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THE ROLE OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN CENTRAL CARDIOVASCULAR REGULATION.

A Thesis submitted for the Degree of
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by

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ABSTRACT

The central effect of nicotine on cardiovascular regulation has been extensively studied. However, due to its unselective nature for nicotinic acetylcholine receptors (nAChR) the involvement of specific nAChRs at sites in the brain, in central nervous cardiovascular regulation remains unclear. The effects of intracerebroventricular (i.c.v.) and intracisternal (i.c.) injections of the α7 selective agonist, PSAB-OFP, and the α4β2 selective agonist, TC-2559, were investigated on blood pressure (BP), heart rate (HR) and renal sympathetic nerve activity (RSNA) compared with nicotine, in the anaesthetised rats.

PSAB-OFP and TC-2559 i.c.v. caused a delayed dose-related increase in BP and RSNA. When given i.c. the action was similar except the rise in BP was more immediate. The possibility that the pressor response was partly due to the agonists causing the release of the vasoconstrictor vasopressin into the circulation was tested by repeating the i.c.v. and i.c. injections of the agonists in the presence of a selective vasopressin V_{1a} antagonist. In the presence of V_1 antagonist (i.v.), PSAB-OFP and TC-2559 (i.c.v.) now induced no change in BP or RSNA; however i.c., the increase in BP and RSNA was delayed with TC-2559, while PSAB-OFP caused a decrease in BP and no change in RSNA. The cardiovascular effects of i.c.v. PSAB-OFP and TC-2559 in the presence of V_1 receptor antagonist (i.c.v.) were also completely blocked. PSAB-OFP and TC-2559 (i.c.) in rats pre-treated with V_1 antagonist (i.c.), no longer produced an increase in BP and RSNA. However, the delayed fall in BP caused by PSAB-OFP was potentiated.

Nicotine i.c.v. caused a dose-related increase in BP and renal sympathoinhibition; while i.c. the rise in BP was larger and now associated with a bradycardia. In the presence of V_1 antagonist (i.v.), nicotine's (i.c.v.) cardiovascular effects were blocked; however nicotine i.c. caused a decrease in BP, RSNA and HR. In the presence of V_1 antagonist (i.c.v.), nicotine caused no change in RSNA, but BP still increased. In the presence of V_1 antagonist (i.c.), nicotine i.c. induced a decrease in RSNA and HR, with no change in BP.

This study indicates that activation of α4β2 and α7 nAChRs in the hindbrain cause an increase in BP due to vasopressin release into the periphery, which is mediated by a central vasopressinergic pathway, possibly involving a pathway from the hindbrain to the PVN. Interestingly, the renal sympathoexcitation was also prevented by blocking V1a receptors in the periphery as well as in the brain. A possible mechanism is discussed whereby vasopressin or its antagonist accesses the brain via the circumventricular organs and then indirectly influence the PVN, supra-medulla or supra-spinal vasopressin neurons influencing the renal sympathetic outflow.

Nicotine also causes an increase in BP by vasopressin release into the periphery, by acting on nAChR in the forebrain and the hindbrain. Interestingly, the decrease in RSNA is still observed when in the presence of V_1 antagonist, suggesting that the decrease in RSNA is partly mediated by an action of nicotine on nAChRs at sites in the hindbrain such as the NTS.

PUBLICATIONS

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ABBREVIATIONS

α,	alpha
β,	Beta
δ,	delta
γ,	gamma
ε,	epsilon
μ,	micro
α-BgT,	α-Bungarotoxin
3HC,	3'-Hydroxycotinine
5-HT,	5- hydroxytryptamine
Acetyl-CoA,	Acetyl Co-enzyme A
ACh,	Acetylcholine
AChE,	Acetylcholinesterase
AD,	Alzheimer's disease
ANOVA,	Analysis of variance
ANS,	Autonomic nervous system
BP,	Blood pressure
Ca^{2+},	Calcium
ChAT,	Choline acetyltransferase
CNS,	Central nervous system
Ctx,	Conotoxin
Cys,	Cysteine
Cyt,	Cytisine
CVLM,	Caudal Ventrolateral medulla
DA,	Dopamine
Dhβ E,	Dihydro-β-erythrodine
DMPP,	Dimethyl-4-Phenyl-Piperazinium
DMVN,	Dorsal vagal motor nucleus
GABA,	γ-aminobutyric acid
HR,	Heart rate

i.c.,	Intracisternal injection
i.c.v.,	Intracerebroventricular injection
Int. RNA,	Integrative renal nerve activity
i.v.,	Intravenous injection
KO,	Knock-out
LGIC's,	Ligand gated ion channels
ILM,	Intermediolateral cell column
MAP,	Mean arterial blood pressure
Mec,	Mecamylamine
min,	Minutes
MLA,	Methyllycaconitine
nAChR,	Nicotinic acetylcholine receptor
NCA,	Non-competitive activator site
NCB,	Non-competitive blockers
Nic,	Nicotine
NAmb,	Nucleus ambiguus
NTS,	Nucleus tractus solitarius
PD,	Parkinson's disease
PVN,	Paraventricular nucleus of the hypothalamus
RNA,	Renal nerve activity
RVLM,	Rostral Ventrolateral medulla
s,	Seconds
S.E.M.,	Standard error of the mean
SN,	Substantia nigra
SON,	Supraoptic nucleus of the hypothalamus
VLM,	Ventrolateral medulla
VTA,	Ventral tegmental area
Wt,	Wild type

CHAPTER 1 GENERAL INTRODUCTION.

1.1. Regulation of cardiovascular system.

The key function of the cardiovascular system is transport, most importantly, oxygen to all tissues and the removal of carbon. The cardiovascular system is also involved in transporting nutrients from the sites that they are absorbed, or synthesised, to the tissue cells where they are used, as well as transporting hormones from endocrine organs, where they are produced, to target cells where they act. Finally, the cardiovascular system is important in the transport of protective cells and substances to sites of tissue damage or infection, so allowing a defensive response and repair to be initiated. In order for this to take place the transport medium required is blood and for the cardiovascular system to be in a state of homeostasis it depends upon the blood flow to all regions of the body being appropriate for the metabolic demands of each region, therefore the cardiovascular system is regulated by both peripheral and central structures, which are responsible for maintaining the internal environment of the body. A number of factors are monitored by peripheral sensors which include blood pressure, cardiac output, blood volume and blood gas tensions, which when activated results in an appropriate compensatory response, leading to a number of complex feedback loops known as cardiovascular and respiratory reflexes. The sensory receptors involved in the reflex feedback loops known to regulate the cardiovascular and respiration system are divided in to two, mechanoreceptors and chemoreceptors. Mechanoreceptors are located in the aortic arch and carotid sinus (Figure 3a) and detect changes in blood vessel wall tension which is dependent on changes in arterial pressure (further explanation is given in section 1.1.3.1.). Mechanoreceptors have also been located in the atria and ventricles and in the endings of great veins (Spyer, 1990). Chemoreceptors (section 1.1.3.2.) are located in the carotid body (Figure 3a), and also the ventral surface of the medulla, and are sensitive in particular to O_2 and CO_2 tensions, as well as the pH of arterial blood (see Daly, 1997).

Overall these receptors relay information to central cardiovascular centres in the central nervous system (CNS) where they are integrated to cause the appropriate regulation of the heart and blood vessels, as well as the respiratory system.

1.1.1. The autonomic nervous system

Regulation of the cardiovascular system by central cardiovascular centres requires axons between the CNS and the effector cells located on smooth and cardiac muscle, as well as glands and the gut. This is termed the autonomic nervous system (ANS) and is divided into three parts known as; the sympathetic system, the parasympathetic system, and the enteric system. Both the sympathetic and the parasympathetic have neuronal cell bodies located in both the CNS and peripheral nervous system (PNS), whereas the neurons of the enteric system are located exclusively in the walls of the gut (see Hardman J.G *et al.*, 2001; see Tortora & Grabowski, 2000).

The ANS pathways are made up of preganglionic and postganglionic neurones. The arrangement of the ganglia is one way of differentiating between sympathetic and parasympathetic neurones of the ANS. The sympathetic preganglionic cell bodies are located in the intermediolateral (IML) cell column of the spinal cord which leaves the CNS in segmental thoracic and lumbar roots (levels T1 to L2, see Figure 1) and forms a chain of sympathetic ganglia on either side of the spinal cord, known as the paravertebral ganglia. The cell bodies of the postganglionic neurons are located close to those of the pre-ganglionic neurones, and at a distance from the target organs observed by long postganglionic fibres. However, parasympathetic preganglionic neurones are located in the brainstem with axons travelling in the cranial nerves III, VII, XI and X, as well as the sacral region of the spinal cord (levels S2 - S4) (Figure 1). Unlike the sympathetic ganglia, the parasympathetic ganglia are found near or in their target organs (see Hardman J.G *et al.*, 2001; see Tortora & Grabowski, 2000).

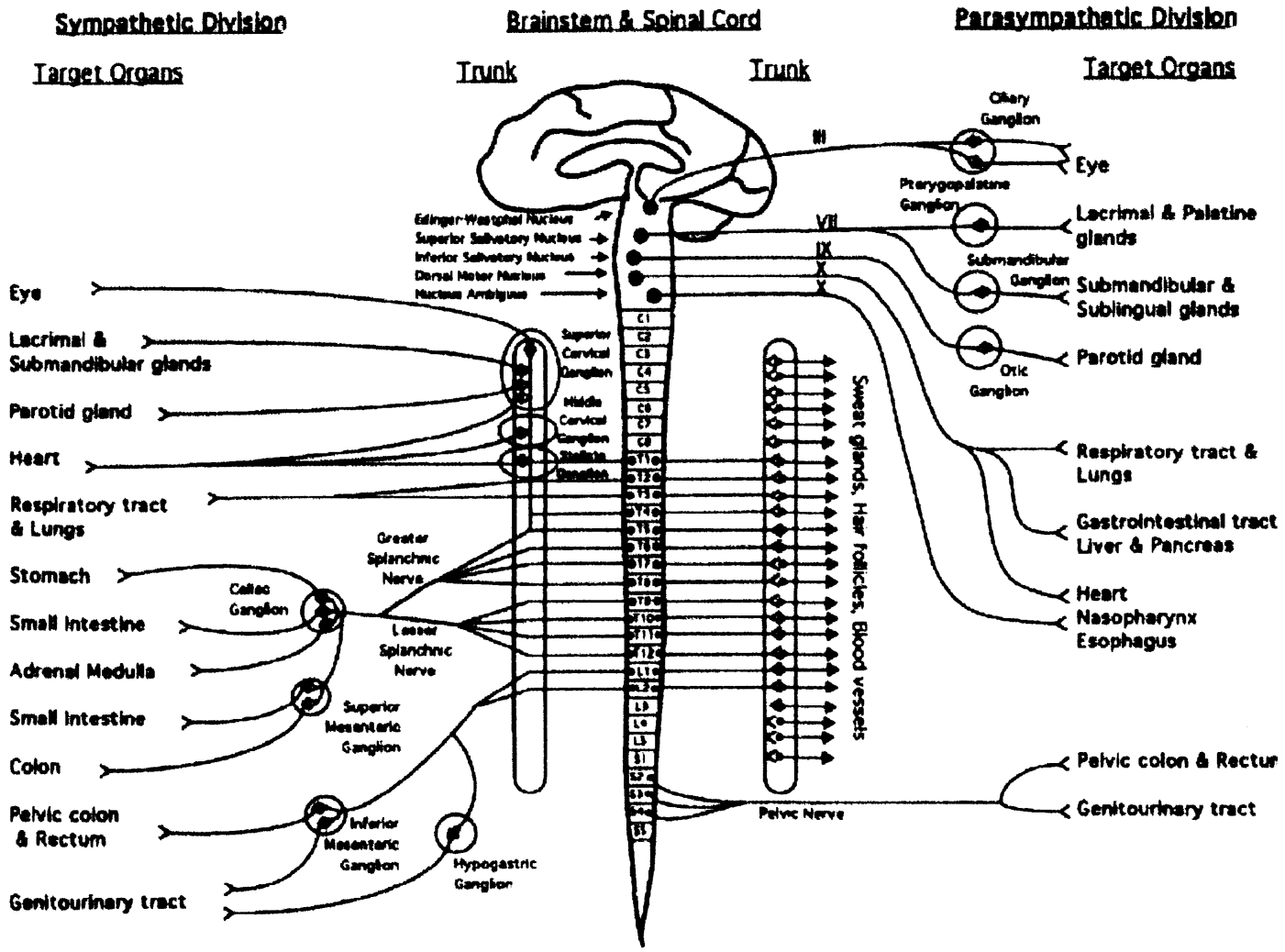


Figure 1. Diagram of the sympathetic and parasympathetic divisions of the ANS. The paravertebral chain of the sympathetic division is illustrated on both sides of the spinal outflow in order to demonstrate the full range of target structures innervated (modified from Rang *et al.*, 1999)

As you can observe in Figure 1, the sympathetic nerves are distributed widely throughout the body, innervating areas such as the heart and lungs via the superior cervical ganglia, as well as the stomach and lower intestine which are innervated via the celiac and inferior mesenteric ganglion respectively. Sympathetic nerve fibres also innervate the smooth muscle of most blood vessels via the superior cervical ganglion. In addition, Figure 1 demonstrates the location of the parasympathetic neurones, which appears to have a restricted distribution. For example, the Edinger-Westphal nucleus located in the midbrain innervates the eye via the IIIrd cranial nerve. The VIIth cranial nerve and the IXth nerve, located at the superior and inferior salivatory nucleus respectively, in the medulla innervate the lucrimal, nasal and parotid salivary glands. The vagus nerve (Xth cranial nerve) innervates the heart, lungs and gut from the dorsal vagal nucleus (DVN) and nucleus ambiguus, which leaves the sacral plexus to innervate the colon, rectum, bladder and external genitalia via the pelvic splanchnic nerve.

Not only do sympathetic and parasympathetic differ in the location of their ganglia, they also vary in the neurotransmitter used. In both sympathetic and parasympathetic divisions, the major neurotransmitter released between pre- and post-ganglionic neurons is acetylcholine, acting on nicotinic receptors. In sympathetic neurones the major neurotransmitter between the postganglionic neurone and the target organs is noradrenaline acting on α and β adrenoceptors, although in some cases depending on species it can be acetylcholine acting on muscarinic receptors e.g. sweat glands. While the postganglionic parasympathetic neuron release acetylcholine acting on muscarinic receptors (see Katzung B.G, 2001; see Rang *et al.*, 1999; see Tortora & Grabowski, 2000).

1.1.2. Brain areas involved in cardiovascular regulation.

1.1.2.1. The nucleus tractus solitarius.

The nucleus tractus solitarius (NTS) is an elongated nucleus that extends throughout the dorsomedial part of the medulla oblongata from the level of the caudal edge of the facial motor nucleus to the cervical spinal cord. The NTS is essentially involved in cardiovascular and respiratory regulation due to it being the principle site of termination of primary afferent fibres arising from cardiovascular receptors such as the arterial baroreceptors, chemoreceptors and lung stretch afferents, which provide essential information on the vascular system. Therefore the neurons within the NTS are essential components of cardiovascular reflexes (see sections 1.1.3.1. – 1.1.3.2)(Spyer, 1990).

In addition to the primary afferents, the NTS receives innervation from all brain levels including the paraventricular nucleus (PVN) and lateral nuclei of the hypothalamus, amygdala, periaqueductal grey (PAG), area postrema (AP), raphe nuclei and A5 cell group. Furthermore the NTS projects to various regions in the spinal cord, lower brainstem, midbrain and forebrain that are known to play an important role in cardiovascular control (Figure 2). These regions include the IML cell column, the rostral and caudal ventrolateral medulla, the PVN and lateral hypothalamus, amygdala, PAG, raphe nuclei, parabrachial complex, AP, nucleus ambiguus and dorsal vagal nucleus (DVN), suggesting that the NTS has similar reciprocal connectivity (see Dampney, 1994).

Previous studies observing different areas of the brain innervating the NTS have shown that electrical stimulation of the PVN inhibits neurons in the NTS (Duan *et al.*, 1999). Studies have shown that stimulation of the amygdala and AP results in excitation of neurons in the NTS (Cox *et al.*, 1986; Hay & Bishop, 1991a; 1991b).

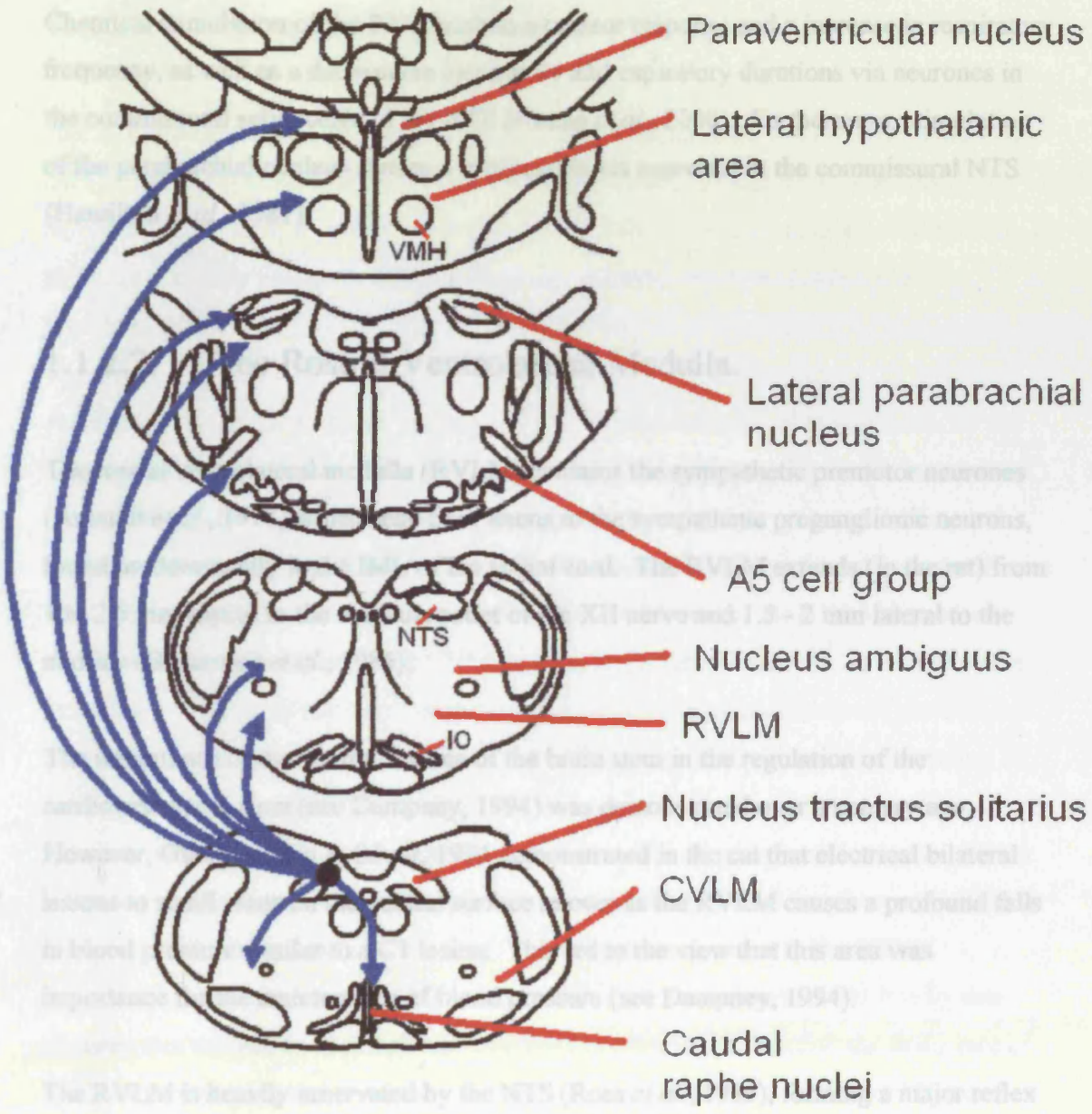


Figure 2. Diagram of brain areas known to have a cardiovascular function and their innervation by efferent projections from the nucleus tractus solitarius (NTS). In addition there are also projections (not shown) to other targets such as the nucleus periaqueductal grey, amygdala and regions surrounding the anteroventral part of the 3rd ventricle. CVLM, caudal ventrolateral medulla; IO, inferior olive; RVLM, rostral ventrolateral medulla; VHM, ventromedial nucleus of hypothalamus (modified from Dampney, 1994)

Chemical stimulation of the PAG leads to a pressor response and a increase in respiratory frequency, as well as a decrease in inspiratory and expiratory durations via neurones in the commissural subnucelus of the NTS (Huang *et al.*, 2000). Furthermore, stimulation of the parabrachial nucleus causes a bradycardia via neurones in the commissural NTS (Hamilton *et al.*, 1981).

1.1.2.2. The Rostral Ventrolateral Medulla.

The rostral ventrolateral medulla (RVLM) contains the sympathetic premotor neurones (Amendt *et al.*, 1978) which send their axons to the sympathetic preganglionic neurons, found predominantly in the IML of the spinal cord. The RVLM extends (in the rat) from 1 to 2.5 mm rostral to the median rootlet of the XII nerve and 1.5 - 2 mm lateral to the midline (Benarroch *et al.*, 1986).

The importance of the ventral surface of the brain stem in the regulation of the cardiovascular system (see Dampney, 1994) was demonstrated over a century ago. However, Guertzenstein & Silver, 1974 demonstrated in the cat that electrical bilateral lesions to small areas on the ventral surface known as the RVLM causes a profound falls in blood pressure similar to a C1 lesion. This led to the view that this area was importance for the maintenance of blood pressure (see Dampney, 1994).

The RVLM is heavily innervated by the NTS (Ross *et al.*, 1985), forming a major reflex pathway (Figure 2). Furthermore, the RVLM is also innervated by the AP (Ross *et al.*, 1985; Shapiro & Miselis, 1985), which may relay information on circulating hormones to the RVLM, as well as major inputs from the PAG, the PVN and lateral hypothalamic area, which are though to be involved in reflex modulation and hypertension (Ross *et al.*, 1985).

1.1.2.3. The Caudal Ventrolateral Medulla.

The caudal ventrolateral medulla (CVLM) is found between the nucleus ambiguus and lateral reticular nucleus (Figure 2) (see Dampney, 1994; see Sun, 1995)and was demonstrated to have an affect on cardiovascular function when application of nicotine to the ventral surface in the cat caused a decrease in blood pressure (Feldberg & Guertzenstein, 1976).

Additional studies have shown that electrolytic lesions or chemical inactivation, by the GABA receptor antagonist, muscimol, in the CVLM resulted in a rise in blood pressure, whereas electrical stimulation and chemical stimulation, by the excitatory amino acid glutamate, in the CVLM produced sympathoinhibition and a fall in blood pressure (Cravo *et al.*, 1991; Gordon, 1987; Jeske *et al.*, 1993). The A1 area of the CVLM has been proposed to be the location of the CVLM sympathoinhibitory neurons, as lesions cause an increase in blood pressure, whereas electrical or chemical stimulation of the CVLM area causes a depressor response (Ross *et al.*, 1985), however more precise mapping of the depressor sites in the CVLM of the rat has indicated that the CVLM sympathoinhibitory neurons are located outside the A1 cell group (see Dampney, 1994). Interestingly, it has been observed that there is an inhibitory GABAergic synapse from the sympathoinhibitory pathway in the CVLM depressor neurons to the RVLM sympathetic premotor neurons (Cravo *et al.*, 1991). It was suggested that it is by this pathway that activation of peripheral baroreceptors decrease or abolish the firing rate of RVLM sympathetic premotor neurons (Agarwal *et al.*, 1990; Cravo *et al.*, 1991; see Dampney, 1994; Gordon, 1987), therefore indicating that the CVLM elicits effects on the baroreflex by inhibiting neurons in the RVLM and causing sympathoinhibition and vasodepressor responses.

Originally it was believed that the NTS was the source of the GABAnergic input but studies have shown that the central baroreceptor pathway consists of a pathway from the NTS to the CVLM, probably by a glutamatergic projection, and then from CVLM to RVLM by a GABAergic input (Agarwal *et al.*, 1990). The involvement of NMDA

receptors in the CVLM was demonstrated by the use of selective NMDA receptor antagonists, which when applied to the CVLM abolished synaptically-mediated depressor responses caused by aortic nerve stimulation, indicating that neural transmission of aortic baroreceptor information in the CVLM is mediated by activation of NMDA receptors. The neurotransmitter released at the CVLM synapse may be an excitatory amino acid, possibly glutamate (Gordon, 1987).

1.1.2.4. Nucleus Ambiguus & Dorsal Vagal Nucleus.

A key area in the regulation of the heart is the vagus (X^{th}) nerve, (see section 1.1.1). Location of cardiac vagal preganglionic neurones are in two main cell groups called the dorsal vagal nucleus (DVN) and the nucleus ambiguus (Bennett *et al.*, 1981; Kalia & Mesulam, 1980; Nosaka *et al.*, 1979; Stuesse, 1982). Those with myelinated axons are found in the ambiguus and are the main neurones for control of the heart while the small group of unmyelinated axons do project from the DVN and only play a small role in the parasympathetic control of the heart (Figure 2), These nuclei received input from other brain regions such as the parabrachial complex, the PVN and the medullary reticular formation (Deuchars & Izzo, 2006; Hunt & Schmidt, 1978).

Studies of the nucleus ambiguus in the cat and rabbit have demonstrated that the low spontaneous firing rate produced by the cardiac vagal preganglionic neurones had a strong respiratory rhythm, firing during the post-inspiratory phase but was inhibited during the inspiration phase (Gilbey *et al.*, 1984; Jordan *et al.*, 1982; McAllen & Spyer, 1978) and thus responsible for sinus arrhythmia.

1.1.2.5. The hypothalamus

The hypothalamus has many autonomic and homeostatic roles. The anteroventral third ventricular region participates in functions such as thermoregulation and drinking (Spyer, 1994). The PVN has diverse functions including secretion of releasing hormones, such as vasopressin, but also has various projections to cardiovascular areas (Spyer, 1994). In addition, electrical and chemical stimulation of the PVN can increase or decrease blood pressure (Yang *et al.*, 2001; Yang & Coote, 1998). The lateral hypothalamic area has a sympathoexcitatory role, and stimulation causes hypertension and tachycardia (Coote *et al.*, 1998).

In addition to the above, the dorsomedial hypothalamic nucleus (DMH) plays a key role in the cardiovascular response. The classic defence response, such as a human subjected to a acute alerting stimulation, i.e. a loud noise, is characterised by a increase in blood pressure, heart rate and skeletal muscle blood flow, accompanied by vasoconstriction of renal and splanchnic vascular beds (Hilton, 1975). This response increases cardiac output and redistributes blood towards skeletal muscle beds which is appropriate for an animal that may need to fight or flee from a threatening situation.

Electrical stimulation of a region in the hypothalamus referred to as the 'defence area' elicited a cardiovascular response very similar to that described above (Hilton, 1975). Furthermore, microinjection of either excitatory amino acid or GABA receptor antagonist in the DMH results in a cardiovascular response very similar to the defence reaction, as well as inhibition of DMH causing a decrease in pressor and tachycardia response to evoked stress in the conscious rat (DiMicco *et al.*, 1996).

The DMH receives innervations from areas such as the amygdale and innervates areas such as the RVLM and the NTS (Fontes *et al.*, 2001). Innervation of the NTS and RVLM suggests why the stimulation of the hypothalamic 'defence' area modulates the baroreceptor reflex (Spyer, 1994) causing an increase in blood pressure but attenuating

the reflex bradycardia, distributing the blood to required areas of the body in response to a threatening situation.

1.1.3. Short term modulation of blood pressure

1.1.3.1. The baroreflex.

As previously mentioned arterial baroreceptors are also known as mechanoreceptors and respond to the stretch of arterial vessel walls and are essential for maintaining blood pressure on a short term basis.

The arterial baroreceptors are located in two main areas known as the carotid sinus and the aortic arch (Figure 3a). The carotid sinus is a portion of the carotid artery which supplies blood to the head and neck. High in the neck, the common carotid arteries is divided into two smaller arteries, at this division the wall of the artery is thinner than usual and contains a large number of branching nerve endings. These nerve endings are highly sensitive to stretch and upon a change in blood pressure, alter the stretch of the artery wall which stimulates a response. In addition, another baroreceptor is located on the arch of the aorta and is termed the aortic arch, which is again sensitive to stretch of the artery wall (Spyer, 1990) (Figure 3a).

At normal blood pressure, baroreceptors afferents fire at tonic, pulse related rate, allowing reflex changes to occur if the afferent firing rate changes in either direction. Similarly, cardiac vagal afferents and sympathetic vasoconstrictor efferents fire at a tonic rate, allowing reflex increase or decrease to occur (Spyer, 1990).

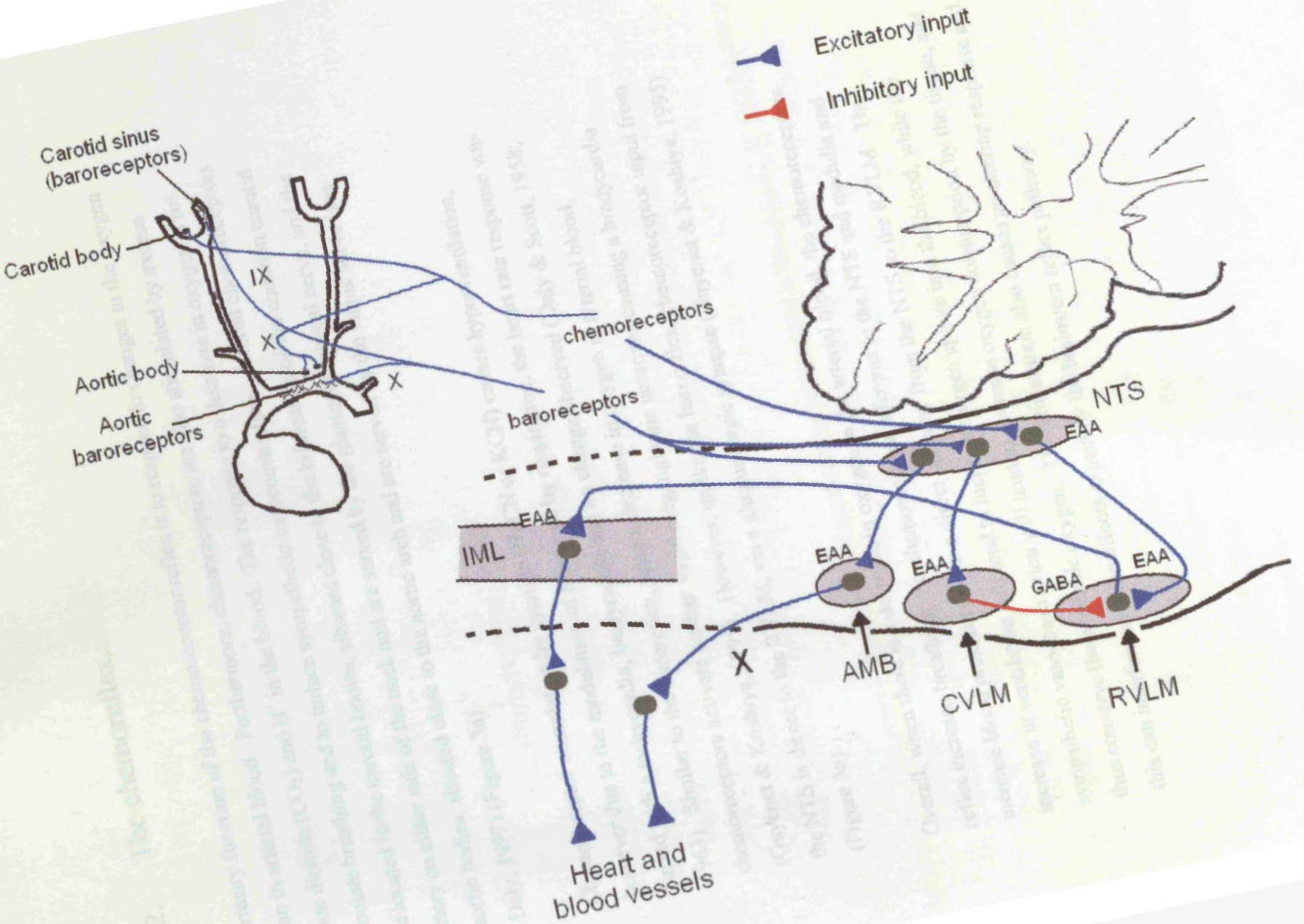
An increase in blood pressure results in an increase in the firing rate of baroreceptors through stretch activation, the aortic nerve and carotid sinus baroreceptor afferents have

been shown to project to the NTS (Donoghue *et al.*, 1982;1984; Spyer, 1990) and excite second-order neurons via a glutamatergic synapse. NTS neurons conveying baroreceptor signals then project to and excite (again via a glutamatergic synapse) neurons within the CVLM. The CVLM neurons project to and inhibit the sympathoexcitatory neurons in the RVLM via a GABAergic synapse (Agarwal *et al.*, 1990; Dampney *et al.*, 1982; Guyenet *et al.*, 1987; Sun, 1995). Therefore baroreflex sympathoinhibition involves switching off the tonic activity of the RVLM, and consequently ILM neurons, abolishment of this inhibitory synapse in the RVLM blocks the baroreflex demonstrating the pivotal role of this group of neurons in the baroreceptor reflex. In addition, the NTS also innervates the nucleus ambiguus thus modulating heart rate via the vagus (Figure 3a). Overall, when blood pressure is elevated, baroreceptors afferents increase their firing inhibiting sympathetic outflow to the heart, skeletal muscle, arterioles and increase the firing rate of cardiac vagal neurones, lowering heart rate. This results in a drop in total peripheral resistance and cardiac output and helps return the blood pressure to near normal.

However, if blood pressure drops the reverse occurs, the firing rates of the carotid sinus and aortic arch baroreceptors decrease resulting in an increase in sympathetic tone due to disinhibition of the CVLM by the NTS, therefore the RVLM is not inhibited and increases sympathetic tone to the heart and blood vessels, as well as inhibiting the firing rate of the cardiac vagal preganglionic neurones. Overall resulting in a increase in cardiac output (due to a increase in heart rate and stroke volume) and a increase in total peripheral resistance (blood vessel vasoconstriction) returning the blood pressure towards normal.

As previously noted (section 1.1.2.4) the baroreflex is able to be modulated under certain circumstances, for example respiration. Studies have shown that the baroreflex bradycardia is subject to respiratory modulation (Haymet & McCloskey, 1975). This is believed to occur at the level of the nucleus ambiguus, via the inhibition by inspiratory neurons.

Figure 3A. Diagram illustrating proposed pathways within the lower brainstem and spinal cord that are involved in regulation of arterial blood pressure by the baroreceptor and chemoreceptor reflex. Pathways utilise neurotransmitters γ-aminobutyric acid (GABA) or an excitatory amino acid (EAA). The blue triangles indicate excitatory inputs and the red triangles inhibitory synapse inputs. AMB, nucleus ambiguus; CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla; IML, intermediolateral cell column of the spinal cord; NTS, nucleus tractus solitarius; X, vagus nerve (modified from Dampney, 1994) .


Excitatory input
Inhibitory input
Carotid sinus
(baroreceptors)
Carotid body
IX
X
Aortic body
X
Aortic
baroreceptors
chemoreceptors
baroreceptors
NTS
EAA
EAA
IML
EAA
EAA
GABA
EAA
X
AMB
CVLM
RVLM
Heart and
blood vessels

1.1.3.2. The chemoreflex.

The primary function of the chemoreceptor reflex is to respond to changes in the oxygen tension in arterial blood. Furthermore, chemoreceptors are also stimulated by excess carbon dioxide (CO_2) and H^+ in the blood. The response to a decrease in oxygen is to stimulate breathing and to induce sympathetic vasoconstriction. Arterial chemoreceptors are located in the carotid bodies, situated close to the bifurcation of the common carotid artery on either side of the neck and are served by the glassopharyngeal nerve, and the aortic bodies, situated close to the aortic arch and are served by the vagus nerve (ses Daly, 1997) (Figure 3a).

Chemoreceptor stimulation by cyanide (NaCN or KCN) causes hyperventilation, however due to the modulation of heart rate by respiration, the heart rate response was varied with a tachycardia, bradycardia and no change observed (Daly & Scott, 1958; 1963). Similar to the baroreflex, upon a decrease in oxygen in arterial blood, chemoreceptors activate cardiac vagal preganglionic neurones causing a bradycardia (Guyenet & Koshiya, 1995). However, unlike the baroreflex, chemoreceptor input from the NTS is direct to the RVLM, via a glutamatergic synapse (Guyenet & Koshiya, 1995) (Figure 3a).

Overall, when there is a decrease in oxygen in the arterial blood, the chemoreceptors reflex increase breathing, via chemoreceptor afferents to the NTS and medulla and increase blood pressure due to a direct pathway from the NTS to the RVLM. The increase in ventilation will tend to increase oxygen uptake into the blood, while the sympathetic vasoconstriction will tend to decrease oxygen consumption by the tissue, and thus conserve the available oxygen. This stimulation also causes an altering response and this can interfere with the effects expect by the brainstem reflex pathway.

1.1.4. Long term modulation.

Baroreflex and chemoreflex are short term modulators of arterial blood pressure, activated instantly by any blood pressure change and attempt to restore blood pressure rapidly to normal. The major factor for long term modulation is blood volume. Blood volume is a major determinant of arterial blood pressure because it influences in turn venous pressure, venous return, end-diastolic volume, stroke volume and cardiac output. Thus an increased blood volume increases arterial blood pressure.

1.1.4.1. Sodium reabsorption

Sodium is a major extracellular solute, changes in total-body sodium results in similar changes in extracellular volume. Since extracellular volume comprises plasma volume and intestinal volume, plasma volume is also significantly affected by total-body sodium. Plasma volume is an important determinant of the pressure in the veins, cardiac chambers and arteries, therefore low total-body sodium leads to low plasma volume, which leads to low cardiovascular pressure and vice versa.

Upon a decrease in total-body sodium, resulting in a decrease in blood pressure, a number of mechanisms occur to 1) increase sodium in the plasma; and 2) maintain a normal blood pressure. A major mechanism in the decrease of total body sodium is the control of sodium reabsorption. One of the key pathways is the renin-angiotensin system, with the rate limiting step being the rate of secretion of the protein renin, which needs to be stimulated to be released from juxtaglomerular cells of the kidneys into the blood(Reid, 1998).

Stimulation of the release of renin occurs by at least two distinct inputs to the juxtaglomerular cells; 1) the renal sympathetic nerves, and 2) intra-renal baroreceptors. The effect of the renal sympathetic nerve input makes sense since a reduction in body sodium and plasma volume lowers blood pressure, and via baroreceptors external to the

kidneys, triggers an increase in sympathetic discharge to the juxtaglomerular cells, stimulating renin release. The controlling of renin release by intra-renal baroreceptors is contained within the kidneys and requires no external neuroendocrine input. The juxtaglomerular cells are located in the walls of the afferent arterioles and are themselves sensitive to the pressure within these arterioles, and so function as intra-renal baroreceptors. When renal arterial pressure decreases, as would occur when extracellular volume is down, these cells secrete more renin. Thus normally, the juxtaglomerular cells respond simultaneously to the combined effects of sympathetic input, triggered by baroreceptors external to the kidneys, and to their own pressure sensitivity (Reid, 1998).

The release of renin is involved in the renin-angiotensin system which ultimately leads to the synthesis and release of aldosterone, which controls the re-absorption of sodium. Renin acts as an enzyme to split off a small polypeptide, angiotensin I, from a large plasma protein, angiotensinogen, which is synthesized by the liver and is always present in the blood. Angiotensin I then undergoes further cleavage to form angiotensin II. This conversion is mediated by an enzyme called angiotensin converting enzyme (ACE), which again is always present in amounts adequate to convert all angiotensin I to angiotensin II. Angiotensin II is a potent stimulator of aldosterone secretion and acts on the adrenal cortex, which controls the production and release of this hormone (Reid, 1998)(Figure 3b).

Aldosterone stimulates sodium reabsorption by the cortical collecting ducts, controlling the reabsorption of the last remaining sodium by inducing the synthesis of proteins that function as sodium channels in the luminal membrane and proteins that constitute the sodium and potassium-ATPase pumps (Dolman & Edmonds, 1975; Halevy *et al.*, 1986; Schultz, 1984). Overall by the release of renin, which leads to the release of aldosterone, there is an increase in sodium reabsorption which increases the total-body sodium, and thereby plasma volume indicating that the renin-angiotensin system contributes to the control of arterial blood pressure.

Interestingly, this is not the only way in which the renin-angiotensin system influences blood pressure. Angiotensin II is a potent vasoconstrictor of arterioles and this effect on

peripheral resistance increases arterial blood pressure (Werner & Bohm, 2003) (Figure 3b). In addition, angiotensin II may also be having a central cardiovascular effect, inducing a sustained increase in renal sympathetic nerve activity and increasing arterial pressure. Studies have shown that angiotensin II is able to act centrally by acting on areas of the rat brain that lack a blood brain barrier, known as the circumventricular organs (CVO), in particular the subfornical organ (SFO) and AP (Ferguson & Wall, 1992). A key central area that is believed to be affected by angiotensin II is the PVN. Activation of PVN neurons by inputs from the SFO has been observed to be modulated, in part by, angiotensin (AT_1) receptors, as well as activation of this pathway increasing arterial blood pressure (Ferguson & Washburn, 1998). The PVN is believed to be an important site mediating sustained increases in sympathetic activity in response to a variety of responses, such as stress, which is consistent with the PVN receiving inputs originating from higher centres and peripheral receptors, as well as the CVO (Badoer, 2001; Blair *et al.*, 1996; see Dampney, 1994). Therefore, it is proposed that the PVN is activated by increasing levels of circulating angiotensin II, as well as additional stimuli, resulting in stimulation of the RVLM directly or indirectly increasing sympathetic output (Coote *et al.*, 1998; Yang *et al.*, 2001).

1.1.4.2. Water reabsorption.

Upon a decrease in plasma volume, a increase in aldosterone secretion, via the renin-angiotensin system is observed, however there is also a mechanism involving the reabsorption of water which increase the plasma volume and therefore increases arterial blood pressure. The decrease in plasma volume and hence arterial pressure triggers the release of vasopressin (also known as anti-diuretic hormone; section 1.10) from the posterior pituitary , via a neural input to the vasopressin-secreting cells from several baroreceptors in the cardiovascular system, possibly via the A1 noradrenergic neurones (Blessing & Willoughby, 1985; Holmes *et al.*, 2003). Upon a decrease in firing from the baroreceptors due to a decrease in blood pressure that occurs when blood volume decreases, fewer impulses transmit from the baroreceptors, via afferent neurons to

ascending pathways to the hypothalamus, resulting in an increase in vasopressin release evoking the retention of water and ultimately increasing plasma volume. Hence an increase in blood pressure by an increase in plasma volume causes more firing by the baroreceptors resulting in a decrease in vasopressin secretion and increasing water excretion, therefore decreasing plasma volume.

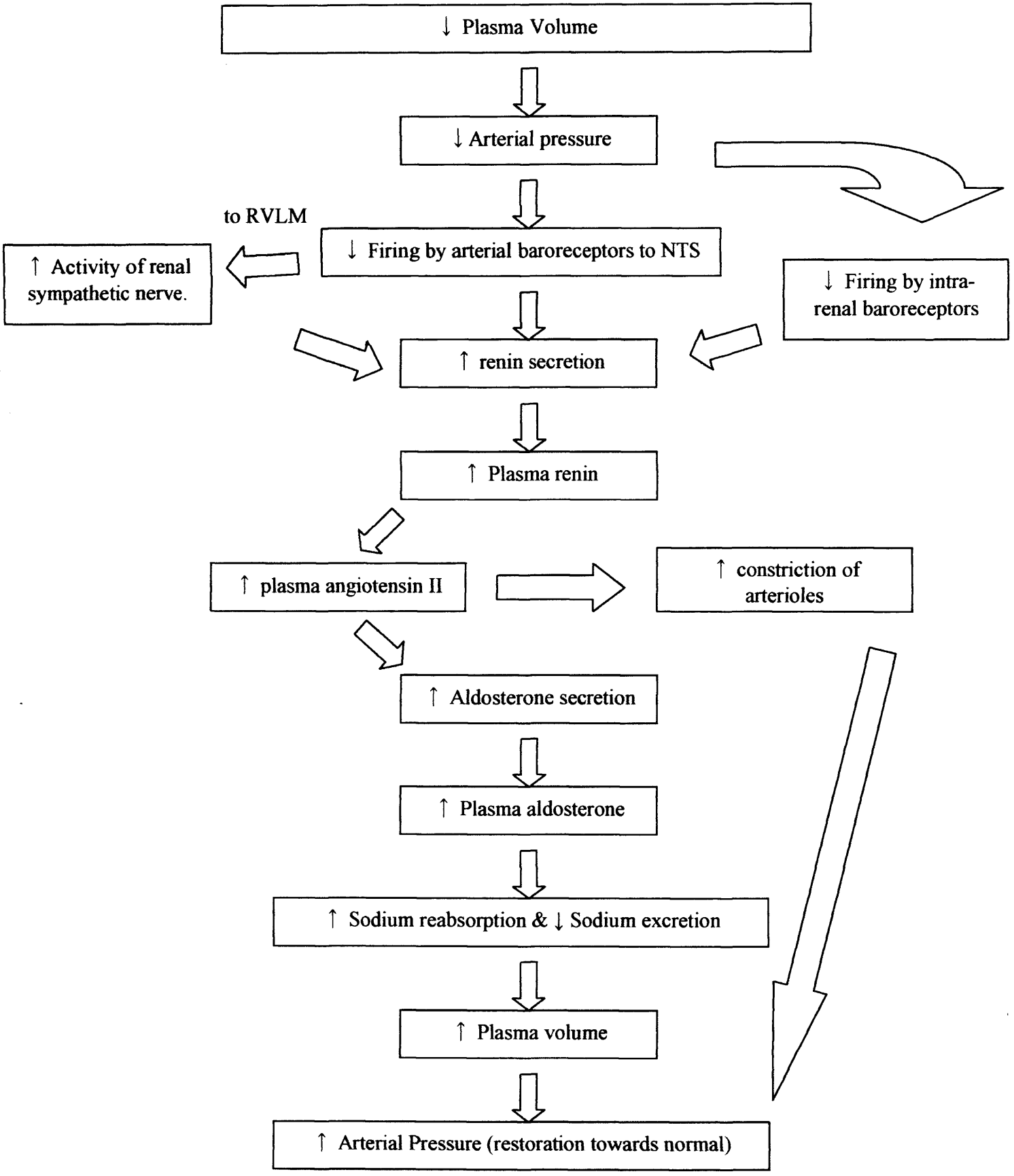
Vasopressin releases into the periphery has a two-fold effect 1) by acting on V_{1a} receptors found on the smooth muscle of blood vessels, causing vasoconstriction inducing a increase in blood pressure, which maintains blood pressure due to the decrease in blood volume; and 2) acts on V_2 receptors found on the kidneys, and induces the appearance of a population of vessels containing water channels on vasopressin-sensitive membranes of the kidney collecting tubules. The insertion of the water channels give rise to the reabsorption of water, which increase plasma volume and blood pressure to normal limits (Holmes *et al.*, 2003; Howl & Wheatley, 1995; Macfarlane *et al.*, 1967).

1.2. Nicotinic acetylcholine receptor structure.

1.2.1. Structure.

Nicotinic ACh receptors (nAChR) belong to a superfamily of ligand gated ion channels, which include muscle and neuronal-type nicotinic ACh receptors, 5-hydroxytryptamine type 3 (5-HT_3) receptors, γ-aminobutyric acid type A ($GABA_A$) and $GABA_C$ receptors, glycine receptors and invertebrate glutamate receptors. This superfamily are also known as Cys-loop receptors because all family subunits contain in their amino N-terminal, a pair of disulphide-bonded cysteines, which are separated by 13 residues (see Absalom *et al.*, 2004; see Karlin, 2002). Apart from structural homologies, fundamental differences exist within this superfamily of receptors, including ionic selectivity between cationic (nAChR and 5-HT_3) and anionic ($GABA_A$, $GABA_C$, glycine receptor and glutamate receptor) permeable channels (see Absalom *et al.*, 2004; see Corringer *et al.*, 2000).

Figure 3B. Flow diagram representing pathways by which decreased plasma volume leads, via the renin-angiotensin system and aldosterone, to increase sodium absorption and hence decrease sodium excretion. Hence increasing plasma volume and returning arterial blood pressure to near normal. NTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla.


↓ Plasma Volume
↓ Arterial pressure
to RVLM
↓ Firing by arterial baroreceptors to NTS
↑ Activity of renal sympathetic nerve.
↓ Firing by intra-renal baroreceptors
↑ renin secretion
↑ Plasma renin
↑ plasma angiotensin II
↑ constriction of arterioles
↑ Aldosterone secretion
↑ Plasma aldosterone
↑ Sodium reabsorption & ↓ Sodium excretion
↑ Plasma volume
↑ Arterial Pressure (restoration towards normal)

The study of nicotinic receptors has been made possible by the use of the electric organ of the electric ray *Torpedo marmorata*, and electrical eel, *Electrophorus*, which contains an abundance of nicotinic synapses and receptors, the composition of which corresponds to the nicotinic receptors found at the neuromuscular junction (see Corringer *et al.*, 2000; Miyazawa *et al.*, 2003). Studies of nAChR on vertebrate neuromuscular junctions, *Torpedo* or electrical eel, have provided large amounts of information on peripheral nicotinic receptors which has lead to the identification of numerous neuronal nAChR in the mammalian central nervous system (see Sargent, 1993; see Schmitt, 2000). Additionally, these studies have demonstrated that neuromuscular and neuronal nAChR possess similar structural and functional properties. Purification of neuromuscular nicotinic receptors, revealed a glycoprotein complex with a molecular weight of approximately 300 kDa. Dissociation of the protein results in four different subtypes, termed α1, β1, γ and δ, according to their increasing molecular weight (see Itier & Bertrand, 2001). Additional studies showed that the muscle nAChR is heteropentameric in structure and is composed of two α1 subunits and one of each of the three subunits, β1, γ and δ (Anand *et al.*, 1991; see Lee, 2003; see Unwin, 2003). It has been shown that in foetal muscle the receptor is composed of $(\alpha 1)_2\beta 1\gamma\delta$ subunits, whereas, in the adult muscle nAChR, the composition is $(\alpha 1)_2\beta 1\varepsilon\delta$ (Figure 4b)(see Karlin, 2002; see Kimura, 1998; see Lee, 2003).

Neuronal nicotinic receptors vary when compared to muscle nAChRs, in that there are twelve known receptor subtypes, labelled α2-α10 and β2-β4. The neuronal nAChR pentameric receptors can be expressed as either a homomeric assembly, comprised of 5 subunits of α7-α9, or a heteromeric assembly, comprised of two or more distinct nAChRs. In the latter, α2-α6 and α10 subunits form functional complexes only when co-expressed with a β subunit or with other α subunits (Figure 4b)(see Galzi & Changeux, 1995; see Itier & Bertrand, 2001). In both muscle and neuronal nicotinic receptors, the five membrane spanning subunits are arranged in a circular order (Figure 4a). At the oligmer centre is a pore that constitutes the ion conductance and gate, like a barrel staves around a central channel (Figure 4a)(Alexander *et al.*, 2004; Cooper *et al.*, 1991; see Galzi & Changeux, 1995; see Karlin, 2002; see Schmitt, 2000).

The overall structure of the nicotinic receptor consists of a 5-subunit macromolecule with an overall length of 120 Å, of which, 60 Å is extracellular, 40 Å transmembrane and 20 Å intracellular. The maximum width of the receptor is ~ 80 Å (Figure 5)(see Lee, 2003). All nAChR subunits exhibit significant homology in their amino acid sequence, as well as, similar distribution of hydrophilic and hydrophobic amino acids along their aligned sequence (see Galzi & Changeux, 1995; see Schmitt, 2000). Overall it has been determined that each nAChR subunit contains four hydrophobic segments that form the transmembrane-spanning domains, termed M1-M4. The four hydrophobic stretches compose the transmembrane part of the protein and some of them line the ion channel (Figure6b). Figure 6a shows the other key structures on the nicotinic receptor; 1) a long N-terminal on the extracellular surface, which presents a glycosylation site and ligand binding domains, 2) a large intracellular domain between transmembrane spanning domain M3 and M4 presenting phosphorylation sites, and 3) a short C-terminal domain which originates from M4 (see Corringer *et al.*, 2000; see Galzi & Changeux, 1995; see Itier & Bertrand, 2001; see Unwin, 2003).

1.2.2. The N-terminal and ACh binding site.

The N-terminal of the nACh receptor contains the so-called Cys-loop, a characteristic feature of this superfamily, with the presence of the cysteine doublet believed to be involved in the ACh binding site. The affinity-labelled residues are a pair of adjacent amino acids, α-Cys192 and α-Cys193, which form a highly unusual disulphide bond (Figure 6a), which is only found on the α-subunits, the other subunits also contain long N-terminals but without the cysteine loops (see Absalom *et al.*, 2004; see Corringer *et al.*, 2000).

Studies of ACh binding sites have revealed that neighbouring subunits to the α subunit are also involved in the ACh binding, as heterologous expression of muscle α1 subunits alone does not produce ACh-binding sites (see Karlin, 2002). Muscle nicotinic receptors containing the subunit combination $(\alpha 1)_2\beta 1\delta\gamma/\varepsilon$, have been shown to contain two binding

sites each, one site located at the αδ interface and the other at the αγ interface (Figure 6b) (see Lee, 2003). Neuronal nAChRs exhibit differences in binding sites due to different subunit combinations when compared to muscle nAChRs (Figure 6b). Heteromeric nicotinic receptors, which have the combination of $(\alpha)_2(\beta)_3$, carry two binding sites situated between the α and β subunits, and homomeric receptors with the subunit confirmation of $(\alpha)_5$, have 5 binding sites situated between each α subunit (see Schmitt, 2000).

The structure of the extracellular domain and in particular the ACh binding site, on the nicotinic receptor is a very difficult area to study. Little was known about this region until the discovery of a homologous pentameric soluble protein, that efficiently binds ACh, called ACh-binding protein (AChBP) secreted by the glial cells of the snail, *Lymnaea stagnalis* (see Absalom *et al.*, 2004; see Alexander *et al.*, 2004; Brejc *et al.*, 2001). The use of AChBP, allowed the first crystallisation of a protein that resembles the N-terminal ligand binding domain of the nAChR, the structure also showed the five identical subunits arranged in a cyclinder of 80 Å in diameter with a central pore of ~ 20 Å (Dutertre & Lewis, 2004). Studies using AChBP confirmed the existence of an interface between the α subunit and the adjacent subunit. More importantly X-ray diffraction analyses of the AChBP indicated that six loops termed A-F where involved in the formation of the binding sites (Figure 7)(see Alexander *et al.*, 2004; Dutertre & Lewis, 2004). The α-subunit is believed to contain three loops (A, B and C) and three loops from the adjacent subunits (γ, δ or ε for the muscle and β for the neuronal nicotinic receptor) comprising D, E and F (see Arias, 2000; Brejc *et al.*, 2001; see Itier & Bertrand, 2001). Site-directed mutagenesis studies revealed the amino acids which contribute to the neurotransmitter binding area, with a decrease in affinity for agonists in α7 neuronal nicotinic, muscle and *Torpedo* receptors and therefore showed the functional importance in neurotransmitter binding for the loops A-F (Figure 7)(see Arias, 2000; see Galzi & Changeux, 1995; see Itier & Bertrand, 2001). A large part of these data comes from experiments using the muscle-type receptor; however, sequence comparison indicates that residues in loop A-D are highly conserved in neuronal-type nAChR, while residues in loop E and F are not (Romanelli & Gualtieri, 2003).

Overall, by the use of X-ray diffraction of the AChBP crystal structure and modelling of the three dimensional structure of the nAChR it has been demonstrated that the ACh binding site is located between two subunits, which form a 'pocket'. High resolution electron microscope techniques indicated that these ACh-binding sites are found at each α subunit. Between the receptor sites and the outer borders the distance is approximately 50 Å apart and about 35 Å above the surface membrane (Figure 5)(Corringer *et al.*, 2000; see Lee, 2003; Valenzuela *et al.*, 1994). Ligands that move into the binding site of the nicotinic ACh receptor must penetrate down into the pocket to form appropriate chemical bridges and initiate the opening of the channel. The ligand binding domains are each organised around two-sets of β-sheets, packed into a curled β-sandwich and joined through a disulphide bridge (Brejc *et al.*, 2001), the encircling chains together create a long, ~ 20 Å diameter, central vestibule (see Karlin, 2002; see Kimura, 1998).

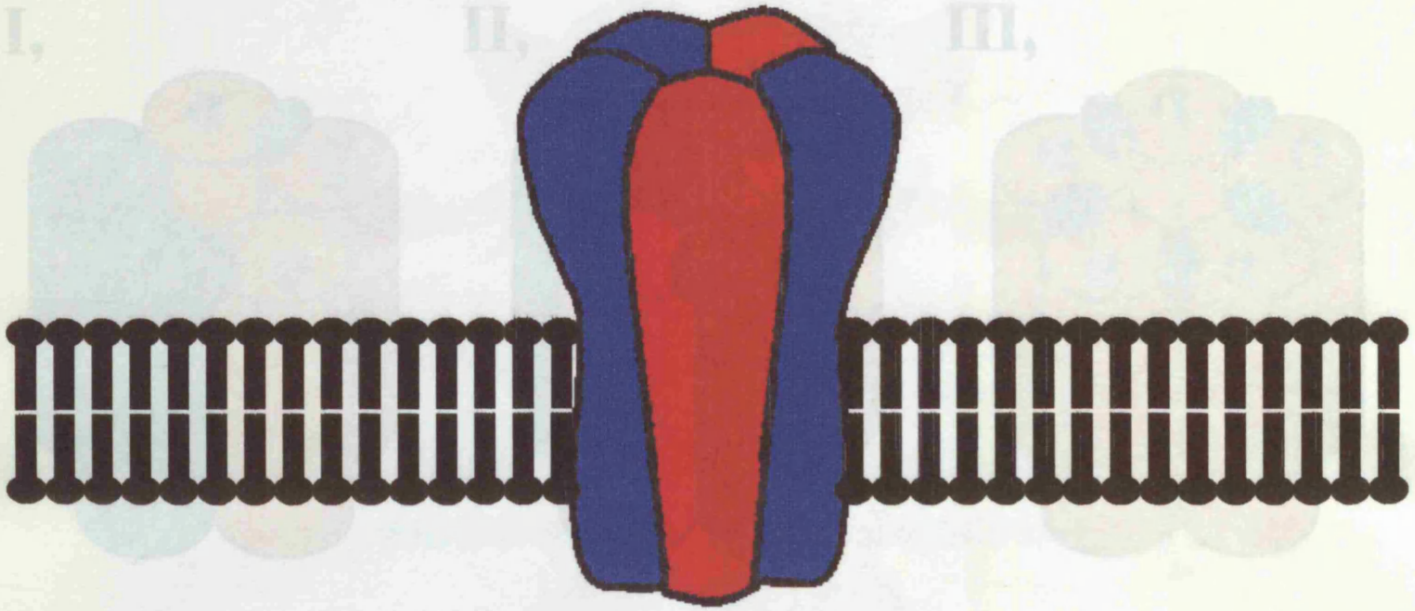


Figure 4A. Diagram representing the generic pentameric structure of nicotinic ACh receptor (nAChR).

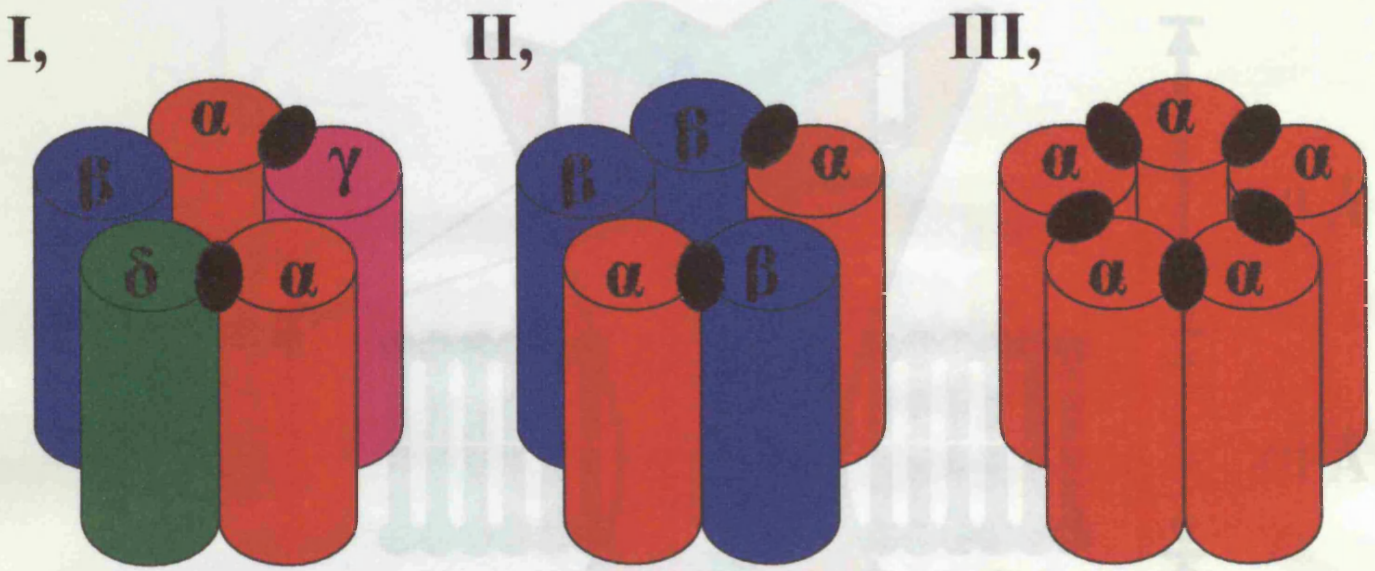


Figure 4B. Diagramatic representation of the quaternary organisation of the muscle-type I, the heteromeric II, and the homomeric, III, neuronal nAChRs. The dark rings indicating the binding site for ACh.

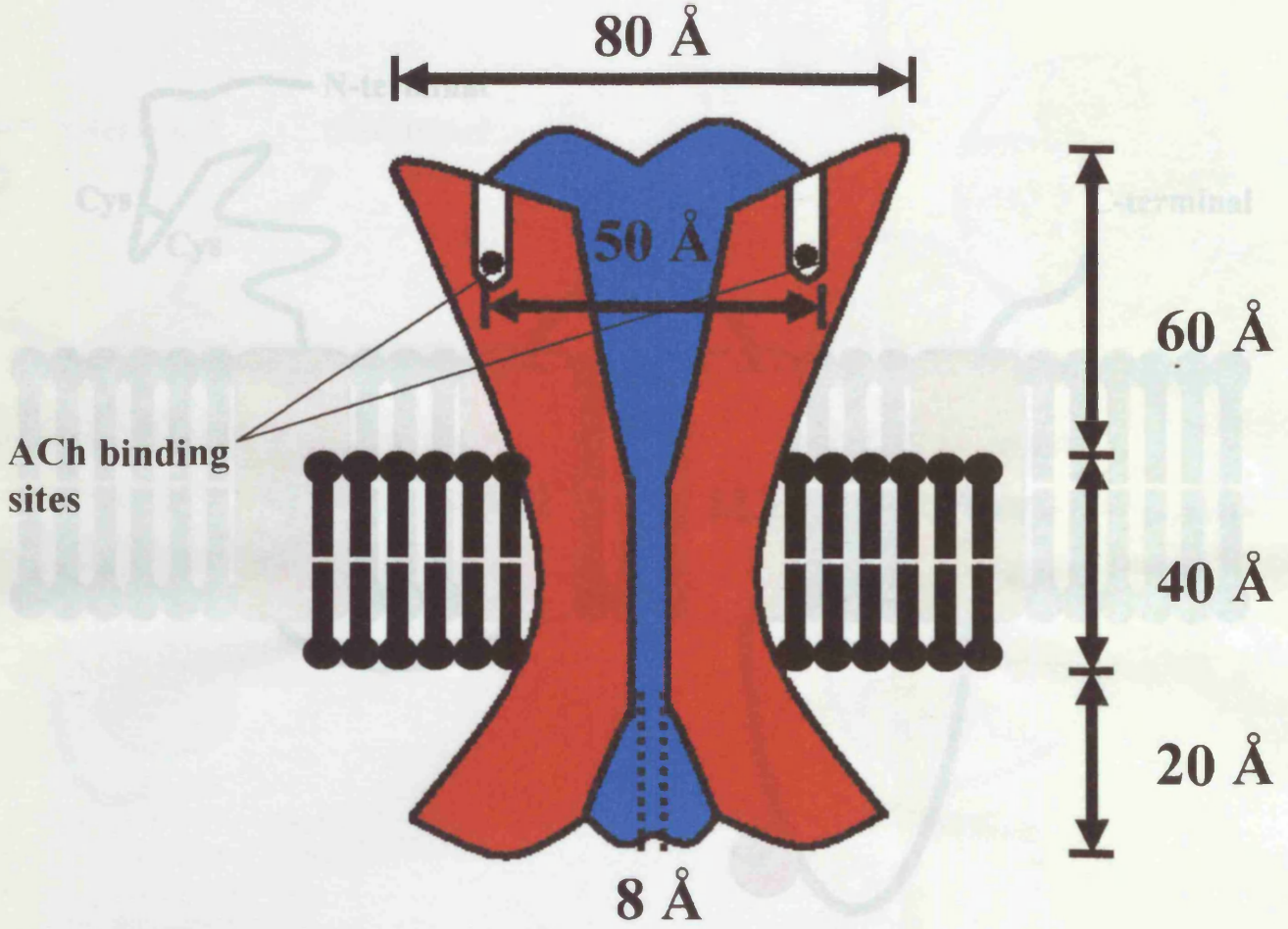


Figure 5. Model of the nicotinic ACh receptor including scale and ACh binding sites

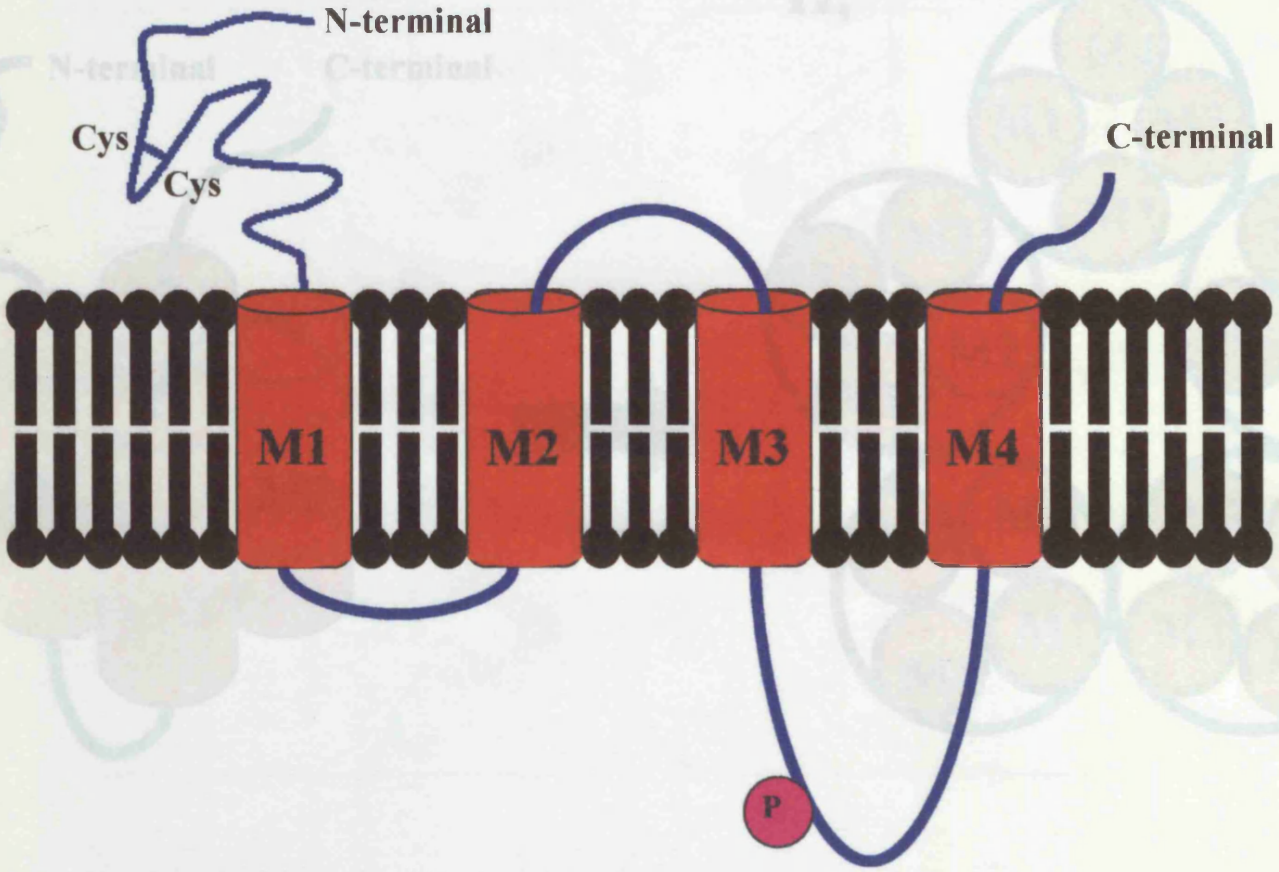


Figure 6A. Diagram of the receptor subunit structure demonstrating the general topology of all nAChRs: the large hydrophobic N- terminal (with cysteine loop), the four hydrophobic transmembrane domains (M1-M4). The intracellular cytoplasmic loop and the short C-terminal.

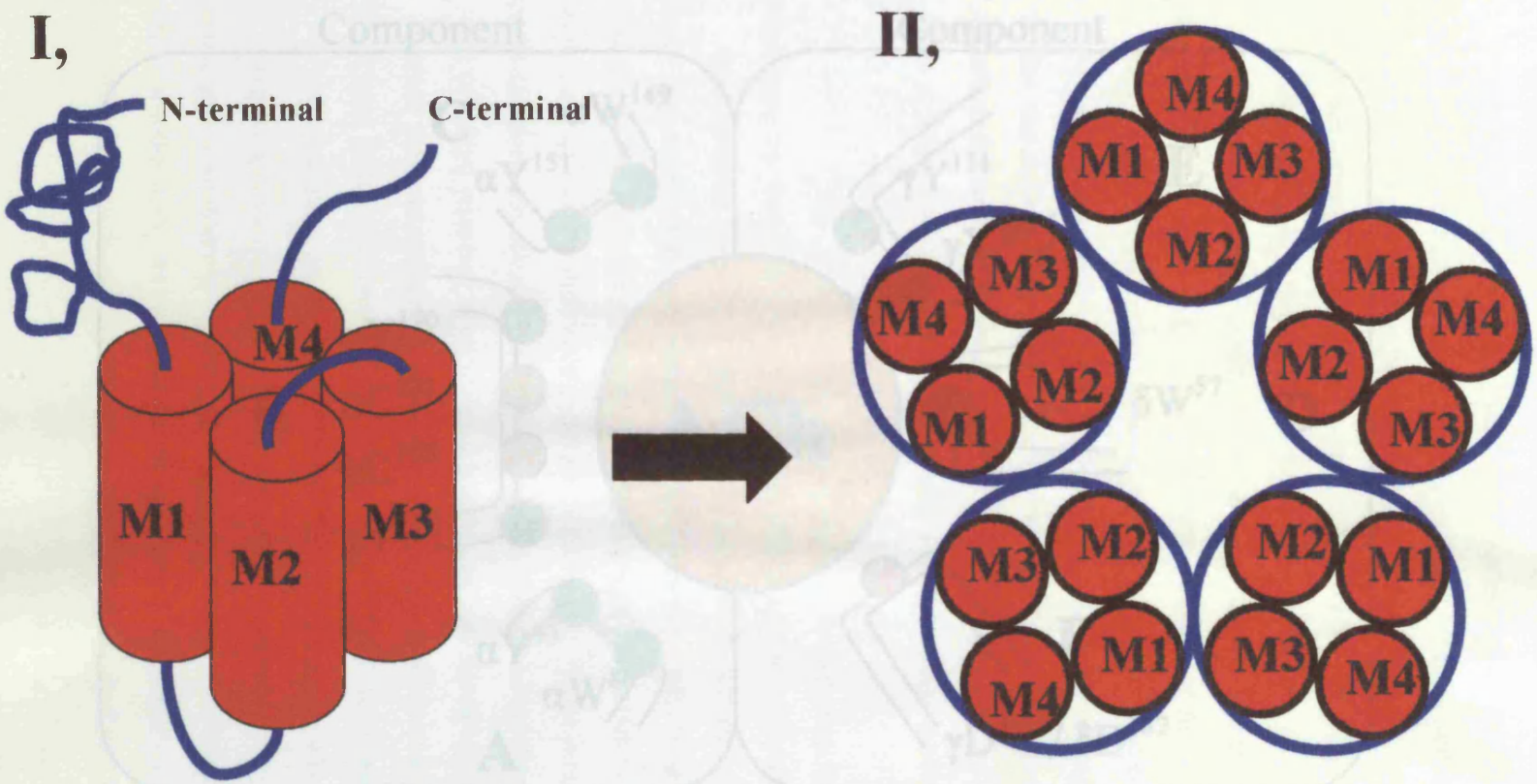


Figure 6B. (I) and (II) indicate the orientation of the four transmembrane domains in forming the nAChR subunits and how the M2 region of each subunit contributes to form the ion pore

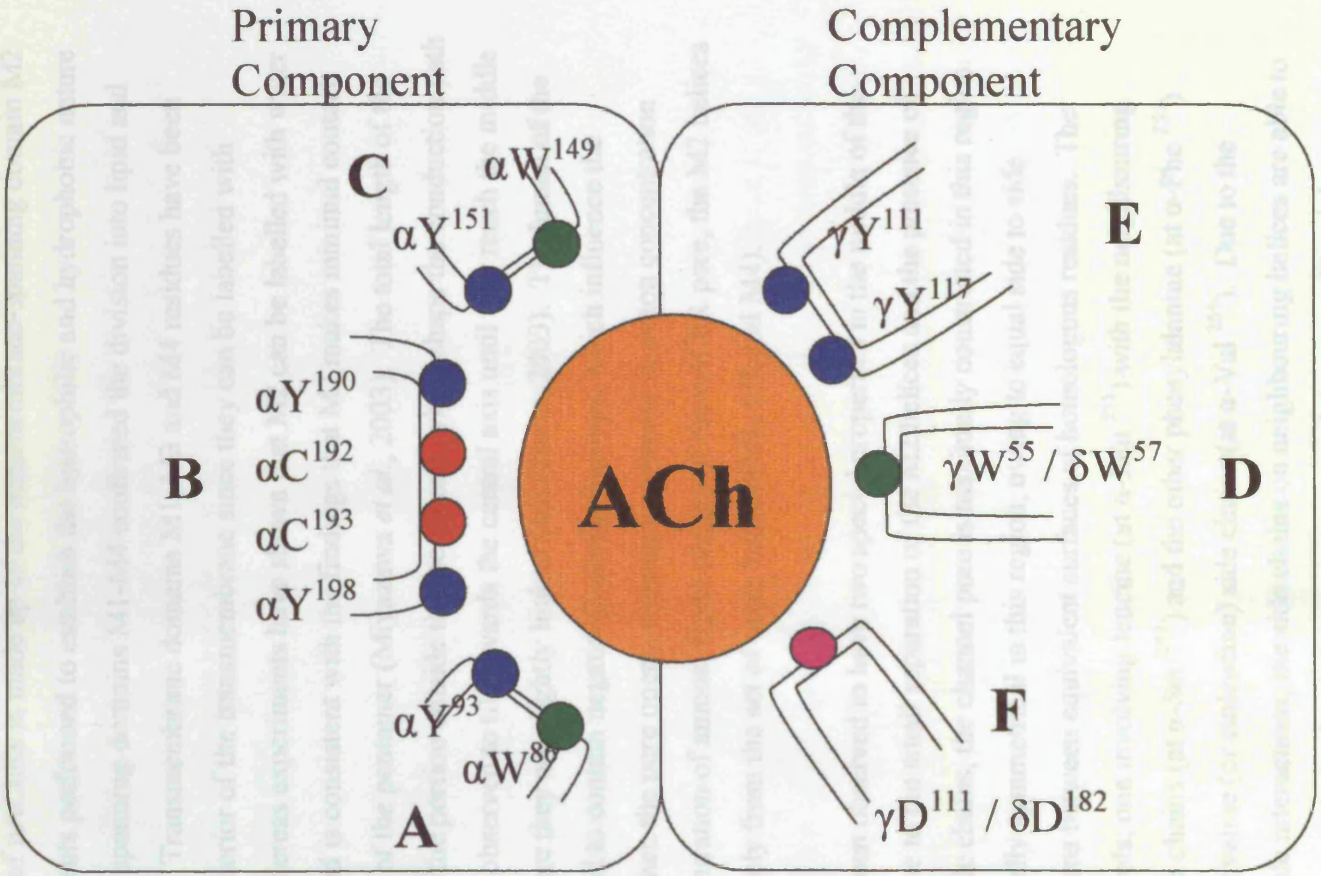


Figure 7. Ligand binding site for agonist on the muscle nAChR.
Residues identified by affinity labelling and site-direct mutagenesis are included in the primary site present on the α subunits and on the complementary site present on δ or γ subunit on muscle receptors or on α/β subunits for neuronal nAChR. Unlike muscle nAChRs, neuronal nAChRs only contain Loop A-E

1.2.3. Channel pore.

The inner pore lining of nAChRs is made up of the transmembrane-spanning domain M2 (Figure 6b). Experiments performed to establish the hydrophilic and hydrophobic nature of the transmembrane spanning domains M1-M4 confirmed the division into lipid and water facing surfaces. Transmembrane domains M1, M3 and M4 residues have been observed facing the interior of the transmembrane since they can be labelled with lipophilic reagents; whereas experiments have shown that M2 can be labelled with water soluble compounds and is consistent with the findings that M2 makes minimal contact with the outer surface of the pentamer (Miyazawa *et al.*, 2003). The total length of the M2 helices, including the portion outside the membrane, which shape the conduction path are 40 Å long and are observed to tilt towards the central axis until they reach the middle of the membrane, where they are slightly kinked (see Unwin, 2003). The lumen of the pore has been observed to contain negatively charged groups, which influence the transport of cations when the pore opens, causing an increase in cation concentration while lowering concentration of anions. Upon closing of the nAChR pore, the M2 helices come together separately from the set of outer helices (M1, M3 and M4).

The closed pore has been observed to have two special properties in the middle of the membrane. Firstly, due to the small separation of the M2 helices and the presence of bulky hydrophobic side chains, the channel pore is maximally constricted in this region. Secondly, it is essentially symmetrical in this region, owing to equal side to side hydrophobic interactions between equivalent surfaces of homologous residues. The contacts are at two levels; one involving leucine (at α-Leu ^{251}) with the neighbouring alanine (or serine) side chains (at α-Ser ^{252}) and the other phenylalanine (at α-Phe ^{256}) with the neighbouring valine (or isoleucine) side chains (at α-Val ^{255}). Due to the symmetrical side to side interactions, the side chains on neighbouring helices are able to come together to make a tight hydrophobic restraint around the pore. The tight hydrophobic restraint around the pore causes it to constrict to a diameter of ~ 6 Å, which is too constricted for a sodium or potassium ion to pass through and therefore the hydrophobic restraint creates an energetic barrier to ion permeation across the lipid

bilayer (Miyazawa *et al.*, 2003). There are no other types of barrier along the conduction path or possible protein occlusion that would block the flow of ions hence the hydrophobic barrier is identified as the gate of the nAChR pore. The involvement of the hydrophobic chains as a pore barrier was firmly established when mutations of the leucine to serine or threonine, in any of the subunits, increased the sensitivity of the channel (Filatov & White, 1995; Labarca *et al.*, 1995; Schaeppi, 1968), indicating that damage to the hydrophobic chains weakens the hydrophobic barrier and increases the relative stability of the open pore.

1.2.4. Opening the channel pore.

By the use of AChBP, it is now possible to obtain a more detailed description of the conformational changes in the nicotinic receptor which allow the pore to open.

The ligand binding domain has been observed to be built around two sets of β-sheets packed into a curled β-sandwich and joined through a disulphide bridge (Brejc *et al.*, 2001). Activation of the nicotinic receptor, by two molecules of ACh, has been shown to cause the pore to widen in the middle of the membrane (Unwin, 1995) which is evoked by a breakdown of the hydrophobic barrier and allows the ions to flow through the channel. This is induced by a very simple mechanism. First, binding of ACh to the ligand binding domain causes an allosteric change in the α-subunit, which brings about the rotation of the inner β-sheets. Second, the rotations of the inner β-sheets are transmitted to the M2 helices via interaction between the β1/β2 loop and the end of M2. The rotational movement of the inner sheets of the α-subunits communicate through this connection and along the pore lining M2 helices to the hydrophobic barrier at the middle of the membrane. Third, the twisting movement weakens the hydrophobic side to side interactions that hold the barrier together, which results in the helices collapsing back against the outer protein wall, making alternative hydrophobic interactions with the channel lining and allows the passage of ions through the channel (Miyazawa *et al.*, 2003;Unwin, 1995; see Unwin, 2003).

1.3. Inhibition of nicotinic receptors.

There are currently three recognised mechanisms by which a compound can inhibit nicotinic receptors; competitive antagonists, non-competitive antagonists and channel blockers, all of which bind to different parts of the nAChR (Figure 8).

1.3.1. Competitive Antagonists.

Competitive antagonists are defined as diverse ligands which compete for the neuronal and neuromuscular binding sites and inhibit channel opening (see Arias, 2000). The most widely used competitive antagonist is the snake venom α-neurotoxin, α-bungarotoxin, which is obtained from the venom of the Taiwanese krait *Bungarus multiunctus*. Other α-neurotoxins come from a family of toxins from the venoms of Elapidae snakes (which include cobras, kraits, mambas and coral snakes) and *Hydrophidae* (also known as sea snakes) (seeAlexander *et al.*, 2004; see Arias, 2000). Observations of the α-neurotoxin structure indicated a similar conformation between all α-neurotoxins. They have been termed the 'three-finger' toxin family due to three adjacent loops or 'fingers' (I, II and III) that emerge from a hydrophobic core, which is cross-linked by four disulphide bridges (Figure 9)(see Tsetlin, 1999). The α-neurotoxin family structure is essentially a flat 'leaf-like' molecule with a slight concavity (see Arias, 2000), containing practically no α-helical segments. Instead its confirmation is determined by a triple-stranded β-structure formed by the two segments of the central loop II and by the fragment of loop III (see Tsetlin, 1999). Interestingly α-bungarotoxin (α-BgT), unlike other α-neurotoxins, exists exclusively as a dimer, both in the crystal form and in solution, whereas, other α-neurotoxins are essentially monomeric.

Studies including electron paramagnetic resonance (EPR), chemical modification and site-directed mutagenesis, have demonstrated that amino acid residues located on the concave side of loops II and III are involved in specific neurotoxin binding to the nAChR. From these studies it was proposed that amino acid residue K^{27}, which is found

on the concave side of loop II (Figure 8 & 9), makes contact with the α subunit through receptor amino acid residue V^{188} – D^{200} (on loop C) at the αδ and/or αγ subunit interface, in the case of neuromuscular nAChRs, or between α/α for neuronal homomeric nAChRs or α/β for neuronal heteromeric nAChRs. Other amino acid residues are also believed to be important in neurotoxin binding, which include R^{33} (Figure 9) (which is located in loop II) which has a strong energetic coupling for receptor amino acid residue αV^{188} (on loop C) and may stabilise the neurotoxin-nAChR complex (see Arias, 2000; Osaka *et al.*, 1999). In a model investigating the interaction of the α-neurotoxins and nAChRs, it was proposed that 18% of the accessible surface of the free toxin is buried in the binding site. Loop II of the toxin appeared to be bound in the binding site cavity between adjacent subunits, with the rest of the toxin extended radially from the outside of the cylindrical pentamer, away from the axis (see Karlin, 2002).

In addition to α-neurotoxins, there is another family of peptide toxins which have been observed to be competitive antagonist for nAChRs. This family are known as conotoxins (Ctx) (Janes, 2003). The toxins come from the venom of marine snails of the genus *Conus*. There are 3 different Ctx families which have been identified and observed to target nAChRs, of these three families α-Ctx is the largest (Dwoskin & Crooks, 2001). α-Ctx can be further divided into structural families depending on the number of amino acids between the 2nd and 3rd cysteine residue and the 3rd and 4th cysteine residue (Millard *et al.*, 2004; Nicke *et al.*, 2004). The α-Ctx subfamilies have four cysteine residues in the following conserved arrangement CCX_3CX_5C (which is termed the $\alpha_{3/5}$ subfamily), CCX_4CX_3C (the $\alpha_{4/3}$ subfamily) and CCX_4CX_7C (the $\alpha_{4/7}$ subfamily), where X represents the indicated number of amino acids between cysteine residues (see Arias & Blanton, 2000; Millard *et al.*, 2004). Studies of α-Ctx subfamilies have indicated that α-Ctx are able to discriminate between muscle and neuronal nAChRs, with $\alpha_{3/5}$ Ctx (α-Ctx MI) found to be selective for muscle nAChRs, while $\alpha_{4/3}$ Ctx (α-Ctx ImI) and $\alpha_{4/7}$ Ctx (α-Ctx MII) are selective for neuronal nAChRs (Dwoskin & Crooks, 2001; McIntosh *et al.*, 1999; Millard *et al.*, 2004; Nicke *et al.*, 2004). Binding sites of α-Ctx to nAChRs, like those of other competitive antagonists, are located at the interface of α and non-α subunits (Dutertre & Lewis, 2004). Three-dimensional visualisation of the binding sites has

indicated that α-Ctx appear to bind mainly to loop C on the α-subunit, but also seem to bind to other parts of the receptor.

Similar to the α-neurotoxin binding to nAChRs, it was observed that specific amino acid residues found on loop I are energetically coupled with their corresponding pairs found on the α subunit, whereas amino acid residues on loop II interact with the non α subunit (see Arias & Blanton, 2000) stabilising the αCtx – nAChR complex, and preventing ACh from binding and opening the nAChR. Also, it is observed that binding of only one toxin molecule is sufficient to block receptor function (Dutertre & Lewis, 2004; Nicke *et al.*, 2004).

A well known antagonist for the muscle nAChR is d-tubocurarine (Chiara & Cohen, 1997; Dwoskin & Crooks, 2001) which is isolated from the plant *Chondodendron tomentosu* and has also been observed to inhibit human neuronal nAChRs in *Xenopus* oocytes. Studies such as photoaffinity labelling indicated that d-tubocurarine causes antagonism of the muscle nAChR by not only binding to the α subunit, but also the γ and δ subunits, suggesting that each ACh binding site is at an interface between the two subunits (Pedersen & Cohen, 1990; Sullivan & Cohen, 2000), which correlates with the results obtained for α-neurotoxins and α-Ctx binding to the muscle and neuronal nAChRs.

1.3.2. Positive and negative allosteric modulators.

Studies into the effects of agonists and competitive antagonists have shown that they bind to the same binding site as the neurotransmitter ACh. Such studies have also indicated the involvement of a variety of compounds which are able to modulate the function of neuronal and neuromuscular nAChRs. These compounds, which include physostigmine, steroids and calcium channel blockers, can activate or block nAChRs, but do not bind to the classical ACh site but to a number of structurally distinct allosteric sites which are in turn insensitive to ACh (Hogg *et al.*, 2005).

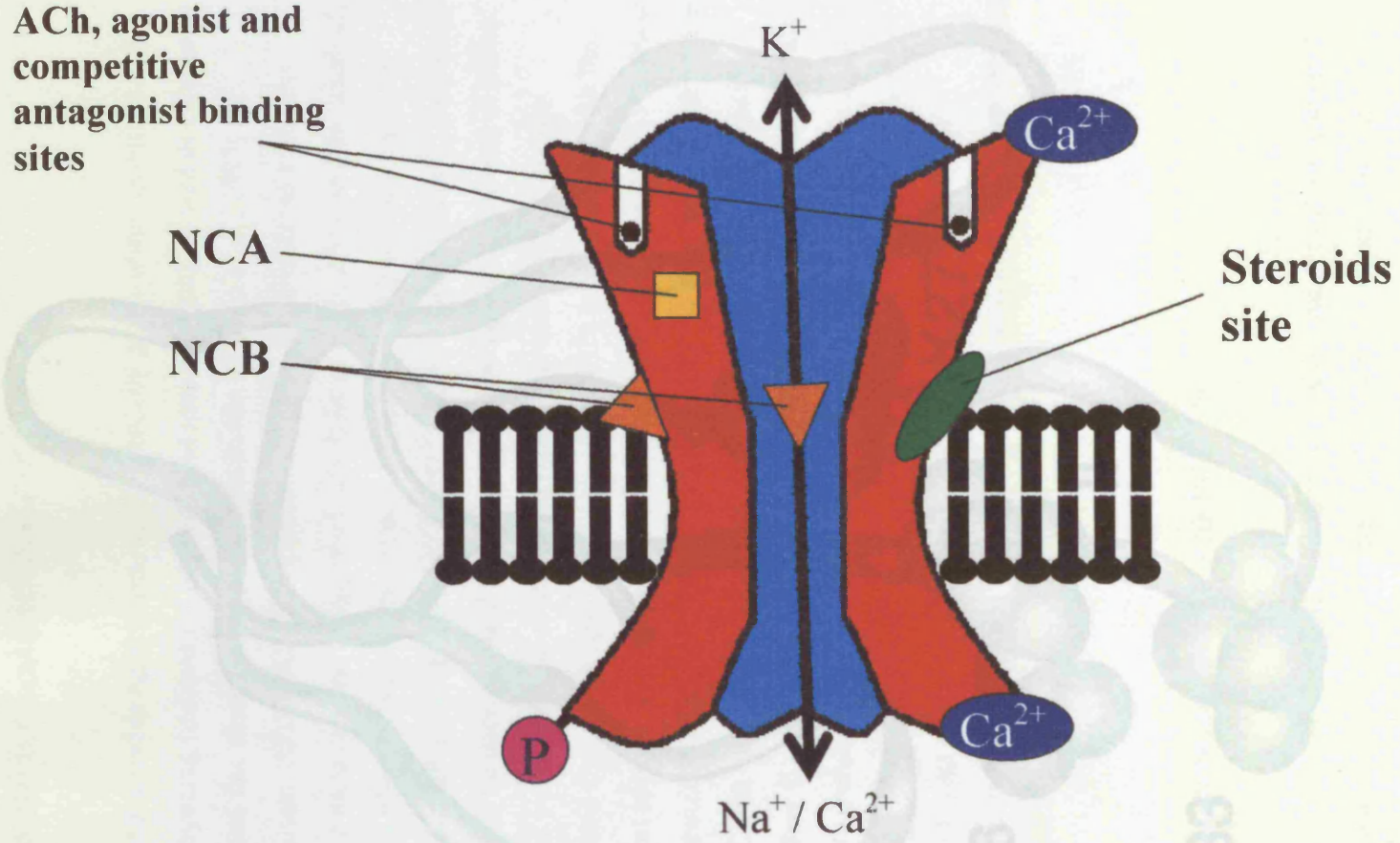


Figure 8. Diagram of the nAChR showing the ion channel, the ACh binding site and different allosteric sites positioned on the nAChR. Allosteric sites include the non-competitive activator site (NCA); non-competitive negative allosteric site (NCB); binding sites for Ca^{2+} and steroid and phosphorylation sites (P)

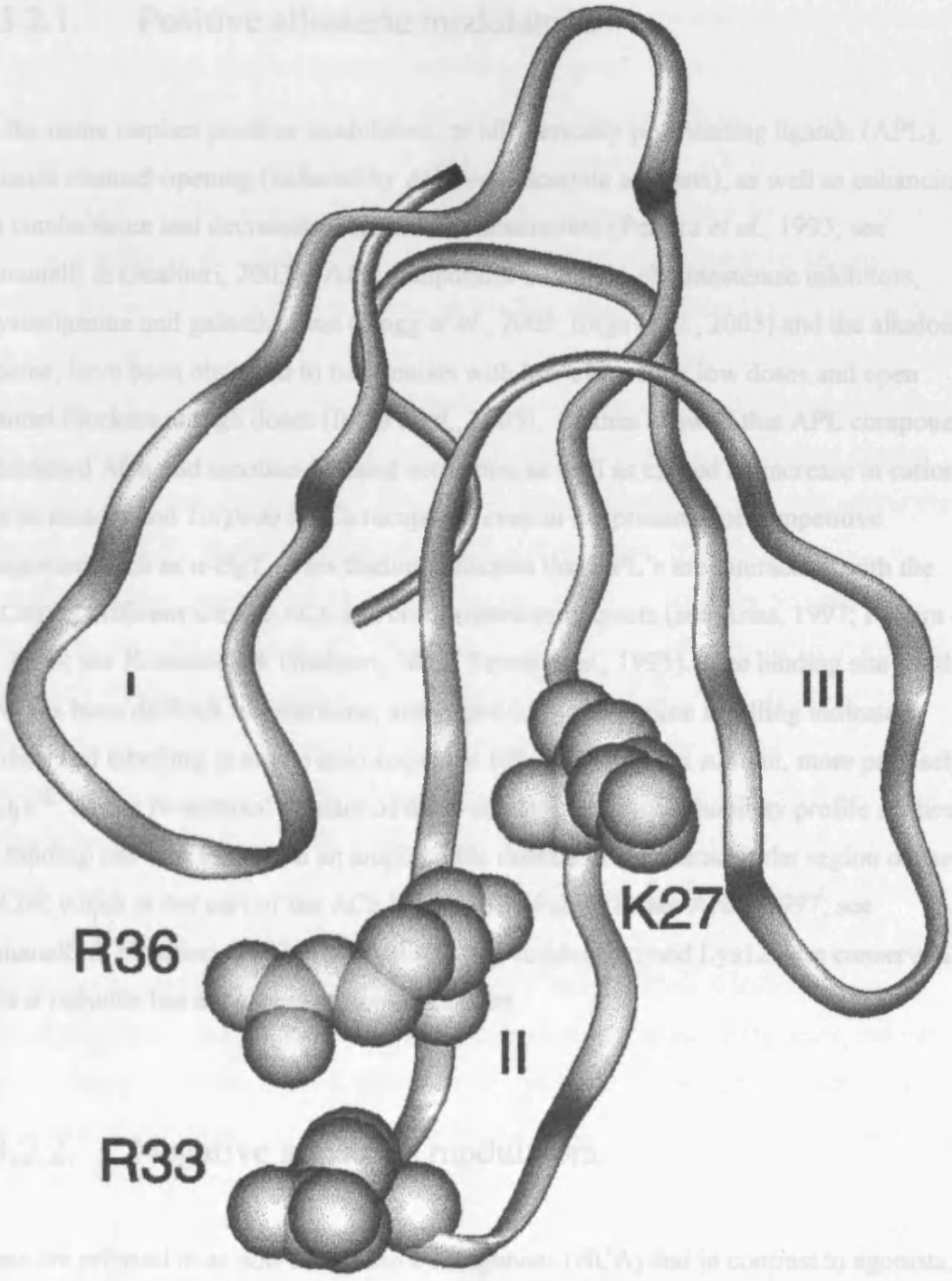


Figure 9. A diagram of the structure of the α-neurotoxin *Naja mossambica mossambica* (NmmI), which is shown with mutated side chains on loop II. The concave face of the toxin is facing the viewer. Taken from Osaka *et al.*, 1999.

1.3.2.1. Positive allosteric modulators.

As the name implies positive modulators, or allosterically potentiating ligands (APL), enhance channel opening (induced by ACh and nicotinic agonists), as well as enhancing ion conductance and decreasing receptor desensitisation (Pereira *et al.*, 1993; see Romanelli & Gualtieri, 2003). APL compounds including cholinesterase inhibitors, physostigmine and galanthamine (Hogg *et al.*, 2005; Iorga *et al.*, 2005) and the alkaloid, codeine, have been observed to be agonists with low efficacy at low doses and open channel blockers at high doses (Iorga *et al.*, 2005). Studies showed that APL compounds potentiated ACh and nicotine-induced activation as well as caused an increase in cation flux in muscle and *Torpedo* nACh receptors, even in the presence of competitive antagonists such as α-BgT. This finding indicates that APL's are interacting with the nAChR at different sites to ACh and competitive antagonists (see Arias, 1997; Pereira *et al.*, 1993; see Romanelli & Gualtieri, 2003; Storch *et al.*, 1995). The binding site for the APL has been difficult to determine, although (-) physostigmine labelling indicated binding and labelling at amino acid sequence 109 – 151 of the α subunit, more precisely at Lys^{125} on the N-terminal domain of the α-subunit. By hydrophobicity profile studies, the binding site was located in an amphipathic domain at the extracellular region of the nAChR which is not part of the ACh binding site(Figure 8)(see Arias, 1997; see Romanelli & Gualtieri, 2003). Interestingly the residues around Lys125 are conserved in most α subunits but are absent in non-α subunits.

1.3.2.2. Negative allosteric modulators.

These are referred to as non-competitive antagonists (NCA) and in contrast to agonists, competitive antagonists and APL compounds, binding of NCAs inhibit ion channel function either by blocking the ion pore by a steric mechanism (in which the compound enters into the open channel lumen, binds and plugs it like a cork in a bottleneck) or by an allosteric mechanism (whereby the NCA binds to a specific site not located in the channel lumen; Figure 7)(see Arias, 1997). NCAs make up a diverse range of compounds

including the neuroleptic chlorpromazine, the hallucinogen phencyclidine (PCP), local anaesthetics, ethanol, barbiturates and nicotinic antagonists hexamethonium and mecamylamine, which exert their blocking action on ion channel without changing maximal agonist binding (Blanton *et al.*, 2000b; Giraudat *et al.*, 1986; see Romanelli & Gualtieri, 2003; Ziebell *et al.*, 2004). In studies trying to determine the binding sites of NCAs it was shown that there are two binding sites at which NCAs have different affinities for, which differ from competitive binding sites. One site binds NCAs with a high affinity and is thus termed 'high affinity NCA sites', whereas, the second site binds NCAs with low affinity and as such is labelled 'low affinity NCA site'. Interestingly, upon binding of one molecule of high affinity another interaction of a second similar ligand does not occur (for example, binding of a local anaesthetic NCA will inhibit the interaction of PCP), this is termed mutually exclusive action. Studies using photolabelling and photocrosslinking, to determine if the high affinity binding site existed, demonstrated that one NCA molecule is able to block the channel activity and is in contact with the five subunits of the nAChR (Giraudat *et al.*, 1986; Ziebell *et al.*, 2004). Analyses of the NCA-nAChR complex indicated that the homologous residues of serine (Ser) from the transmembrane-spanning domain M2 of each subunit were the preferred targets of several NCAs. Labelling with the NCA chlorpromazine showed that Ser residues α^{248}, δ^{262}, β^{254} and γ^{257} were labelled, whereas, the NCA lipophilic cation $TPMP^{+}$ (triphenylmethylphosphonium) labelled Ser residues at position α^{248}, δ^{262} and β^{254} (Giraudat *et al.*, 1986). The serine ring is part of a series of rings which span the ion channel from the extracellular to the intracellular channel portion in the order; valine ring, leucine ring, serine ring and threonine ring, and form an uncharged locus. The importance of this serine ring in NCA binding to the ion channel was demonstrated using site directed mutagenesis, in combination with patch-clamp techniques. Mutagenesis affected the pharmacological activity of QX-222 (which is a local anaesthetic) when a Ser-rich domain was mutated causing the NCA-nAChR bound state to be significantly shorter and affected the binding affinity (see Arias, 1997; Changeux & Edelstein, 2001; Giraudat *et al.*, 1986; Hogg *et al.*, 2005; Osaka *et al.*, 1999). Mutation of the serine ring also affected the binding of other NCA's such as PCP, barbiturates and lipophilic substances such as TID (3-(3-iodophenyl)-3-trifluoromethyl-^{3}H-diazirine) (Blanton *et al.*, 2000b). The importance of the serine ring, with respect to NCA binding, was indicated

when mutation of αSer^{252} (not found on the serine ring) produced no change in channel conductance. Mutations other than on the M2 (e.g. Cyt^{418} on αM4) caused no alteration in PCP non-competitive inhibition (see Arias, 1997). NCA inhibition of ion channels is greatly facilitated by agonist activation of the receptor causing opening of the channel and allowing the NCA to move in and block the ion conductance by simple steric hindrance. The hypothesis that one molecule of NCA binds and blocks the ion channel was proven by computational modelling which showed the NCA fitted into the channel on a parallel axis within a crevice formed by two adjacent nAChR subunits. The low affinity binding sites which bind NCA ligands (>100 μM) are believed to be situated at the interface between the receptor protein and the lipid membrane (Figure 8)(Hogg *et al.*, 2005). Binding of NCA ligands to the multiple sites present on each receptor (10-20 sites) causes acceleration in desensitisation of the receptor-ion channel, resulting in receptor inhibition (Nagata *et al.*, 1996). Studies observing the effect of the NCA, ethanol, on nAChR function indicated that ethanol was able to reduce mean open time of channels in cultured PC12, as well as, inhibiting ACh-induced currents of α7 nAChR in *Xenopus oocytes*, without affecting the affinity of ACh for the receptor (Nagata *et al.*, 1996).

1.4. Neuronal nAChR discovery

The first discovery of neuronal nAChR subunits was recorded by Nef *et al.* (1986) and Boulter *et al.* (1986) who identified the chick α2 subunit and the rat α3 subunit, respectively. This was accomplished by the use of muscle-type nAChR antibodies (Abs), which although specific for muscle nAChRs, were believed to be potentially useful in identifying neuronal nAChR subunits, since both proteins are encoded for by the same homologous genes (see Deneris *et al.*, 1991; see McGehee & Role, 1995; see Sargent, 1993). By the use of monoclonal antibodies (mAb) mAb35, which were raised against nicotinic receptors from the electric organ of the electric ray and the electrical eel, and mAb210, which were raised against nicotinic receptors from bovine muscle, the question of whether neuronal nAChRs exist was examined (Whiting & Lindstrom, 1986). It was

observed that mAb35 bound and purified a neuronal nAChR from the chick brain which was observed to bind with a high affinity to [^{3}H]-nicotine but not [^{125}I]-αBgT (Whiting & Lindstrom, 1987; Whiting *et al.*, 1987b). Interestingly, mAb35 binding indicated two subunits which had a molecular weight (Mr) of 48/49 kDa and 59 kDa.

Since this approach was successful, other mAbs were raised against the chicken brain for immunoprecipitation experiments, including mAb270 and mAb284. mAb270 labelled all the proteins labelled by mAb35, plus some additional proteins which included purifying a subunit with a Mr of 75 kDa, which was also observed to be labelled by mAb284 (Whiting & Lindstrom, 1987; Whiting *et al.*, 1987b). From these studies it was suggested that neuronal nAChRs only have two different subunits, unlike neuromuscular nAChR which have four different subunits (Whiting & Lindstrom, 1986). All of these mAb's bound to nAChRs that were observed to have a high affinity for [^{3}H]-nicotine (Whiting & Lindstrom, 1986), for example, mAb270 depleted over 90% of nicotine binding sites, whilst, mAb35 was observed to remove around 50% of [^{3}H]-nicotine binding sites (Whiting *et al.*, 1987b). These mAb's which were raised against nAChRs in the chicken brain were also able to detect nAChRs in the rat brain. Monoclonal Ab270 purified a receptor containing two subunits with a Mr of 51 kDa and 79 kDa. Originally the subunits were labelled by Whiting as α and β (Whiting *et al.*, 1987a; 1987b; 1991a; Whiting & Lindstrom, 1986; 1987), depending on their increasing molecular weight. These subunits corresponded with the ACh binding subunits, identified by [^{3}H]-MBTA labelling, (Whiting *et al.*, 1987a; 1987b) located on the larger subunits (see Sargent, 1993). Whiting *et al.* (1987) suggested that, by the use of mAb, there are two neuronal nAChRs, found in the chicken brain which have identical α subunits but different β subunits and can be composed of α(n=2-3)β(n=2-3) (Whiting *et al.*, 1987b).

As well as immunoprecipitation studies looking into nAChR subtypes, experiments using recombinant technology to identify and clone genes (cDNA) of the nAChR in the brain were also performed. Using the gene encoding the α subunit in the chicken muscle nAChR, three neuronal nAChR subunits were discovered, which were very similar in structure, in that they all contained adjacent cysteine residues at positions 192 and 193 within the ACh binding site (see McGehee & Role, 1995; Nef *et al.*, 1988; see Sargent,

1993). The three nAChR subunits were termed α2, α3 and α4 which contradicted Whiting's nomenclature as the 75 kDa band produced by mAb270 and mAb284 had an N-terminal amino acid sequence which corresponded to the α4 subunit (Nef *et al.*, 1988; Whiting *et al.*, 1991b). This was also the case with the 49 kDa band produced by mAb270 which was termed an α subunit by Whiting but was shown to correspond with the N-terminal amino acid sequence of the avian β2 subunit identified by cDNA (Nef *et al.*, 1988; see Sargent, 1993; Schoepfer *et al.*, 1988). The classifications of the nAChR were changed from Whiting's original nomenclature to correspond to the cDNA nomenclature, in which subunits containing the cysteine 192/193 pair are referred to as α subunits and those without the cysteine pair where referred to as β subunits.

Using both the immunoprecipitation and the cDNA probe methods, a wide range of neuronal nAChR subunits were discovered in the chicken and rat which range from α2-α7 and β2-β4 (Anand & Lindstrom, 1990; Balestra *et al.*, 2000; Boulter *et al.*, 1987; Couturier *et al.*, 1990b; Keyser *et al.*, 1993; see Lukas *et al.*, 1999; Nef *et al.*, 1988; see Sargent, 1993;Wada *et al.*, 1988;Whiting *et al.*, 1987a; 1991a). Human neuronal nAChR subunit clones have also been produced with subunits ranging from α2-α10 and β2-β4 (Anand & Lindstrom, 1990; Changeux & Edelstein, 1998; Chini *et al.*, 1992; Elliott *et al.*, 1996; Le & Changeux, 1995; see Sargent, 1993). The neuronal nAChR subtypes purified from neuronal tissue had a sedimentation coefficient of 10-11S (Whiting & Lindstrom, 1986; 1987; Whiting *et al.*, 1987b), which corresponds to a molecular weight of approximately 300 MDa, and suggests a pentameric subunit arrangement in comparison with the muscle nAChR (Anand *et al.*, 1991; Cooper *et al.*, 1991; Whiting *et al.*, 1991a).

It has been previously discussed that mAb270 precipitated nAChRs which consisted of α4 and β2 subunits and that mAb270 caused removal of over 90% of [^{3}H]-nicotine binding, which suggested that α4β2 nAChRs may account for a majority of the high affinity [^{3}H]-nicotine binding (Whiting & Lindstrom, 1986). On the other hand, it has been shown that nicotinic antagonist α-BgT binds with high affinity to areas of the brain that are distinct from high [^{3}H]-nicotine affinity areas (Clarke *et al.*, 1985; Perry *et al.*, 1992; Rubboli *et al.*, 1994a; Spurden *et al.*, 1997). These α-BgT binding sites were

shown to be mostly α7 nAChR subunits and are found in many areas of the mammalian CNS (Keyser *et al.*, 1993). This demonstrated that at least two classes of nAChR are expressed in the nervous system; however molecular biology studies of cloned nAChR subunits have indicated that a very large number of nAChR subtypes are theoretically possible.

1.5. Expression of neuronal nAChRs

Studies using *Xenopus* oocytes and human embryonic kidney (HEK) cells have examined the expression of neuronal nAChRs. These studies have indicated that neuronal nAChRs fall into two categories depending on the formation of subunits in the functional receptor. These receptors are termed heteromeric nAChRs, which are made up of two or more different neuronal nAChR subunits, and homomeric nACh receptors, which are made up of five of the same subunit. Possible combinations of nAChR subunits forming functional nicotinic receptors are shown in table 1.

1.5.1. Heteromeric neuronal nAChR

The expression of neuronal nAChRs was observed first in the *Xenopus* oocytes when Wada *et al.* (1988) injected messenger RNA (mRNA) encoding the cDNA for α2, α3, α4 and β2 subunits. It was observed that these subunits injected alone into the cell would not produce a functional ion channel but when α2 and β2 mRNA where added together a response to ACh was observed, which was blocked by the nicotinic antagonist d-tubocurarine (Wada *et al.*, 1988). Additional experiments have also shown that when α2-4 and β2 or β4 are expressed in pairs, the functional receptors which are produced have different pharmacological and physiological profiles; these include α2β2, α2β4, α3β2, α3β4, α4β2 and α4β4 (Anand *et al.*, 1991; Couturier *et al.*, 1990b; Luetje & Patrick, 1991; Nef *et al.*, 1988; Vibat *et al.*, 1995; Wada *et al.*, 1988; Wang *et al.*, 1996). Later studies were done with cell lines such as HEK-293, M10 cells and SH-SY5Y

Nicotinic receptor subtype	Subunits involved	Nicotinic ACh receptor subunit compositions
Muscle-type	α1, β1, γ, δ, ε	α1,β1,γ,δ α1,β1,ε,γ
Neuronal type (α-BgT-insensitive)	α2 – α6, β2 - β4	α2β2 α2β4 α3β2 α3β4 α4β2 α4β4 α6β2 α6β4 α2α5β2 α3α5β2 α3α5β4 α3α6β2 α3α6β4 α3β3β4 α4α5β2 α5α6β2 α6β3β4 α3α5β2β4 α3α6β3β4 α4α5α6β2 α4β2β3β4
Neuronal type (α-BgT-sensitive)	α7 – α10	α7 α8 α9 α7β2 α7β3 α7α8 α9α10 α5α7β2 α5α7β4

Table 1. The composition and assembly of muscle-type and neuronal-type nicotinic ACh receptors. Taken from (see Millar, 2003).

neuroblastoma cells injected with chick, rat and human mRNA coding for neuronal nAChR subtypes α2-4, β2 and β4, which showed stable expression when applied in pairs, especially α4β2 and α3β2, but produced no functional channel when the subunits were injected into the cell alone (Chavez-Noriega *et al.*, 1997; 2000; Gopalakrishnan *et al.*, 1996; Perry *et al.*, 2002; Sabey *et al.*, 1999; Warpman *et al.*, 1998; Whiteaker *et al.*, 1998; Whiting *et al.*, 1991a; Yeh *et al.*, 2001). These observations indicate that this group of subunits can only form heteromeric receptors.

Functional studies on these neuronal nAChRs have indicated that both the α and β subunits contribute to the sensitivity of the neuronal nAChR to agonists and antagonists, channel opening times and pharmacological and functional profiles (de Fiebre *et al.*, 1995; Luetje & Patrick, 1991; Papke *et al.*, 1997; Sabey *et al.*, 1999; Sharples *et al.*, 2000; Warpman *et al.*, 1998; Warpman & Nordberg, 1995). As shown in Tables 2 – 5, when the receptor is comprised of different neuronal nAChR subunits then the EC_{50}, K_i and order of potency of different agonists and antagonists are different (Chavez-Noriega *et al.*, 1997; 2000; see Court JA *et al.*, 1994; de Fiebre *et al.*, 1995; Papke *et al.*, 1997; Sharples *et al.*, 2000; Warpman *et al.*, 1998). For example α2β2 was 5 fold more sensitive to nicotine than to ACh, while α3β2 was 17-fold less sensitive to nicotine than to ACh (Table 2 and 3)(Luetje & Patrick, 1991). Differences in antagonist affinity is also effected by varying composition of the neuronal nAChR, for example, DhβE was observed to have a high affinity for α4β2 and α2β2 but not for α3β4, α2β4 and α7 (Tables 2, 4b and 5b)(Chavez-Noriega *et al.*, 1997; 2000).

Other differences include desensitization rate, upregulation, channel conductance and average channel opening times (Alkondon & Albuquerque, 1993; Gopalakrishnan *et al.*, 1996; see McGehee & Role, 1995; Peng *et al.*, 1994; see Sargent, 1993; Warpman *et al.*, 1998; Whiting *et al.*, 1991a; Zwart *et al.*, 1994). Although it has been demonstrated that α and β subunits both affect the function of the nAChR, it has been suggested that the β subunit has the major influence in the receptor. This was suggested when it was observed that α2-4 bound with β2, had 14-100 fold less sensitivity to cytisine than to ACh, whereas, α2-4 bound to β4 were 3-17 fold more sensitive to cytisine that to ACh (Luetje & Patrick, 1991). It was also observed that α4β2 and α3β2 had very different affinity to

agonist and antagonist when compared to α4β4 and α3β4 (Tables 2–5) (Chavez-Noriega *et al.*, 1997; Luetje & Patrick, 1991; Papke *et al.*, 1997; Stetzer *et al.*, 1996; Wheeler *et al.*, 1993).

Apart from neuronal nAChRs α2-4, β2 and β4, neuronal nAChR subunits α5 and α6 have also been cloned. Unlike the other α subunits that are able to form functional receptors alone, or with a β subunit, α5 and α6 subunits have been shown to be unable to form functional ion channels when they are injected into a cell alone or with any β subunit. However they are able to bind with functional nACh receptors such as α3β2 and α4β4 and form functional receptors such as α3β2α5 and α4β4α6 (Anand *et al.*, 1991; Couturier *et al.*, 1990b; Evans *et al.*, 2003; Wang *et al.*, 1996). In the case of the α5 subunit, when it is bound to α3β2 to form α3β2α5, there is an increase in affinity for nicotine from 50% for α3β2 receptors, to 100% on α3β2α5 receptors (Wang *et al.*, 1996). It was shown that both α3β2 and α3β2α5 receptors when expressed in *Xenopus* oocytes, had a sedimentation size of 11S, which is in accordance with the size reported for the native α3 containing receptors in human neuroblastoma cell lines (Wang *et al.*, 1996). Thus, α3β2α5 is approximately the same size as all nACh receptors and suggests that it is composed of a five subunit nAChR (Chini *et al.*, 1992; Wang *et al.*, 1996). Heteromeric α4β4α6 receptors were observed to be expressed in HEK-293 cells and have a high affinity for epibatidine, which was similar to α4β4. It was also demonstrated that α4β4α6 was sensitive to inhibition by α-conotoxin MII, whereas, α4β4 was insensitive (Evans *et al.*, 2003), indicating differences in the two neuronal nAChRs and providing a useful starting block for determining the pharmacological profile of the wild type α6 containing nAChR.

The most extensively studied heteromeric nAChR is α4β2, which was shown to be the cause of high nicotine binding in the rat and human cell lines and tissue (Table 4 – 5) (Vibat *et al.*, 1995; Warpman & Nordberg, 1995). It was shown that this neuronal nAChR not only has a pentameric structure, but is comprised of two α4 nAChR subunits and three β nAChR subunits (Anand *et al.*, 1991), which is the general nomenclature of heteromeric neuronal nAChRs.

Agonists	Human EC_{50} (μM)				Rat EC_{50} (μM)				Chick EC_{50} (μM)
	α4β2	α3β2	α3β4	α7	α4β2	α3β2	α3β4	α7	α4β2
Nicotine	3.5 - 5.5	6.8 - 7.7	80.3	113.3	0.3 - 14	28		47	0.35 - 0.84
ACh	44 - 100	28 - 443	203.1	180 -322	3 - 270	30 - 65	207	274 - 316	
Cytisine	2 - 38.2	67.1 - 72	0.9	71.4					0.031 – 1.23
DMPP	2.5 - 18	6.7 - 55.9	18.67	30.9					0.073
Epibatidine	0.017 - 0.043	0.19			0.016	0.1	0.1	1	
ABT-418	10.6				6	119	188	155	
TC-2559	0.18	>100	>30	>100					
GTS-21				109				81	

Table 2. Showing the functional potency of nicotinic agonists for neuronal nAChR in human and rat transfected cells.

Neuronal nACh receptor	[^{3}H] – nicotine		[^{3}H] – epibatidine		[^{3}H] – cytisine		[^{125}I] – αBgT		Order of potency
	Kd (nM)	Bmax	Kd (nM)	Bmax	Kd (nM)	Bmax	Kd (nM)	Bmax	
α4β2	[0.4 ^]	[544 ^]	0.033 ^	1564 ^	0.21 ^ [0.12 ^]	1359 ^ [383 ^]			TC-2559 ≈ Epib > Cyt ≈ DMPP ≈ Nic > ABT-418 > ACh (1) Epib > Cyt ≈ Nic ≈ DMPP > ACh (2)
α3β2	5.4 *		0.12 * 0.15 ~ 0.007 ^	1564 ^					Epib > DMPP ≈ Nic > Cyt > ACh (1) DMPP ≈ Cyt ≈ Nic > ACh (3)
α3β4			4.9 *						Epib > Cyt ≈ DMPP > Nic > ACh (1) DMPP > Cyt ≈ Nic > ACh (3)
α7							0.71 ^ [0.48 °]	973 ^ [3.42 °]	DMPP > Cyt > GTS-21 ≈ Nic > ACh (1) DMPP > Cyt ≈ Nic ≈ ACh (3)

Table 3. Showing the binding of nicotinic agonists for neuronal nAChR in human and rat transfected cells, including the order of potency of agonists for neuronal nAChRs.

Bmax values as fmol/mg of protein
* = expressed in *Xenopus* oocytes
^ = expressed in HEK-293 cells
~ = expressed in SH-SY5Y
° = expressed in GH_4C_1 cells
[ ] = Rat nAChRs observed
(1) = Taken from EC50 in Table 2.
(2) = Taken from (Chavez-Noriega *et al.*, 2000)
(3) = Taken from (Chavez-Noriega *et al.*, 1997)
Abbreviations: Epib – Epibatidine, Nic – Nicotine, Cyt – Cytisine, ACh – Acetylcholine

Agonists	Human Ki (nM)					Rat Ki (nM)		Chick Ki (nM)	
	α4β2	α3	α3β2	α3β4	α7	α4β2	α7	α4β2	α7
Nicotine	1.05 [e*]				1610 [b*]	0.43 [e*] 4.1 [f#]	400 [b#] 820-900 [b^]	3.9 [d∞] 7.7 [d∞]	
ACh	2.66 [e*]	19.3 [a~]	35.4 [a~]		9910 [b*]		6200-8900 [b^]		
Cytisine	0.43 [e*]	16.5 [a~]	13.1 [a~]	270 [d*]	3883 [b*]	3.3 [e∞] 1.7 [d*]	1800-2300 [b^]	0.14 [a∞] 3.3 [d∞]	
DMPP	10.71 [e*]	14.7 [a~]	16.3 [a~]				160-670 [b^]	9.4 [d∞]	
Epibatidine	0.07 [e*]		0.035 [d*]	0.59 [d*]	20.6 [b*]	0.061 [d*]		0.018 [d∞]	
(+) Anatoxin-a	0.55 [e*]				63.3 [b*]			4.2 [d∞]	
ABT-418	7.89 [e*]							17 [d∞]	
UB-165								0.44 [d∞]	
Carbachol	68 [e*]				21500 [b*]		9300 – 13000 [b^]	360 [a∞]	
(-) Lobeline	1.92 [e*]				13100 [b*]				

Table 4a. Showing the binding affinity of nicotinic agonists for neuronal nAChR in human, rat and chick transfected cells.

Antagonists	Human Ki (nM)		Rat Ki (nM)		Chick Ki (nM)	
	α4β2	α7	α4β2	α7	α4β2	α7
DhβE	60.1 [e*]	57900 [b*]	22 [e*]		270 [d∞]	
α-BgT	4729 [e*]	0.48 [b*]		0.23-0.95 [b^]		
MLA	3205 [e*]	10.3 [b*]		3.1-3.9 [b^]	1600 [d∞]	
Mec	>10000 [e*]	>100000 [b*]			>1000 [d∞]	

Table 4b. Showing the binding affinity of antagonists for human, rat and chick transfected cells.

[a] = [^{3}H] – nicotine
[b] = [^{125}I] – αBgT
[c] = [^{3}H] – MLA
[d] = [^{3}H] – epibatidine
[e] = [^{3}H] – cytisine
f = [^{3}H] - MCC

* = HEK-293 cells
# = *Xenopus* oocytes expression
∞ = M10 cells
^ = GH_4C_1 cells
~ = SH-SY5Y cells

Abbreviations: ACh – Acetylcholine, DMPP – Dimethylphenylpiperazinium , DhβE – Dihydro-β-ethroydine, α-BgT – α-Bungarotoxin, MLA – Methyllcaconitine, Mec – Mecamylamine.

Agonists	Human Ki (nM)		Rat Ki (nM)		Chick Ki (nM)	
	α4β2	α7	α4β2	α7	α4β2	α7
Nicotine	8-16 [a]	1700 [b]	0.6–15 [a] 3–10 [d] 0.6-3.5 [e]	400-14000 [b] 450-6100 [c]	2.4-6.7 [a] 38 [d]	
ACh			14.5 [e]	4000-7600 [b]	57 [d]	
Cytisine	0.8-1.6 [a]		0.56 [d] 0.12-1.8 [a] 0.16-0.46 [e]	1400-2800 [b]	0.14-1.2 [a] 2.7 [d]	
DMPP	6500 [a]		8.7-40 [a] 8.2 [e]	250 [b]	40 [d]	
Epibatidine			0.02-0.06 [d] 0.04 [e] 0.021 [a]	230-233 [b] 170 [c]	0.046 [d]	
+ Anatoxin-a			1.25 a 1.27 d	1840-2040 [b] 707 [c]		
ABT-418			46 [a]			
UB-165			0.27 [a]	2760-2790 [b]		
TC-2559			5 [a]			

Table 5a. Showing the binding affinity of agonists for human, rat and chick tissue.

[a] = [^{3}H] - nicotine
[b] = [^{125}I] - αBgT
[c] = [^{3}H] - MLA
[d] = [^{3}H] - epibatidine
[e] = [^{3}H] - cytisine

Abbreviations: ACh – Acetylcholine, DMPP – Dimethylphenylpiperazinium

Antagonists	Human Ki (nM)		Rat Ki (nM)		Chick Ki (nM)	
	α4β2	α7	α4β2	α7	α4β2	α7
DhβE	1900 [a]		13-13.9 [a] 14.5-15.5 [d] 14.8 [e]	2500 [b]		
α-BgT			>10,000 [a]	0.35-1.1 [b] 1.78-7 [c]		0.73 [b]
MLA			1560-3700[a]	1-7.9 [b] 0.98-2.3 [c]	3700 [a]	5.4 [b]
Mec				>20,000 [c]		

Table 5b. Showing the binding affinity of antagonists for human, rat and chick tissue.

[a] = [^{3}H] - nicotine
[b] = [^{125}I] - αBgT
[c] = [^{3}H] - MLA
[d] = [^{3}H] - epibatidine
[e] = [^{3}H] - cytisine

Abbreviations: DhβE – Dihydro-β-ethroydine, α-BgT – α-Bungarotoxin, MLA – Methyllcaconitine, Mec - Mecamylamine

Observations from expression studies suggested that heteromeric receptors have an important role in calcium signalling. Firstly, it has been seen in HEK-293 cells injected with rat α3, β4, α4 and β2 mRNA, that heteromeric receptors are not only permeable to cations such as Na^+, but also to Ca^{2+} (Sabey *et al.*, 1999; Stetzer *et al.*, 1996). Secondly, extracellular calcium affects the heteromeric receptors which was demonstrated when increases in extracellular calcium increased α4β2 nAChR's response to nicotine, whereas, a decrease in extracellular calcium decreased nicotine's response (Sabey *et al.*, 1999).

1.5.2. Homomeric receptors

Contrary to heteromeric receptors, which form functioning neuronal nicotinic AChRs by two or more different subunits, homomeric receptors are composed of a single type of subunit which form a functional pentameric structure and can be composed of either α7, α8 or α9 neuronal nAChR subunits (Couturier *et al.*, 1990a; Gerzanich *et al.*, 1994; Seguela *et al.*, 1993). Although, observed to be similar in size to the heterologous nAChRs, the homomeric neuronal nAChR's have different pharmacological and physiological profiles (Table 2 - 5). Chick and rat homomeric neuronal nAChRs have been expressed in *Xenopus* oocytes and it has been shown that α7 is sensitive to nicotine, with an order of potency of nicotine > cytisine > DMPP > ACh for the rat and chick neuronal nAChR. In contrast the order of potency for human α7 neuronal nAChR in *Xenopus* oocytes was observed to be DMPP > cytisine > nicotine > ACh (Table 2 and 4) (Chavez-Noriega *et al.*, 1997; Gerzanich *et al.*, 1994; Seguela *et al.*, 1993). The order of potency observed for chick α8 neuronal nAChR's expressed in *Xenopus* oocytes was shown to be nicotine ≈ cytisine > ACh > DMPP, indicating similarities between α7 and α8 neuronal nAChRs. However, there are also differences, such as α8 has a higher sensitivity to agonists than α7. In addition, it has been shown that there are also similarities between α7 and α8 homomeric receptors expressed in *Xenopus* oocytes compared with the native α7 and α8 neuronal nAChRs (Gerzanich *et al.*, 1994), for example, radioligand binding of α7 nAChR in HEK-293 cells noted the agonists affinity for α7 was nicotine > cytisine > ACh, which is observed to be very similar to α7

expressed in *Xenopus* oocytes (Table 4) (Gopalakrishnan *et al.*, 1995; Zhao *et al.*, 2003). Rat pituitary clonal cell lines, GH4Cl, expressed α7 nAChR stably and had pharmacological and functional properties similar to α-BgT nAChRs in rat brain (Quik *et al.*, 1996). The homomeric receptors, especially α7, are observed to be blocked by nanomolar concentrations of α-BgT. Therefore the high affinity of α7 nAChRs for α-BgT suggested that α7 subunits may play a major role in determining the properties of the rat brain α-BgT binding studies (Tables 2 – 5) (Gerzanich *et al.*, 1994; Gopalakrishnan *et al.*, 1995; Quik *et al.*, 1996; see Sargent, 1993; Seguela *et al.*, 1993; Zhao *et al.*, 2003).

Interestingly, similar to heteromeric neuronal nAChRs, homomeric nAChRs have been shown to not only be permeable to cations such as Na^+ but also to Ca^{2+} (Gerzanich *et al.*, 1994; Gopalakrishnan *et al.*, 1995; Seguela *et al.*, 1993). Calcium ions have also been shown to have an effect on α7 and α8 nAChRs, whereby it was observed that extracellular Ca^{2+} positively regulates α7 and α8 nAChR channel opening, since human α7 nAChR responses to nicotine were suppressed when there was a reduction in external Ca^{2+} (Gopalakrishnan *et al.*, 1995; Zhao *et al.*, 2003). Although it has been stated that homomeric subunits can not co-assemble with any other subunit, it is now believed that in some cases co-assembly occurs. This arose from the suggestion that α7 nAChRs, found in rat hippocampal interneurons, were shown to have properties unlike those seen in homomeric α7 nAChRs, for example, these native α7 containing receptors desensitize more slowly and have a smaller single channel conductance. It was shown that α7 and β2 subunits can co-assemble and form functional heteromeric nAChRs with functional and pharmacological properties different from those of homomeric α7 channels (Khiroug *et al.*, 2002).

1.6. Distribution of neuronal nAChR

1.6.1. Autoradiography techniques

By the use of methods which included radioligand binding, *in situ* hybridisation and immunohistochemistry, using subunit specific antibodies, the locations of neuronal nAChRs were determined. The first method used for isolating nAChRs was radioligand binding in autopsy tissue homogenates, or for greater anatomical detail, autoradiography of tissue sections (Wada *et al.*, 1989). The first compounds used in the autoradiography technique were [^{3}H]-nicotine, as this has been demonstrated to bind with high affinity to cell lines expressing neuronal nAChRs (Table 4 & 5), and [^{125}I]-α-bungarotoxin ([^{125}I]-αBgT) which was shown to block nicotinic cholinergic transmission and bind to rat brain membranes with high affinity (Table 4 & 5) (Clarke *et al.*, 1985; Hunt & Schmidt, 1978; Morley *et al.*, 1977; Segal *et al.*, 1978). As well as these nicotinic compounds, [^{3}H]-ACh (in the presence of atropine) was also used (Table 6 & 7) (Adem *et al.*, 1987; Perry *et al.*, 1992). Interestingly, it was observed that there were two distinct patterns of distribution for the [^{3}H]-nicotine and [^{125}I]-αBgT binding, with little overlapping, whereas [^{3}H]-nicotine and [^{3}H]-ACh binding were almost identical (Clarke *et al.*, 1985), indicating that [^{3}H]-nicotine and [^{3}H]-ACh probably labelled the same neuronal nAChRs (Table 6 & 7).

In the rat brain, [^{3}H]-nicotine binding was observed in high density in the thalamus as well as the hippocampus and cerebral cortex (Clarke *et al.*, 1984; 1985; Hunt & Schmidt, 1978; Morley *et al.*, 1977; Oswald & Freeman, 1981). These high density binding sites in the rat brain correlate with autoradiography studies in the human brain, which showed high [^{3}H]-nicotine binding sites in the subiculum, presubiculum and entorhinal cortex areas of the hippocampus (see Court JA *et al.*, 2000; Perry *et al.*, 1992; Rubboli *et al.*, 1994a) and within the thalamus, in areas such as the lateral dorsal, median and lateral geniculate and anterior nuclei (Rubboli *et al.*, 1994a; Spurden *et al.*, 1997). Other areas of high [^{3}H]-nicotine binding in the human brain were observed in the basal ganglia

which included the putamen and caudate (Table 6 & 7) (Adem *et al.*, 1989; see Court JA *et al.*, 2000; Rubboli *et al.*, 1994a).

Studies looking into [^{125}I]-αBgT binding in the human brain showed that the areas high in nicotine binding were observed to have low [^{125}I]-αBgT binding especially in the basal ganglia, hippocampus areas and an overall low binding in the thalamus (Clarke *et al.*, 1984; see Court JA *et al.*, 2000; Rubboli *et al.*, 1994a; Spurden *et al.*, 1997) apart from in the reticular thalamic nuclei which showed very high [^{125}I]-αBgT binding (Breese *et al.*, 1997; Spurden *et al.*, 1997). High density binding of radioligand [^{125}I]-αBgT in the human brain was observed in the hypothalamus in areas such as the paraventricular nucleus (PVN), supraoptic nucleus (SON) and mammillary bodies (Table 6). Similar binding was also shown by autoradiography performed on the rat brain (Breese *et al.*, 1997; Clarke *et al.*, 1985; Hunt & Schmidt, 1978).

Overall, autoradiography studies using the two radioligand compounds [^{3}H]-nicotine and [^{125}I]-αBgT indicated that there are at least two different neuronal nACh receptors present in the human and rodent brain. These receptors were referred to as α-BgT insensitive nAChRs, which were observed to be selective for the nicotine agonist but not the α-BgT antagonist, and α-BgT sensitive nAChRs which has a low selectivity for nicotine but were selective for α-BgT. Although this categorised the nAChRs and indicated where they are located in the brain, information regarding nACh receptor composition was still unclear, which was due to unavailable subtype selective ligands.

As a result of extensive research into nAChR composition, function and binding affinity, agonists were discovered that are selective to certain nAChR subtypes, for example, [^{3}H]-cytisine was shown to label α3, α4 and β4 subunits, whilst [^{3}H]-epibatidine labelled α3, α4 and α7 nAChR subunits (Marutle *et al.*, 1998; Rubboli *et al.*, 1994a; Rubboli *et al.*, 1994b; Spurden *et al.*, 1997). By the use of partially selective ligands, it was observed by autoradiography that there is an overall high binding of cytisine and epibatidine in the thalamus, with moderate binding in the basal ganglia (Marutle *et al.*, 1998; Rubboli *et al.*, 1994a; Sihver *et al.*, 1998) and varied results with the binding in the cerebral cortex (Table 7) (Sihver *et al.*, 1998; 1999).

Brain Region	[^{125}I]-αBgT binding	[^{3}H]- Nicotine binding
Cerebral Cortex		
- Frontal Cortex	+	++
- Occipital Cortex	± / +	+ / +++
- Parietal Cortex	±	++
- Temporal Cortex	+	+ / ++
- Cingulate Cortex	+ / +++	+++
- Insular Cortex	± / +	+ / ++
- Neocortex		+ / ++
Limbic Areas		
-Hippocampus		
- Overall		+ / ++
- Pyramidal layer CA1	+ / ++	++
- CA2 & CA3	+ / ++	± / +
- Mol layer of dentate gyrus	+	
- Granular layer of dentate	+ / ++	±
- Presubiculum	-	+++
- Subiculum	+	+++
- Entorhinal Cortex		+++
- Dentate gyrus	++	++ / +++
- Amygdala	++	
Basal Ganglia		
- Putamen	- / +	++ / +++
- Caudate	- / +	++ / +++
- Claustrum		++
Thalamus		
- Overall	- / +	+++
- Midline n	+	+++
- Anterior n	±	++
- Dorsal medial n	±	
- Ventral lateral n	±	++
- Reticular n.	++	+ / ++

Hypothalamus		
- PVN.	++ / +++	
- SON.	+++	
- Mammillary bodies	++	
- Substantia nigra		
- pars compacta	- / +	
Midbrain		
- Superior colliculus	+++	
- Inferior colliculus	+++	
- Raphe	+++	+++
- Reticular formation	++	
Cerebellum		
- Overall	- / ±	
- Molecular layer		++
- Granular layer		+++
- Deep cerebellum n.	++	
Hindbrain & Medulla		
- Medulla overall	+	
- Inferior olive complex	+	
- Parabrachial n.	+++	

Table 6. Comparing the expression of [^{3}H]-nicotine and [^{125}I]-α-Bungarotoxin radioligand binding in the human brain.

Symbols indicate:

- = Not Detected
± = Very Weak Signal
\+ = Weak Signal
++ = Moderate Signal
+++ = High Signal

Brain Region	[^{3}H]- Nicotine binding	[^{3}H]- ACh binding	[^{3}H]- Epib binding	[^{3}H]- Cyt binding
Cerebral Cortex				
- Frontal Cortex	++		+ / +++	++
- Occipital Cortex	+ / +++		+ / +++	+ / ++
- Parietal Cortex	++		++	++
- Temporal Cortex	+ / ++		+ / +++	+
- Cingulate Cortex	+++	++		+
- Insular Cortex	+ / ++			+
- Neocortex				
- All layers	+ / ++			
Limbic Areas				
- Hippocampus				
- Overall	+ / ++	+	+ / ++	
- Pyramidal layer CA1	++			
- CA2 & CA3	± / +			±
- Dentate granular layer	±			±
- Presubiculum	+++			++
- Subiculum	+++			++
- Entorhinal Cortex	+++			++
- Dentate gyrus	++ / +++			
Basal Ganglia				
- Globus Pallidus		+		
- Putamen	++ / +++	++	++	++
- Caudate	++ / +++	++	++ / +++	+ / ++
- Claustrum	++			
Thalamus				
- Overall	+++	+++	+++	++
- Pulvinar n.				+
- Lateral dorsal	+++			
- Medial geniculate	+++			
- Lateral geniculate	+++			
- Anterior n	++			
- Lateral posterior n.	+++			++
- Ventral anterior n.	++			
- Ventral lateral n.	++			
- Ventroposteromedial n.	++			
- Ventroposterolateral n.	+ / ++			++

- Reticulum n. - Centromedial n. - Dorsomedial n. - Reticular hotspots - Subthalamic n.	+ / ++ + / ++ - +++			+ ++ +++
Hypothalamus - Overall		++		
Substantia nigra - Overall - Pars compacta - Pars reticulate	 +++ ++ / +++	++		
Midbrain - Raphe n. - Periaqueductal grey matter - Locus coeruleus	 +++ ++ +++			
Cerebellum - Overall - Molecular layer - Granular layer	 ++ +++		++	
Striatum	+++			
White matter	-			

Table 7. Comparing the expression of [^{3}H]-nicotine, [^{3}H]-ACh, [^{3}H]-epibatidine (Epib) and [^{3}H]-cytisine (Cyt) radioligand binding in the human brain.

Symbols indicate:

- = Not Detected
± = Very Weak Signal
+ = Weak Signal
++ = Moderate Signal
+++ = High Signal

It was demonstrated that there was similar distribution of [^{3}H]-nicotine, [^{3}H]-cytisine and [^{3}H]-epibatidine in the cerebral cortex, indicating that they are all binding to a similar receptor; however, the levels of binding were shown to be different. Similar binding of all three was observed in the parietal cortex, with nicotine and epibatidine having high binding sites in laminar layers I, III and IV, although nicotine was also observed to have high binding in laminar VI, unlike epibatidine. Cytisine had moderate binding throughout laminar I to VI. High binding in the frontal cortex was indicated with nicotine and epibatidine compared with moderate to low binding with cytisine. In laminar III of the frontal cortex both nicotine and epibatidine were shown to have high binding and similarities in binding density at all other layers (Table 7). This similarity in site and binding density indicate they are binding to the same receptor, most likely the α4 nAChR. However, differences in binding densities in certain areas of the cerebral cortex, indicate that there may also be another high affinity nACh receptor involved, most possibly the α3 nAChR (Sihver *et al.*, 1998; 1999).

Overall, it was demonstrated that at least two classes of neuronal nAChR are present in the human brain which are distinguishable by their affinities for [^{3}H]-nicotine, [^{3}H]-ACh, [^{3}H]-epibatidine and [^{125}I]-αBgT.

1.6.2. Neuronal nAChR subunit distribution

Although autoradiography is accurate it is unable to specifically state what nAChR subtypes are present in the human brain. By the use of molecular genetics, the primary structure of nAChR subunits expressed in the brain which combine to form nAChRs were discovered and it was noted that different genes make up the individual subunits making what is known today as an nAChR gene family (Rubboli *et al.*, 1994b; Wada *et al.*, 1989). Originally, the genes encoding rat neuronal nAChR subtypes were identified and genes encoding for α2-9 and β2-4 were discovered (Breese *et al.*, 1997; Rubboli *et al.*, 1994b; Shioda *et al.*, 1997a; Wada *et al.*, 1989; Whiting *et al.*, 1991a) and by 2003, twelve nAChR subunit genes had been identified (α2-10, β2-4) (Graham *et al.*, 2003). It

was shown that mRNA encoding certain nAChR subunits, injected into *Xenopus* oocytes formed functional neuronal nAChRs such as α4β2 and α2β2 (Wada *et al.*, 1989; Whiting *et al.*, 1991a). *In situ* hybridisation enabled the various subunit mRNAs required to make nAChRs, to be identified in both the rat and human brain. Table 8 shows the distribution of α3, α4 and β2 mRNA subunits in the rat brain. There was high density of mRNA in the thalamus for all three of the subunits, interestingly, it was observed that the α4 and β2 subunit seem to have very similar densities in most of the CNS including the hippocampus, cerebral cortex, especially laminar II and V, and thalamus, indicating that these two subunits could bind and form functional nAChRs (Nakayama *et al.*, 1995; Wada *et al.*, 1989). *In situ* hybridisation and RT-PCR enabled studies into the expression of α4, α3, β2 and α7 nAChR subunits in the human brain (Table 8 & 10) (Dursun *et al.*, 1994; Guan *et al.*, 2002; Hellstrom-Lindahl *et al.*, 1999; Rubboli *et al.*, 1994b; Schroder *et al.*, 1995; Wada *et al.*, 1989; Whiting *et al.*, 1991a). It was shown that α4 mRNA was found throughout the cerebral cortex especially in the frontal and temporal cortex (Hellstrom-Lindahl *et al.*, 1999; Wevers *et al.*, 1994), whereas, α3 is found in high amounts in the frontal and parietal cortex, with moderate mRNA density in the temporal cortex (Hellstrom-Lindahl *et al.*, 1999). This correlates with the high nicotine and epibatidine binding demonstrated by autoradiography and indicates that the α4 nAChR subunit is a predominant subunit in the cerebral cortex. The mRNA data for α3 also correlate with the nicotine and epibatidine high density binding in different areas of the brain shown by autoradiography (Sihver *et al.*, 1998; 1999; Wevers *et al.*, 1994). Surprisingly, high density α7 nAChR subunit mRNA was detected in the cerebral cortex which contradicts the autoradiography studies which showed very little [^{125}I]-αBgT binding in all regions of the cerebral cortex (Breese *et al.*, 1997; Hunt & Schmidt, 1978; Utsugisawa *et al.*, 1999). This may be due to several factors, such as age and disease, causing varying levels of [^{125}I]-αBgT binding sites (Agulhon *et al.*, 1999;Hellstrom-Lindahl *et al.*, 1998) but it is important to note, that mRNA expression studies localise the mRNA encoding the receptor and not the receptor protein, which might move from the site and form a receptor in another area (Wada *et al.*, 1989). Studies observing α7 mRNA expression were recorded in the hippocampus, particularly in the pyramidal layers CA1 to CA3, entorhinal cortex and subiculum (Table 9 & 10) (Agulhon *et al.*, 1999; Breese *et al.*, 1997; Rubboli *et al.*, 1994a).

Using immunohistochemistry and RT-PCR, it was shown that the α7 nAChR are present in pyramidal layers CA1/CA3 as well as in the subiculum of the hippocampus in the human and rat brain (Dominguez del *et al.*, 1994; Graham *et al.*, 2003). Immunohistochemistry studies in the hippocampus have also shown that low protein expression is seen in the pyramidal layer, CA1, for α4 and β2 subunits with low to high expression observed in CA2 to CA4. Although moderate expression of α3 protein subunit is observed in CA1/2, there is a low to high expression in CA3/4. Both α4 and α3 protein expression is observed in the subiculum, although the expression is not as dense as with the α7 nAChR subunit (Table 8 - 10) (Graham *et al.*, 2003). Low mRNA expression for the α7 subunits is seen in the overall thalamus, apart from moderate expression in the reticular thalamic nucleus, which was also shown to have high [^{125}I]-αBgT binding (Breese *et al.*, 1997). Interestingly, it was shown that there was high α7 mRNA expression in the thalamus in a 25 week old human brain. However, other studies have shown less expression in older brains, indicating that loss of mRNA may occur with age (Agulhon *et al.*, 1999; Schroder *et al.*, 2001; Wade & Timiras, 1980). Other mRNA subunits that are expressed in the thalamus are α3 and β2 and are expressed in different intensities at the thalamus. α3 was shown to have moderate to high expression in the ventroposterolateral nucleus, lateroposterior nucleus and the dorsomedial nucleus, in the human and rat brain (Rubboli *et al.*, 1994a; 1994b; Terzano *et al.*, 1998; Wada *et al.*, 1989), while, β2 mRNA has low expression in the human brain, although high expression is shown in the rat brain, indicating that the functional nAChR formed probably contain a α3 nAChRs subunit but not the β2, suggesting the possibility of β4 (Table 8 & 9) (Rubboli *et al.*, 1994a).

The cerebellum mRNA subunit expression in human and rat brain is indicated to have overall moderate to high expression of the α3, α4 and β2 subunits, whereas, there is low to moderate expression of α7 nAChR subunit. It was shown that there is a higher expression of α3 and β2 in the granular layer than the molecular layer, although there was an equal expression to α7 mRNA (Table 8 – 10). When this was compared with immunohistochemistry studies done on the cerebellum, it was observed that α4 protein expression was greater in the molecular layer than granular layer. For α3, α7 and β2, although there was a greater protein expression in the granular layer than in the molecular

Brain Region	α3 Distribution	α4 Distribution	β2 Distribution
Cerebral Cortex			
- Frontal Cortex	+++	+ / ++	+++
- Occipital Cortex			
- Parietal Cortex	+++	+ / ++	
- Temporal Cortex	++	(++ / +++)	+
- Cingulate Cortex	±		-
- Insular Cortex	±		±
Limbic Areas			
- Hippocampus			
- Overall	+++	++ / +++	++ / +++
- Pyramidal layer			
- CA1	+ (± / ++)	(- / +)	+ (- / +)
- CA2	+ (± / ++)	(± / +++)	+ (± / +++)
- CA3	± / + (± / ++)	(± / +++)	+ (± / +++)
- CA4	± / + (± / ++)	(± / +++)	+ (± / +++)
- Dentate granular layer	± / +		
- Presubiculum	± / +	(-)	
- Subiculum	± / + (+ / ++)	(- / ++)	
- Entorhinal Cortex		± / +++	
- Dentate gyrus	+++		
Basal Ganglia			
- Globus Pallidus			
- Putamen	-		+ / ++
- Caudate n.	-		+ / ++
Thalamus			
- Overall	+++		+
- Pulvinar n.	+ / ++		±
- Lateral dorsal n.			
- Medial geniculate n.			
- Lateral geniculate n.			
- Anterior n.			
- Lateral posterior n.	++ / +++		-
- Ventral anterior n.			
- Ventral lateral n.			
- Ventroposteromedial n.			

- Ventroposterolateral n. - Reticulum n. - Centromedial n. - Dorsomedial n.	++ / +++ ++ + / ++ +		± - - ±
Hypothalamus - PVN - SON - Suprachiasmatic n. - Ventromedial n.		++ +++ ++ ++	
Substantia nigra - Overall		(+ / ++)	+ (++)
Midbrain - Reticular Formation	+ / ++		
Cerebellum - Overall - Molecular layer - Granular layer	++ / +++ ± (±) + / +++ (-)	++ / +++ (± / ++) (+)	++ / +++ +++ (+ / ++) ++ (±)
Spinal Cord	+++	+++	+ / ++

Table 8. Comparing the expression of neuronal nAChR α3, α4 and β2 subunit mRNA and protein expression in the human brain

Symbols indicate:

- = Not Detected
± = Very Weak Signal
\+ = Weak Signal
++ = Moderate Signal
+++ = High Signal

() indicates protein subunit expression

Brain Region	α3 mRNA Distribution	α4 mRNA Distribution	β2 mRNA Distribution	α7 protein Distribution
Rhinencephalon				
- Olfactory bulb	- / +	±	- / +	- / ++
- Olfactory tubercle	- / +++	+ / ++	+ / ++	- / ++
Cerebral Cortex				
- Laminar I	-	+	±	- / +
- Laminar II	-	++	++	- / +
- Laminar III	-	++	±	- / +
- Laminar IV	+++	+	±	- / +
- Laminar V	±	++	+ / ++	++ / +++
- Laminar VI	+ / ++	-	± / +	+ / ++
Limbic Areas				
- Hippocampus				
- Overall				
- Pyramidal layer				
- CA1	+	++	+	+ / ++
- CA2				+ / ++
- CA3	-	+	± / ++	+ /+++
- Presubiculum	-	± / +++	± / ++	
- Subiculum	± / ++	± / ++	± / ++	+ / ++
- Entorhinal Cortex	-	± / ++	± / ++	- / ++
- Dentate gyrus				
- Molecular layer	-	±	±	±
- Granular layer	-	-	++	++ / +++
- Amygdala	-	+	++	++
Basal Ganglia				
- Globus Pallidus	-	++	±	+ / ++
- Putamen	-	-	±	-
- Caudate n.	+	-	±	-

Thalamus				
- Medial habenula n.	+++	+++	++	++ / +++
- Lateral dorsal n.	++	+++	++	+ / ++
- Lateral posterior n.	+	+++	+++	+ / ++
- Medial geniculate n.	++ / +++	+++	+++	++
- Lateral geniculate n.	± / +++	+++	+ / +++	++ / +++
- Anterior n.	+++	+++	+ / ++	++
- Ventral anterior n.	+++	+++	+++	++
- Ventral lateral n.	+++	+++	+++	++
- Ventral posterior n.	++	+++	+++	++ / +++
- Centromedial n.	-	+	+ / ++	-
- Dorsal medial n.	++ / +++	+ / ++	++	+++
- Reticulum n.	±	++	+	++ / +++
Hypothalamus				
- PVN	-	- / +	+ / ++	- / +
- SON	-	-	++	++ / +++
- Suprachiasmatic n.	-	-	+	+ / ++
- Mammillary body	- / +++	- / +	+ / ++	+++
- Preoptic area	-	+	± / +	- / +
Substantia nigra				
- Pars compacta	+	+++	+++	++ / +++
- Ventral tegmental area	++	+++	+++	- / +
Midbrain				
- Superior colliculus	± / +	± / ++	± / ++	- / ++
- Inferior colliculus	± / +	+ / ++	+ / ++	++ / +++
- Raphe	+	- / ±	+	- / +
- Reticular Formation	-	+	+	- / ++
- Superior olivary complex	-	+ / ++	++	++ / +++
- Periaqueductal grey matter	-	+	+	++
- Locus coeruleus	±	+++	++	++ / +++
- Pontine nucleus	+	-	+	±

Cerebellum				
- Deep nuclei	-	+++	++	++ / +++
- Molecular layer	-	-	±	-
- Granular layer	+	±	+	- / +
Hindbrain & Medulla				
- Parabrachial				+++
- Inferior olivary complex				+ / ++
- Red nucleus				+++
- Cochlear				++ / +++
Spinal Cord	-	+	+ / ++	- / +++

Table 9. Comparing the expression of neuronal nAChR subunits α3, α4 & β2 mRNA expression in the rat brain

Symbols indicate:

- \- = Not Detected
- ± = Very Weak Signal
- \+ = Weak Signal
- ++ = Moderate Signal
- +++ = High Signal

Brain Region	α7 Distribution
Cerebral Cortex	
- Frontal Cortex	++ / +++
- Occipital Cortex	++
- Parietal Cortex	+ / ++
- Temporal Cortex	++ / +++
- Cingulate Cortex	+
- Insular Cortex	++ / +++
Limbic Areas	
- Hippocampus	
- Overall	+++
- Pyramidal layer	
- CA1	++ / +++ (++ / +++)
- CA2	++ / +++ (++ / +++)
- CA3	++ / +++ (++ / +++)
- Dentate granular layer	++
- Mol. layer of dentate gyrus	++
- Presubiculum	±
- Subiculum	± / ++ (+ / ++)
- Entorhinal Cortex	++
- Amygdala	++
Basal Ganglia	
- Globus Pallidus	±
- Putamen	± / +
- Caudate n.	- / ++
Thalamus	
- Overall	
- Pulvinar n.	+ / ++
- Midline n.	-
- Medial geniculate n.	
- Lateral geniculate n.	
- Anterior n.	
- Lateral posterior n.	
- Ventral anterior n.	
- Ventral lateral n.	+
- Ventroposteromedial n.	
- Ventroposterolateral n.	+

- Reticulum n. - Centromedial n. - Dorsomedial n.	+ / ++ - ± / +	
Hypothalamus - PVN - Mammillary bodies	 + +	
Substantia nigra - Pars compacta	 -	
Midbrain - Superior colliculus - Inferior colliculus - Reticular formation - Pontine nucleus - Superior olivary complex	 - - + ++ / +++ +++	
Cerebellum - Overall - Molecular layer - Granular layer	 + / ++ +++ ++ / +++	 (+ / ++) (+)
Spinal Cord	+++	
White Matter	±	

Table 10. Comparing the expression of neuronal nAChR α7 subunit mRNA and subunit protein expression in human brain.

Symbols indicate:

- \- = Not Detected
- ± = Very Weak Signal
- \+ = Weak Signal
- ++ = Moderate Signal
- +++ = High Signal

() indicates protein subunit expression

layer, the amount of expression was greatly reduced compared with mRNA expression (Graham *et al.*, 2003), but overall indicating that nAChRs are present in the cerebellum (Table 8 - 10). This is an example that although mRNA subunits are expressed here it does not indicate that the protein receptors form at the same site and instead it is possible that they may migrate to other areas, which may account for the reduction in expression.

There is increasing evidence that nAChRs are not only found in the CNS, but also in the autonomic nervous system (ANS), as well as, non-neuronal cells

1.6.3. Distribution of nAChRs in the ANS.

In order for the CNS to integrate sensory input and send the commands back to the organs it is required to go through the ANS. The autonomic ganglia are the peripheral sites for the control of the organs and tissues by the nervous system. Unlike nAChRs in the CNS, ganglionic nAChRs possess different functional and pharmacological characteristics due to expression of various nAChRs subunits. However, there are common features found between neuronal and autonomic nAChRs; for example, relatively slow gating, moderate calcium permeability and significant rectification of the post-synaptic responses (Seguela *et al.*, 1993).

Autonomic ganglia have high levels of mRNA for α3, and β4 subunits, as well as, transcripts for α4, α5, α7 and β2 nAChRs subunits (Poth *et al.*, 1997; Xu *et al.*, 1999a; 1999b). Research looking into the nAChRs involved in the neurotransmission in the intracardiac ganglia indicated, by *in vivo* and *in vitro* studies, that there are at least two nAChR subtypes present; which are the α3β4 and α7 nAChR (De *et al.*, 2000a; Xu *et al.*, 1999b). Studies found that α3 subunits in the rat combine mostly with the β4 subunit and that the α3β4 subunit causes an increase in heart rate upon application of nicotine (Ji *et al.*, 2002). The α7 subunits have been located in both the parasympathetic and sympathetic neurons that innervate the heart and upon activation cause a decrease in heart rate. Studies have indicated that in the intrinsic cardiac neurons of the rat, α7 nAChRs

may not be homomeric but instead interact with other nAChR subunits to form heteromeric nAChRs (Ji *et al.*, 2002a).

Additional studies into the involvement of nAChRs in the ANS have located nAChRs in organ systems such as the gut and bladder. Unfortunately, very little is known about the subunit composition of nAChRs in these organs (De *et al.*, 2000b). However by the use of knockout studies, it was observed that removal of the β2 and β4 subunits caused a disruption of gut motility (Xu *et al.*, 1999b). Additional studies have also shown the presence of α3 nAChRs in the mouse bladder, such that α3 knockout mice have abnormalities in the bladder which include enlarged bladder (or megabladder), bladder infection and dribbling urination (see Picciotto *et al.*, 2001). Interestingly, it was shown that certain nAChR subtypes may be able to mediate ganglionic transmission in the bladder, particularly the α3β4 nAChR. Furthermore, bladder smooth muscle non-responsive to nicotine was observed in α3, β2 and β4 knockout mice (Xu *et al.*, 1999a; 1999b).

1.6.4. Distribution of nAChRs in non-neuronal cells.

There are increasing studies demonstrating the presence of nAChRs, other than those involved in synaptic transmission in the central and peripheral nervous system which, upon activation, modulate certain cellular functions. A number of non-neuronal cells have been observed to express functional nAChRs such as skin keratinocytes (Grando, 1997), bronchial epithelial (Maus *et al.*, 1998), aortic endothelial cells and immune tissue (Table 11) (Nguyen *et al.*, 2000; Skok *et al.*, 2003). Studies involving α-subunit specific antibodies in β-lymphocyte cell lines identified that α4 and α7 nAChRs were present (Skok *et al.*, 2003). *In situ* hybridisation studies revealed that the α9 and α10 subunits are distributed in the lymphoid system (tonsil, thymus and spleen) (Baker *et al.*, 2004; Elgoyhen *et al.*, 2001). The α9 subunit is expressed in sensory organs and in particular in outer hair cells where it modulates the encoding of auditory stimuli (Elgoyhen *et al.*, 2001). The localization of nAChRs in lymphocytes is a special interest because it would

demonstrate the participation of α7 nAChRs in the promotion of tumor cell proliferation, as well as in antibody production (Skok *et al.*, 2003).

Cell type	Subunit	Reference
Lymphocytes	α7, α4, α9, and α10	Skok *et al.*, 2003
Vascular endothelial cells (human)	α3, α5, α7, β2 and β4	(Macklin *et al.*, 1998)
Bronchial epithelium (human, rat)	α3, α5, α7, β2 and β4	Maus *et al.*, 1998
Keratocytes	α5 and α3	Grando *et al.*, 1997

Table 11. Distribution of nAChRs in non-neuronal cells

1.7. Neuronal nAChR acting as auto-and heteroreceptors.

It has been discussed that neuronal nAChRs are found on post-synaptic nerve terminals and are involved in fast synaptic responses (see Colquhoun & Patrick, 1997; see McGehee & Role, 1995; see Role & Berg, 1996), however there are other areas of the nerve where nAChRs are also found, for example on pre-synaptic nerve teminals, numerous studies have investigated the role of nAChR at these locations. Pre-synaptic nAChRs were first identified by [^{3}H]-nicotine and [^{125}I]-α-BgT binding studies which detected binding sites along the axons and the terminal field of the CNS (see McGehee & Role, 1995; see Sargent, 1993). By the use of focal lesions and quantitative radioligand binding, it was shown that a large amount of [^{3}H]-nicotine and [^{125}I]-α-BgT binding sites were found pre-synaptically (see Jones *et al.*, 1999). These nAChRs have been shown to facilitate in the release of neurotransmitters and have been termed autoreceptors and heteroreceptors (see Wonnacott, 1997).

Autoreceptors are receptors which facilitate the increase or reduction of its own neurotransmitter release by acting on receptors located axonally or terminally, for example, ACh acting on nACh autoreceptors to decrease or increase the release of ACh

from nerve terminals (Balfour, 1982; see Balfour & Fagerstrom, 1996; see Chesselet, 1984). However, heteroreceptors are characterised as receptors that facilitate the increase or decrease in the release of a different neurotransmitter, for example, ACh acting on nACh heteroreceptors causing an increase in the release of dopamine (see Balfour & Fagerstrom, 1996; see Colquhoun & Patrick, 1997; see Wonnacott, 1997). Since the discovery of autoreceptors and heteroreceptors a great amount of interest and research has gone in to this area and it has been documented that nAChRs modulate the release of nearly every neurotransmitter including dopamine (DA), glutamate (Glu), gamma-aminobutyric acid (GABA), noradrenaline (NA), 5-hydroxytyramine (5-HT) and ACh (Alkondon *et al.*, 1997; Clarke & Reuben, 1996; see Dani, 2001; Girod *et al.*, 2000; Lapchak *et al.*, 1989b; Lena *et al.*, 1993; Mihailescu *et al.*, 2002; see Radcliffe & Dani, 1998; Schilstrom *et al.*, 2003; Tani *et al.*, 1998; Wonnacott *et al.*, 1989).

Not only has it been noted that there are differences in the pre-synaptic receptors i.e. auto-and heteroreceptors but it has also been shown that the localisation of these receptors can vary. Pre-synaptic receptors in the presence of tetrodotoxin (TTX), a sodium channel blocker, caused inhibition of some nAChRs but not all (see Dani, 2001; see Wonnacott, 1997). Further studies which included looking at GABAnergic axons in rat interpeduncular nucleus (Lena *et al.*, 1993) and glutamatergic axons in rat cerebellum, (De *et al.*, 2001) showed that in the presence of TTX, the increase in frequency of post-synaptic currents caused by a nicotinic agonist was inhibited. It has also been shown that neurotransmitter release can occur in the presence of TTX, for example, indicating that they are TTX-insensitive pre-synaptic receptors, this was shown with NA release by nicotine in rat hippocampal synaptosomes (Clarke & Reuben, 1996) and an increase in post-synaptic currents by nicotine in the medial habenula and interpeduncular nucleus neurons resulting in the release of glutamate (Girod *et al.*, 2000). Studies have suggested that there are different localisations for nAChR on the neuron. The first is believed to be found on the axon (Figure 10) (see Dani, 2001; see Wonnacott, 1997), whereby, neurotransmitter release is believed to occur by the opening of nAChR ion channels, resulting in the influx of Na^+, causing the opening of voltage-gated calcium channels and the influx of Ca^{2+}, which evokes the exocytosis mechanisms and the release of neurotransmitter (Figure 11) (see Jones *et al.*, 1999; see McGehee & Role, 1995;

Wonnacott *et al.*, 1989). It has also been shown that due to the high permeability of Ca^{2+} through the ion channel of the nAChR, the influx of Na^{+} to open the voltage-gated ion channel is not always required and exocytosis of neurotransmitters can occur by the Ca^{2+} influx through the nAChR ion channel alone (see Dajas-Bailador & Wonnacott, 2004; Girod *et al.*, 2000).

Unlike axonal auto-and heteroreceptors, which are believed to be found on the nerve bouton, terminal nAChR are believed to be found on the terminal regions of the axon but not in the proximity of a single synaptic bouton (Figure 10) (see Dani, 2001; see Wonnacott, 1997). As previously mentioned terminal nAChR can be identified by the use of TTX, whereby inhibition by this sodium channel blocker indicates terminal nAChRs. In contrast to axonal nAChR activation, terminal nAChR activation causes TTX-sensitive spikes in the terminal portion of the axons, resulting in depolarisation of the membrane locally, leading to activation of voltage-dependent Na^{+} channels that result in action potentials (Alkondon *et al.*, 1997; Lena *et al.*, 1993). These action potentials presumably run down the axon, causing voltage-gated Ca^{2+} channels to open in the pre-synaptic bouton and inducing the exocytosis mechanism, resulting in the release of the neurotransmitter (see Dajas-Bailador & Wonnacott, 2004).

1.7.1. Neuronal nACh autoreceptors involvement in ACh release.

It has been clearly demonstrated that the administration of nicotine intravenously (i.v.) into cats resulted in an increase in the release of ACh in the brain, in particular the parietal cortex, although it was originally unclear how this occurred (Balfour, 1982). Due to extensive research, it has been shown that nicotine and other nicotinic compounds are able to act on autoreceptors and cause the release of ACh from nerve terminals (Lapchak *et al.*, 1989b; Tani *et al.*, 1998).

A particular area of interest for studying nicotinic autoreceptors is the cholinergic input from the medial septal nucleus to the hippocampus (Mesulam, 1995; Mesulam & Geula, 1988). It has been shown *in vivo* in free moving rats (Tani *et al.*, 1998), as well as, in hippocampal synaptosomes and slices (Araujo *et al.*, 1988; see Chesselet, 1984; Wilkie *et al.*, 1996), that nicotinic agonists stimulate the release of ACh. In the hippocampus of free moving rats, α4β2 selective nicotinic agonist, ABT-418, enhanced the release of ACh, while GTS-21, a α7 selective nicotinic agonist, had no effect on ACh release (Tani *et al.*, 1998), indicating that the autoreceptor present was the α4β2 receptor. This is emphasized in hippocampal synaptosomes, slices and *in vivo* studies which demonstrated that ACh release by nicotinic agonists is blocked by the α4β2 antagonist, DhβE, but not by the α7 antagonists α-BgT and MLA (Araujo *et al.*, 1988; Wilkie *et al.*, 1996; Wonnacott *et al.*, 1989). These findings also correlate with the high levels of α4 and β2 subunit transcripts that have been found in the cell bodies of the medial septum (Wada *et al.*, 1989). The location of the nicotinic receptor has been demonstrated to be axonal due to it being largely TTX-insensitive and the nicotinic-induced release of ACh is believed to be calcium dependent (Colquhoun & Patrick, 1997).

Other areas of the CNS that demonstrate nicotinic AChR-induced ACh release include the cerebellum, which is innervated by ACh containing neurons from the medullary tegmental nucleus. Upon administration of nicotinic agonists into the rat cerebellum there is a increase in ACh release (De *et al.*, 2005; Lapchak *et al.*, 1989a; 1989b) which was blocked by the α4β2 antagonist, DhβE, but was insensitive to atropine or α-BgT indicating the presence of α4β2 receptors, but not α7 or muscarinic receptors (Lapchak *et al.*, 1989b). As with neuronal nAChRs found in the hippocampus, cerebellum autoreceptors are TTX-insensitive, suggesting an axonal location.

1.7.2. Dopamine release by activation of neuronal nACh heteroreceptors.

Dopamine (DA) release plays an important role in mediating the reinforcement effect of natural rewards, as well as that of various drugs of abuse (see Jones *et al.*, 1999; Marshall *et al.*, 1997; Schilstrom *et al.*, 1998; 2003). One such drug of abuse is nicotine inhalation via cigarette smoking which has been shown to cause the release of DA which results in the stimulation that smokers feel (Wu *et al.*, 2004) and the addiction (Champtiaux *et al.*, 2003). Due to this effect, the release of DA by nicotine has been extensively studied to confirm that nicotine can induce DA release. One technique is the use of striatal synaptosome preparations. The striatum has been extensively studied because it has been shown by lesion studies there is a high number of pre-synaptic nAChRs present. Lesion studies on nigrostriatal neurons, which make up the dopaminergic projection from the substantia nigra to the striatum, caused a significant decrease in nicotine binding sites in the striatum (see Colquhoun & Patrick, 1997). Previous studies demonstrated that the striatum has no detectable nicotinic genes, therefore the decrease in nicotinic binding sites is not due to loss of receptors on the striatal neuron, and instead it is believed that the decrease in nicotinic binding sites is due to the loss of presynaptic nAChRs on innervating nigral axons. A large number of nAChR subunits from the nigral efferent to the striatum have been identified, including α3, α4, α5, β2 and β3 (Wada *et al.*, 1989).

Striatal synaptosome preparations showed that nicotinic agonists induce the release of DA from the perfused synaptosome preparation preloaded with radiolabelled DA (Rapier *et al.*, 1988; 1990). This release of DA by nicotinic agonists occurred at very low concentrations and was inhibited by the non-selective nicotinic antagonist, mecamylamine (Rapier *et al.*, 1988). More specifically, the nicotinic-mediated DA release was inhibited by the α4β2 selective antagonist, DhβE, although there was no observed inhibition by α-BgT or atropine, indicating α7 and muscarinic receptors are not involved (Grady *et al.*, 1992; Rapier *et al.*, 1990). The release of DA is believed to be calcium-dependent, this is based on the finding that there is a reduction in DA release in the absence of Ca^{2+} (Grady *et al.*, 1992; Rapier *et al.*, 1988). This finding, together with

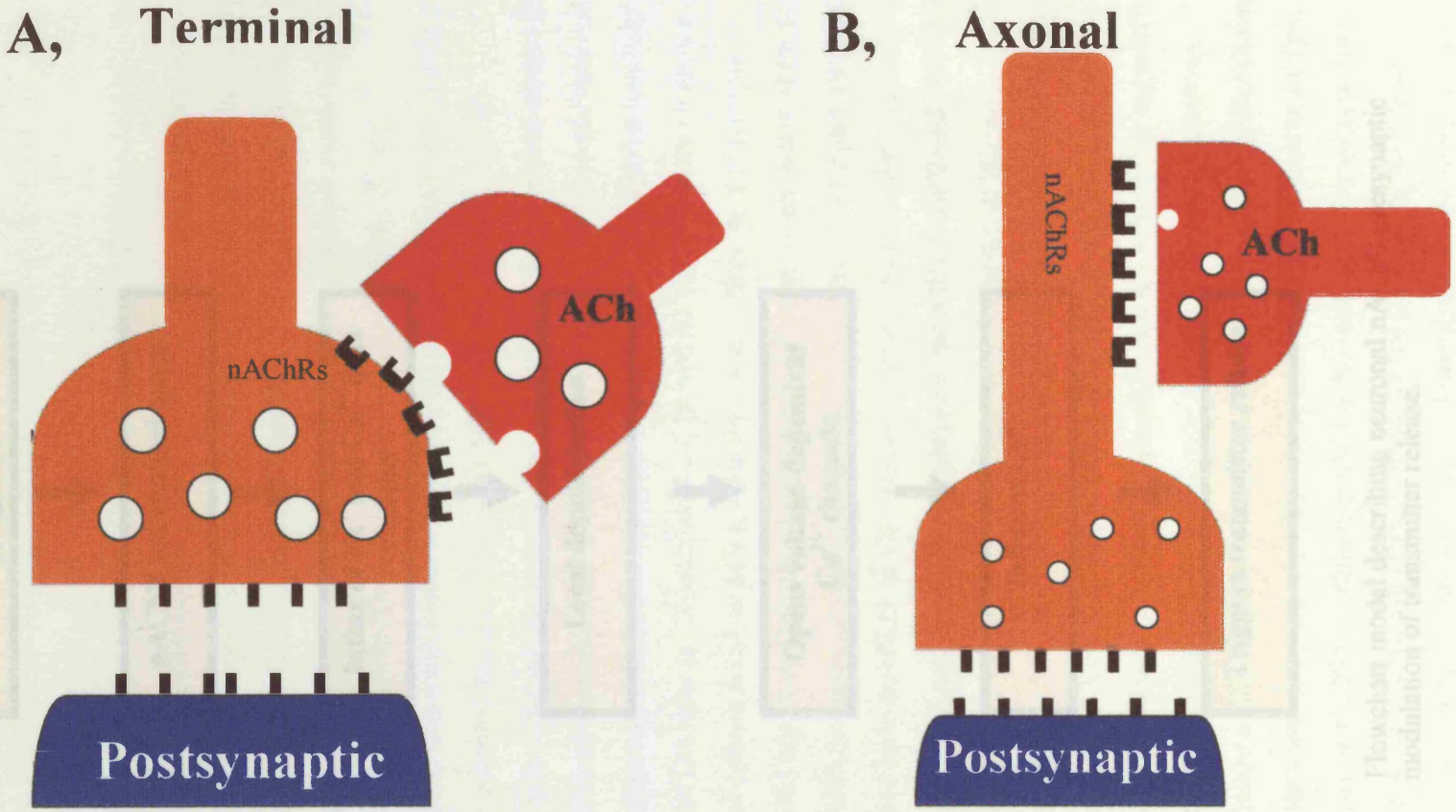


Figure 10. Diagrammatic representation of the location of A, terminal nAChRs; and B, axonal nAChR.

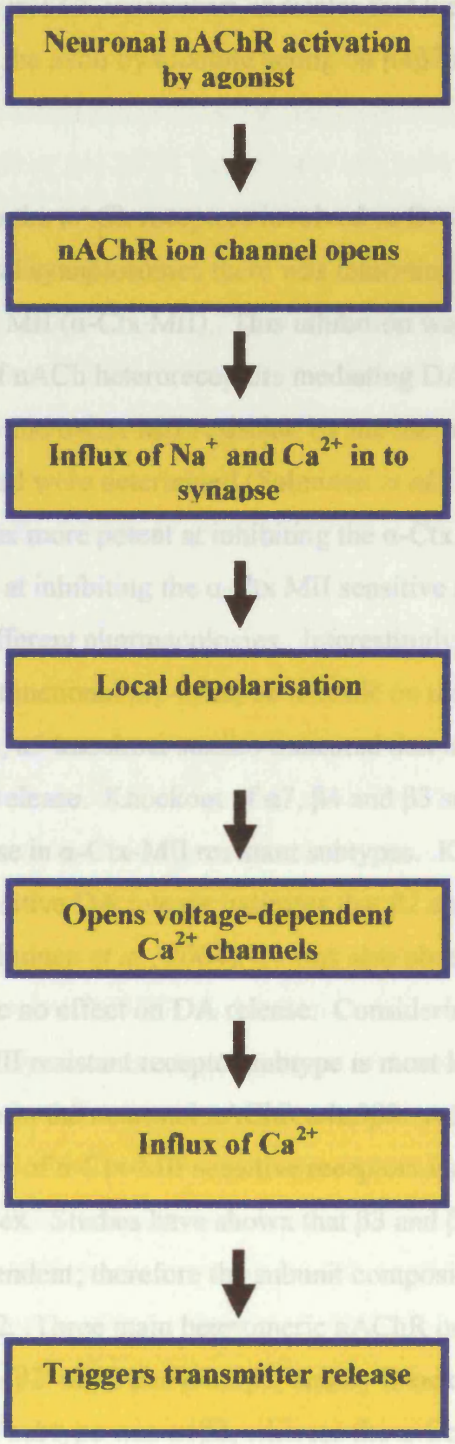


Figure 11. Flowchart model describing neuronal nAChR presynaptic modulation of transmitter release.

the observation that release is TTX-insensitive, indicates that the release of DA occurs via direct depolarization of the axon by nicotine acting on α4β2 receptors located axonally.

Further studies looking into the nACh receptors involved in DA-mediated release, showed that in mouse striatal synaptosomes there was inhibition of nicotinic-mediated DA release by α-conotoxin MII (α-Ctx MII). This inhibition was only partial, indicating that there are two classes of nACh heteroreceptors mediating DA release, which are termed α-Ctx MII sensitive and α-Ctx MII resistant, by the use of subunit deletion, the particularc nAChRs involved were determined (Salminen *et al.*, 2004). It was demonstrated that DhβE was more potent at inhibiting the α-Ctx MII resistant subtype and MLA was more potent at inhibiting the α-Ctx MII sensitive subtype, clearly indicating nAChRs with different pharmacologies. Interestingly, knock-out of α4 and β2 subunits caused a block of functional pre-synaptic nAChR on the dopaminergic terminal of the striatum. In addition, α5 knockout studies indicated that α5 may also be involved in α-Ctx-MII resistant DA release. Knockout of α7, β4 and β3 subunits were all shown to have no effect on DA release in α-Ctx-MII resistant subtypes. Knockout of receptor subtypes for α-Ctx-MII sensitive DA release indicates that β2 and β3 are essential for DA release (Cui *et al.*, 2003;Salminen *et al.*, 2004), α4 was also observed to be important but again α7 was shown to have no effect on DA release. Considering these data it could be concluded that the α-Ctx MII resistant receptor subtype is most likely α4β2 and may also involve the α5 subunit to form the neuronal nAChR α4α5β2. Additional studies determining the composition of α-Ctx-MII sensitive receptors indicated that the subunit composition is more complex. Studies have shown that β3 and β2 are essential, whereas α6 and α4 are partially dependent; therefore the subunit composition has been suggested to be α4α6β3β2 and α6β3β2. Three main heteromeric nAChR on the mouse striatum were identified which are α4β2, α6β2 and α4α6β2, and by knockout studies it was shown that the α-Ctx-MII resistant subtype was α4β2, whereas the α-Ctx-MII sensitive subtype was α6β2 (Champtiaux *et al.*, 2003). Although α3β2 is the most selective subtype for α-Ctx-MII (Sharples *et al.*, 2000) there is little evidence for the presence of α3 nAChRs in the striatum (Wada *et al.*, 1989). Additionally, *in situ* hybridisation only demonstrated very low concentrations of α3 mRNA in DA neurons (Champtiaux *et al.*, 2003). Using

UB-165 (Karig *et al.*, 2003;Sharples *et al.*, 2000) and (±) anatoxin-a (Soliakov *et al.*, 1995), which are selective agonists of the α4β2 receptor, an increase in DA release was observed in striatal synaptosomes and provided evidence that the α-Ctx-MII insensitive subtype is α4β2 (Champtiaux *et al.*, 2003; Salminen *et al.*, 2004; Sharples *et al.*, 2000; Soliakov *et al.*, 1995). Studies suggested the nAChR-mediated DA release was calcium dependent and located axonally (Grady *et al.*, 1992; Rapier *et al.*, 1988). However, it is also possible that some terminal nAChR are present since it was shown that there was 40% inhibition in the presence of TTX (Rapier *et al.*, 1988; Soliakov *et al.*, 1995). Interestingly, studies *in vivo*, demonstrated that TTX completely blocked the nicotine-induced DA release, suggesting that the localisation of the nAChR may be found terminally rather than axonally (Marshall *et al.*, 1997).

Additional areas in the brain have been studied, including the midbrain dopaminergic nuclei, which apart from the substantia nigra, also include the ventral tegmental area (VTA). The VTA plays an important role in mediating the effects of natural rewards (Klink *et al.*, 2001; Schilstrom *et al.*, 2003) as well as various drugs of abuse, including nicotine, through an increased dopaminergic transmission to the nucleus accumbens (Schilstrom *et al.*, 1998; Wu *et al.*, 2004). Nicotinic agonists applied to the rat VTA, resulted in an increase in DA release in the nucleus accumbens (Schilstrom *et al.*, 1998). In later studies by Schilstrom *et al.* (2003) nicotine increased burst firing and firing rate of dopaminergic neurons. Involvement of the neuronal nACh α7 receptor was demonstrated by the use of the selective antagonist, MLA, which was observed to block burst firing but not firing rate, whereas, the α4β2 selective antagonist, DhβE, caused inhibition of firing rate but did not inhibit burst firing (Schilstrom *et al.*, 2003). Using the α4β2 selective agonist, TC-2559, it was shown that α4β2 nAChRs are present in the VTA and that they facilitate the release of DA (Chen *et al.*, 2003), which was inhibited by the α4β2 selective antagonist, DhβE. Studies performed by Klink *et al.* (2001) indicated that DA release by nAChRs is in fact a consequence of more diverse nAChRs such as $\alpha4\alpha5(\beta2)_2$ (Klink *et al.*, 2001).

1.7.3. 5-HT release by activation of neuronal nACh heteroreceptors.

The largest serotonergic innervation of the forebrain originates from the raphe nucleus, with the dorsal raphe nucleus containing the largest amount of serotonergic neurons in the brain (see Balfour & Fagerstrom, 1996; see Chesselet, 1984; Li *et al.*, 1998; Mihailescu *et al.*, 2002). Although it has been shown that serotonin can control its own release in slices such as the cortex and hypothalamus (see Balfour & Fagerstrom, 1996; see Chesselet, 1984), it has been shown that administration of nicotinic agonists on *in vitro* dorsal raphe neurons of the rat, caused an increase in the discharge rate of the serotonergic neurons and resulted in the release of 5-HT (Li *et al.*, 1998), which was MLA-insensitive, suggesting that the α7 nAChR subtype was not involved (Li *et al.*, 1998). Other studies have shown that nicotine administrated into the dorsal raphe nucleus of the rat midbrain also increases the release of serotonin (Mihailescu *et al.*, 2002). Li *et al.* (1998) demonstrated that the nicotine-induced serotonin release is TTX-insensitive, suggesting that neuronal nACh receptors are present on the axon rather than a terminal location. Neuronal nAChR subtype studies have shown a high expression of α4 and α7 present in these neurons (Bitner & Nikkel, 2002), although further study is required to determine which neuronal nACh receptor is activated to facilitate in the release of serotonin. The effects of nicotine-invoked serotonin release on other areas of the brain, such as the hippocampus, showed that nicotine did modulate its release but the dose required was very high, and therefore the effect is probably of little pharmacological significance (Balfour, 1982).

1.7.4. Noradrenaline release by activation of neuronal nACh heteroreceptors.

Noradrenaline (NA) release has been studied in detail in many areas of the brain, the most extensively studied areas are the hippocampus (Colquhoun & Patrick, 1997) and

hypothalamus (Hall & Turner, 1972), where it was shown that the application of nicotinic agonists evoked NA release. Studies of rat hippocampal synaptosomes have shown that nicotinic agonists cause the release of NA, which can be abolished by the nicotinic antagonist, mecamylamine indicating the presence of neuronal nACh heteroreceptors. It was observed that cytisine, a very selective agonist for α3 and β4, was as effective at causing NA release as nicotine (Alkondon & Albuquerque, 1993; Chavez-Noriega *et al.*, 1997; Colquhoun & Patrick, 1997). Additionally, the selective antagonists DhβE and MLA were not as effective at inhibiting nicotinic stimulated NA release. These results therefore suggest the presence of an α3 or β4 subunit (Anderson *et al.*, 2000; Clarke & Reuben, 1996). Innervation by NA-containing neurons of the hippocampus is derived from the locus coeruleus (see Chesselet, 1984; Lena *et al.*, 1999; see Wonnacott, 1997), *in situ* hybridization and immunohistochemistry studies showed that the NA-containing neurons contained an extremely high abundance of α7 and α3 nAChR subunits (Bitner & Nikkel, 2002), as well as moderate levels of β4 and very little α4 subunit (Lena *et al.*, 1999; Seguela *et al.*, 1993; Wada *et al.*, 1989). It is believed that the heteroreceptors present that cause the release of NA are α7 and α3β4, although this is not completely proven. The nicotine evoked NA release was unaffected by TTX therefore suggesting the location of these heteroreceptors are on the axon (Clarke & Reuben, 1996). Additionally, it has been observed, in cell lines, that the release of NA by the activation of DMPP is caused by depolarization of the cell resulting in activation of L-type calcium channels (Vaughan *et al.*, 1993) and the influx of Ca^{2+} enhance evoking levels of intracellular calcium, which would theoretically cause the exocytosis of NA from the synaptic terminal and release NA (see Dajas-Bailador & Wonnacott, 2004; Vaughan *et al.*, 1993; Wonnacott *et al.*, 1989).

Studies of the hypothalamus, have shown NA release upon nicotine administration both by i.v. and i.c.v. injection (see Hall & Turner, 1972). In hypothalamic tissue slices, nicotine was demonstrated to cause an increased release of NA which is believed to be a calcium-dependent process (see Hall & Turner, 1972). In hypothalamus synaptosomes nicotine, even at very low doses, causes the increased release of NA (Balfour, 1982).

Additional areas of the brain have been studied to observe if neuronal nAChRs are causing the release of NA, and it has been shown that nicotinic agonists can cause the release of NA from different areas of the brain. However, it was found that the concentration of agonist required to elicit a response was too great and are therefore unlikely to be of any physiological signficance (see Balfour, 1982) and makes it less plausible that it naturally occurs in these other sites of the CNS.

1.7.5. GABA release by activation of neuronal nACh heteroreceptors.

The substantia nigra receives a huge GABAergic input from the caudate nucleus, unfortunately, not much research has gone into looking at the effects of nicotine-induced GABA release in this area. One area that has been studied in great detail in terms of nicotine-induced GABA release is the rat hippocampus. It has been observed in CA1 neurons that application of nicotine results in the release of GABA from interneurons. These nicotinic responses were blocked by nicotinic antagonists, indicating that the GABA-mediated post-synaptic currents and slow decaying nicotinic currents were mediated by nAChRs (Alkondon *et al.*, 1997). The GABA-mediated post-synaptic currents were not blocked by either of the α7 antagonists α-BgT or MLA, whereas, the slow decaying nicotinic currents recorded from the interneurons were blocked by the α4β2 nAChR selective antagonist DhβE (Alkondon *et al.*, 1997). Nicotine-induced release of GABA has also been observed in CA3 whereby it was suggested that nAChRs modulated giant depolarizing potentials (GDP) (Maggi *et al.*, 2001). Nicotine enhanced the GDP frequency and was blocked by the α4β2 antagonist, DhβE, and partially inhibited by the α7 antagonist, MLA. Selective agonists for α7 also increased GDP frequency (Maggi *et al.*, 2001). It was therefore suggested that GABA release in the rat hippocampus is mediated by at least two nAChRs, most likely α4β2 and α7, especially as high levels of staining for the α7 subunit were observed on GABAergic interneurons (Kawai *et al.*, 2002). The CA1 was shown to be TTX-sensitive, indicating that the

neuronal nACh heteroreceptors are found terminally (Alkondon *et al.*, 1997; Lena *et al.*, 1993).

Another area of the rat brain that has a significant role in nAChR-induced GABA release is the IPN, due to its cholinergic innervation derived from the medial habenula and medio-basal forebrain through the fasciculus retroflexus (Mesulam & Geula, 1988; see Woolf, 1991). This area has been shown to have radioligand binding sites for [^{3}H]-nicotine and [^{125}I]-α-BgT (Spurden *et al.*, 1997; Whiting *et al.*, 1991). Nicotine applied to IPN neurons caused an increase in frequency of post-synaptic GABAnergic currents, which could be inhibited by the α4β2 antagonist, DhβE (Lena *et al.*, 1993). There are a larger number of neuronal nAChR subunits found in the IPN, with the α2 subunit gene having the highest expression in the CNS (Wada *et al.*, 1989). Other subunit genes expressed include α3, α4, α5, β2 and β4 (Wada *et al.*, 1989), indicating that there could be a large variety of nAChR involved in GABA release (Lena *et al.*, 1993). Interestingly, the nicotinic agonist-induced GABA release was inhibited by TTX (Lena *et al.*, 1993), as previously mentioned indicating that the neuronal nAChR are located on the terminal. Activation of these terminal nACh receptors triggers TTX sensitive spikes in the terminal portion of the GABAnergic axon which produces action potentials that initiate GABA release from multiple presynaptic terminals (Alkondon *et al.*, 1997; Lena *et al.*, 1993). In the chick mesencephalic lateral spiriform nucleus, nicotinic agonists caused an increase in the frequency of spontaneous post-synaptic currents, which are inhibited by bicuculline, indicating GABAnergic currents (McMahon *et al.*, 1994). The nicotine-induced effects are inhibited by DhβE and the nicotinic agonist-induced post-synaptic currents are blocked by TTX, indicating a α4β2 nAChR located on the terminal (Lena *et al.*, 1993; McMahon *et al.*, 1994).

1.7.6. Glutamate release by activation of neuronal nACh heteroreceptors.

Glutamate has been observed to be most abundant in the hippocampus and on the medial habenula projection to the IPN nucleus (see Jones *et al.*, 1999). The medial habenula – IPN (MHN-IPN) synapse was observed to increase frequency of glutamatergic spontaneous post-synaptic currents by 57% when nicotine was applied. Observations that the nAChRs were TTX-insensitive indicated that the neuronal nACh heteroreceptors were located on the axon (Girod *et al.*, 2000). Due to the high gene expression on the MHN and IPN with almost all the known nicotinic gene products found, it is difficult to determine which nAChRs are present (Wada *et al.*, 1989), additionally it is highly possible that multiple receptor subtypes exist. Studies of the MHN-IPN synapse suggests that α7 may be involved in glutamate release from observations of α-BgT inhibition of nicotine-induced effects. Additionally, deletion of the α7 subunit diminished the magnitude of nicotine-elicited calcium signalling into the pre-synaptic neuron. However, complete inhibition of calcium signalling did not occur. This is likely to be due to the extensive expression of other receptor subunits, which would compensate for the loss of a specially deleted subtype by increasing their calcium signalling. Interestingly, the amount of nicotine required to activate α7 receptors is very low compared to other α7 receptor studies which usually require a 100 fold higher nicotine concentration for activation. This implies that the α7 may be co-assembled with other nicotinic subunits altering its pharmacology (Khiroug *et al.*, 2002). Activation of α7 nAChRs on the MHN neurons has shown that glutamate release is due to an influx of Ca^{2+} into the synaptic bouton. However, α7 receptors are able to cause this release without depolarization of the bouton and activation of voltage-gated calcium channels, but by Ca^{2+} flow directly through neuronal nACh heteroreceptors on the axon into the synaptic bouton (see Dajas-Bailador & Wonnacott, 2004; Girod *et al.*, 2000; Seguela *et al.*, 1993).

Nicotine-induced glutamate release has been observed in many other areas of the brain, including the rat cerebellum which showed an increase in post-synaptic currents and activation of somatic currents when ACh was applied. This was shown to be due to

glutamate release when the ACh-induced post-synaptic currents were completely blocked by glutamate antagonists. The somatic currents were shown to be mediated by non-α7 receptor activation, due to MLA having no effect (De *et al.*, 2001) but were blocked by the α4β2 antagonist, DhβE, indicating α4β2 nACh receptors are involved, whereas postsynaptic currents are induced by α7 receptor activation as MLA and α-BgT both blocked ACh-induced glutamate release. Similar to the MHN-IPN nucleus, the ACh-induced release was inhibited by TTX (TTX-sensitive) suggesting that the nicotinic receptors are located on the terminal (De *et al.*, 2001; Girod *et al.*, 2000). In the olfactory bulb and amygdala, which both have large cholinergic innervation, nicotinic-induced glutamate release is observed, which is shown to be α-BgT sensitive indicating α7 nAChRs and are resistant to TTX localising the nAChR to the axon (see Chesslet., 1984; see Radcliffe & Dani., 1998).

The hippocampus, which is the centre for learning and memory, responds to nicotine by an increase in glutamate release which is abolished by α7 antagonists α-BgT and MLA and was shown to be inhibited by TTX indicating the α7 receptors are found terminally (Aramakis & Metherate, 1998; see Radcliffe & Dani, 1998). Similar to the MHN-IPN neurons and cerebellum, the nicotine-induced glutamate release was shown to be calcium-dependent, with nicotine directly increasing the presynaptic concentration of Ca^{2+} due to α7 nAChRs being highly permeable to Ca^{2+} (Aramakis & Metherate, 1998; Girod *et al.*, 2000; see Radcliffe & Dani, 1998; Wonnacott *et al.*, 1989). This suggests that the α7 subtype is involved, but it is also believed that other subtypes are also involved due to the high expression of other subunits at these sites (Rubboli *et al.*, 1994a; Seguela *et al.*, 1993; Wada *et al.*, 1989).

1.8. Knockout Studies.

Research into the function of neuronal nAChRs has taken place by the use of electrophysiological and pharmacological studies, which have provided a vast amount of data on the involvement of neuronal nAChRs in a number of processes. Unfortunately,

there have been limitations in the research, due to the lack of sufficient selective agonists and antagonists. However, due to relatively new techniques the function of neuronal nAChR subunits has been possible to study, with the use of knockout mice, whereby nACh receptor subunits are removed or "knocked out" from the mice and observations are made on the development of the mice. This provides a powerful tool in understanding of the role of individual nAChR subunits in the CNS.

The initial work on knockout mice was completed by Thomas & Capecchi in 1987 and since then has helped in understanding the function of receptors. Knockout technology involves embryonic stem cells grown in vitro and genetically modified by the substitution of a non-functional copy of a given gene. By the use of mouse blastocysts, which are injected with the engineered embryonic stem cells, they are re-implanted in a host mother's uterus. Of the resulting chimeric mice, some contain the mutated embryonic stem cells in the germ cell layer allowing the mutation to be passed onto the next generation. The resulting heterozygote mice can then be inter-bred to produce homozygote mutant knockout mice (see Marubio & Changeux, 2000; Wang *et al.*, 2003; Xu *et al.*, 1999b). Below are described studies involving nAChR subtype knockout (KO) mice and the observed effects caused by the KO studies (Table 14).

1.8.1. α3 Knockout studies.

The α3 subunit has been observed to be found extensively in the autonomic system and is expressed in all autonomic ganglia. Expression of the α3 is also observed in the medial habenula; dorsal medulla and retina (Picciotto *et al.*, 2001). Studies have indicated that α3 can combine with either β2 or β4 to form a functional nAChR (Luetje & Patrick, 1991), and that α3 subunit-containing nAChRs are critical for fast-EPSP in the ANS, providing unique physiological and pharmacological properties (Table 14) (see Wang *et al.*, 2002b).

Knockout mice lacking the α3 subunit mostly died during the first week of life, with the remaining dying within the next 6-8 weeks (see Cordero-Erausquin *et al.*, 2000; see Picciotto *et al.*, 2001; see Wang *et al.*, 2002b). The α3 KO mice had a number of deformities, such as retarded growth whereby α3 KO mice had a mean weight approximately 40% of the wild-type (Wt) mice. In addition to this, there were observed abnormalities in the bladder which included; enlarged bladder (or megabladder), bladder infections, dribbling urination and urinary stones. Observations of non-responsive bladder strips to nicotine were also recorded *in vitro* (Xu *et al.*, 1999b). The symptoms caused by the knockout of α3 have been found to be similar to a human autosomal recessive disease called Megacytis-Microcolon-Intestinal-Hypoparistalsis Syndrome (MMHIS), patients suffering from this disease have a mean survival of 3.6 months (see Cordero-Erausquin *et al.*, 2000). Studies looking at these patients discovered that no α3 mRNA was detected in the small bowel, suggesting that the α3 subunit gene mutation may be responsible for the disease.

In addition, KO studies of the α3 subunit caused defects in the visual system of mammals, suggesting that the α3 nAChR subtype may be involved in retinal development (Table 14). Mutant mice have been recorded to suffer from small ocular globes, very widely dilated pupils with no constriction in response to light and reduction in function of the refinement of retinothalamic projections (which occur during early postnatal development). These studies indicated that the impairments observed in α3 KO mice could be due to autonomic ocular innervations.(Champtiaux & Changeux, 2004; see Wang *et al.*, 2002b). Altogether, the α3 subunit has been shown to be an essential component for survival as it is important in the construction of nAChRs in the autonomic ganglia, such as the nAChRs that mediate fast synaptic transmission.

1.8.2. α4 Knockout studies.

Upon α4 KO, there was observed no detected binding sites for [^{3}H]-nicotine, [^{3}H]-epibatidine and [^{3}H]-cytisine in most rat brain regions suggesting that high affinity

binding sites for nicotinic compounds represent nAChRs containing the α4 subunit (see Marubio & Changeux, 2000). Interestingly mutant mice that lack the α4 subunit have been observed to be normal in size, fertility and home cage behaviour when compared to Wt mice (Table 14) (Ross *et al.*, 2000). It could be suggested that the involvement of another subunit, substituting itself for the lack of α4, may be involved and playing the role that α4 would be playing. This was demonstrated when in α4 KO mice high [^{3}H]-nicotine binding is still observed in certain areas of the brain including the interpeducular nucleus (IPN), as well as, high [^{3}H]-epibatidine binding in substantia nigra and superior colliculus, suggesting the presence of another α subunit which can co-assemble with a β subunit and facilitate in the role that α4 should be playing (see Picciotto *et al.*, 2001; Ross *et al.*, 2000), however, there are observed difference between the α4 KO mice and Wt mice.

Previous studies have shown that nicotine acts like an analgesic in Wt mice for both the hot plate test (which tests the primary brain-mediated pain response) and the tail flick test (which tests the primary spinal cord mediated pain response). Studies observing the nicotine-induced analgesia effect in α4 KO mice showed a decrease in nicotine-induced analgesia in the hot plate test and a reduced analgesic response in the tail flick test (Champtiaux & Changeux, 2004; see Picciotto *et al.*, 2001). These studies suggest that α4 may play a role in nicotine induced analgesia (Table 14). Further studies indicated that there was a decrease in [^{3}H]-nicotine and [^{3}H]-epibatidine binding in the thalamus and the raphe magnus (which both play an important role in the nicotinic antinociceptive pathway) in α4 KO mice, as well as a decrease in nicotine-elicited currents in the 5-HT neurons of the raphe magnus and neurons in the thalamus. Altogether, it was indicated that the α4 subunit may be involved with the nicotinic antinociceptive pathway (see Cordero-Erausquin *et al.*, 2000; see Marubio & Changeux, 2000).

In addition, it was demonstrated that α4 KO mice may be a good model for neurodegenerative diseases such as the dopamine neurodegeneration associated with PD. Studies showed that in α4 KO mice, upon acute nicotine treatment there was no protection against methamphetamine-induced neurodegeneration of striatal dopaminergic terminals, unlike in Wt mice (Champtiaux *et al.*, 2003; Champtiaux & Changeux, 2004).

In mice lacking the α4 subunit, the extent of the lesion caused by methamphetamine was similar to that observed in the Wt mice, but unlike Wt mice, it could not be prevented by nicotine (Table 14). This established the α4 subunit to have a role in nAChR neuroprotective effects by nicotine (Champtiaux *et al.*, 2003; Champtiaux & Changeux, 2004; see Picciotto *et al.*, 2001).

Interestingly, α4 KO studies have shown that α4 KO mice are prone to higher levels of anxiety when compared to Wt mice (Ross *et al.*, 2000). Studies have demonstrated that α4 KO mice have been observed to spend less time on the open arms of the elevated-plus maze, as well as making fewer open arm entries when compared with Wt mice, indicating that α4 may be involved in modulating the mammalian stress level (see Picciotto *et al.*, 2001; Ross *et al.*, 2000).

1.8.3. α5 Knockout studies.

Unfortunately, there has been limited study into α5 KO. The α5 has been most extensively studied in the chick, whereupon it was discovered that the α5 is expressed most highly in deep layers of the cortex, SN and VTA, as well as in the autonomic ganglia (see Picciotto *et al.*, 2001; see Wang *et al.*, 2002b).

Studies in to the effects of α5 knockout revealed that α5 KO mice grew to normal size showing no obvious physical or neurological deficits. Compared to the Wt mice, the α5 KO mice are normal in thermoregulation, pupil size and resting heart rate under physiological conditions (see Cordero-Erausquin *et al.*, 2000; Wang *et al.*, 2002a). Studies observing if there were any abnormalities in α5 KO mice compared to Wt mice indicated impairment in cardiac parasympathetic ganglionic transmission during high frequency vagal stimulation. It was noted that Wt mice suffer from cardiac arrest due to high frequency stimulation, whereas α5 KO mice were more resistant (Wang *et al.*, 2002a). Furthermore, the α5 subunit may be involved in the affinity and sensitivity of agonists and antagonists in native receptors, due to observations that α5 KO mice had a

significant increase in ileal contractile responses to nicotinic agonists cytisine and epibatidine. Additional studies showed that in α5 KO mice had an increased sensitivity to low concentrations of hexamethonium, which nearly blocked the bradycardia in response to vagal stimulation. This implies that α5 modulates the pharmacological activity of nAChRs in autonomic ganglia *in vivo* (Wang *et al.*, 2002a). In addition, studies suggested that α5 containing nAChRs are involved in nicotine-induced seizures as α5 KO mice were more resistant to nicotine-induced convulsions (Kedmi *et al.*, 2004).

1.8.4. α7 Knockout studies.

There is limited information about studies involving α7 KO mice. The α7 transcripts are found to be abundant as early as embryonic day (E)6 in the embryonic sympathetic system, and increase until E10 and then have been observed to considerably decrease thereafter (see Marubio & Changeux, 2000). Interestingly, α7 nAChRs have a high permeability to Ca^{2+} and therefore have been suggested to be involved in embryonic development due to possible influences on a variety of calcium-dependent events in neurons, such as second messenger cascades and neuronal survival or apoptosis. In addition, to high permeability to Ca^{2+}, α7 nAChRs are believed to have a fast desensitisation and sensitive blockade of ACh-induced currents by α-BgT (see McGehee & Role, 1995; see Sargent, 1993). Knockout studies have shown, that in comparison to Wt mice, α7 KO mice are found not to contain nicotine-induced fast desensitising currents confirming α7 nAChR are responsible for these responses (see Wang *et al.*, 2002b). Additional studies have suggested that, the high Ca^{2+} permeability exhibited by α7 nAChRs might possibly contribute to neurodegeneration. This is suggested as in the developing chick embryo α-BgT infusions rescued motorneurons from naturally occurring cell death, although no differences were observed between α7 Wt and KO animals in respect to signs of neuronal injury or cell death in the hippocampus (Champtiaux & Changeux, 2004; see Cordero-Erausquin *et al.*, 2000).

Franceschini *et al.* (2000) investigated the role of α7 nAChRs in cardiac autonomic function by measuring baroreflex-mediated responses in α7 KO mice. It was observed that, α7 KO mice, had impaired sympathetic responses to vasodilation which was demonstrated by sodium nitroprusside infusion, causing a 48% heart rate increase in Wt but only a 21% increase observed in α7 KO mice. The α7 KO mice developed supersensitivity to adrenoceptor agonists while baroreflex mediated parasympathetic responses were normal in α7 KO mice. The decreased baroreflex-mediated tachycardia in α7 mutant mice indicates that α7-containing nAChRs participate in the autonomic reflex that maintains blood pressure homeostasis (Franceschini *et al.*, 2000).

Interestingly, knockout studies have suggested that α7 may be involved in regulation of inflammation (Wang *et al.*, 2003). Excessive inflammation is usually prevented by regulation of the magnitude of innate immune responses by highly conserved, endogenous mechanisms. The nervous system, through the vagus nerve, can inhibit significantly and rapidly the release of macrophage TNF and attenuate inflammatory responses. This mechanism, termed the 'cholinergic anti-inflammatory pathway', has many implications in immunology and therapeutics, however the receptor that responds to vagus nerve signals was previously unknown (Wang *et al.*, 2003a). Studies completed with α7 KO mice indicated that electrical stimulation of the vagus nerve inhibits macrophage TNF synthesis in Wt mice but failed to inhibit TNF synthesis in α7 KO mice, suggesting α7 nAChRs are essential for inhibiting cytokine synthesis by the cholinergic anti-inflammatory pathway. Additional knockout studies have also implied that α7 nAChRs may be involved in some aspect of nicotine dependence, which was indicated when Wt mice showed an increase in hyperalgesia upon nicotine withdrawal, whereas α7 knockout mice did not (Grabus *et al.*, 2005).

Including studies of α7 KO mice, studies have taken place whereby 'knockin' α7 subunit Leu247Thr point mutation has been generated, which involves a leucine to threonine mutation in the leucine ring of the M2 region of the α7 nAChR (see Cordero-Erausquin *et al.*, 2000). Studies with knockin α7 mice have shown that it dramatically effects neuronal survival, with homozygous mice for the α7L247T point mutation showing extensive apoptosis throughout the somatosensory cortex and the majority dying within 24 hours of

birth (Champtiaux & Changeux, 2004). Interestingly, knockin α7 nAChR contain an increase in nicotinic agonist affinity and a decrease in the rate of desensitisation in nicotine-elicited currents in hippocampal neurons. These are surprising results as previously observed, α7 KO mice develop normally, whereas α7 knockin mice die within 24 hours (Table 14). This may be an indication that in α7 KO mice additional receptors compensate for the lost.

1.8.5. β2 Knockout studies.

Studies observing the function of β2 subunits in the CNS β2 KO studies have suggested that β2 may be involved in functions such as learning and memory, the reinforcing properties of nicotine and nicotine-mediating antinociception (see Picciotto *et al.*, 2001; Ross *et al.*, 2000).

Nicotine administration has been shown to improve information acquisition and memory retention in rodents and information processing in humans (Table 14) (Levin, 1992). The involvement of the β2 in memory and learning has been examined by the use of β2 KO studies and a test known as the passive avoidance test, which measures an animal's latency to perform a highly probable response (in this case, entry from a well lit chamber in to an adjacent dark chamber) for which it had been previously punished during the training session, (see Marubio & Changeux, 2000) and the effects of β2 KO mice were studied compared to Wt mice. Studies demonstrated that β2 KO mice where significantly different to Wt mice when tested for the retention of an avoidance response 24 hours later. Administration of nicotine increased the latency of entry into the dark chamber in Wt rats but did not change the performance in β2 KO mice These studies suggest that β2 subunits are an important component in mediating the positive effect of nicotine in this test (see Cordero-Erausquin *et al.*, 2000). Observations that β2 subunit KO mice also exhibit an increased latency to the dark compartment suggests that the β2 subunit may mediate effects of endogenous ACh on learning and memory (see Picciotto *et al.*, 2001).

As previously observed with α4 subunit KO, β2 subunits may also play a role in nicotine reinforcement properties (or nicotine addiction). Experiments observing the involvement of β2 subunits in nicotine reinforcement demonstrated that in microdialysis experiments, nicotine elicited a dose-dependent increase in striatal dopamine release in Wt but not in β2 knockout mice. Nicotine also elicited an increase in the discharge frequency of dopamine-containing neurons of the SN and VTA in Wt but not in β2 KO mice (Champtiaux & Changeux, 2004; see Cordero-Erausquin *et al.*, 2000). Furthermore, the effect of β2 KO on nicotine self administration was tested. Wild type and KO mice both demonstrated cocaine self administration during the training session. However, KO mice progressively ceased self administration when the cocaine was switched for nicotine whereas Wt mice continued. This provides more data to the suggestion that β2 nAChR subtypes contribute to the reinforcing properties of nicotine.

Studies have showed that nicotine had antinociceptive effects when measuring mice on the hot plate test or tail-flick test. β2 KO showed that mutant mice had a greatly decrease nicotine-induced analgesia in the hot plate test and a low analgesic response in the tail flick test (Table 14) (Champtiaux & Changeux, 2004; see Picciotto *et al.*, 2001). Further studies looking into β2 involvement in the antinociceptive pathway were carried out, by looking at neurons in the thalamus, raphe magnus and dorsal horn of the spinal cord. Radioligand binding indicated that there was a loss of high affinity [^{3}H]-nicotine binding sites in all these areas after β2 KO. Consistent with these finding, patch clamp recordings from serotonergic neurons in the raphe magnus and neurons in the thalamus demonstrated a loss of nicotine-elicited currents in β2 KO mice suggesting the involvement of these subunits. As previously noted, including β2, α4 is also involved in the antinociceptive pathway and it is suggested that the functional receptor α4β2 is involved in the nicotine pain pathway as disruption of either of these subunits results in attenuation of nicotine antinociceptive effects, indicating the importance of both subunits (see Cordero-Erausquin *et al.*, 2000).

Observations into the cause of degenerative diseases such as PD, AD and dementias have demonstrated that the β2 subunit protects against the impaired cognitive performance associated with aging. β2 KO studies have shown that in two year old animals there is

observed region-specific alternations in cortical structures, which include neocortical hypotrophy, neocortical and hippocampal gliosis, and cell loss in the CA3 hippocampal subfield, as well as, elevated serum corticosterone levels (Champtiaux & Changeux, 2004; see Cordero-Erausquin *et al.*, 2000). Furthermore, learning studies done on Wt and one year old β2 KO mice showed no significant difference in localizing the hidden platform in a water maze, however two year old β2 KO mice learned at a significantly slower rate. This may be possibly due to young animals being able to compensate for the deficit of β2 nAChRs whereas in the adult mouse brain, the deficit reveals the effects of the absence of β2-nAChRs in a spatial learning task. The β2 KO mice have anatomical and behavioural deficits similar to those found in pathological aging, therefore they may serve as a useful model for the study of dementias (Champtiaux & Changeux, 2004; see Marubio & Changeux, 2000).

1.8.6. β4 Knockout studies.

Unlike β2 subunits, β4 nAChR subunits are prominently expressed in the autonomic ganglia (see McGehee & Role, 1995; see Sargent, 1993), with the role of β4, believed to be in ganglionic transmission. The β4 KO mice behave normally, and their autonomic responses, i.e. tachycardia under stress, are apparently intact. As β4 is an integral part of normal ganglionic nicotinic receptors, their lack, must be well compensated for (i.e. β2 replacing β4). However, these new receptors respond differently under some conditions from Wt β4 containing receptors (Wang *et al.*, 2003) (Table 14).

Electrical stimulation of the vagus nerve results in a frequency dependent bradycardia in both Wt and β4 KO mice, although at high rates of stimulation a greater bradycardia was produced in Wt when compared with β4 KO mice (Table 14) (Wang *et al.*, 2003). Additionally, when β4 KO mice were compared to Wt mice they had a significantly higher heart rate (Sack *et al.*, 2005). Further studies have demonstrated that supramaximal stimulation produced cardiac arrest in all Wt mice but in none of the β4 KO mice. In addition to these studies, observations in *in vitro* experiments in the ileal

contractile responses to nicotine, cytisine and DMPP indicated differences between Wt and β4 KO mice. The β4 KO mice had significantly reduced ileal contractile responses to all of these agonists. In addition to the ileal observations, the effects of nicotine on bladder strip contractions indicated reductions in β4 KO compared to Wt mice (Table 14) (Xu *et al.*, 1999b). Whole cell current studies in the superior cervical ganglion neurons reported profound reductions in nicotine-induced currents in β4 KO mice (see Wang *et al.*, 2002b; Wang *et al.*, 2003). These results suggest that β4 deficit results in an impairment of cholinergic conductance in both sympathetic and parasympathetic ganglia. Therefore, it is suggested that β4 subunits are important subunits for normal ganglionic transmission.

Knockout studies involving the β4 subunits suggested that the β4 subunit may play a part in the maintenance of core body temperature. Mice deficient in β4 subunits demonstrated diurnal and nocturnal baseline body temperature significantly lower than those of Wt mice (Table 14) (Sack *et al.*, 2005). In addition, β4 KO mice also demonstrated a reduced nicotine-induced hypothermic response and impaired desensitisation following repeated nicotine exposure, which is believed to be due to the reduction in receptor sensitivity to nicotine, due to the deficiency of the β4 subunit (Sack *et al.*, 2005), which has been shown in other studies (see Wang *et al.*, 2002b; Wang *et al.*, 2003).

1.8.7. β2 and β4 Knockout studies.

Studies involving the knockout of either β2 or β4 subunits have produced mice which are viable and develop normally. Surprisingly, the double KO of β2 and β4 show a severe phenotype which is similar to the mice lacking the α3 subunits. The β2 / β4 double KO mutants survive to birth but have impaired growth and increased postnatal mortality, with death usually occurring during the first 3 weeks of life from severe autonomic dysfunction. Other dysfunctions include no papillary response to light, enlarged bladder with dribbling urination and the development of urinary infections and bladder stones (Xu *et al.*, 1999b). In addition bladder strips from β2/β4 KO mice did not respond to

nicotine (Table 14) (Xu *et al.*, 1999b). Therefore, since mice with a single KO of β2 or β4 subunit do not exhibit a similar phenotype, it is suggested that β subunits play a compensatory role for the absence of the other β subunits (i.e. β2 replacing β4), and that a functional synergy between both β subunits is necessary for survival. The similar effects evoked by α3 KO and β2/β4 KO suggests that α3 can be functional if co-expressed with either β2 or β4 but if both β subunits are lost then α3 cannot form effective ion channels and the α3 containing nAChR are lost (Table 14) (Wang *et al.*, 2002b; Wang *et al.*, 2003)

1.9. Cardiovascular actions of nicotine.

1.9.1. Peripheral cardiovascular actions.

The administration of nicotine intravenously (i.v.) has been performed in the rat, cat, dog and human. Nicotine administered i.v. in both conscious and anaesthetised rats produces a large pressor response followed by either a tachycardia or bradycardia (Aubert *et al.*, 1987; Bisset & Fairhall, 1995; Marano *et al.*, 1999). A dose-dependent, short lasting pressor response was observed with increasing concentrations of nicotine administered i.v. in to conscious rats which was associated with a dose-dependent tachycardia response (Marano *et al.*, 1999). At 3 μg kg^{-1}, nicotine (i.v.) caused a 5 mmHg increase in blood pressure associated with an increase of 10 beats min^{-1} in heart rate. Increasing concentrations of nicotine (10 and 30 μg kg^{-1}) caused an increase in blood pressure of 15 and 25 mmHg respectively, associated with a increase in heart rate of 25 and 65 beats min^{-1} respectively, with the highest dose of nicotine (100 μg kg^{-1}) increasing blood pressure by 65 mmHg and heart rate by 80 beats min^{-1} (Marano *et al.*, 1999). Nicotine i.v. infusion for 15 min, at 20 μg min^{-1}, in conscious rats caused a significant increase in blood pressure of 22 mmHg associated with a significant bradycardia. Upon the rise in blood pressure by nicotine infusion, blood pressure remained stable at this height until nicotine infusion was ceased (Aubert *et al.*, 1987).

Nicotinic AChR subunit	Cellular / morphological basal phenotype	Nicotine-induced behaviour	Survival
α3	ANS defects; megacystis, mydriasis and altered ACh-mediated responses in SCG neurones	ND	Lethal 1-8 weeks postnatal
α4	Loss of high-affinity nic binding, loss of nic-mediated responses to thalamus and raphe magnus.	Reduced nic-elicited antinociception	Adult
α7	Lack of rapidly desensitising nicotinic currents in hippocampal neurons	ND	Adult
α7 Leu247Thr	Slowly desensitising nicotinic currents in hippocampal neurons	ND	Lethal 24 hour post-natal
α9	Abnormal efferent innervation of hair cells in the cochlea	Suppression of cochlear responses	Adult
β2	Loss of high affinity nic binding; loss of nic-induced DA release; accelerated aging; loss of presynaptic receptor activity	Enhanced passive avoidance responses; loss of nic self-admin activity; reduced nic-elicited antinocoception	Adult
β3	Loss of α-conotoxin MII-senistive rec in striatal synaptosomes	Increased locomotor activity; decreased acoustic startle	Adult
β4	Reduced nic-elicited current in SCG neurons	ND	Adult
β2 and β4	ANS defects: enlarged bladder and dilated ocular pupils	ND	Lethal 1-3 weeks post-natal

Table 12. Effects of nicotinic AChR subunit knockout on behaviour and pharmacology of nicotine.

Abbreviations: ACh: acetylcholine; DA: dopamine; Nic: nicotine; SCG: superior cervical ganglion; ND: not determined.

Anaesthetised rats, administered with 100 μg kg^{-1} nicotine, demonstrated a significant rise in blood pressure of 30 mmHg. When compared with nicotine administration in conscious rats, at the same dose, a pressor response was observed to increase to 65 mmHg, indicating that the effects caused by nicotine may be reduced when the animal is under an anaesthetic (Bisset & Fairhall, 1995; Marano *et al.*, 1999).The pressor response observed upon nicotine administration in anaesthetised rats, was not affected when pre-treated the with homomeric neuronal nAChR antagonist α-BgT (400 ng kg^{-1}, i.c.v.) suggesting that the nicotine responses observed are not caused by the activation of central homomeric nicotinic receptors (Bisset & Fairhall, 1995). Other nicotinic agonists tested in anaesthetised rats included cytisine, which upon i.v. administration produced a brief rise in blood pressure followed by a prolonged and significant depressor response (Bisset & Fairhall, 1995).

Experiments performed on anaesthetised cats demonstrated that blowing tobacco smoke into the lungs resulted in an increase in blood pressure, which matched the pressor response produced by nicotine administered i.v.(Armitage & Hall, 1967). The experiment suggested that the nicotine intake per puff of cigarette is 1 – 2 μg kg^{-1}, as this correlated with an increase in blood pressure following i.v. injections of nicotine at that dose. Nicotine administered i.v. in bolus injections was observed to cause a dose dependent increase in blood pressure, similar to experiments preformed on conscious rats, although the rise in blood pressure in the cat is observed to be greater than in rats (Castro de & Rocha E Silva, 1977). At 5, 10 and 20 μg kg^{-1}, a pressor response of 28, 60 and 78 mmHg respectively was observed. Higher doses of 50 and 100 μg kg^{-1} produced large rises in blood pressure of 90 and 145 mmHg respectively, and was associated with a significant tachycardia (Castro de & Rocha E Silva, 1977).

Interestingly, in anaesthetised dogs, nicotine (10 μg kg^{-1}, i.v.) caused a pressor response of 113 mmHg, but only after an initial depressor response of 38 mmHg. The initial depressor response was also observed with other nicotinic agonists such as DMPP, which at 5 μg kg^{-1} produced a depressor response of -41 mmHg followed by a pressor response of 88 mmHg (Domino, 1969). The decrease in sensitivity of nicotine i.v. in anaesthetised rats, compared to conscious rats (Bisset & Fairhall, 1995; Marano *et al.*, 1999), has also

been observed in experiments performed on dogs. Before pentobarbital anaesthesia, nicotine administered i.v., at a dose of 10 μg kg^{-1}, produced a significant depressor - pressor response of -38 and 113 mmHg, respectively. After administration of an anaesthetising dose of pentobarbital (30 mg kg^{-1}, i.v.), the depressor - pressor response caused by nicotine (i.v.) was almost abolished, producing a depressor - pressor response of only -10 mmHg and 20 mmHg, respectively. The nicotinic agonist DMPP was also affected by pentobarbital anaesthesia, with a reduction in the depressor and pressor response from -40 and 88 mmHg respectively, to -15 and 22 mmHg, indicating that the cardiovascular responses to i.v. nicotine and DMPP were markedly reduced by pentobarbital anaesthesia. The effects of anaesthesia on nicotine and DMPP responses were suggested to be due to decreases in sensitivity of the anesthetised animal to the agonists, as increasing doses of nicotine (20 and 40 μg kg^{-1}) showed increasing depressor-pressor responses of -19 to 25 mmHg and -42 to 64 mmHg respectively, and increasing doses of DMPP (10 and 20 μg kg^{-1}) progressively restored the depressor-pressor response to levels similar to those prior to pentobarbital anaesthesia (Domino, 1969).

The effect of nicotine i.v. has also been studied in human patients. Two concentrations of nicotine were investigated in conscious humans. At 0.25 μg kg^{-1} min^{-1} nicotine caused a rise in blood pressure but it was only at 0.5 μg kg^{-1} min^{-1} that a significant increase in systolic blood pressure was observed (Newhouse *et al.*, 1990).

The administration of nicotine i.v. has shown varied results, but it has been noted that the cardiovascular response to nicotine is complex. Thus, the classic response of elevated blood pressure and tachycardia observed after inhalation of nicotine into cats (Armitage & Hall, 1967) or after i.v. administration in to the rat, cat or dog (Bisset & Fairhall, 1995; Castro de & Rocha E Silva, 1977; Domino, 1969) was suggested to be the result of several different and opposing effects of nicotine on the sympathetic and parasympathetic system. As well as nicotine having opposing effects, changes in nicotine's effects on the cardiovascular system were also observed by the use of different general anaesthetics, spinal anaesthetics and comparisons between conscious and unconscious species (Bisset & Fairhall, 1995; Domino, 1969; Marano *et al.*, 1999). Due to species variance

conflicting results have been produced; for example in the dog, a rise in blood pressure was observed, upon i.v. injection of nicotine, although produced after an initial depressor response (Domino, 1969), which is not observed in cat or rat studies (Castro de & Rocha E Silva, 1977; Marano *et al.*, 1999). Differences have been observed in the same species, e.g. in the rat, nicotine (i.v.) administration was observed to cause a pressor response, associated with a tachycardia in conscious unrestrained rats (Marano *et al.*, 1999), but in conscious restrained rats, nicotine was shown to have a pressor response associated with a bradycardia (Aubert *et al.*, 1987), therefore indicating that changes in conditions, i.e. restrained or free moving models, can effect the cardiovascular effects produced by nicotine.

The pressor response of nicotine (i.v.) and its modes of action have been extensively researched. Two mechanisms that are believed to cause these effects have been suggested; first, by nicotine-mediated vasopressin release. Vasopressin or anti-diuretic hormone, as it is also known, has been shown to be synthesised in the neurons of the supraoptic and paraventricular nuclei (SON and PVN) and is released from the pituitary gland (see Holmes *et al.*, 2003; see Howl & Wheatley, 1995; see Jard, 1998). Vasopressin, as well as retaining water by acting on vasopressin receptors found in the kidney, acts on V_{1a} vasopressin receptors found on blood vessels and upon activation by vasopressin results in vasoconstriction. Nicotine is believed to cause the release of vasopressin, which results in vasoconstriction and the pressor responses observed. Intravenous injections of nicotine have been demonstrated to cause the release of vasopressin in cats (Castro de & Rocha E Silva, 1977) and rats (Aubert *et al.*, 1987; Bisset & Fairhall, 1995). Although this is a possibility, it has been shown that when nicotine is in the presence of a V_{1a} vasopressin antagonist (i.v.) there was no change in the pressor response when compared to nicotine-alone (Aubert *et al.*, 1987; Marano *et al.*, 1999). This suggests two possibilities; one, that vasopressin release, caused by nicotine, is having no effect on blood pressure, or two, that nicotine-induced vasopressin release, could be acting via a different mechanism, i.e. vasopressin acting centrally, not peripherally. Another mechanism in which nicotine may cause the pressor response is by sympathoexcitation. Although it has been well documented that sympatho-adrenergic activation is involved in the nicotine-mediated blood pressure pressor response (Milton &

Paterson, 1974; Wigoda *et al.*, 1995), it was Marano *et al.* (1999) who demonstrated, by the use of chemical sympathectomy (which destroys post ganglionic sympathetic nerve endings but not the adrenal medulla), and α-adrenoceptor antagonists, that nicotine i.v. evokes the pressor response via sympathetic nerve endings and not the adrenal medulla. It was observed that the nicotine-induced blood pressure rise was inhibited in the presence of an α-adrenoceptor antagonist, although it had no effect on the tachycardia, indicating that the nicotine-induced pressor response is due to α-adrenergic-mediated vasoconstriction and the nicotine-induced tachycardia alone is unable to raise blood pressure, suggesting that the tachycardia effects of nicotine are not obligatory for the occurrence of the blood pressure rise. How the blood pressure rise was caused by nicotine-induced sympathoexcitation is not fully understood, although studies have indicated that central nicotinic receptors are capable of stimulating sympathetic cardiovascular neurons (Buccafusco & Yang, 1993; Khan *et al.*, 1994), Therefore, theoretically nicotine-activated neuronal nAChRs could cause sympathetic activation, by stimulating the release of NA, which binds to vascular α-adrenoceptors resulting in vasoconstriction of blood vessels and a rise in blood pressure, causing the observed effects of i.v. administration of nicotine on the cardiovascular system.

1.9.2. Central actions of nicotine.

1.9.2.1. I.c.v., i.c. and topical administration of nicotine.

Intracerebroventricular (i.c.v.) injections through the cerebral ventricles, allows the injected compound to have access to many structures that form the inner and outer surface of the brain, therefore it is difficult to localize anatomically the compounds' pharmacological actions. However, this approach has been used extensively to establish the cardiovascular effect of nicotine in the CNS.

Nicotine administered by i.c.v. injections into the lateral ventricle of anaesthetised rats produced a large rise in blood pressure, of over 40 mmHg which was observed to be a greater increase than the muscarinic receptor agonist, carbachol (Bisset & Chowdrey, 1984). The rise in blood pressure produced by nicotine (100 μg kg^{-1}, i.c.v.) was completely inhibited when in the presence of the nicotinic antagonist, hexamethonium (2.5 μg kg^{-1}, i.c.v.). In conscious rats, i.c.v. injections of nicotine (10 μg kg^{-1}, i.c.v.) caused a small, but significant, increase in blood pressure, of up to 10 mmHg (Iitake *et al.*, 1986). Nicotine at doses 0.1, 1 and 10 μg kg^{-1} (i.c.v.) were observed to have a significant decrease in heart rate, with 10 μg kg^{-1} shown to cause a bradycardia of up to 40 beats min^{-1} (Hoffman, 1979; Iitake *et al.*, 1986). Pre-treatment with the nicotinic antagonist, hexamethonium (10 μg kg^{-1}, i.c.v.) was shown to block the pressor and bradycardia effects of nicotine (Hoffman, 1979 ;Iitake *et al.*, 1986).
Intracerebroventricular injection of choline, a precursor of ACh, in anaesthetised rats caused a dose-related increase in blood pressure. Choline at doses 50, 100 and 150 μg kg^{-1} caused an increase in blood pressure ranging from 10 - 20 mmHg, with the responses peaking within 3 - 4 mins after injection and returning to baseline within 15 mins (Li & Buccafusco, 2004). Choline (50 and 150 μg kg^{-1}, i.c.v.) administered in conscious rats, produced similar results to anaesthetised rats with 50 μg kg^{-1} causing an increase in blood pressure of 10 mmHg and 16 mmHg for 150 μg kg^{-1} (Savci *et al.*, 2002). In both anaesthetised and conscious rats, choline's effects at 150 μg kg^{-1} were blocked by pre-treatment with the nicotinic antagonists, mecamylamine and α-BgT, but not by the muscarinic antagonist, atropine, suggesting that choline is acting on the nicotinic receptor and not the muscarinic receptor (Li & Buccafusco, 2004; Savci *et al.*, 2002). Choline is believed to be a selective endogenous agonist for the α7 neuronal nAChR, when studied in the presence of the α7 selective antagonist, MLA, the increase in blood pressure produced by choline was abolished. Observations that the α4β2 antagonist, DhβE, failed to significantly inhibit the pressor response produced by 150 μg kg^{-1} choline (Li & Buccafusco, 2004), suggest that choline was activating the α7 nAChR, rather than the α4β2 nAChR.

Interestingly, i.c.v. injections of nicotine (10 μg kg^{-1}) in anaesthetised cats, caused a slight increase in blood pressure followed by a slight decrease in blood pressure. When

the dose of nicotine was increased to 100 μg kg^{-1}, the slight increase in blood pressure was followed by a larger depressor response than that seen at 10 μg kg^{-1} (Schaeppi, 1968). Additional studies demonstrated that nicotine (0.1 & 1 mg kg^{-1}, i.c.v.) produced a pressor response of around 10 mmHg for both concentrations and was followed by a depressor response of around 30 - 40 mmHg (Bisset *et al.*, 1975; Bisset & Feldberg, 1973). Nicotine and nicotinic agonists when administered centrally by i.c.v. injections were observed to cause a rise in blood pressure associated with a bradycardia in both conscious and anaesthetised rats (Bisset & Chowdrey, 1984; Bisset & Fairhall, 1995; Iitake *et al.*, 1986; Li & Buccafusco, 2004; Savci *et al.*, 2002). These effects are associated with an elevation in vasopressin release suggesting that nicotine may cause this release of vasopressin by acting at sites reached from the ventricles, possibly the PVN and SON which have been shown to contain neuronal nAChR (Hatton & Yang, 2002; Zaninetti *et al.*, 2000). The involvement of nAChRs in vasopressin release is demonstrated when the nicotine-induced release of vasopressin, as well as the increase in blood pressure, is inhibited by nicotinic antagonist hexamethonium (Bisset & Chowdrey, 1984; Iitake *et al.*, 1986). Additional nicotinic agonist, such as cytisine, and choline, both caused the release of vasopressin which is inhibited by a nicotinic, but not a muscarinic, antagonist (Bisset & Fairhall, 1995; Li & Buccafusco, 2004; Savci *et al.*, 2002). This release of vasopressin was believed to be due to nicotine acting directly on the SON, due to demonstrations that the SON contains neuronal nAChRs (Hatton & Yang, 2002; Zaninetti *et al.*, 2000) and direct application of nicotine causes, a increase in rate of firing on the supraopticohypophysial tract (Castro de & Rocha E Silva, 1977), as well as nicotine microinjection into the SON resulting in the increase in vasopressin release (Ota *et al.*, 1992). Bisset *et al.* (1975) demonstrated that although there was a increase in vasopressin release when nicotine was administered by i.c.v. injection, the release was inhibited when nicotine was prevented access to the fourth ventricle suggesting that nicotine may not be acting directly on the PVN and SON to cause the release of vasopressin (Bisset *et al.*, 1975; Castro de & Rocha E Silva, 1977). The mechanism of action of the nicotine-induced rise in blood pressure, caused by i.c.v. injection, is not specifically known and may involve pathways innervating the hypothalamus or other cholinergic pathways, although conflicting data make it difficult to interpret.

The decrease in blood pressure caused by i.c.v. nicotine, in anaesthetised cats, was observed to be similar to nicotine injected intracisternally in anaesthetised rats (Bisset & Chowdrey, 1984). Intracisternal (i.c.) injections are injections in to the cisterna magna, from this site injected compounds are able to move around the subarachnoid space and come into contact with brain areas such as the brain stem. Nicotine (2-8 $\mu g\ kg^{-1}$, i.c.) was observed to cause a decrease in blood pressure in anaesthetised rats, which was blocked by nicotinic antagonist hexamethonium, but not by the muscarinic receptor antagonist atropine (Bisset & Chowdrey, 1984), suggesting that the decrease in blood pressure is due to nicotine acting on nAChR in cardiovascular regulating areas in the brain stem.

Although intravertebrae (i.vert) injections are not directly injected into the brain like i.c.v. and i.c., it was observed, by the use of a dye, that injections into the vertebral artery results in the compound coming into contact with the brainstem especially areas in the lower brain stem such as the medulla and pons (Castro de & Rocha E Silva, 1977; Schaeppi, 1968). Anaesthetised cats injected with increasing concentrations of nicotine (0.1, 1, 10, 100 $\mu g\ kg^{-1}$) into the vertebrae artery, showed similar results to nicotine given i.c.v in cats. At 0.1 $\mu g\ kg^{-1}$, nicotine given by i.vert injection caused a slight depressor response in blood pressure. At 1$\mu g\ kg^{-1}$, nicotine caused a transient increase in blood pressure which then continued to become a slight decrease, this was also observed at 10 $\mu g\ kg^{-1}$, although the biphasic pattern was still a transient increase in blood pressure, it was followed by a substantial decrease in blood pressure, which lasted as long as 15 min. At 100 $\mu g\ kg^{-1}$, nicotine produced a moderate increase in blood pressure followed by a large depressor response (Schaeppi, 1968). Additional studies observing the increasing effects of nicotine (2.5, 10, 20 and 50 $\mu g\ kg^{-1}$, i.vert) in anaesthetised cats, had mixed results. At lower doses of 2.5 and 10 $\mu g\ kg^{-1}$, nicotine produced a dose-dependent fall in blood pressure. The onset of the decrease in blood pressure at these concentrations was rapid but the time needed for the blood pressure to return to normal was slow, requiring around 10-20 min (Castro de & Rocha E Silva, 1977). At the higher doses of 20 and 50 $\mu g\ kg^{-1}$, nicotine produced varying results. Over half of the experiments showed a depressor response, whereas an increase in blood pressure was observed with the remaining animals. Heart rate was observed to significantly decrease using nicotine concentrations from 10-50 $\mu g\ kg^{-1}$ but these decreases were only observed to be small,

with 50 μg kg^{-1} nicotine causing a drop of less than 20 beats min^{-1} (Castro de & Rocha E Silva, 1977).

To determine if the depressor effects produced by nicotine injected by i.c. and i.vert involved the brainstem, experiments were carried out studying the effects of topical application of nicotine to the ventral surface of the brainstem (Bisset & Chowdrey, 1984; Feldberg & Guertzenstein, 1976). These experiments involved the use of paired perspex rings attached to a holder. Upon exposure of the ventral surface of the brainstem, the paired perspex rings are placed across the medulla each at the same distance from the midline and compounds are placed inside each ring. The rings are moved along the ventral surface of the medulla in longitudinal and lateral directions, which allows the effects of applying a drug to different areas of the surface to be investigated. In order to determine the areas on the ventral surface which have been covered by the nicotine solution placed in the paired rings, they are filled at the end of the experiment with dye and the stained areas on the ventral surface are measured with a divider, using as references the caudal border of the trapezoid and the midline. Nicotine applied topically to the ventral surface of the brainstem of the cat, induced no significant change in blood pressure when the uppermost limit of the oval areas were just caudal to the trapezoid bodies (Bisset & Chowdrey, 1984; Feldberg & Guertzenstein, 1976). As the perspex rings were moved away from the trapezoid bodies, a fall in blood pressure was observed. Depressor responses were seen when rings were placed more caudally, and the uppermost limits of the oval areas were about 6 mm caudal to the trapezoid bodies, in this area application of nicotine resulted in a depressor response of 38 mmHg (Bisset *et al.*, 1975). Movement of the perspex rings by a further 0.5 mm caudal to the trapezoid bodies caused a further increase in depressor response, to -90 mmHg. Application of nicotine further down the brainstem to a position of 8.5 and 9 mm caudal to the trapezoid bodies resulted in a depressor response of -74 and -40 mmHg, respectively. Further movement of perspex rings away from the nicotine sensitive area (6-6.5 mm caudal to the trapezoid bodies), resulted in depressor responses but which were reduced in size (Bisset *et al.*, 1975). Additional experiments observing the effect of topical application of nicotine to the ventral surface of the brainstem, by the use of perspex rings, suggested that the depressor response produced by the application of nicotine were most sensitive at 5-6 mm

caudal to the trapezoid bodies (Feldberg & Guertzenstein, 1976). Unilateral application of nicotine to this area produced a depressor response of 15 mmHg at a concentration of 0.33 mg ml^{-1} (a threshold concentration). Increasing concentrations of nicotine (0.5, 1, 2 and 4 mg ml^{-1}) bilaterally to the perspex rings produced a dose-related decrease in blood pressure. Blood pressure fell more steeply with each increase in the nicotine concentration, with 2 mg ml^{-1} causing a depressor response of -40 mmHg. Upon application of nicotine at concentrations of 4 and 8 mg/ml, did not lower the blood pressure any further than the dose of 2 mg ml^{-1} (Feldberg & Guertzenstein, 1976). The depressor response was rapid with a fall observed 5-18 seconds after application and full recovery of blood pressure following 4 mg ml^{-1} nicotine was 30 - 40 min, indicating a slow recovery (Feldberg & Guertzenstein, 1976). Application of the nicotinic antagonist, hexamethonium, both during nicotine recovery and prior to nicotine administration blocked the nicotine-induced depressor response, whereas the muscarinic antagonist, atropine (Feldberg & Guertzenstein, 1976). By the use of unilateral application of 2 mg ml^{-1} nicotine, using only one ring, the localization of the nicotine sensitive areas were determined. Although there was variation from cat to cat and therefore some overlapping, it was suggested that the maximum response to nicotine was obtained from the region around the rootlets of the XII^{th} cranial nerve (Bisset *et al.*, 1975; Feldberg & Guertzenstein, 1976).

The nicotine-induced fall in blood pressure, which was administered onto or near the brainstem, was suggested to be due to carotid sinus nerve activity; this is due to the similarity observed between carotid sinus nerve stimulation, which causes a decrease in blood pressure which is converted to a rise when given an overdose of pentobarbitone, and a fall in blood pressure caused by nicotine which also converted to a rise by pentobarbitone (Douglas *et al.*, 1950; Katz & Thesleff, 1957a). The rise in blood pressure caused by nicotine administration by i.c.v. injection could be caused by stimulation of the chemosensory pressor fibres in the carotid sinus and aortic nerves in the region of the brainstem, whereas the nicotine-induced decrease in blood pressure could result from stimulation of barosensory depressor fibres (Bisset *et al.*, 1975; Katz & Thesleff, 1957a), therefore nicotine could be affecting sympathetic tone by indirect action on the vasomotor centre. With the help of microinjections of nicotine and nicotinic

agonists into specific areas of the brain, the effects of these compounds could be observed with more accuracy.

The release of vasopressin upon nicotine administration, by i.c.v., i.c. injection and topically to the ventral surface of the brainstem, has been demonstrated to be caused by nAChR, due to inhibition by nicotinic antagonists. It has been suggested that the medullary areas is the cause for nicotine-induced vasopressin release (Bisset *et al.*, 1975; Castro de & Rocha E Silva, 1977) rather than the forebrain area, as vasopressin release induced by nicotine administered by i.c.v. can be inhibited when the nicotine is prevented from reaching the brainstem (Bisset *et al.*, 1975). Including topical and i.c. administration of nicotine, i.vert injection of nicotine cause vasopressin release, again, with the nicotine observed to reach the lower brainstem especially the medulla and pons. Observations that topical and i.vert nicotine administration do not go near the SON or PVN, (indicating by dye that i.vert injected nicotine remains in the brainstem area and topical administration remains in the area applied) suggests that nicotine does not act on the neurosecretory cell directly, but is stimulating vasopressin release by activation of some other pathway, i.e. upon application of nicotine to the medulla. However, Bisset *et al.* (1975) noted that there was no correlation between the decrease in blood pressure and the release of vasopressin and especially since vasopressin release could still be obtained after the sinus and vagus nerves were cut, therefore it was suggested that nicotine-induced fall in blood pressure may not due to vasopressin release (Bisset *et al.*, 1975; Castro de & Rocha E Silva, 1977).

1.9.2.2. Nucleus Tractus Solitarius.

The nucleus tractus solitarius (NTS) is an elongated nucleus that extends throughout the dorsomedial part of the medulla oblongata from the level of the caudal edge of the facial motor nucleus to the cervical spinal cord. The NTS receives baroreceptor afferents and plays a central role in mediating the reflexes and regulating cardiovascular responses (section 1.1.) (see Sun, 1995). Areas of the NTS receiving baroreceptor and

chemoreceptor afferent projections, as well as pulmonary stretch receptors and atrial ventricular receptors which terminate in the NTS, have neuronal projections which innervate levels of the ventrolateral medulla such as the rostral and caudal ventrolateral medulla, evoking baroreceptor reflexes (Anand *et al.*, 1993; see Dampney, 1994). It is from the NTS that the caudal ventrolateral medulla (CVLM) neurons are innervated by glutamatergic pathways, which then project to the rostral ventrolateral medulla (RVLM) by GABAergic pathways (an inhibitory projection) (Agarwal *et al.*, 1990; Cravo *et al.*, 1991; Jeske *et al.*, 1993). From the RVLM, neurons project on to the immediolateral cell column of the spinal cord, which helps in maintaining normal resting blood pressure (Brown & Guyenet, 1984; Ross *et al.*, 1985). In addition to projections to the CVLM, the NTS has been shown to project to the lateral hypothalamus, some forebrain nuclei, the dorsal motor nucleus of vagus, the ambiguus nucleus, the area postrema and immediolateral cell column of the spinal cord (section 1.1.2.1.) (see Dampney, 1994; see Sun, 1995).

The NTS has been shown to contain ChAT immunoreactive cholinergic neurons in the region that receives baroreceptor afferent fibres (Armstrong *et al.*, 1988; Ruggiero *et al.*, 1990; Winzer-Serhan & Leslie, 1997), suggesting that ACh may have some involvement in the baroreflex and that the NTS may also be rich in cholinergic nerve terminals (Ruggiero *et al.*, 1990). Additional studies showed that ChAT activity in the NTS was decreased by removal of the nodose ganglion, which contains cholinergic cell bodies, thus implying that certain afferent nerve terminals have the ability to release ACh (Shiraki *et al.*, 1997). Radioligand binding studies using [^{3}H]-nicotine and [^{125}I]-α BgT have also demonstrated that nicotinic binding sites are present in the NTS of rats, cats and humans (Adem *et al.*, 1989; Breese *et al.*, 1997; Clarke *et al.*, 1985; Maley & Seybold, 1993; Wada *et al.*, 1989), with binding observed in the areas of the NTS where cardiovascular and respiratory afferent fibres terminate (Maley & Seybold, 1993), although there is observed a higher binding affinity for [^{125}I]-α-BgT in cats than [^{3}H]-nicotine (Maley & Seybold, 1993).

Due to the importance of the NTS in cardiovascular regulation, the effects of nicotine on the NTS have been examined by microinjection. Microinjections of nicotine into the

NTS of anaesthetised rats has been shown to produce a dose-dependent decrease in blood pressure (Dhar *et al.*, 2000; Ferreira *et al.*, 2000; Tseng *et al.*, 1993; 1994), with rapid effects observed immediately after microinjection, with the maximum effect occurring 29-36 sec after injection and a duration from 5-8 min (Ferreira *et al.*, 2000). The effect on heart rate was similar to blood pressure, with increasing concentrations causing significant decreases. Dhar *et al.* (2000) demonstrated that at 0.5 μM, nicotine microinjected in to the NTS resulted in a fall of around -45 mmHg. However, nicotine microinjected into the medial NTS (mNTS) caused a greater decrease in blood pressure, at considerably lower doses, with 0.1 pmol kg^{-1} causing a 10 mmHg decrease, with 1 and 1000 pmol kg^{-1} resulting in a 28 mmHg and over 40 mmHg decrease in blood pressure, respectively (Ferreira, Jr. *et al.*, 2002).

Pre-treatment with homomeric nicotinic antagonist α-BgT (100 pmol kg^{-1}) had no effect on nicotine-induced blood pressure changes in NTS (Ferreira *et al.*, 2000;Tseng *et al.*, 1994), however, at higher concentrations of α-BgT (1 μM), nicotine was observed to have a small decrease (20%) in blood pressure of -21 ± 3 mmHg (Whiteaker *et al.*, 1998). When the concentration of α-BgT was increased to 250 and 500 μM, there was no further decrease in nicotine-induced depressor responses (Dhar *et al.*, 2000). Pre-treatment with the α4β2 antagonist, DhβE, was shown to have no effect on the nicotine-induced depressor response at 1 pmol kg^{-1}. However, at concentrations of 10 and 100 pmol kg^{-1} there was a ~30 % inhibition in the depressor response, whereas at 1000 pmol kg^{-1}, an inhibition of nearly 70 %, in the nicotine-induced decrease in blood pressure was recorded (Ferreira, Jr. *et al.*, 2002). The neuronal nAChR selective antagonist, α-conotoxin ImI, which is selective for the α7 nACh receptor at 1 μM, was observed to significantly decrease the nicotine-induced depressor response by 38% (Dhar *et al.*, 2000), in addition, the α7 selective antagonist, MLA, caused a significant inhibition of the depressor response (Ferreira *et al.*, 2001). Studies using the α7 antagonists, α-BgT, α-conotoxin ImI and MLA suggest that only a small proportion of nAChR containing α7 are involved in mediating the depressor responses induced by microinjection of nicotine into the mNTS, but indicated that there are also α4β2 nAChRs present (Dhar *et al.*, 2000; Ferreira *et al.*, 2000; 2001). Neuronal nAChR selective antagonists α-conotoxin AulB and hexamethonium (1 μM) are selective to α3β4 nAChRs and caused significant

decreases in nicotine-induced depressor responses, with α-conotoxin AulB causing a 79% decrease, which remained similar even when the concentration was increased to 100 μM, causing a 74% reduction. Mecamylamine caused a reduction in the nicotine-induced depressor response of 69% and hexamethonium cause a 47% decrease in depressor response (Dhar *et al.*, 2000). The large inhibition of depressor response produced by α3β4 antagonists suggests a relatively high proportion of α3β4 nAChRs are present, which are involved in mediating depressor responses induced by microinjection of nicotine into the NTS. Studies demonstrating that α7, α4β2 and α3β4 antagonists cause inhibition of the nicotine-induced affects, suggest that there are more that one type of nAChR present. This was confirmed when the α7 antagonist, α-BgT, and α3β4 antagonist, mecamylamine, were added together and caused the nicotine-induced depressor effect to be almost completely blocked with around 98.2 % inhibition (Dhar *et al.*, 2000). Thus it was concluded that the predominant type of nAChR in the NTS is α3β4, with a smaller proportions of α7 and α4β2 nAChRs present.

Studies into whether neuronal nACh heteroreceptors may cause the release of glutamate in the mNTS (the neurotransmitter of vagal afferent nerve terminals in the NTS is believed to be glutamate), resulting in the depressor response, were demonstrated by nicotine microinjectioned in to the NTS, pre-treated with the glutamate receptor antagonist kynurenic acid. It was observed that the glutamate receptor antagonist did not inhibit the nicotine-induced decrease in blood pressure (Ferreira, Jr. *et al.*, 2002; Mosqueda-Garcia *et al.*, 1991), suggesting that nicotine is not acting on neuronal nACh heteroreceptors causing the release of glutamate, which results in the decrease in blood pressure.

Nicotine has been suggested to cause the depressor response in blood pressure by activation of nAChRs on the NTS, which evokes excitation of secondary NTS neurons that, in turn, excite neurons located in the CVLM. Excitation of the CVLM neurons results in inhibition of neurons in the RVLM, via a GABAnergic mechanism which decreases the excitatory input to sympathetic preganglionic neurons located in the immediolateral cell column of the spinal cord (ILM) and induces a depressor response. This was suggested due to the nicotine-induced depressor response being unaffected by

cervical vagotomy (Ferreira *et al.*, 2000; Ferreira, Jr. *et al.*, 2002) and therefore is presumably due to inhibition of sympathetic nervous system activity.

1.9.2.3. The Ventrolateral Medulla

Studies observing the effects of blood pressure control showed that the ventrolateral medulla was an essential component in blood pressure maintenance and reflex cardiovascular control (Feldberg & Guertzenstein, 1976; 1986; Guertzenstein & Lopes, 1984). The ventrolateral medulla was observed to contain two distinct areas, the rostral ventrolateral medulla (RVLM), which was demonstrated to be responsible for maintaining tonic excitation of sympathetic preganglionic neurons (Calaresu & Yardley, 1988; Guertzenstein & Silver, 1974), and the caudal ventrolateral medulla (CVLM), which was demonstrated to be a depressor area involved in reflex regulation of blood pressure (Feldberg & Guertzenstein, 1976; Guertzenstein & Lopes, 1984).

1.9.2.3.1. The Rostral Ventrolateral Medulla.

The rostral ventrolateral medulla (RVLM) is important in the regulation of the cardiovascular system (see section 1.1). This was demonstrated over a century ago when experiments performed in rabbits showed a profound fall in blood pressure upon bilateral destruction of a portion of the ventral medulla, near the level of the facial nucleus, but not by the destruction of a large dorsal portion of medulla oblongata (see Dampney, 1994). The RVLM is a pressor area and one of the sympathetic premotor neurons (Amendt *et al.*, 1978), which extends (in the rat) from 1 to 2.5 mm rostral to the median rootlet of the XII nerve and 1.5-2 mm lateral to the midline (Benarroch *et al.*, 1986). The RVLM vasomotor neurons, which are located immediately caudal to the posterior end of the facial motor nucleus and ventral to the nucleus ambiguus complex (see Sun, 1995), receive afferent input from the lateral tegmental field and the CVLM, and were shown to

be a major vasomotor area which projects pre-ganglionic sympathetic fibres directly to the immediolateral cell column of the spinal cord (see section 1.1).

Studies into the role of the RVLM in cardiovascular regulation demonstrated that electrical stimulation produced an increase in blood pressure and heart rate, whilst, chemical stimulation, by microinjections of glutamate, caused an increase in sympathetic nerve activity and blood pressure (Ross *et al.*, 1984). Additional studies demonstrated that chemical inactivation, by inhibitory amino acids, such as glycine and GABA, and electrolytic lesions of the RVLM, caused a decrease in blood pressure and an immediate decrease in sympathetic nerve activity (Anand *et al.*, 1993; Dampney & Moon, 1980; Granata *et al.*, 1983; Guertzenstein, 1973; Guertzenstein & Silver, 1974) suggesting that the RVLM is essential for the maintenance of the basal sympathetic vasomotor tone.

RVLM-spinal vasomotor neurons function as an essential relay, transferring signals and commands to the sympathetic preganglionic neurons with blockade of this transmission abolishing sympathetic and cardiovascular responses (see Dampney, 1994; Ross *et al.*, 1984). Glutamate and glutamatergic receptors are known to be involved (Campos & McAllen, 1999; Dampney *et al.*, 1982). Studies have also demonstrated nicotine's effects at sites close to the rootlet of the XII^{th} cranial nerve and nicotinic receptor involvement in glutamate release, therefore it has also been suggested that neuronal nAChRs may participate in the regulation of the cardiovascular system by acting at the RVLM. Indeed, microinjection studies injecting nicotine into the RVLM, produced a dose-related and long lasting increase in blood pressure (Tseng *et al.*, 1994), additional studies also demonstrated a long lasting pressor response and tachycardia (Tseng *et al.*, 1993). The pressor effects of nicotine were completely inhibited by prior microinjections of hexamethonium, but were not affected by the muscarinic antagonist, atropine, (Tseng *et al.*, 1994). Studies also demonstrated that pre-treatment with the α and β adrenoceptor antagonist, labetalol, caused up to a 96% decrease in the nicotine-induced pressor response in Spaque Dawley (SD) rats, whilst the, α_1-adrenoceptor antagonist, prazosin, inhibited the nicotine pressor response by 44% in SD rats (Tseng *et al.*, 1993). The inhibition of the nicotine-induced pressor response, by the use of adrenoceptor antagonists suggests that the pressor response may be caused by adrenergic pathways in

the RVLM acting on the sympathetic nerves, which in turn, stimulate receptors on the blood vessels resulting in vasoconstriction. Therefore, studies suggest that nicotine microinjections within the RVLM cause activation of neuronal nAChRs, which results in an increase in blood pressure and renal sympathetic nerve activity (Tseng *et al.*, 1993). Interestingly, it was observed that the pressor effect of nicotine microinjected into the RVLM was more pronounced in spontaneously hypertensive rats (SHR) than in SD rats and Wistar (WKY) rats suggesting that enhanced cardiovascular responses of the RVLM neurons to nicotine might contribute to hypertension. However, it has been shown that hypertensive animals generally show exaggerated cardiovascular responses to reflex and centrally evoked pressor responses (Chan *et al.*, 1990), probably due to damaged integration by the control system.

1.9.2.3.2. The Caudal Ventrolateral Medulla.

The caudal ventrolateral medulla (CVLM) is found between the nucleus ambiguus and lateral reticular nucleus (see Dampney, 1994; see Sun, 1995) and was first demonstrated to have an affect on cardiovascular function when application of nicotine to the ventral surface in the cat caused a decrease in blood pressure (Feldberg & Guertzenstein, 1976). Additional studies have shown that electrolytic lesions or chemical inactivation, by the GABA receptor antagonist, muscimol, in the CVLM resulted in a rise in blood pressure, whereas electrical stimulation and chemical stimulation, by the excitatory amino acid glutamate, in the CVLM produced sympathoinhibition and a fall in blood pressure (Cravo *et al.*, 1991; Gordon, 1987; Jeske *et al.*, 1993). The A1 area of the CVLM has been proposed to be the location of the CVLM sympathoinhibitory neurons, as lesions cause an increase in blood pressure, whereas electrical or chemical stimulation of the CVLM area causes a depressor response (Ross *et al.*, 1985), however more precise mapping of the depressor sites in the CVLM of the rat has indicated that the CVLM sympathoinhibitory neurons are located outside the A1 cell group (see Dampney, 1994). Interestingly, it has been observed that there is an inhibitory GABAergic synapse from the sympathoinhibitory pathway in the CVLM depressor neurons, to the RVLM

sympathetic premotor neurons (Cravo *et al.*, 1991). It was suggested that it is by this pathway that activation of peripheral baroreceptors decrease or abolish the firing rate of RVLM sympathetic premotor neurons (Agarwal *et al.*, 1990; Cravo *et al.*, 1991; see Dampney, 1994; Gordon, 1987), therefore suggesting that CVLM elicits effects on the baroreflex by inhibiting neurons in the RVLM and causing sympathoinhibition and vasodepressor responses (see section 1.1).

Originally it was believed that the NTS was the source of the GABAnergic input but studies have shown that the central baroreceptor pathway consists of a pathway from the NTS to the CVLM, probably by a glutamatergic projection, and then from CVLM to RVLM by a GABAergic input (Agarwal *et al.*, 1990). The involvement of NMDA receptors in the CVLM was demonstrated by the use of selective NMDA receptor antagonists, which when applied to the CVLM abolished synaptically-mediated depressor responses caused by aortic nerve stimulation, indicating that neural transmission of aortic baroreceptor information in the CVLM is mediated by activation of NMDA receptors. The neurotransmitter released at the CVLM synapse may be an excitatory amino acid, possibly glutamate (Gordon, 1987).

Although the affect of application of nicotine to the CVLM has been demonstrated by Feldberg & Guertzenstein, (1976), the involvement of neuronal nAChRs in CVLM cardiovascular regulation has not been studied in great detail. The microinjection of increasing concentrations of nicotine into the CVLM was shown to cause depressor effects in both blood pressure and heart rate. Significant decreases of blood pressure and heart rate were observed at 1 mM of nicotine resulting in a decrease of 34 mmHg in blood pressure and 64 beats min^{-1} in heart rate, with increasing concentrations of nicotine not causing any greater effect (Aberger *et al.*, 2001). The effect of microinjections of nicotine was studied in the presence of mecamylamine ($\alpha3\beta4$ antagonist) and α-BgT (homomeric selective nicotinic antagonist), these compounds were shown to block the depressor effect and block the decrease in heart rate (Aberger *et al.*, 2001). From these findings, Aberger *et al.* (2001) suggested that $\alpha3\beta4$ and $\alpha7$ nAChRs are present in the CVLM and upon activation by nicotine cause a decrease in blood pressure and bradycardia, unfortunately, these two antagonists were added at the same time and not

individually, therefore it can not be stated conclusively that both receptors are present and in fact only one may be present and that inhibition of this neuronal nAChR is causing blockade of the pressor response. Overall, it can be theorised that activation of neuronal nAChRs in the CVLM results in inhibition of neurons in the RVLM via GABAnergic neurons. The inhibition of the RVLM neurons decreases excitatory inputs to sympathetic preganglionic neurons located in the spinal cord, which results in a depressor and bradycardia response (Agarwal *et al.*, 1990; Brown & Guyenet, 1984; Cravo *et al.*, 1991; see Dampney, 1994; Gordon, 1987).

1.9.2.4. Nucleus Ambiguus & Dorsal Motor Vagal Nucleus.

Additional areas of the brain that receive afferent innervations from the NTS are the nucleus ambiguus (NAmb) and the dorsal vagal motor nucleus (DMVN), which also receive afferent input from other brain regions such as the nuclei of the hypothalamus and the medullary reticular formation (see section 1.1) (Hunt & Schmidt, 1978). The NTS innervates the NAmb and DMVN and may mediate various vagal visceral reflexes. Innervations from the NTS to NAmb have been shown to project to the cardiopulmonary areas, whilst projections from the DMVN project to cardiac tissue. An arc of cardiac preganglionic neurons extending from the ventrolateral medulla to the DMVN have also been shown to receive anterograde projections from the NTS. Cardiac vagal neurons play a crucial role in the control of heart rate and cardiac function. These neurons, which are primarily located in the NAmb and DMVN, dominate the neural control of heart rate under normal conditions. Cardiac vagal neurons are intrinsically silent and therefore rely on synaptic inputs to control their firing. There are three major synaptic inputs to cardiac vagal neurons. One of these is cholinergic, with ACh exciting the cardiac vagal neurons via three mechanisms, one, activating a direct ligand-gated post-synaptic nAChR, a second, enhancing post-synaptic non-NMDA currents, and a third, pre-synaptically by facilitating transmitter release (Sahibzada *et al.*, 2002). The enhancement by nicotine is dependent upon activation of pre-and postsynaptic P-type voltage-gated calcium channels (Sahibzada *et al.*, 2002). Not much data are available concerning neuronal nAChRs and

the NAmb, although microinjection of nicotine into the NAmb was shown to cause a decrease in blood pressure in dogs, although there were mixed results with heart rate, producing both bradycardia and tachycardia. Studies using the nicotinic antagonist mecamylamine, (Shiraki *et al.*, 1997) demonstrated the inhibition of the nicotine-induced depressor response suggesting that nicotinic ACh receptors are distributed in the region of the NAmb.

Studies using anti-ChAT staining in the DMVN revealed that every DMVN neuron stained for ChAT. Using antibodies against ChAT, immunocytochemistry demonstrated that the DMVN contains many cholinergic neurons, as well as projections to the vagus nerve, which is accepted to be cholinergic in nature(Ferreira *et al.*, 2001). Radioligand binding has shown that there was an intense degree of binding of [^{125}I]-αBgT in the DMVN (Eilers *et al.*, 1997). Additional studies also showed that DMVN neurons possess nicotinic not muscarinic ACh receptors (Neff *et al.*, 1995). Further studies demonstrated that α7 nAChRs are present in the DMVN neurons, shown by inhibition of ACh evoked currents of up to 60-75% by α7 antagonists α-BgT and MLA (Sahibzada *et al.*, 2002), and by the use of anti-α7 staining in the DMVN (Ferreira *et al.*, 2001). DMVN neurons, once degenerated by unilateral cervical vagotomy, had no binding sites for α-BgT, therefore suggesting α-BgT binding were post-synaptic on DMVN neurons. Although α7 nAChRs were identified in the DMVN there were pharmacological and recovery differences between α7 nAChR studies in *Xenopus* oocytes and the studies of native α7 on the DMVN (Ferreira *et al.*, 2001). It was suggested that the α7 present in the DMVN appear to be functionally and pharmacologically distinct from heterologously expressed α7 and the α7 expressed in most neural tissue. This raises the possibility that the α7 nAChR at the DMVN may exist as a heteromeric complex, comprised of the α7 with an, as of yet unknown, nicotinic receptor subunit, possibly the β2 subunit (Wang *et al.*, 2001). Additional studies have shown that the nAChRs not inhibited by α-BgT were blocked by DhβE, suggesting the presence of α4β2 in the DMVN. Cytisine, which is selective for β4, was observed to evoke significant currents, which suggest the possibility of additional nAChR such as α3β4 nAChR on DMVN neurons (Sahibzada *et al.*, 2002).

Microinjections of nicotine, administered in a dose range from 10 to 1000 pmol kg^{-1}, into the DMVN had no significant effect on blood pressure causing a decrease of only 7.7 mmHg at 100 pmol kg^{-1} (Ferreira *et al.*, 2000). To observe if NTS is communicating with the DMVN to exert its effects on blood pressure, interneurons and neurotransmitters implicated in communicating information between these nuclei where studied, including nitric oxide, GABAergic, glutamatergic and noradrenergic systems (Ferreira, Jr. *et al.*, 2002). Additionally, the effect of specific antagonists, microinjected into the DMVN, was also studied. Bicuculline, a $GABA_A$ antagonist, kynurenic acid, a glutamate antagonist, and yohimbine, an α_2 adrenoceptor antagonist, were observed not to alter the decrease in the blood pressure produced by nicotine microinjected in to the NTS (Ferreira, Jr. *et al.*, 2002), suggesting that the NTS is not communicating with the DMVN to exert its effects on blood pressure.

1.10. Nicotine induced secretions.

The effects of nicotine and nicotinic compounds on the release of hormones has been observed in some detail with studies demonstrating nicotine causing the release of a number of hormones including anti-diuretic hormone, also known as vasopressin (Bisset & Chowdrey, 1984; Bisset & WALKER, 1957; Reaves, Jr. *et al.*, 1981), oxytocin (Bisset & WALKER, 1957), adrenocorticotropic hormone (Cam *et al.*, 1979; Matta *et al.*, 1997; 1998), prolactin and growth hormone (Andersson *et al.*, 1983; Davis *et al.*, 1982; Davis & Davis, 1980; Seyler, Jr. *et al.*, 1986). The nicotine-induced affects observed upon injection, which include changes in blood pressure and heart rate, may be due to release of the compounds named above.

1.10.1. Vasopressin.

Nicotine-induced vasopressin release has been extensively studied, and because of this an explanation of this compound and its mechanism of action are required. In 1953, the

chemical structure of vasopressin was discovered as was the synthesis of this peptide (see Jard, 1998). The neurophypophysial nonapeptide hormone vasopressin, is believed to be encoded by a gene found on chromosome 20p and contains a disulfide bridge between 2 cysteine amino acids (see Holmes *et al.*, 2003). Vasopressin is synthesised by neurons whose cells bodies are located in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. Axons from the neurons found in the PVN and SON terminate in the neurohypophysis (posterior pituitary) where, by a process of exocytotic secretion, vasopressin is released into the systemic circulation (see Howl & Wheatley, 1995; see Kombian *et al.*, 2002).

The actions of vasopressin are mediated by stimulation of tissue specific G-protein-coupled receptors which are classified into three major subtypes named V_{1a}, V_{1b} and V_2 (Cotte *et al.*, 2000; see Howl & Wheatley, 1995). The V_{1a} subtype are found in high density on the vascular smooth muscle, V_{1b} subtypes appears to be restricted to anterior pituitary corticotrophs and V_2 subtypes are restricted mainly to the kidneys. Due to the location of different vasopressin receptors it is indicated that the action of vasopressin can be varied. Activation of V_{1a} receptors results in vasoconstriction (Harland *et al.*, 1989; Unger *et al.*, 1984; Waeber *et al.*, 1983), believed to be caused by an increase in intracellular calcium via the phosphatidyl-inositol-bisphosphonate cascade (see Holmes *et al.*, 2003). Activation of V_2 receptors causes the retention of water, and is how vasopressin received its name of anti-diuretic hormone. Vasopressin regulates the excretion from the kidneys by increasing the osmotic water permeability, this is caused by vasopressin binding to V_2 receptors, stimulating adrenylyl cyclase and elevating intracellular cAMP (see Jard, 1998; Macfarlane *et al.*, 1967). This action of vasopressin induces the appearance of a population of endocytic vessels containing water channels in vasopressin-sensitive membranes of the kidney collecting tubules. The insertion of the water channels into the apical membrane gives rise to the re-absorption of water and to the anti-diuresis observed with vasopressin (see Holmes *et al.*, 2003; Howl & Wheatley, 1995; Macfarlane *et al.*, 1967). Unfortunately, very little is known about the V_{1b} receptor, due to its rarity, although it is observed to be a G-protein-coupled pituitary receptor. One mechanism of action of the V_{1b} receptor seems to be in signal transduction, for example, vasopressin causing the secretion of adrenocorticotropic hormone (ACTH)

from the anterior pituitary cells in a dose-dependent manner, through activation of PKC (Liu *et al.*, 1994). Interestingly the V_{1b} receptor is over expressed in ACTH-hypersecreting tumours (Thibonnier *et al.*, 1997).

The importance of vasopressin is emphasised when it was demonstrated to act as a neurotransmitter/neuromodulator within the brain and sympathetic nervous system, as well as being shown to be distributed throughout the brain and spinal cord, which includes the hypothalamus, DMVN, NTS, locus coeruleus and nucleus of spinal tract of the trigeminal nerve (Jenkins *et al.*, 1984). As a consequence, vasopressin is believed to influence cardiovascular functions by regulating sympathetic outflow (Unger *et al.*, 1984). Studies have suggested that the peripheral actions of vasopressin, which are believed to be due to changes in systemic vasopressin concentrations, may be neurally evoked. One mechanism involved is by a vasopressinergic projection to the NTS. The NTS has been previously described, and is believed to be first synaptic relay in the brainstem for information on blood pressure levels by sinus and aortic nerve afferents. Depending on the change in blood pressure, the NTS excites other areas of the brainstem such as CVLM, RVLM, DMVN and nucleus ambiguus. Including, projections from the NTS to the amygdala and cortex, the NTS was shown to project to the PVN and SON and cause the release of vasopressin, as well as receive monosynaptic projections from the PVN (Buijs *et al.*, 1978; Nilaver *et al.*, 1980; Sawchenko & Swanson, 1982; Sofroniew & Schrell, 1980) which provides a feedback control loop through which the PVN could modulate afferent cardiovascular inputs coming from the periphery to the NTS (see Michelini, 1994). The PVN is believed to be the only source of vasopressinergic projection to the NTS, with the long descending projection containing as well as vasopressin, oxytocin, enkenphalins and somatostatin (Buijs *et al.*, 1978; Nilaver *et al.*, 1980; Sofroniew & Schrell, 1980). Therefore, the vasopressinergic projection to the NTS are of major importance because they constitute a pathway by which the PVN could directly modulate baroreceptor function (see Michelini, 1994), as well as the possibility that neurohormonal vasopressin could also influence the reflex control of the heart.

Due to the importance of vasopressin in cardiovascular regulation, the effects of nicotine and nicotinic compounds have been observed on modulating vasopressin release.

Nicotine, administered centrally, has been shown to cause a release of vasopressin in both rat (Bisset & Chowdrey, 1984; Iitake *et al.*, 1986) and cat (Bisset *et al.*, 1975; Castro de & Rocha E Silva, 1977). Injections of nicotine into the third ventricle caused a increase of up to thirty fold in the concentration of vasopressin in blood collected 35-155 sec after injection (Bisset & Feldberg, 1973), as well as nicotine injections given into or in close proximity to the SON, causing an increase of vasopressin release of 175%, when compared to control (Milton & Paterson, 1970). The nicotine-induced vasopressin release was observed to be inhibited by nicotinic antagonist hexamethonium, indicating the involvement of nicotinic receptors. The effect of nicotine on the SON and PVN is possible due to the cholinergic cell bodies found just dorsolateral to the SON (Clarke & Pert, 1985), which is thought to be the source of the SON cholinergic innervations. These may innervate neurons through other local circuit neurons, and thus, possibly providing the anatomical and functional basis for the cholinergic effect on neurons in the SON and PVN (Clarke & Pert, 1985).

Nicotine injected i.c. was more effective at inducing vasopressin release than when injected i.c.v in the rat. Compounds injected i.c. cannot reach the cerebral ventricles, whereas drugs injected i.c.v. can readily reach the ventral surface of the brainstem, through the lateral foramina of the fourth ventricle. It was suggested therefore, it is unlikely that nicotine induced vasopressin release by acting directly on the PVN (Bisset *et al.*, 1975; Bisset & Chowdrey, 1984). This became evident when it was demonstrated that nicotine injected into the cerebral ventricles after cannulation of the aqueduct, which prevents nicotine access to the fourth ventricle, no longer produced the vasopressin release (Bisset *et al.*, 1975). Overall, it was suggested that the dorsal and ventral surface of the brainstem is the site of action for nicotine-induced vasopressin release, with the ventral surface more likely as it is reached more readily by the i.c.v. route, which is consistent for both rat and cat (Bisset *et al.*, 1975; Bisset & Chowdrey, 1984). It was finally concluded when topical application to areas of the ventral surface of the brainstem caused the release of vasopressin (Bisset & Chowdrey, 1984; Feldberg & Guertzenstein, 1976).

It is therefore hypothesised that an efferent pathway from the NTS, which receives its stimulus from baro-and stretch receptors, has a nicotine sensitive synapse between the afferent fibres and the central projections to the neurosecretory cells, which release vasopressin from the PVN and SON (Bisset *et al.*, 1975; Bisset & Chowdrey, 1984) by the activation of nAChRs found on the SON and PVN. Autoradiography of the SON and PVN demonstrated the presence of α7 nAChR, due to [^{125}I]-α-BgT binding sites (Zaninetti *et al.*, 2000) whereas cytisine binding studies have indicated the presence of β4 subunits (Bisset & Fairhall, 1995), as well as, strong immunoreactivity for the α4 nAChR subtype, suggesting the presence of the α4β2 nAChR and possibly α4β4 nAChR (Shioda *et al.*, 1997b; Wada *et al.*, 1989).

Nicotine applied to vasopressin containing-neurons in the SON, was observed to cause an increase in Ca^{2+} in the neurons, due to activation of adenylate cyclase by nAChRs (Shioda *et al.*, 1997b). The increase in Ca^{2+} in the rat hypothalamic vasopressin-containing neurons were suggested to be mediated by a cAMP-PKA signal transduction system, whereby nicotine acts through a cAMP-dependent mechanism, probably via a PKA-mediated phosphorylation of calcium channels, to trigger the influx of Ca^{2+} and facilitate vasopressin release (Shioda *et al.*, 1997b).

1.10.2. Oxytocin.

Oxytocin and vasopressin only differ by one amino acid (80% homology) but they have very different physiological actions. Vasopressin, as previously mentioned, is essential in cardiovascular and water homeostasis, whereas oxytocin is important in lactation and sexual behaviour (see Holmes *et al.*, 2003). Oxytocin is also synthesised by neurons whose cells bodies are located in the PVN and SON of the hypothalamus with the axons of the neurons terminating in the neurohypophysis and, upon stimulation, release oxytocin in to the circulation (Acher, 1993).

Oxytocin receptors have been localised in a number of areas, including reproductive and non reproductive tissues such as the heart (Gutkowska *et al.*, 1997) and vascular endothelium (Thibonnier *et al.*, 1999). The oxytocin receptor is a GPCR coupled to $G_{q/11}$ class binding proteins, which stimulate the activity of phospholipase C (Gimpl & Fahrenholz, 2001). Phospholipase C causes the generation of inositol triphosphate, which triggers the release of Ca^{2+} from intracellular stores, and 1,2,-DAG which stimulates PKC to phosphorylate target proteins (Gimpl & Fahrenholz, 2001). The release of Ca^{2+} effects many events such as stimulation of nitric oxide, which helps to produce cGMP and leads to vascular endothelium vasodilatation (Gimpl & Fahrenholz, 2001;Thibonnier *et al.*, 1999). Calcium can also cause smooth muscle contractions and release in neurosecretory cells. The concentration of oxytocin in the human brain and spinal cord, using specific radioimmunoassay, showed interesting results when compared with vasopressin concentrations. It was shown that, in the hypothalamus, the ratio of vasopressin to oxytocin was approximately 3:1. There was also observed a greater amount of vasopressin in the extrahypothalamic areas of the brain, such as the locus coeruleus and periaquaeductal grey. In the NTS, the DMVN and nucleus of spinal tract of the trigeminal nerve there were observed to be higher concentrations of oxytocin than vasopressin, which was also the case in the spinal cord, where oxytocin predominated over vasopressin to an even greater extent (Jenkins *et al.*, 1984). Electrical stimulation of the PVN in lactating guinea pig and rabbit caused milk ejection without a rise in blood pressure suggesting release of oxytocin alone, whereas in the cat, electrical stimulation of the PVN increases the concentration of both hormones in the blood (Bisset *et al.*, 1975).

Studies observing the affects of nicotine on oxytocin release have indicated that in cats, central administration of nicotine caused the release of vasopressin but did not cause the release of oxytocin (Bisset *et al.*, 1975;Bisset & Feldberg, 1973). This provides further evidence that nicotine-induced vasopressin release is not caused by direct action of nicotine on PVN, as stimulation of the PVN would be expected to release both hormones (Bisset & Chowdrey, 1984). Administration of nicotine subcutaneously (s.c.) was demonstrated to cause the release of oxytocin into the blood, and was not blocked by hexamethonium, which is unable to cross the blood brain barrier, indicating that nicotine is acting centrally to cause the release of oxytocin rather than acting peripherally.

1.10.3. Adrenocorticotropic hormone.

Adrenocorticotropic hormone (ACTH) is a polypeptide hormone synthesised and secreted from corticotropes in the anterior lobe of the pituitary gland in response to the hormone corticotrophin-releasing factor (CRF) released by the hypothalamus (H.P.Rang *et al.*, 1999;Hardman J.G, 1996). The CRF neurons are located in the parvocellular region of the PVN of the hypothalamus (pcPVN). ACTH has been observed to stimulate the cortex of the adrenal glands and boost the synthesis of corticosteroids, which are a class of steroid hormones and are involved in a wide range of physiological systems such as the stress response, immune response and regulation of inflammation (see Tortora & Grabowski, 2000; see Rang *et al.*, 1999; see Hardman , 1996; Silverman *et al.*, 2005; Zipser *et al.*, 1976).

The effect of nicotine on ACTH release was observed when tobacco smoking caused an increase in ACTH plasma levels (Kershbaum *et al.*, 1968;Seyler, Jr. *et al.*, 1986). Further studies on the effects of i.p. (Clarke & Pert, 1985), s.c. (Balfour *et al.*, 1975) and i.v. injection (Andersson *et al.*, 1983) of nicotine, observed the release of ACTH from the anterior pituitary corticotropes of adult rats. Studies to determine the mechanism of action of nicotine-induced release of ACTH, were preformed and indicated that systemic nicotine was acting centrally to stimulate the release of ACTH from the pituitary. Nicotine administered i.v. (2.5 – 5 $\mu g\ kg^{-1}$) produced a dose-dependent increase in plasma levels of ACTH which was not inhibited by hexamethonium i.v. (which cannot cross the blood brain barrier) (Asghar & Roth, 1971), but mecamylamine, which is a centrally active antagonist, did block nicotine-induced ACTH release (Matta *et al.*, 1987; 1990). Nicotine i.v. was observed to have no effect on corticotropes *in vitro*, nor did it modulate responses to CRF (Matta *et al.*, 1987). Interesting nicotine administered by i.c.v. injections (0.25 – 5 $\mu g\ kg^{-1}$) into the third ventricle, from which the PVN is immediately accessible, produced a dose-dependent elevation of plasma ACTH (Matta *et al.*, 1987), which was not inhibited when mecamylamine was injected into the PVN, indicating that the PVN itself was not involved (Matta *et al.*, 1987). Matta *et al.* (1990) noted that injections of nicotine in to the fourth ventricle resulted in a dose-dependent increase in

plasma ACTH. Nicotine (i.v.)-induced release of ACTH was blocked by pre-treatment of mecamylamine at the brainstem, suggesting the involvement of brainstem structures in the release of ACTH.

The PVN can mediate ACTH secretion in response to central catecholamine stimulation, Matta *et al.* (1993) hypothesised that nicotine could induce ACTH secretion by activating catecholaminergic afferents projecting to the PVN from the brainstem. Catecholamine release by nicotine has been demonstrated both *in vitro* (see Hall & Turner, 1972; Westfall, 1974) and *in vivo* (Andersson *et al.*, 1983). *In situ* hybridisation has identified specific nAChR subunit mRNA in the brainstem (Wada *et al.*, 1989). The NTS has noradrenergic (NTS - A_1 and A_2 regions) and adrenergic (NTS – C_1 and C_2 regions) inputs into the PVN. Microinjection of nicotine into these regions, indicated that the rank order of sensitivity of plasma ACTH responses to nicotine was A2>C2>A1 and C1 was insensitive (Matta *et al.*, 1993). Overall it was suggested that ACTH secretion can be stimulated by nicotine acting at multiple brainstem catecholaminergic regions, which show differential sensitivity to the neuroendocrine effect of nicotine (Matta *et al.*, 1993; Matta *et al.*, 1997; Seyler, Jr. *et al.*, 1986).

1.11. Diseases associated with neuronal nAChRs.

The role of neuronal nicotinic receptors in neurodegenerative diseases has been established over the last 30 years. These include schizophrenia, Tourettes's syndrome, Parkinson's disease, Alzheimer's disease and epilepsy. Figure 12 shows the areas of the rat brain associated with these neurodegenerative diseases.

1.11.1. Schizophrenia.

Schizophrenia is a severe mental illness that affects 0.6-1.5 % of the population worldwide and is characterized by disorganised thought processes, hallucinations,

delusions, cognitive deficits and impaired learning function (see Arnold & Trojanowski, 1996; see Salamone & Zhou, 2000). The pathology of this disease has only just started to be understood in the last 30 years.

Schizophrenia was first associated with neuronal nAChRs in 1986 when Hughes *et al* reported that schizophrenic patients tended to smoke more cigarettes than the normal population, with a 88% prevalence of smoking in patients with schizophrenia compared with 33% prevalence in the local or national population (see Lohr & Flynn, 1992). To determine if neuronal nAChRs had any involvement in schizophrenia the number of nAChRs was established in the brain of the subjects suffering from the disease. It was observed that there was a significant decrease in neuronal nAChRs in certain brain areas such as the cingulate and orbito-frontal cortex with a loss of α4β2 nAChRs (see Court JA *et al.*, 2000; Graham *et al.*, 2002), as well as a significant decrease in α-BgT binding in brain areas such as in the hippocampus (Freedman *et al.*, 1995) and a decrease in α7 subunit protein expression in the frontal cortex (Figure 12) (Guan *et al.*, 1999).

One of the most significant symptoms of schizophrenia is an effect on sensory gating, which is the failure to discriminate the P50 wave of the auditory evoked response in a paired stimulus conditioning testing paradigm (Adler *et al.*, 1999). This deficit has also been seen in many relatives of the patients, indicating a genetic predisposition is involved (Adler *et al.*, 1992; Tsuang, 1993). Such effects have been reproduced in animals by the use of the α7 antagonist, α-BgT, indicating that inhibition of this receptor could be involved in the schizophrenic condition (Freedman *et al.*, 1995). Further evidence was given by Alder *et al.* (1992, 1999) who demonstrated that the deficit in sensory gating could be reversed by nicotine administration in the form of nicotine-containing gum. Although the effects were only observed for an hour it showed that α7 nAChRs may control auditory sensory gating (Adler *et al.*, 1992). Additional studies performed indicated that the deficit in P50 auditory evoked potential gating has been localized and found to be on chromosome 15, band q14 (Dursun & Reveley, 1997; Freedman *et al.*, 1995), which is also the gene locus of the α7 nAChR (Chini *et al.*, 1994). Taken together with the decreased α7 gene expression observed in the hippocampus, reticular thalamic nucleus and superior olive complex, which are areas that receive sensory information

(Agulhon *et al.*, 1999) these finding suggest that α7 nicotinic receptors are involved in the pathophysiology of schizophrenia (Figure 12) (Agulhon *et al.*, 1999; Freedman *et al.*, 1995).

In conclusion, it has been suggested that the α7 neuronal nAChR could be involved in certain aspects of schizophrenic and that the large number of cigarettes smoked in patients may help compensate for a defect in the α7 receptor. Unfortunately not enough information is known, therefore future studies will be required in order to determine the overall effect of nAChRs in this disease.

1.11.2. Tourette's syndrome.

Tourette's syndrome is a hyperkinetic movement disorder characterized primarily by the expression of sudden, rapid and brief, recurrent, non-rhythmic, stereotyped motor movements (motor tics) or sounds (vocal tics) that are experienced as uncontrollable, but usually can be suppressed for varying lengths of time (Sanberg *et al.*, 1997). The incidence of Tourette's syndrome (TS) in the population is around 0.5/1000, although this is believed to be an underestimate (Dursun & Reveley, 1997). By the use of neuroleptics (Dopamine D_2 antagonists) such as haloperidol and pimozide, the symptoms can be suppressed, however these drugs have side effects such as deterioration in function and compliance which include difficulty in concentration, drowsiness and attentional problems (McConville *et al.*, 1992), therefore new drugs are required to help treat this disorder.

Although there is no direct evidence that nAChRs have an involvement in this disorder, it has been shown that the administration of nicotine causes substantial improvement of tics observed in TS patients. Studies have shown that patients suffering from TS, upon chewing nicotine gum, exhibited a marked reduction in tics, as well as improvements in attention span and a reduction in hyperactivity, which allow them to do certain tasks that they could not do previously (Sanberg *et al.*, 1997). Unfortunately these therapeutic

effects were of short duration and side effects such as bitter taste and nausea made patients have a low compliance. The effects of chewing nicotine gum, when the patient is on haloperidol, was also studied (McConville *et al.*, 1992). It was shown that nicotine potentiated the haloperidol effects on TS patients, but the combination of haloperidol and nicotine gum was more effective at controlling the tic frequency and severity than nicotine gum alone. Due to the side effects mentioned for chewing nicotine gum, other modes of administration were studied; Dursun *et al.* (1994) demonstrated that administration of nicotine by the use of transdermal nicotine patches reduced tics of non-smoking patients with TS with no side effects for up to 4 weeks. This study was important because it was carried out on TS patients whose symptoms were unable to be controlled by haloperidol, indicating the possibility of nicotine being used to treat TS (Dursun *et al.*, 1994; Dursun & Reveley, 1997). Interestingly, patients who have TS and are smokers have less severe symptoms; however, when these patients stop smoking they often display dramatically increase in severity.

Although neuronal nAChRs have not been directly shown to have an involvement in TS, the positive effect of nicotine suggests that nAChRs may play a role in symptom manifestation. Although many mechanisms have been suggested for how nicotine causes its effects, so far none have been proven. One suggested mechanism by which nicotine and neuronal nAChR may affect TS patients is due to nAChRs heteroreceptors found on the dopaminergic neurons modulating the release of DA from the striatum and limbic cortical areas (see Colquhoun & Patrick., 1997; Rapier *et al.*, 1988; 1990). If this is occurring then nicotine may be effective due to desensitization of the heteroreceptors nAChRs, inhibiting the release of NA. Administration of nicotine by transdermal patch for 48 hours continuously, correlates with the time taken to induce receptor desensitization. This indicates why the 48 hour transdermal patch is able to relieve the TS symptoms for up to 4 weeks, whereas, nicotine gum, which is only chewing for 30 mins, is only able to relieve the TS symptoms for a few hours, due to the low desensitization of nAChRs (Dursun *et al.*, 1994; Dursun & Reveley, 1997; Sanberg *et al.*, 1997).

1.11.3. Parkinson's disease

Parkinson's disease was first described by James Parkinson in 1817 and is one of the most common neurodegenerative movement disorders affecting 1% of the population over 65 years of age (Bossy-Wetzel *et al.*, 2004; Maggio *et al.*, 1998; Meissner *et al.*, 2004). The disease is characterized by serious functional disturbances such as tremors at rest, rigidity, akinesia (or postural instability) and bradykinesia (the difficulty or failure to execute voluntary movement) (Fernandez-Espejo, 2004; Meissner *et al.*, 2004). These functional disturbances are caused by the loss of dopaminergic neurons in the substantia nigra pars compacta projecting to the striatum leading to DA deficiency in the basal ganglia (Figure 12). The defining neuropathological feature in Parkinson's disease (PD) is the presence of cytoplasmic α-synuclein positive inclusions (Lewy bodies) in the substantia nigra together with nigra cell loss. Parkinson signs appear when neuronal death exceeds a critical threshold of 70-80% of DA nerve terminals in the striatum (Fernandez-Espejo, 2004; Meissner *et al.*, 2004).

Currently, even with an extensive amount of research looking into PD, the cause of the disease is still unknown, although it is believed that there is a genetic component (see Newhouse *et al.*, 1997). Current treatments, such as L-DOPA, to produce DA, are only symptomatic and do not stop or delay the progressive loss of neurons. Surprisingly, it was observed that the incidence of PD was significantly lower in smokers when compared to non-smokers and this lead to extensive studies observing the effects of nicotine and smoking (Janson *et al.*, 1989;Morens *et al.*, 1995;Newhouse *et al.*, 1997), as well as establishing the involvement of neuronal nAChRs in PD (Burghaus *et al.*, 2003; Guan *et al.*, 2002; Martin-Ruiz *et al.*, 2000b).

There have been extensive studies looking at the changes in nAChRs in PD. Autoradiography studies in rat and human brain tissues have compared healthy brains with patients who suffered from PD and shown that there was a significant decrease in nicotinic agonist binding in patients suffering from PD, the most significant loss was observed in the striatum showing up to 76% loss of nicotinic binding. A decrease in

binding in the hippocampus, cortex and temporal cortices has also been documented (Aubert *et al.*, 1992; Lange *et al.*, 1993; Newhouse *et al.*, 1997; Perry *et al.*, 1995; Schroder *et al.*, 1995). This loss of neuronal nACh receptors in PD are believed to be located pre-synaptically on degenerating cholinergic axons. The nucleus basalis of Meynert has the highest concentration of nicotinic receptors in the human brain and being the principle nucleus of the basal forebrain, degeneration of neurons, which occurs in PD, will reduce pre-synaptic nAChRs which in turn will reduce the nicotine and ACh-induced release of ACh from the cholinergic terminals in the cortex (Figure 12) (Aubert *et al.*, 1992; Lange *et al.*, 1993). Further studies revealed that neuronal nAChR subtypes were decreased in additional areas of the PD brain, for example, in the striatum area, which includes the caudate nucleus and putamen. Studies have shown that there is a significant decrease in [^{3}H]-epibatidine radioligand binding, α3 mRNA and protein expression in the caudate nucleus in PD brains (Guan *et al.*, 2002). Degenerative changes are also observed in the cortices, which play an important role in cognitive deficits of Parkinson's disease patients. It has been demonstrated that there are significantly decreased amounts of α4 and α7 protein subunits in human cortices in PD patients (Burghaus *et al.*, 2003). A decrease in α3 and β2 mRNA and protein expression has also been observed in the temporal cortex in PD brains (Burghaus *et al.*, 2003; Guan *et al.*, 2002). Surprisingly there was no change in any nicotinic subunits (α3, α4, α7, β2 and β4) expression in the putamen when controls (healthy brains) were compared to PD brains (Guan *et al.*, 2002; Martin-Ruiz *et al.*, 2002) suggesting that the receptors that are being reduced are in fact receptor subtypes not examined in this study, possibly α5, α6 and β3 nAChRs may be decreased and be responsible for the high-affinity nicotine binding in PD striatum. This would correlate with the findings that the neuronal nAChRs believed to be involved in the release of DA are the α6β3β2, α6β2 or α6β3 (Champtiaux *et al.*, 2003; Cui *et al.*, 2003; Salminen *et al.*, 2004). Additional suggestions for the decrease in nicotine binding without any obvious change in nAChR expression, may be an increased synthesis of nAChR subunits in the remaining nigrostriatal neurons in PD patients, which are acting as some kind of compensation for the loss of nAChRs but fail to be assembled in degenerative terminals, so maintaining apparent subunit expression when receptor binding is attenuated (Guan *et al.*, 2002; Martin-Ruiz *et al.*, 2000b; 2002).

All the data reported strongly support the involvement of nAChRs in this disease especially when it was noted that smokers were less susceptible to PD than non-smokers (Morens *et al.*, 1995). This lead to research into the effects of nicotine on PD models. Parkinson's disease models were produced in rats and mice by the use of 1,methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is a neurotoxin and has been shown to induce degeneration of nigrostriatal DA neurons (Janson *et al.*, 1992). Other methods of stimulating PD include the use of partial hemitransection made at the mesodiencephalic junction, causing lesions in the nigrostriatal DA neuron (Janson *et al.*, 1989; 1994; Janson & Moller, 1993). Studies showed that chronic treatment with nicotine, sometimes lasting up to 2 weeks, caused neuroprotective effects by preventing lesion-induced upregulation of the high affinity agonist binding sites of the DA D_2 receptor and preventing hypersensitivity of the D_2 receptor which would normally cause rapid release of DA. From these studies it has been suggested that nicotine acts as a neuroprotector by binding to nACh heteroreceptors found on DA neurons causing desensitization of nAChRs and decreasing burst firing of nigral DA neurons which decreases DA utilization, reducing the release of DA. Studies have also shown that depolarization would reduce the release of DA by reducing the influx of Na^+ and Ca^{2+} decreasing the energy demands to maintain homeostasis. In addition a decrease in Ca^{2+} influx would contribute to the survival of DA neurons (Janson *et al.*, 1989; Janson & Moller, 1993), as well as, reduce burst firing of DA neurons on the lesioned side (Janson *et al.*, 1994). All of the above suggest that nicotine would be a useful tool in the neuroprotection of PD, but unfortunately clinical trials of nicotine treatment have lead to controversial results with little or no improvement in cognitive and motor symptoms (Kelton *et al.*, 2000). This is not surprising when it is considered that there is a vast amount of nAChR present in the brain, all activated by an unselective agonist. Therefore until more selective agonist and antagonist are tested for the treatment of PD, it is unknown if neuronal nicotinic agonists will become a useful tool in treating this disease.

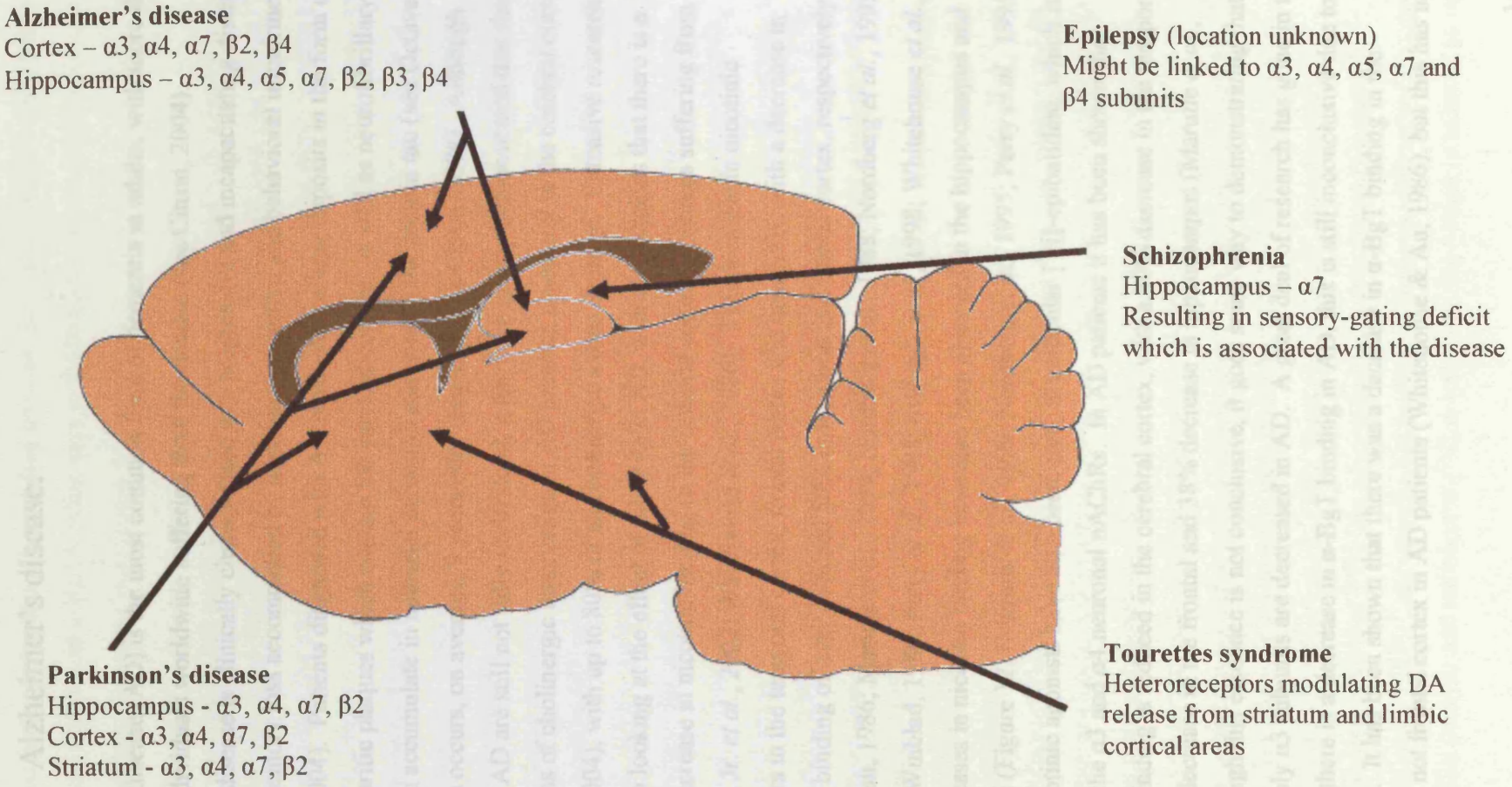


Figure 12. Diagrammatic representation of the possible physiological involvement of nicotinic ACh receptor subunits observed in the rat brain. Modified from Jones *et al.*, 1999.

1.11.4. Alzheimer's disease.

Alzheimer's disease (AD) is the most common form of dementia in adults, with more that 12 million individuals worldwide suffering from the disease (see Citron, 2004). Alzheimer's disease is clinically characterized as a progressive and incapacitating disease that causes memory loss accompanied by additional cognitive and behavioral impairment (Mesulam, 2004). Patients diagnosed with AD have extracellular deposits in the form of diffuse or neuritic plaques which contain the amyloid peptide, as well as neurofibrillary tangles which accumulate in neuronal perikarya and contain the protein tau (see Octave, 1995). Death occurs, on average, 9 years after diagnosis (see Citron, 2004). Although the causes of AD are still not fully understood, it has been clearly demonstrated that there is a severe loss of cholinergic innervation in AD patients, especially in the cerebral cortex (Mesulam, 2004), with up to 80% of all cholinergic axons depleted. Extensive research has gone in to looking at the effect of nAChRs in AD, with observations that there is a significant decrease in nicotine binding in the cerebral cortex in patients suffering from AD (Ferreira, Jr. *et al.*, 2002; Whitehouse *et al.*, 1986). This decrease in nicotinic binding occurs in the temporal cortex, frontal cortex and neocortex, with a decrease in [^{3}H]-nicotine binding of over 44% and 50% in temporal and frontal cortex, respectively (Flynn & Mash, 1986; Marutle *et al.*, 1999; Nordberg *et al.*, 1988; Nordberg *et al.*, 1995; Nordberg & Winblad, 1986; Perry *et al.*, 1995; Sabbagh *et al.*, 1998; Whitehouse *et al.*, 1986). Decreases in nicotine binding have also been observed in the hippocampus and presubiculum (Figure 12) (Marutle *et al.*, 1999; Nordberg *et al.*, 1995; Perry *et al.*, 1995). Different nicotinic agonists have also been used, in particular [^{3}H]-epibatidine, which is selective for the α3 and α4 neuronal nAChRs. In AD patients it has been shown that epibatidine binding is reduced in the cerebral cortex, with a 40% decrease in the temporal cortex, 45% decrease in the frontal and 38% decrease in hippocampus (Marutle *et al.*, 1999). Although this evidence is not conclusive, it goes some way to demonstrating that α4 and possibly α3 subunits are decreased in AD. A great deal of research has gone in to examining if there is a decrease in α-BgT binding in AD, this is still inconclusive due to mixed results. It has been shown that there was a decrease in α-BgT binding in the temporal; but not frontal cortex in AD patients (Whitehouse & Au, 1986), but this has not

been able to be replicated with further studies showing that there is no difference between AD patients and controls in α-BgT binding sites in the temporal cortex (Martin-Ruiz *et al.*, 2000a) and hippocampus (Whitehouse & Au, 1986).

Decreases in nicotinic AChR binding sites have also been correlated with a decrease in ChAT activity, with up to 60% loss in the temporal cortex and noted losses in frontal cortex that were about the same extent as loss of [^{3}H]-nicotine binding sites (Nordberg & Winblad, 1986; Sihver *et al.*, 1999). This parallel reduction in nicotine binding sites and ChAT activity in AD suggests receptors are found pre-synaptically (Ferreira, Jr. *et al.*, 2002), which was also noted in neocortical slices when it was observed that nicotinic agonist [^{3}H]-epibatidine binding was reduced in AD patients, as well as synaptic density. The loss of synaptic density, as well as nicotinic binding indicates that the loss of nAChR binding sites, seen in AD, results from the loss of nerve terminals bearing these receptors (Sabbagh *et al.*, 1998). This provides more evidence for the hypothesis that the nucleus basalis, which provides cholinergic innervation to the cortex and contains the highest concentration of [^{3}H]-nicotine binding sites in the human brain, is degenerated in AD, resulting in a reduction in pre-synaptic receptors on the axons innervating the cortex (Ferreira, Jr. *et al.*, 2002). It is in the cerebral cortex that the substantial loss of cholinergic innervation occurs in AD and is shown to cause the memory loss and other cognitive impairment (Figure 12) (Mesulam, 2004).

Radioligand binding studies have shown that there is a reduction in nAChRs in AD patients, but it has only been over the last 7 years or so that studies into which specific nAChR subtypes are lost in AD have been performed. Studies in the cerebral cortex have shown that there are significant decreases of 40% in the α4 subunit protein expression in AD patients when compared to age-matched controls (Wevers *et al.*, 1999; 2000). Further studies in the temporal cortex provided more evidence of α4 involvement in AD, with data showing α4 nAChR subunit protein levels decrease by 47% in AD patients (Martin-Ruiz *et al.*, 1999; Perry *et al.*, 2000; Sparks *et al.*, 1998). Additionally a reduction in α4 protein level of 35% was observed in the hippocampus (Guan *et al.*, 2000; Mousavi *et al.*, 2003). Interestingly, although there has been an observed decrease in the α4 nAChR subtype and radioligand [^{3}H]-nicotine binding (which is believed to be

most selective for the α4β2 nAChR) there has been no observed decrease in protein levels in the temporal cortex or hippocampus (Guan *et al.*, 2000), indicating another receptor apart from the nAChR α4β2 may be involved. Studies took place to observe if the protein levels of the α3 subunit were affected in AD producing some contradictory results, with some studies showing a reduction in the temporal cortex and hippocampus (Guan *et al.*, 2000;Mousavi *et al.*, 2003), whereas, other studies indicated that protein levels of α3 nAChR remain unchanged (Martin-Ruiz *et al.*, 1999; Perry *et al.*, 2000). Although there is a significant decrease in α4 and α3 protein expression, it has been demonstrated there is no corresponding decrease in mRNA expression for either of these two subunits (Mousavi *et al.*, 2003; Terzano *et al.*, 1998; Wevers *et al.*, 1999; 2000). This decrease in nAChR protein level without a change in mRNA transcription level in AD, supports an abnormality in the translation and/or post translational events in nAChR biosynthesis, whereby the neurons are failing to translate the nAChR mRNA into corresponding proteins and possibly causing the cholinergic deficit in AD.

Studies into the expression levels of α7 nAChR in AD patients have demonstrated a decrease in α7 subunit expression in the cerebral cortex (Burghaus *et al.*, 2000; Wevers *et al.*, 1999; 2000), as well as, the hippocampus (Guan *et al.*, 2000; Mousavi *et al.*, 2003), although there was no change in α7 expression in the temporal cortex (Figure 12) (Guan *et al.*, 2000; Martin-Ruiz *et al.*, 1999; Mousavi *et al.*, 2003; Perry *et al.*, 2000). Interestingly, it has been shown that there was no difference between mRNA expression of the α7 subunit between AD and controls in the temporal cortex (Mousavi *et al.*, 2003) and cerebral cortex (Wevers *et al.*, 1999; 2000), therefore, as with the α4 and α3 nAChR subunits, it appears that there is a problem in translating the mRNA of α7 into it's corresponding protein. Contradictory to this hypothesis, Martin-Ruiz *et al.* (2000a) demonstrated a 65% increase in mRNA expression of α7 in the hippocampus, indicating that a compensation mechanism may be occurring in AD, whereby increasing transcription of α7 mRNA may help to maintain normal α7 subunit protein expression in the majority of cases.

Originally, upon discovery of AD, the belief that it was a cholinergic disease instigated the research and development of cholinergic agents such as anti-acetylcholinesterases

which would prevent the breakdown of ACh and lead to symptomatic improvement (Mesulam, 2004), unfortunately this was not the case and they were demonstrated to only have modest effects. There has been a vast amount of research into AD and one of the areas of research involves looking into drugs which can block the β-amyloid (Aβ) aggregation (see Citron, 2004). One of the characteristics of AD is the development of senile plaques, these are composed of a core of Aβ and it has been hypothesized that accumulation of Aβ causes neurodegeneration and neuronal death (Kihara *et al.*, 1997). Thus it is believed that the neurodegeneration by Aβ could be inhibited by interfering with this sequence of events and hopefully prevent, or at least, treat AD. Due to the effects of AD on nicotinic radioligand binding, the effects of nicotinic compounds were observed on treating the disease, especially by interfering with Aβ accumulation. Nicotinic agonist studies against Aβ cytotoxicity in the cerebral cortex showed that nicotine exposure for 48 hours significantly reduced Aβ cytotoxicity in a concentration dependent manner (Kihara *et al.*, 1997; 1998), the neuroprotective effects of nicotine were shown to be blocked by the non-selective neuronal nAChR antagonist, mecamylamine, which indicates that the effects of nicotine are modulated by nicotinic receptors (Kihara *et al.*, 1997; 1998). Further studies were performed using selective agonists and antagonists to establish if any specific nAChRs are involved. It was shown that the α7 antagonist, α-BgT, blocked the neuroprotective effects of nicotine, as well as selective α7 agonists reducing Aβ cytotoxicity significantly and protecting against Aβ-induced neuronal death (Kihara *et al.*, 1997). Studies looking into α4β2 neuronal nAChRs by using the selective antagonist, DhβE, showed that the neuroprotective effects of nicotine were blocked, as well as selective α4β2 agonists causing a significant reduction of Aβ cytotoxicity (Kihara *et al.*, 1998). This demonstrated that both α4β2 and α7 nAChRs exert a neuroprotective effect against Aβ-induced cytotoxicity. The neuroprotective affects have been shown in hippocampal cell cultures, with nicotine inhibiting the neurotoxicity of Aβ in a dose-dependent manner which is inhibited by the nicotinic antagonist, mecamylamine, indicating that nicotine has a neuroprotective effect via a receptor mediated pathway (Zamani *et al.*, 1997). Studies have shown that the increasing levels of Aβ demonstrated in AD were correlated with the loss of α4 and suggested that there is an involvement of α4 subunits in the early stages of AD (Perry *et al.*, 2000).

The precise mechanism of nicotine's neuroprotection is not clear, although one suggestion is that the application of nicotine causes desensitization of nACh heteroreceptor on glutamate neurons which may reduce the Aβ induced Ca^{2+} influx which causes the excess release of glutamate and results in cytotoxicity by glutamate toxicity (Kihara *et al.*, 1998;Zamani *et al.*, 1997). Other nicotinic treatments have shown that nicotine can help improve visual attentional deficits in AD (Jones *et al.*, 1992;Perry *et al.*, 2000). Overall it has been shown that nicotine or nicotine related compounds could help in the loss of cortical synapses which have been shown to reduce in synaptophysin in correlation with nicotinic binding (Sabbagh *et al.*, 1998) and hopefully help in the treatment of AD.

1.12. Aims of the thesis.

Central administration of nicotine can evoke either an increase or decrease in blood pressure and sympathetic outflow. The unselective nature of nicotine for different neuronal nAChR prevents identification of which nAChR mediates the different cardiovascular responses elicited from each central cardiovascular control region.

The aim of this study was first to determine the effects on the cardiovbascular system of nicotine applied to the forebrain compared to the hindbrain using intracerebroventricular (i.c.v.) and intracisternal (i.c.) injections respectively. Secondly the aim was to identify the nAChR subtype involved in the cardiovascular responses elicted from each site of application and the pathways involved. The studies were carried out in anaesthetised rats amd depend on the recent development of two agonists selective for α4β2 and α7 nAChR subunits. Possible mechanisms for the cardiovascular changes such as the role of central and peripheral vasopressin were also employed.

CHAPTER 2. METHOD.

2.1. Experimental design.

Experiments were performed on the male Sprague-Dawley normotensive rats (250-350g). Anaesthesia was induced by isoflurane (2.5% in oxygen) and maintained with α-chloralose (100 mg kg^{-1}, i.v.). Supplementary doses of α-chloralose (10 – 20 mg kg^{-1}, i.v.) were given as required. Depth of anaesthesia was assessed by the stability of cardiovascular and respiratory variables being recorded. The right carotid artery was cannulated in order to measure blood pressure and for sampling arterial blood for analysis of pH and blood gases. Blood pressure was measured using a pressure transducer (Gould Statham P23XL) and the heart rate was derived electronically from the blood pressure signal (Gould Biotach Amplifier). The left jugular vein was cannulated for the purpose of drug administration and a tracheal cannula was implanted (Figure 13). Body temperature was monitored by a rectal probe and maintained at 36 - 38°C with a homoeothermic blanket system (Harvard). The animals were artificially ventilated (rate 50 strokes min^{-1}, stroke volume 8 ml kg^{-1}) with oxygen enriched room air by the use of a positive pressure pump (Harvard Rodent Ventilator 683) and neuromuscular blockage was produced with decamethonium (3 mg kg^{-1}, i.v.). Blood samples were taken from a T-piece on the carotid arterial cannula and blood gases and pH were monitored with a Corning pH/blood gas analyzer. Blood gases were maintained between 90-130 mmHg $P0_2$, 40-50 mmHg $PC0_2$ and pH 7.3-7.4. Adjustments of the respiratory pump volume were made as necessary to maintain blood gas and pH balance. Once ventilated, the animals were infused (6 ml kg^{-1} h^{-1}) via the jugular vein with a solution comprising 10 ml plasma substitute (gelofusine), 10 ml distilled water, 4 mg glucose, 168 mg sodium bicarbonate and 10 mg decamethonium. This was to prevent the development of non-respiratory acidosis and to maintain blood volume and neuromuscular blockade. During neuromuscular blockade, the depth of anaesthesia was assessed by monitoring the stability of the arterial blood pressure and heart rate and the cardiovascular responses to pinching the paws.

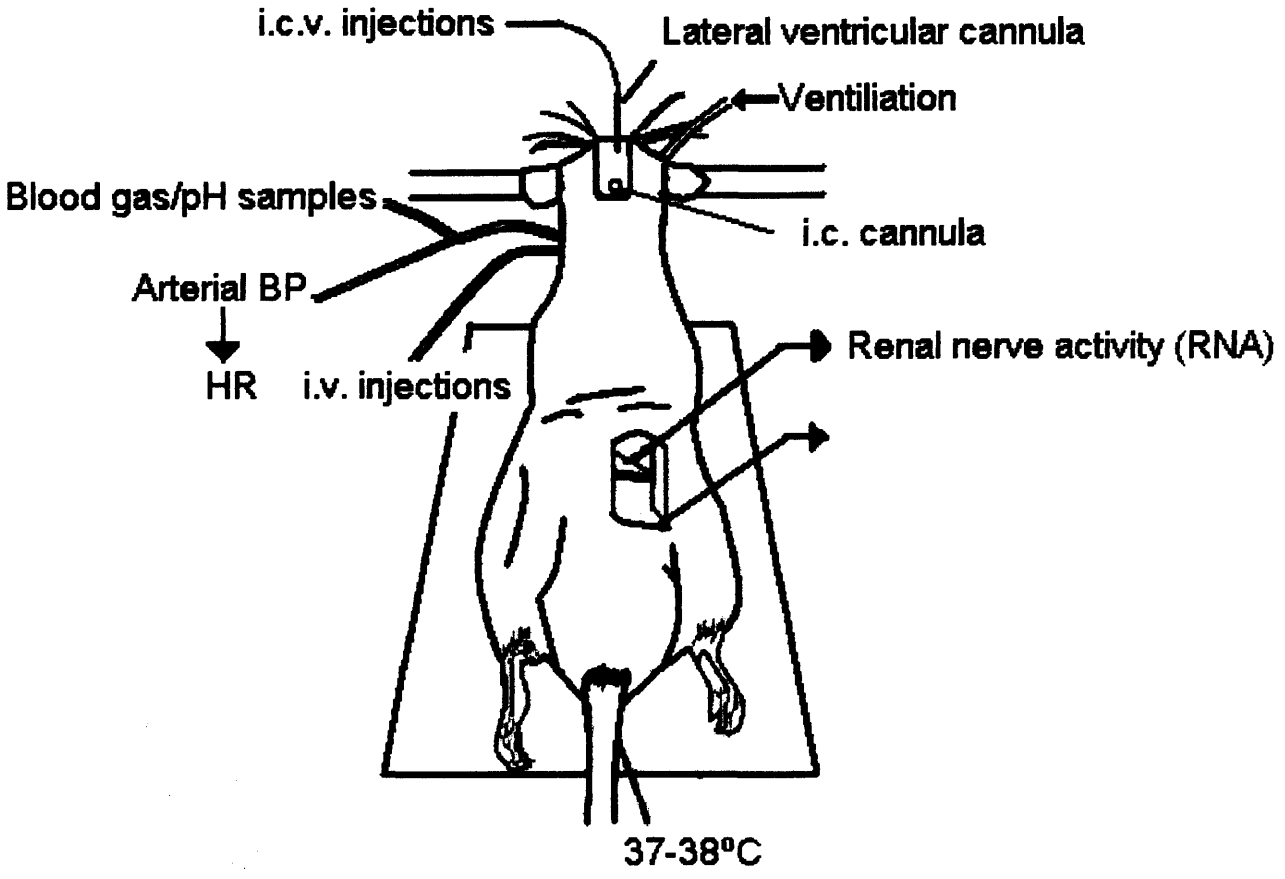


Figure 13. Diagrammatic representation of the animal model used during the study illustrating a rat artificially ventilated. Measured from the right carotid artery were arterial blood pressure (arterial BP) and heart rate (HR). Blood samples were also from the carotid cannula to measure blood gases and pH levels. Intra-venous (i.v.) injections were delivered via cannulation of the left jugular vein. Intracerebroventricular (i.c.v.) injections were delivered via the cannulation of the lateral cerebral ventricles and intracisternal (i.c.) injections were delivered via the cannulation of the cisterna magna. Renal nerve activity (RNA) was detected using bipolar silver hook electrodes.

2.1.1. Cannulation of the lateral cerebral ventricle.

The rats were placed in a sterotaxic head holder and a stainless steel guide cannula (22 gauge) was implanted into the right lateral cerebral ventricle. The co-ordinates used from the bregma were 4 mm ventral, 1.5 mm lateral and 1 mm posterior. Drugs and vehicle solutions were administered through an i.c.v. injection cannula (28 gauge) attached by a length of polythene tubing to a 25 μl syringe (Hamilton). At the end of the experiment, the cannula placement was confirmed by the administration of 5 μl of 2% pontamine sky blue dye.

2.1.2. Cannulation of the cisterna magna.

The rats were positioned in a sterotaxic head holder, and the atlanto-occipital membrane was exposed. A stainless steel guide cannula (23 gauge) was inserted perpendicularly into the membrane to the depth of its bevel to just pierce the membrane. Drugs and vehicle solutions were administered through an i.c. injection cannula (28 gauge) attached by a length of polythene tubing to a 25 μl syringe (Hamilton). Successful cannulation was verified by the filling of stainless steel guide cannula with clear cerebrospinal fluid and at the end of the experiment, by the administration of 5 μl of 2% pontamine sky blue dye.

2.1.3. Recording of renal nerve activity.

The left kidney was exposed by a retroperitoneal approach and was deflected laterally to reveal the renal artery and nerve. The nerve was cleared of connective tissue and positioned on a bipolar silver hook electrode (Figure 13). The renal nerve activity was amplified (Digitimer NL104), filtered (Digitimer NL125, frequency band width 100-500Hz) and quantified by integrating the signal above the background noise over 5

seconds with a solid state integrator (Medical Electronics workshop, Royal Free Hospital School of Medicine) (Anderson *et al.*, 1992). At the end of the experiment 20 mg of pentobarbitone sodium (per animal) was used to validate the integrator threshold.

At the beginning of each experiment the baroreceptor reflex response was tested by observing whether the renal nerve activity and heart rate were reduced by a rise in blood pressure caused by noradrenaline (25 ng per animal, i.v.), and whether they were raised, by a reduction in blood pressure caused by sodium nitroprusside (0.6 μg per animal, i.v.). Only preparations with an intact baroreceptor reflex were used.

2.2. Experimental protocol.

2.2.1. Neuronal nicotinic receptor agonists (i.c.v. & i.c.).

In all the experiments recorded, the rat model preparations were performed on anaesthetised rats, artificially ventilated and neuromuscular blocked, by decamethonium. The preparation was allowed to stabilize for 20 min before the administration of 5 μl saline (i.c.v. or i.c.). Ten minutes after the saline control, a single dose of test compound or saline control was given i.c.v. or i.c. and the response was followed for at least 20 min. A diagrammatic representation of this protocol is shown in Figure 14-1. In each rat the cardiovascular response to a single dose of the test agonist or saline was recorded.

2.2.2. Neuronal nicotinic receptor antagonist ligands (i.c.v. and i.c.).

The preparation was allowed to stabilize for 20 min before flushing the i.c.v. cannula with saline (5 μl). Ten minutes after this flush injection of saline (initial saline), a

nicotinic antagonist (dihydro-β-erythroidine, methyllycaconitine or α-bungarotoxin) was given i.c.v. This was then followed 3 min later by a test drug which was given i.c.v. and the response followed for at least 20 min (Figure 14-2). The control carried out for these experiments was an injection of saline instead of nicotinic antagonist (dihydro-β-erythroidine, methyllycaconitine citrate or α-bungarotoxin) i.c.v. and the response followed for at least 20 min.

In the case of i.c. controls, the preparation was allowed to stabilize for 20 min before flushing the i.c. cannula with saline (5 μl). Ten minutes after this flush injection of saline (initial saline), an antagonist (α-Bungarotoxin) was given i.c. This was then followed 3 min later by a test drug or saline which was given i.c. and the response followed for at least 20 min (Figure 14-2).

2.2.3. Pretreatment with the V_1 receptor antagonist d(CH2)5Tyr(Me)AVP (i.v.).

The V_1 receptor antagonist, d(CH2)5Tyr(Me)AVP, was administered i.v. 5 min after the saline flush, followed 10 min later by the administration of test drug i.c.v. or i.c. The control carried out for these experiments was an injection of V_1 receptor antagonist administered i.v., 5 min after the saline flush, followed 10 min later by the administration of 5 μl saline i.c.v. or i.c (Figure 15-1). These pre-treatment times were chosen to allow stabilization of any changes in the variables being recorded caused by the administration of these substances. In each rat the cardiovascular response of a single dose of the test agonist and saline were recorded.

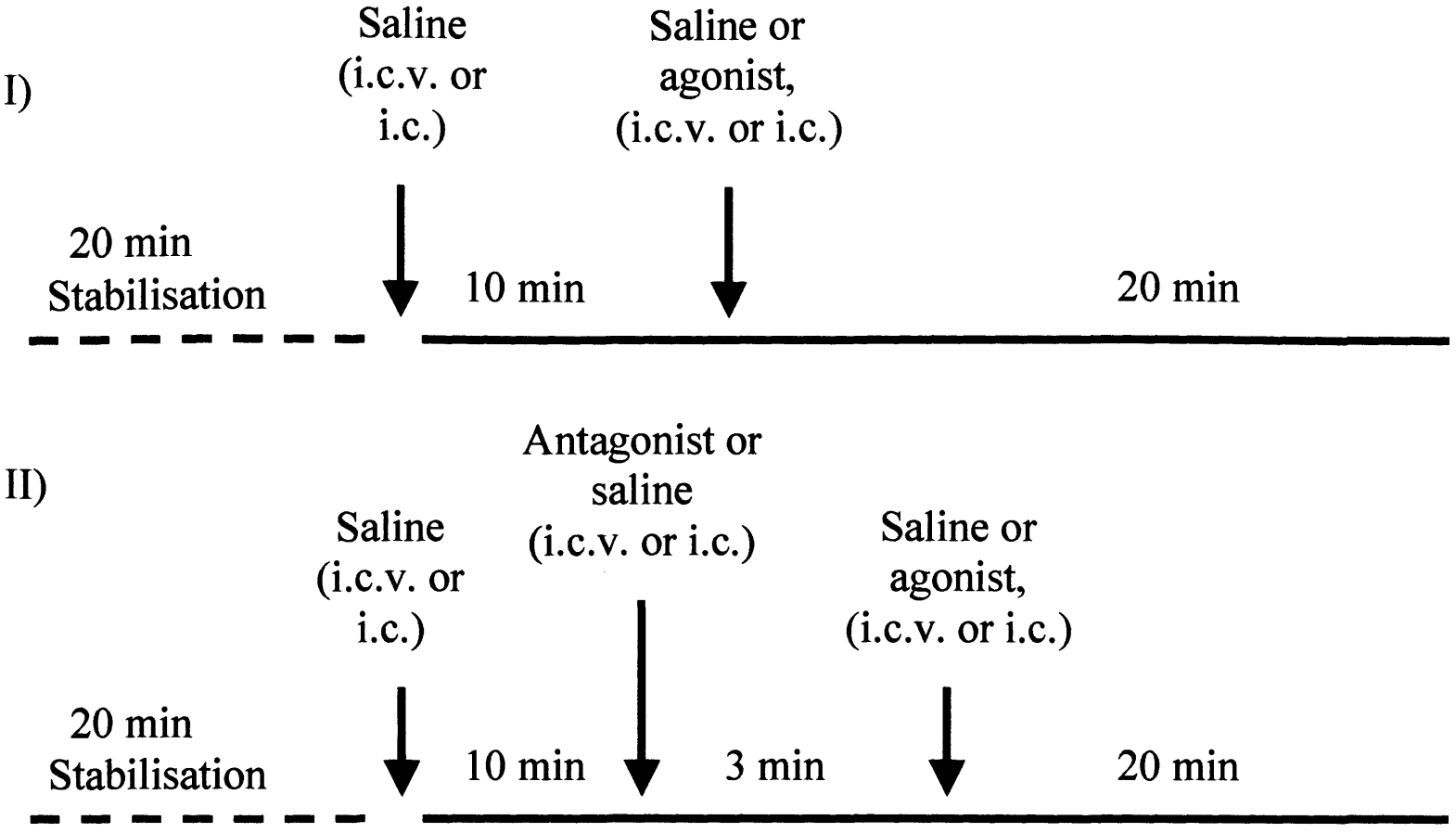


Figure 14. Diagrammatic representation of the experimental protocol, showing the timing of pre-treatment and drug administration. 1) Drug or saline central administration in artificially ventilated neuromuscular blocked rats; 2) Pre-treatment of antagonist then drug or saline administration in artificially ventilated neuromuscular blocked rats.

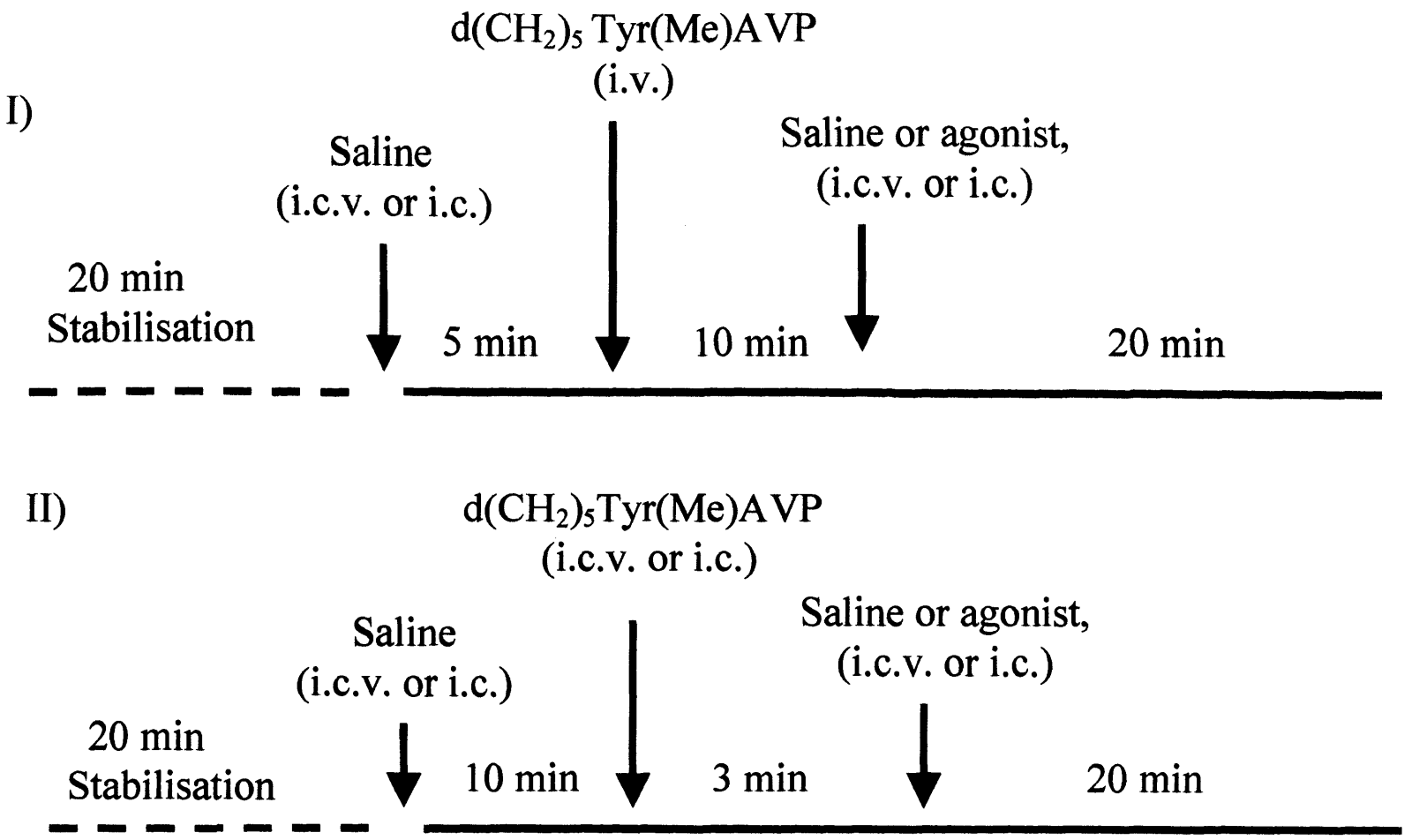


Figure 15. Diagrammatic representation of the experimental protocol, showing the timing of pre-treatment and drug administration. 1I) V_1 pre-treatment (i.v.) followed by agonist or saline administration, 2) V_1 pre-treatment administered centrally (i.c.v. & i.c.) followed by agonist or saline administration, in artificially ventilated neuromuscular blocked rats.

2.2.4. Pretreatment with the V_1 receptor antagonist d(CH2)5Tyr(Me)AVP (i.c.v. & i.c.).

The preparation was allowed to stabilize for 20 min before flushing the i.c.v. or i.c. cannula with saline (5 μl). Five minutes after this flush injection of saline, an V_1 receptor antagonist (d(CH2)5Tyr(Me)AVP) was given i.c.v. or i.c. This was then followed 3 min later by a test drug which was given i.c.v. or i.c. and the response followed for at least 20 min (Figure 15II). The control carried out for these experiments was an injection of saline instead of the test drug after the injection of V_1 antagonist (i.c.v. or i.c.) and the response followed for at least 20 min.

2.2.5. Nicotinic agonist infusion.

The preparation was allowed to stabilize for 20 min then given a 0.5 ml flush of saline in to the i.v. cannula. Five minutes after this flush injection of saline (initial saline), nicotinic agonist was infused over a 3 min period through the i.v. cannula and the response followed for at least 20 min. The control carried out for these experiments was an infusion of saline instead of the test drug for 3 min and the response followed for at least 20 min.

2.3. Analysis of results.

Baseline values were taken 1 min before the addition of the drug or vehicle. All results are expressed as changes from baseline values. Nerve activity was measured as the average of the integrated values over 10 sec for the first 5 min and then as an average of the integrated values over 1 min in arbitrary units and was expressed as the percentage change from baseline. Changes in mean arterial blood pressure, heart rate and renal nerve activity caused by the test drug were compared with time-matched vehicle controls

using two-way analysis of variance and were subsequently analysed using the least significant difference test. All values are expressed as the mean ± s.e. mean; differences in the mean were taken as significant when $P< 0.05$.

All baseline values are shown in Table 17 in the appendix. In addition, Tables 15 and 16 give an overview of the experiments performed and the results recorded throughout the thesis.

2.4. Drugs and solutions.

Drugs were obtained from the following sources: α-chloralose; sodium nitroprusside; nicotine hydrogen tartrate; cytisine, decamethonium bromide; [β-Mercapto-β,β-Cyclopentamethylenepropionyl1,O-Me-Tyr2,Arg8]-Vasopressin, (d(CH2)5Tyr(Me)AVP); dihydro-β-erythroidine, DMPP and α-Bungarotoxin from Sigma Chemical Co., Poole, Dorset, U.K.; noradrenaline acid tartrate from Winthrop, Guildford, Surrey, U.K.; isoflurane from Abbott Labs Ltd, Queenborough, Kent; Gelofusine from Braun Medical Ltd, Aylesbury, Bucks; Methyllycaconitine citrate from Tocris Cookson Ltd., Avonmouth, Bristol, U.K. The following were gifts from Eli Lilly Co. PSAB-OFP ((R)-(-)-5'Phenylspiro[1-azabicyclo[2.2.2] octane-3,2'-(3'H)furo[2,3-b]pyridine) and TC-2559 ((E)-N-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine).

Drugs given i.c.v. and i.c. were dissolved in 0.9% w/v saline. Solutions were administered in dose volume of 5 µl over a 20 sec period. All drugs given i.v. were dissolved in saline.

CHAPTER 3. RESULTS.

3.1. Effect of central administration of neuronal nAChR selective agonists on cardiovascular regulation.

3.1.1. Introduction.

Although the effects on cardiovascular control of nicotine have been studied previously, the role of different types of nAChR in mediating these effects is unknown. With the availability of selective agonists and antagonists for different neuronal nAChR subtypes it is now possible to determine which subtypes are involved.

Two compounds have been shown to be selective for the two most predominant neuronal nACh receptors in the CNS, the α4β2 and α7 receptor (Clarke *et al.*, 1985; Wada *et al.*, 1988). The first, TC-2559 (Bencherif *et al.*, 2000) has been shown to compete with [^{3}H]-nicotine (α4β2 receptors) binding but not with [^{125}I]-αBgT (α7 receptors). The selectivity of TC-2559 was then characterised across six recombinant human nAChRs and was shown to selectively activate α4β2 (EC_{50} = 0.18 μM), while at β4-containing nAChR (α2β4, α4β4 and α3β4) it was 100X weaker, having an EC_{50} in the range of 10-30 μM and no activity at α3β2 and α7 nACh receptors (Chen *et al.*, 2003). TC-2559 was also demonstrated to be selective for α4β2 over α4β4 and α7 subtypes expressed in *Xenopus* oocytes. The effects of TC-2559 have been studied *in vitro* (Bencherif *et al.*, 2000; Chen *et al.*, 2003) and *in vivo* (Wang *et al.*, 2006) particularly on dopaminergic activity in the ventral tegmental area (VTA). These studies have shown that TC-2559 causes an increase in firing of VTA neuronal dopaminergic cells in a dose-dependent manner (*in vitro*), over the same concentration range that activates α4β2 receptor in recombinant cell lines (Chen *et al.*, 2003). This effect was blocked by α4β2 selective receptor antagonist, dihydro-β-erythroidine (DhβE), but not by the α7 selective receptor antagonist, methyllycaconitine (MLA). *In vivo* studies observing the effects of TC-2559 on

spontaneous firing and burst properties of the dopaminergic neurons in the VTA shows that TC-2559 mimics nicotine-evoked excitation of VTA dopaminergic neurones. TC-2559 and nicotine's effects were blocked by DhβE, which implies α4β2 receptors are the dominant nAChR mediating nicotinic excitation of VTA dopaminergic neurones *in vivo* (Wang *et al.*, 2006). Thus TC-2559 is a useful tool for both *in vivo* (Wang *et al.*, 2006) and *in vitro* (Bencherif *et al.*, 2000) studies of the α4β2 nAChR. At present, the effects of central administration of TC-2559 on the cardiovascular system are unknown.

Another novel agonist is PSAB-OFP (Astra IV) which has been shown to be selective for the α7 nicotinic AChR, with studies demonstrating that MLA inhibits PSAB-OFP evoked excitation and burst activities of dopaminergic neurons on the VTA (Broad *et al.*, 2002; Wang *et al.*, 2006). Currently there is limited published data about PSAB-OFP and no published data on the effects of central administrations of PSAB-OFP on the cardiovascular system.

Thus the effect of selective receptor agonists, TC-2559 and PSAB-OFP, given i.c.v. or i.c. were investigated on baseline arterial blood pressure, heart rate and renal sympathetic nerve activity in α-chloralose anaesthetised rats, to determine the role of different neuronal nAChRs in pathways inflencing these cardiovascular variables.

3.1.2. Results.

3.1.2.1. Intracerebroventricular (i.c.v.) administration of selective nACh receptor agonists.

Saline i.c.v. (5 μl, saline control, n=5) had little effect on mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity and these variables remained stable for the duration of the experiment (20 min). Baseline values for MAP and HR were 137 ± 14 mmHg and 380 ± 27 beats min^{-1} (see Figures 16 - 19).

3.1.2.1.1. Effects of PSAB-OFP, a selective α7 nicotinic receptor agonist.

PSAB-OFP (1 μmol kg^{-1}; n=5) had no significant effect on mean arterial blood pressure or heart rate, however there was a significant increase in renal nerve activity at 6 min of 34 ± 7%, reaching a maximum of 70 ± 7% at 10 min (Figure 16). Baseline values for MAP and HR were 122 ± 7 mmHg and 362 ± 44 beats min^{-1}. PSAB-OFP (3 μmol kg^{-1}, n=5) caused a significant pressor response associated with an increase in renal nerve activity at 4 min of 22 ± 6 mmHg and 77 ± 21%, reaching a maximum of 23 ± 6 mmHg and 126 ± 23% after 5 min. There was no significant change in heart rate. Baseline values for MAP and HR were 124 ± 8 mmHg and 356 ± 23 beats min^{-1} (Figure 17 & 18). At the higest dose, 10 μmol kg^{-1} (n=5), PSAB-OFP caused a significant increase in mean arterial blood pressure and renal nerve activity at 4 min reaching 32 ± 9 mmHg and 290 ± 101% at 6 min and 20 min respectively. However a tachycardia was now observed at 6 min reaching a maximum of 43 ± 16 beats min^{-1} at 20 min (Figure 19). Baseline values for MAP and HR were 126 ± 22 mmHg and 350 ± 23 beats min^{-1}.

3.1.2.1.2. Effects of TC-2559, a selective α4β2 nicotinic receptor agonist.

TC-2559 (1 μmol kg^{-1}, n=5) had no significant effect on mean arterial blood pressure or heart rate, however, there was a significant increase in renal nerve activity at 3 min, reaching a maximum of 79 ± 21% at 8 min (Figure 20). Baseline values for MAP and HR were 120 ± 17 mmHg and 368 ± 54 beats min^{-1}. At 3 μmol kg^{-1}, TC-2559 (n=5) caused a significant increase in blood pressure at 3 min of 21 ± 6 mmHg, reaching a maximum of 22 ± 3 mmHg at 5 min. There was a delayed significant increase in renal nerve activity at 5 min reaching a maximum of 249 ± 53% at 9 min, as well as, no significant change in heart rate apart from an increase at 9 min of 29 ± 9 beats min^{-1} (Figure 21 & 22). Baseline values for MAP and HR were 113 ± 16 mmHg and 366 ± 34 beats min^{-1}. At the highest dose, 10 μmol kg^{-1} (n=5), TC-2559 now caused a significant fall in mean arterial blood pressure and renal nerve activity, reaching a maximum of -34 ± 12 mmHg and -75 ± 10% by 3 min. The heart rate was unaffected (Figure 23). Baseline values for MAP and HR were 135 ± 19 mmHg and 380 ± 14 beats min^{-1}.

Figure 16. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (1 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

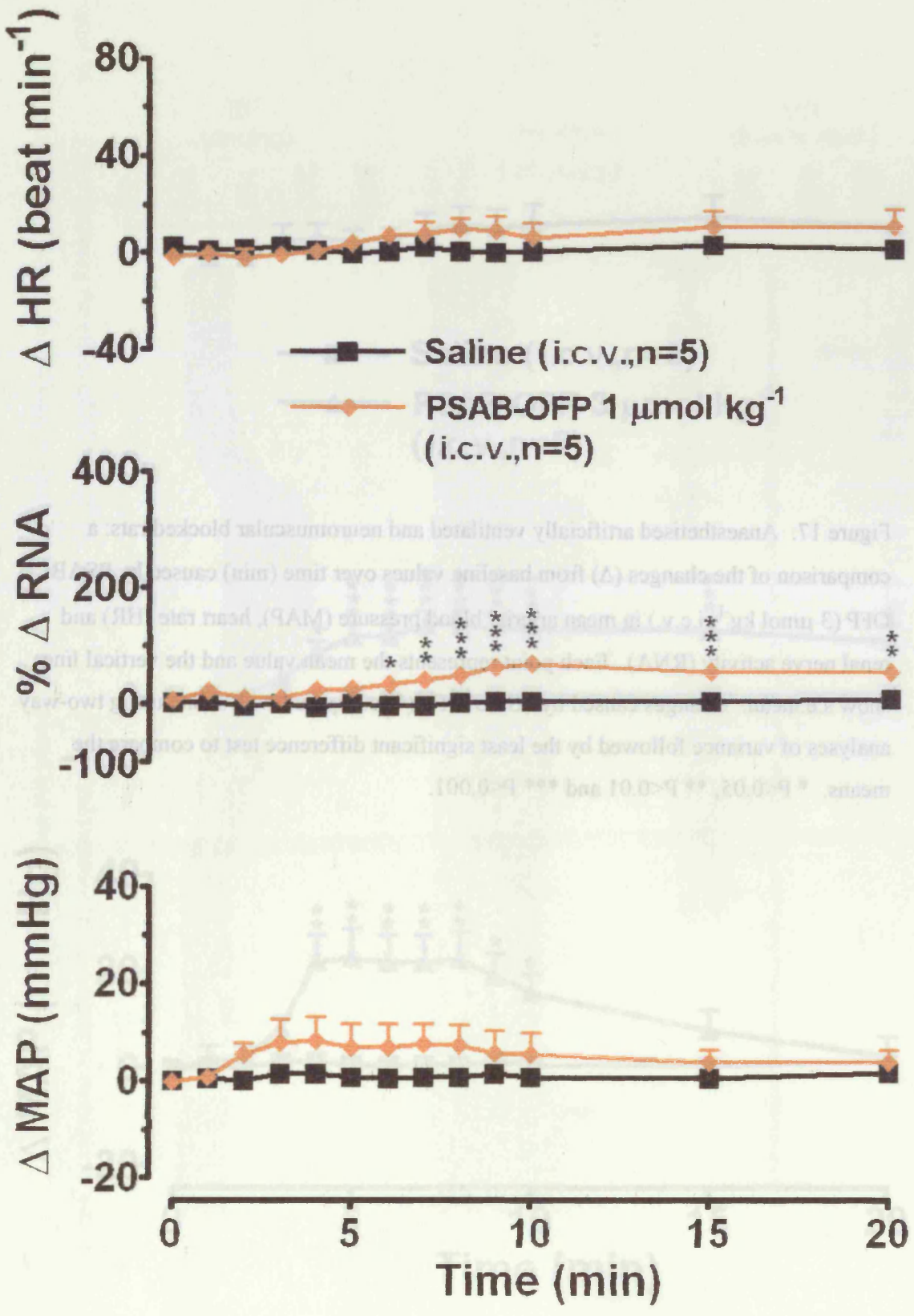
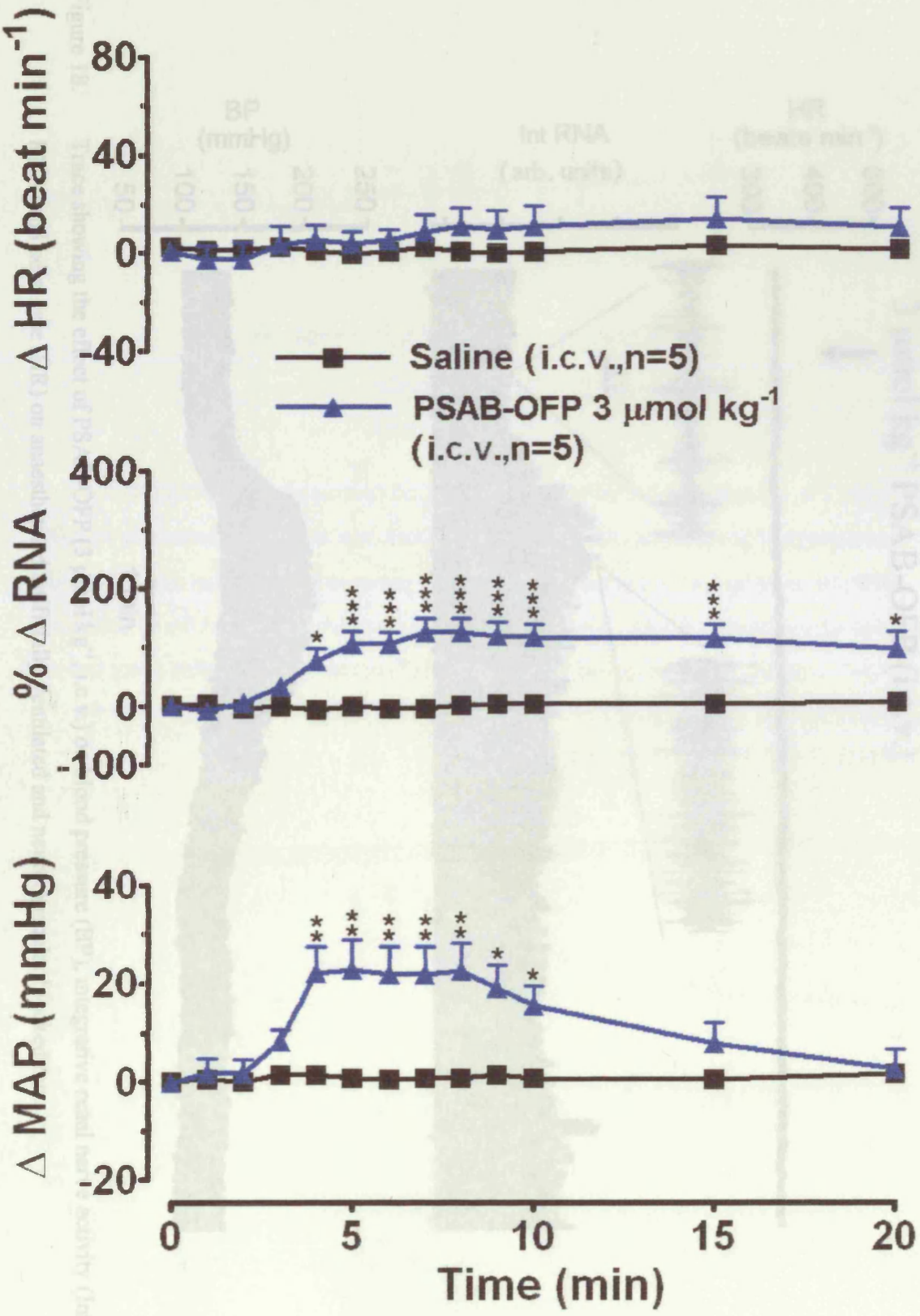

Δ HR (beat min-1)
80
40
0
-40
Saline (i.c.v.,n=5)
PSAB-OFP 1 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 17. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.


Δ HR (beat min-1)
80
40
0
-40
Saline (i.c.v.,n=5)
PSAB-OFP 3 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

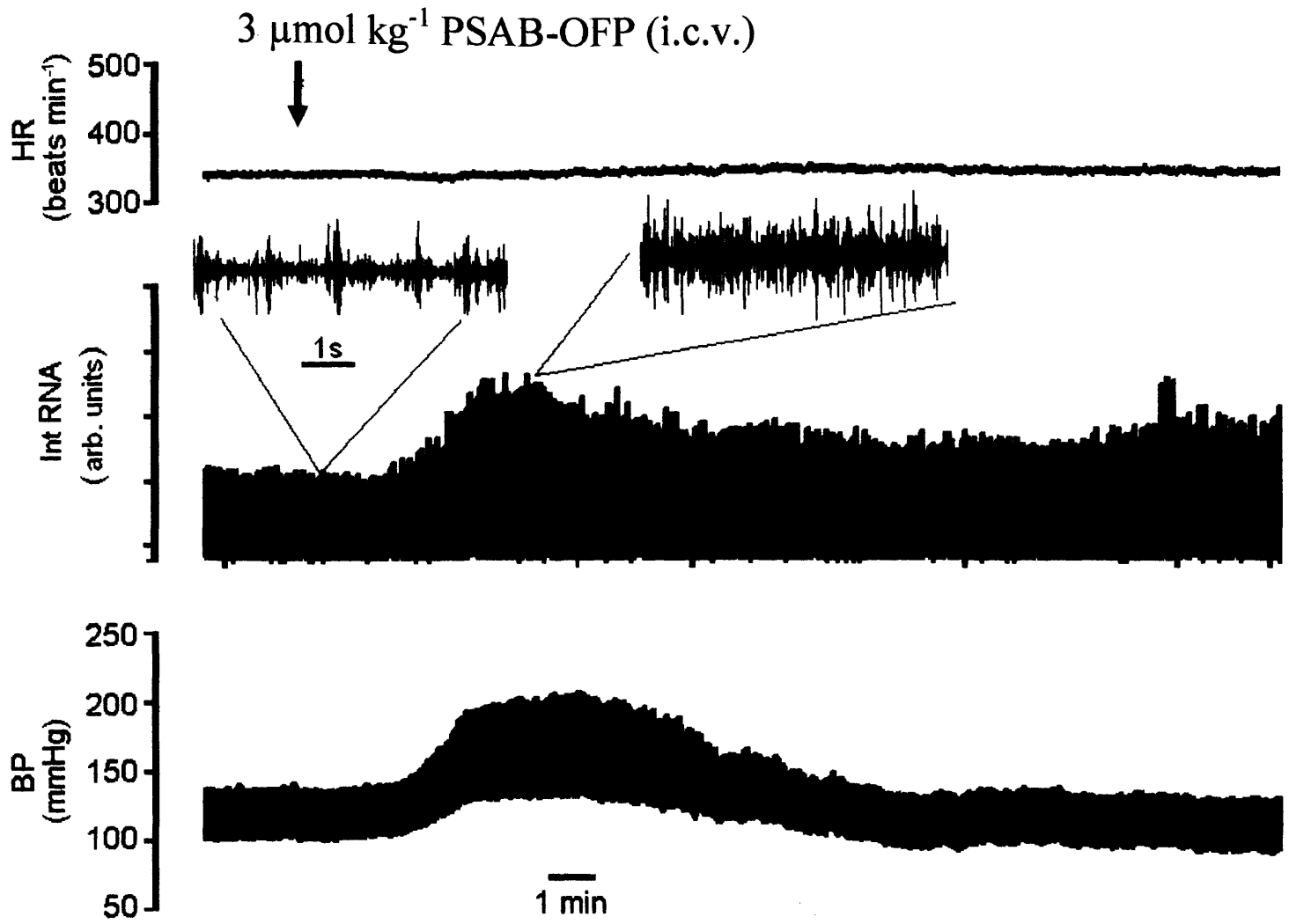


Figure 18. Trace showing the effect of PSAB-OFP (3 µmol kg^{-1}, i.c.v.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 19. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (10 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

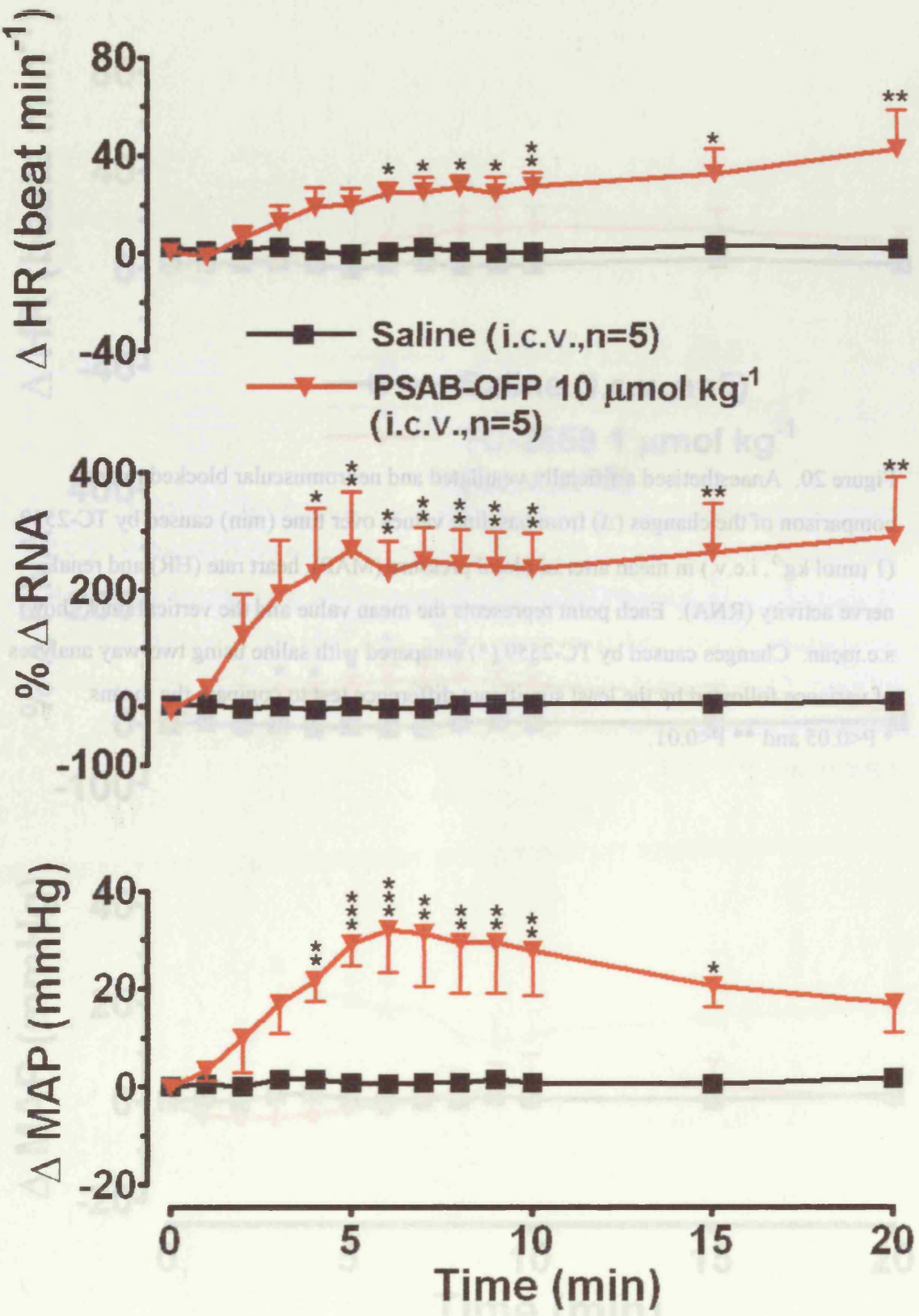

Δ HR (beat min-1)
80
40
0
-40
Saline (i.c.v.,n=5)
PSAB-OFP 10 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 20. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (1 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means.
* $P<0.05$ and ** $P<0.01$.

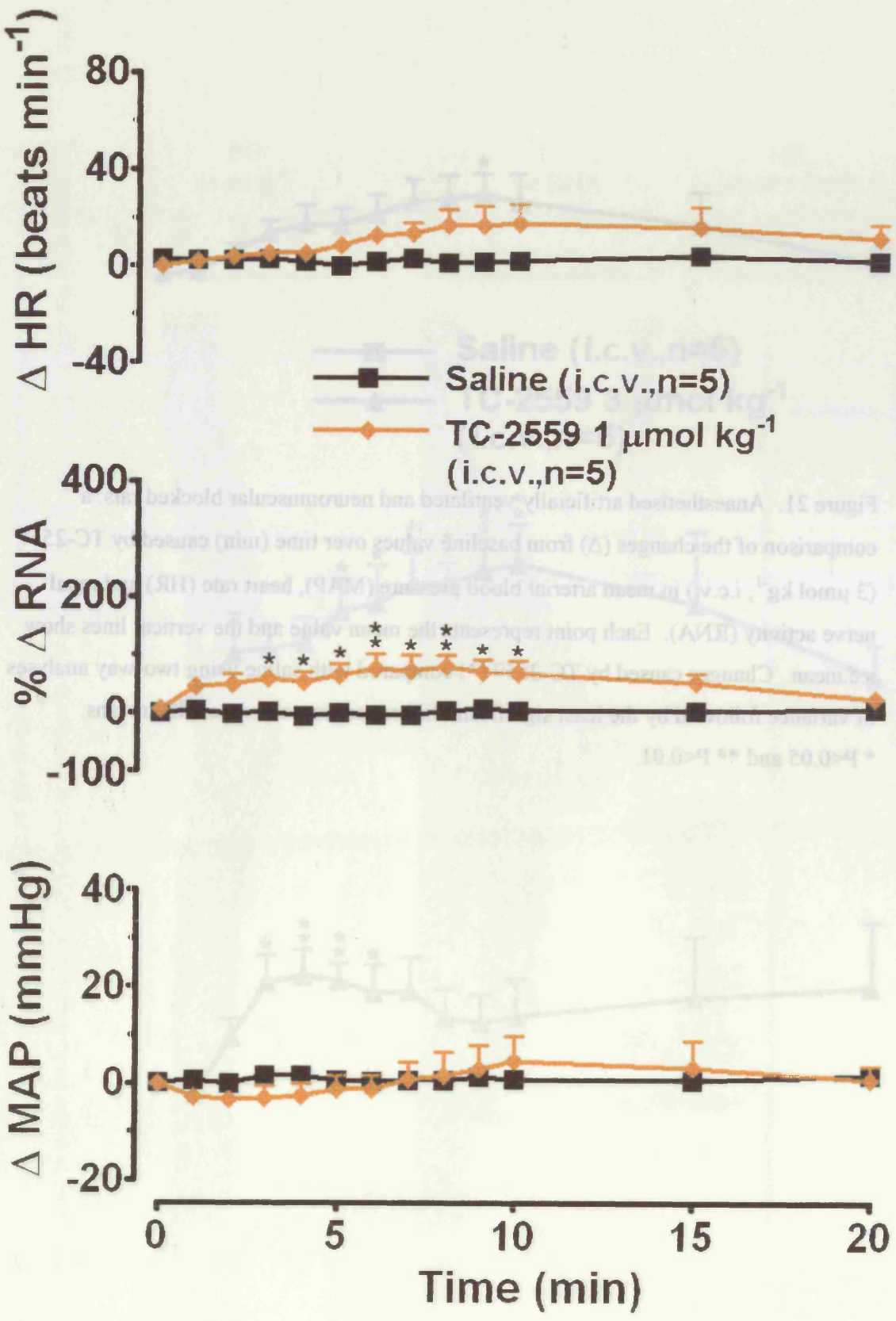
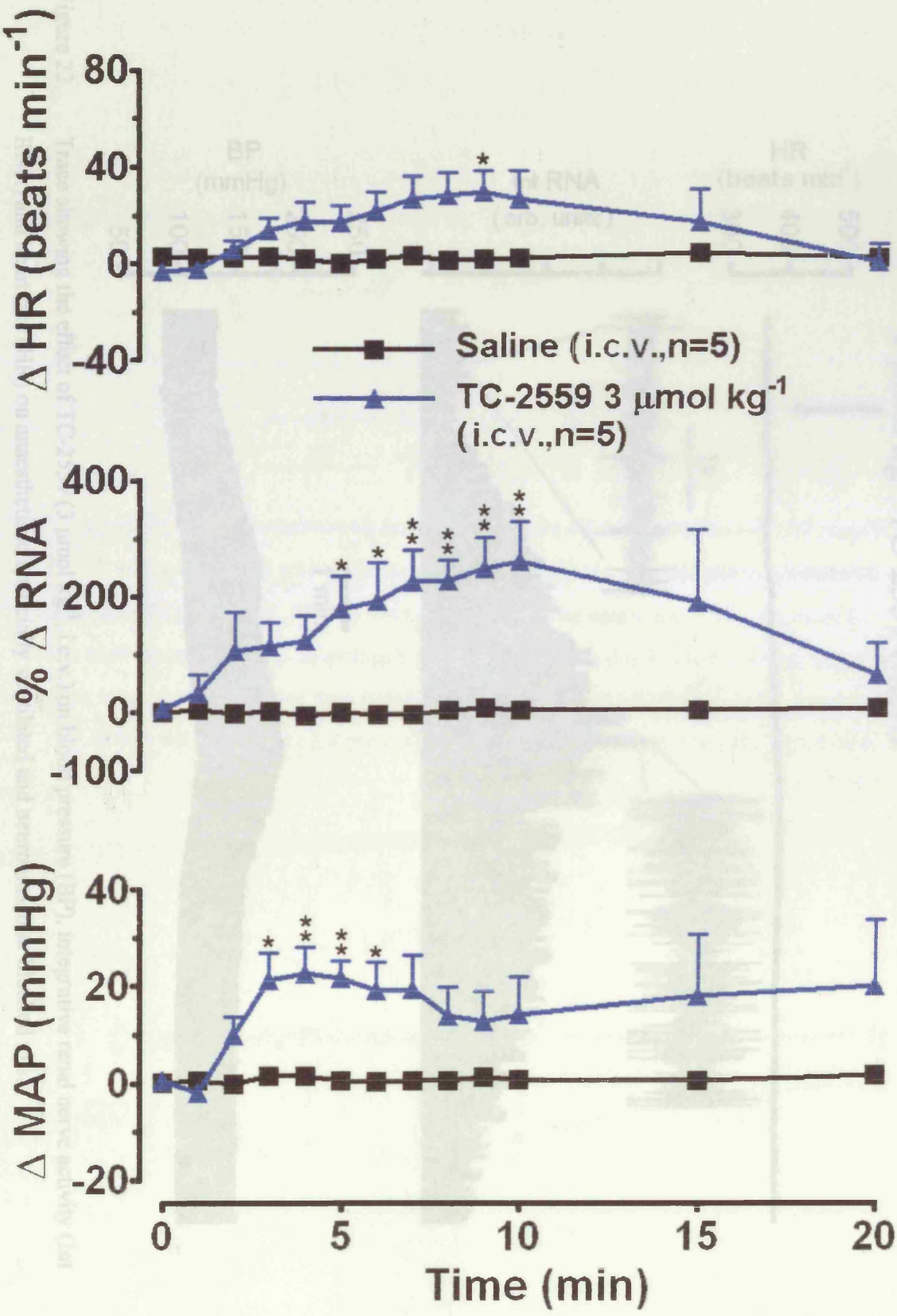

Δ HR (beats min-1)
80
40
0
-40
Saline (i.c.v.,n=5)
TC-2559 1 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 21. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means.
* $P<0.05$ and ** $P<0.01$.


Δ HR (beats min-1)
80
40
0
-40
Saline (i.c.v.,n=5)
TC-2559 3 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

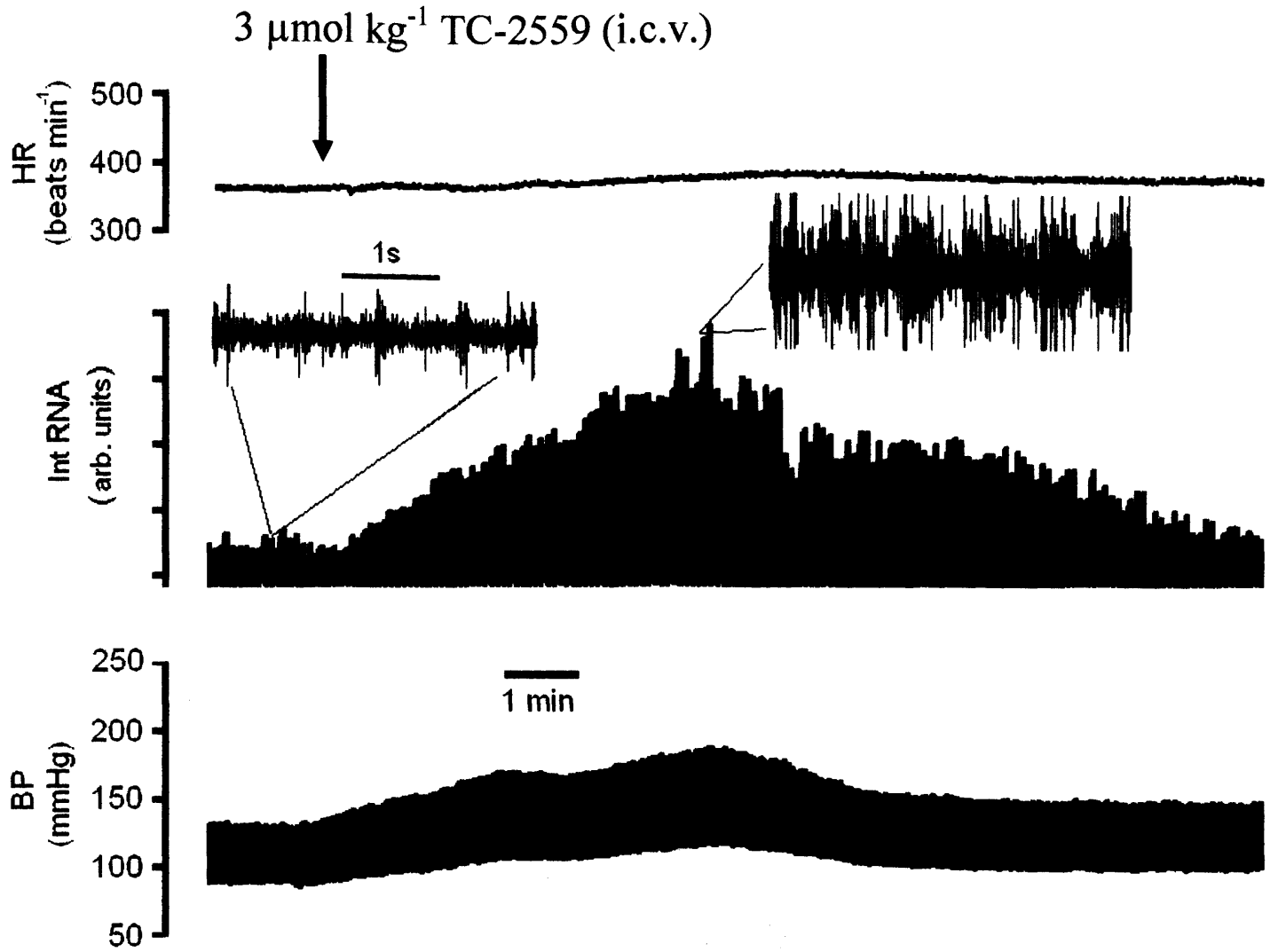
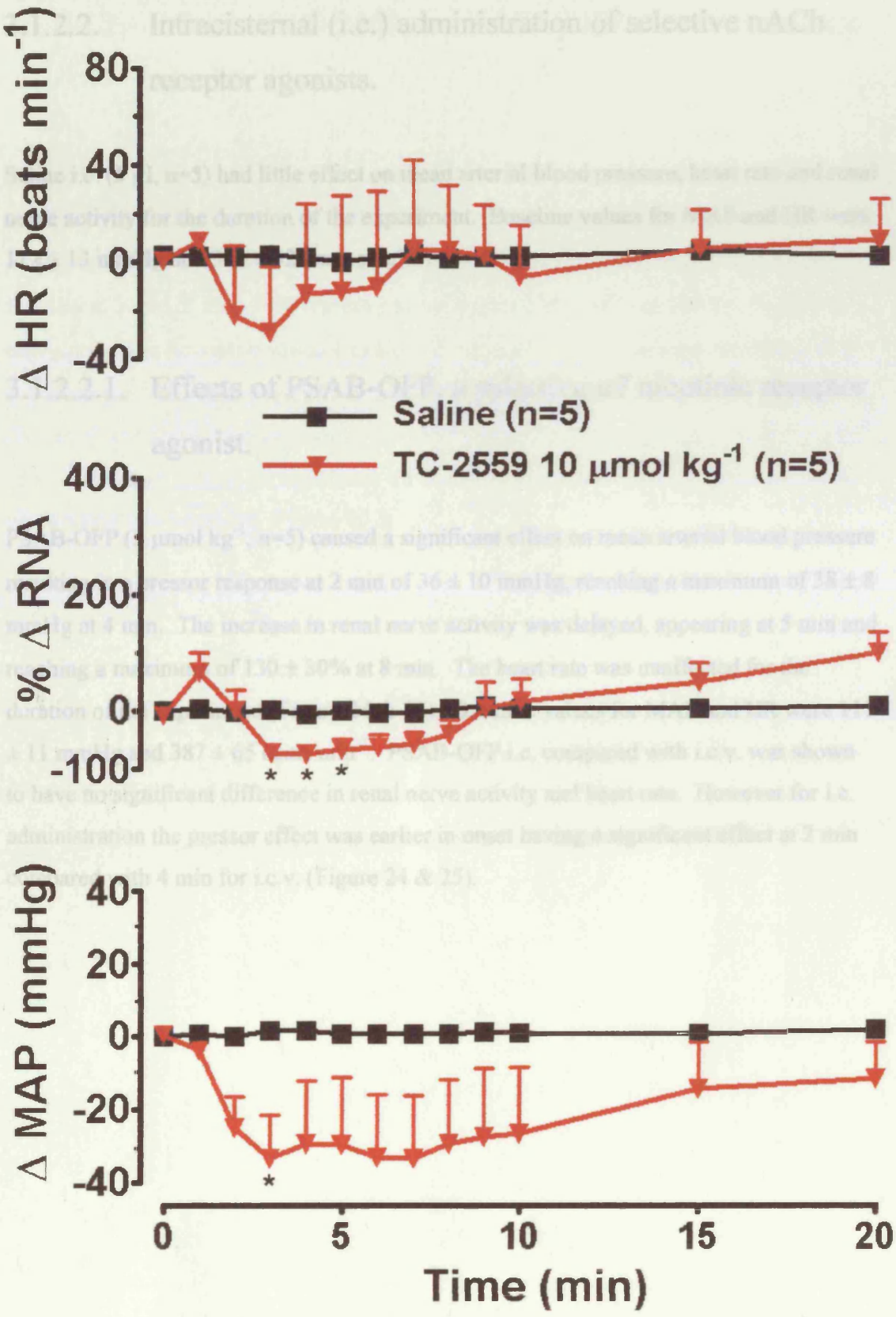


Figure 22. Trace showing the effect of TC-2559 (3 μmol kg^{-1}, i.c.v.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 23. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (10 μmol kg^{-1}, i.c.v.,) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means.
* $P<0.05$.


Δ HR (beats min-1)
80
40
0
-40
Saline (n=5)
TC-2559 10 μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
*
*
*
Δ MAP (mmHg)
40
20
0
-20
-40
*
0
5
10
15
20
Time (min)

3.1.2.2. Intracisternal (i.c.) administration of selective nACh receptor agonists.

Saline i.c. (5 μl, n=5) had little effect on mean arterial blood pressure, heart rate and renal nerve activity for the duration of the experiment. Baseline values for MAP and HR were 112 ± 13 mmHg and 385 ± 32 beats min^{-1}.

3.1.2.2.1. Effects of PSAB-OFP, a selective α7 nicotinic receptor agonist.

PSAB-OFP (3 μmol kg^{-1}, n=5) caused a significant effect on mean arterial blood pressure resulting in a pressor response at 2 min of 36 ± 10 mmHg, reaching a maximum of 38 ± 8 mmHg at 4 min. The increase in renal nerve activity was delayed, appearing at 5 min and reaching a maximum of 130 ± 30% at 8 min. The heart rate was unaffected for the duration of the experiment (Figure 24 & 25). Baseline values for MAP and HR were 111 ± 11 mmHg and 387 ± 65 beats min^{-1}. PSAB-OFP i.c. compared with i.c.v. was shown to have no significant difference in renal nerve activity and heart rate. However for i.c. administration the pressor effect was earlier in onset having a significant effect at 2 min compared with 4 min for i.c.v. (Figure 24 & 25).

3.1.2.2.2. Effects of TC-2559, a selective α4β2 nicotinic receptor agonist.

TC-2559 (3 μmol kg^{-1}, n=5; i.c.) had a significant effect on mean arterial blood pressure resulting in a pressor response at 2 min of 13 ± 3 mmHg, reaching a maximum of 21 ± 7 mmHg at 5 min. The increase in renal nerve activity was delayed, with a significant increase at 5 min of 78 ± 34% reaching a maximum of 86 ± 28% at 10 min. However, a tachycardia was now observed at 4 min of 17 ± 5 beats min^{-1} reaching a maximum of 24 ± 4 beats min^{-1} at 7 min (Figure 26 & 27). Baseline values for MAP and HR were 113 ± 14 mmHg and 392 ± 59 beats min^{-1}. TC-2559 administered i.c. compared with TC-2559 i.c.v. was shown to have no significant difference in mean arterial blood pressure, renal nerve activity or heart rate (Figure 26 & 27).

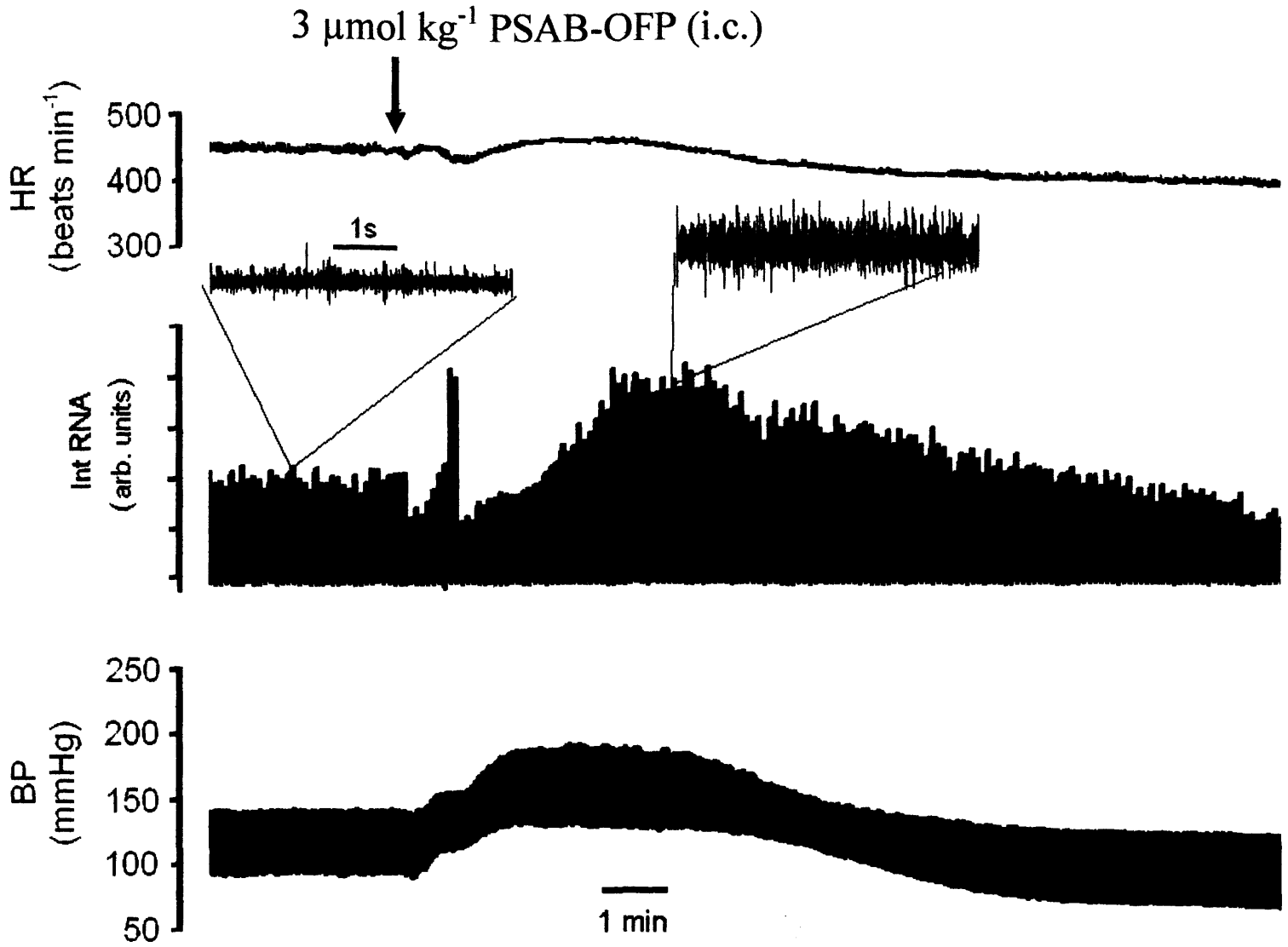
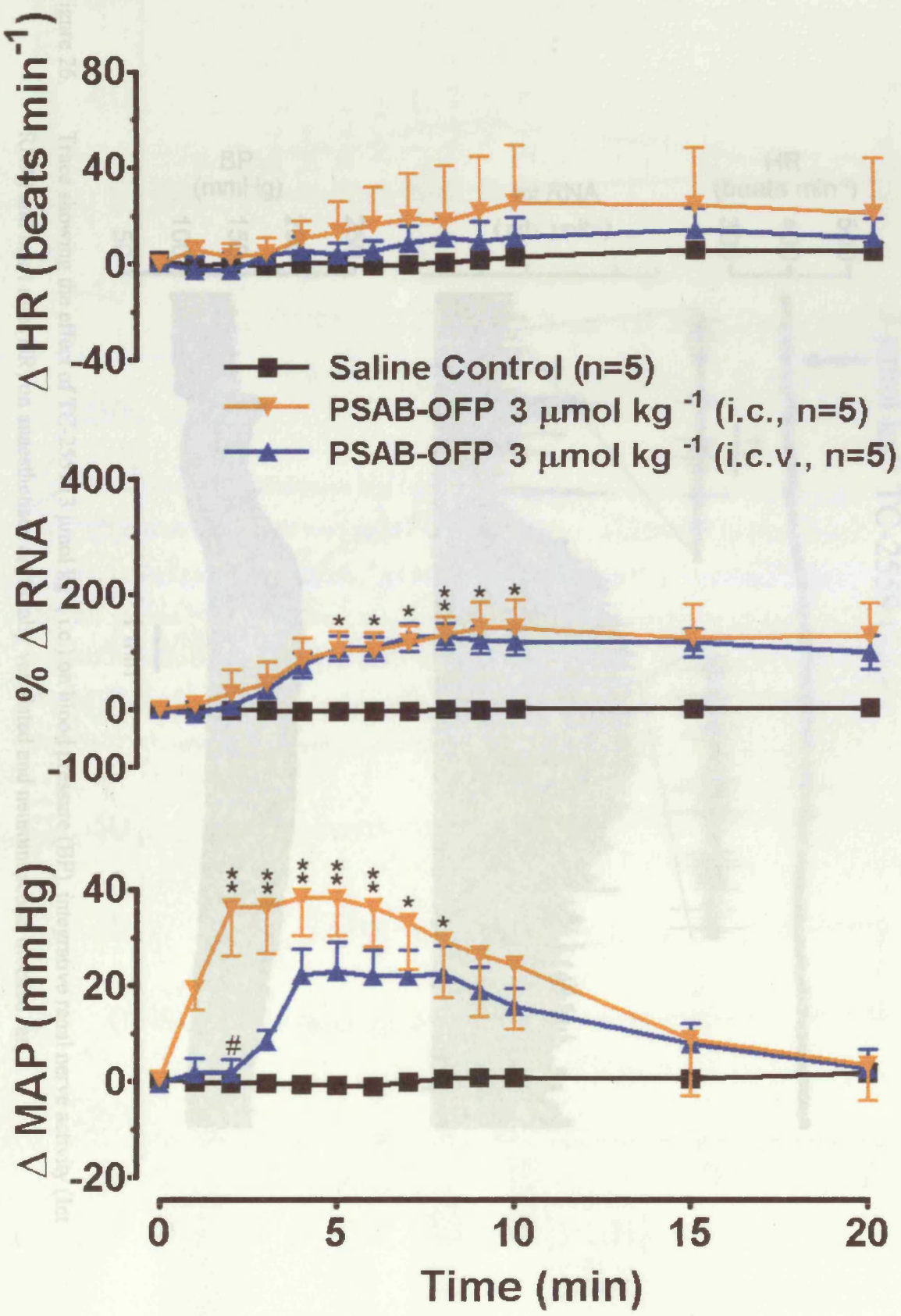


Figure 24. Trace showing the effect of PSAB-OFP (3 µmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 25. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.) and PSAB-OFB (3 μmol kg^{-1}, i.c.v.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFB i.c. (*) compared with saline and changes caused by PSAB-OFB i.c. (#) compared with PSAB-OFB i.c.v. by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and ** $P<0.01$.


Δ HR (beats min-1)
80
40
0
-40
Saline Control (n=5)
PSAB-OFP 3 μmol kg -1 (i.c., n=5)
PSAB-OFP 3 μmol kg -1 (i.c.v., n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
#
0
5
10
15
20
Time (min)

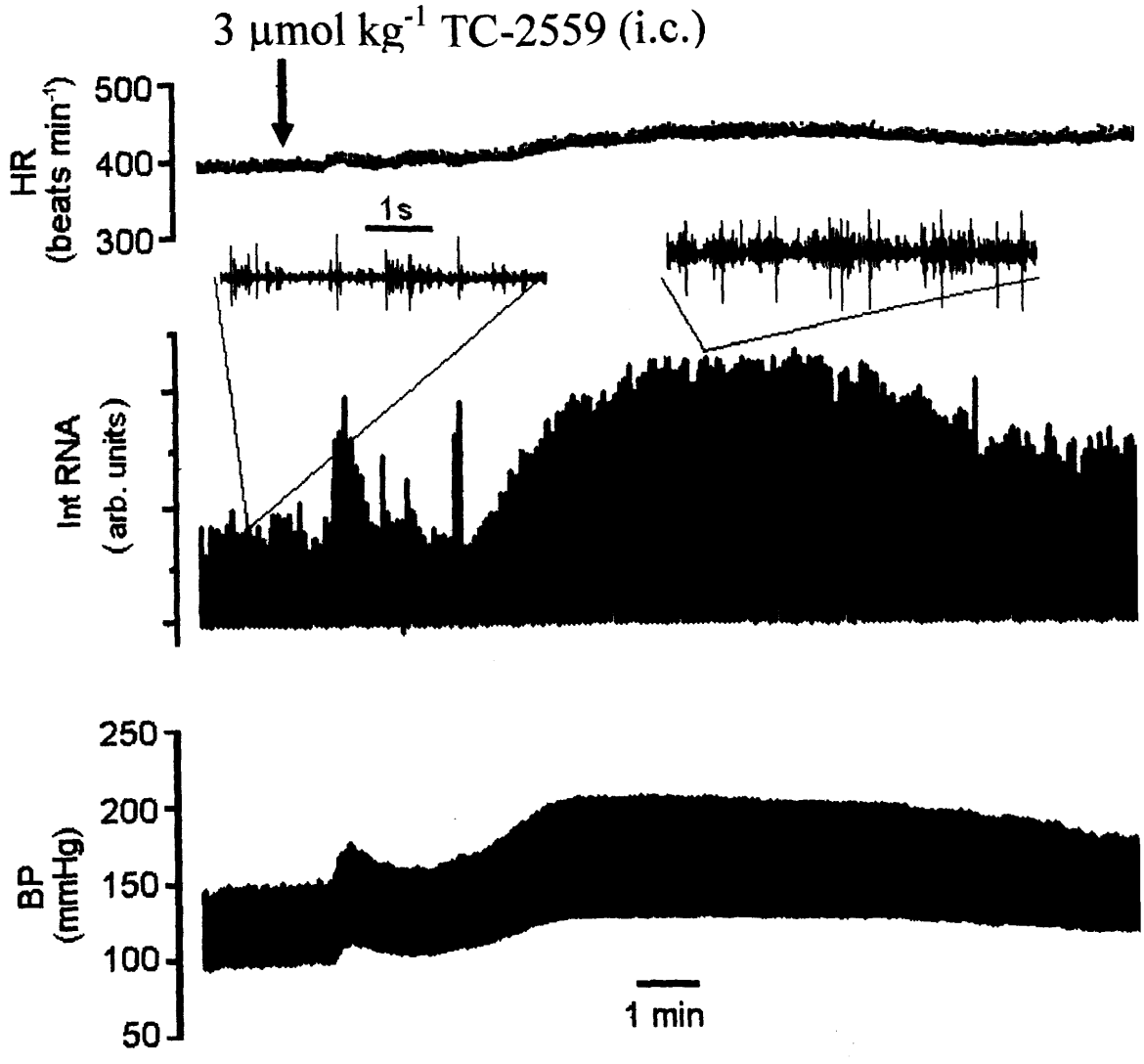
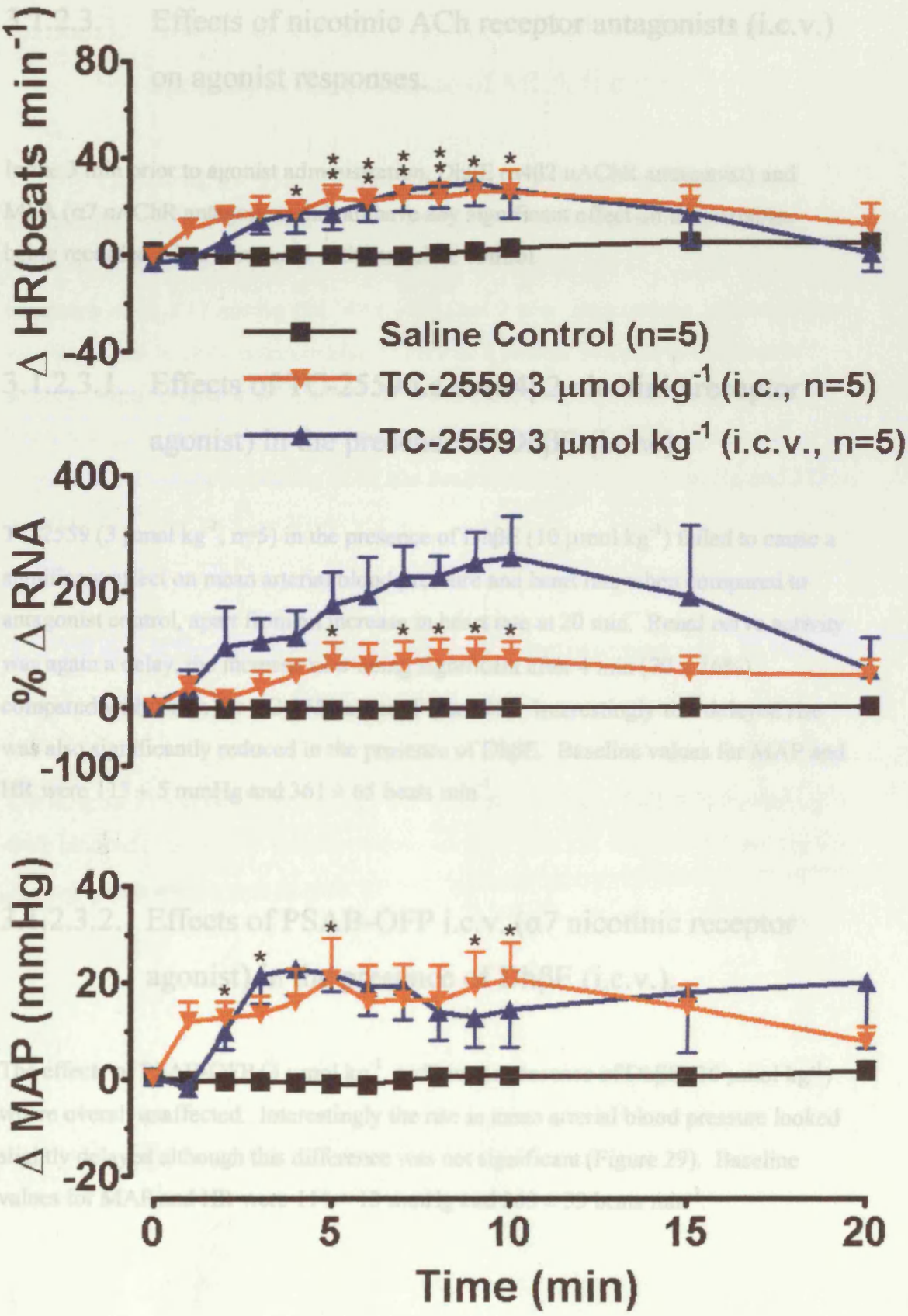


Figure 26. Trace showing the effect of TC-2559 (3 µmol kg^{-1}, i.c.) on blood pressure (BP), integrative re
RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked

Figure 27. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.) and TC-2559 (3 μmol kg^{-1}, i.c.v.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 i.c. (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$ and ** $P<0.01$.


Δ HR(beats min-1)
80
40
0
-40
Saline Control (n=5)
TC-2559 3 μmol kg-1 (i.c., n=5)
TC-2559 3 μmol kg-1 (i.c.v., n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.1.2.3. Effects of nicotinic ACh receptor antagonists (i.c.v.) on agonist responses.

In the 3 min prior to agonist administration, DhβE (α4β2 nAChR antagonist) and MLA (α7 nAChR antagonist) did not have any significant effect on the variables being recorded when compared with the saline control.

3.1.2.3.1. Effects of TC-2559 i.c.v. (α4β2 nicotinic receptor agonist) in the presence of DhβE (i.c.v.).

TC-2559 (3 μmol kg^{-1}, n=5) in the presence of DhβE (10 μmol kg^{-1}) failed to cause a significant effect on mean arterial blood pressure and heart rate when compared to antagonist control, apart from an increase in heart rate at 20 min. Renal nerve activity was again a delay, the increase now being significant after 4 min (70 ± 16%) compared with 5 min for TC-2559 alone (Figure 28). Interestingly this delayed rise was also significantly reduced in the presence of DhβE. Baseline values for MAP and HR were 113 ± 5 mmHg and 361 ± 65 beats min^{-1}.

3.1.2.3.2. Effects of PSAB-OFP i.c.v. (α7 nicotinic receptor agonist) in the presence of DhβE (i.c.v.).

The effects of PSAB-OFP (3 μmol kg^{-1}, n=5) in the presence of DhβE (10 μmol kg^{-1}) where overall unaffected. Interestingly the rise in mean arterial blood pressure looked slightly delayed although this difference was not significant (Figure 29). Baseline values for MAP and HR were 114 ± 13 mmHg and 363 ± 33 beats min^{-1}.

3.1.2.3.3. Effects of TC-2559 i.c.v. (α4β2 nicotinic receptor agonist) in the presence of MLA (i.c.v.).

TC-2559 (3 μmol kg^{-1}; n=5) in the presence of MLA (0.5 μmol kg^{-1}) had a very similar effect to TC-2559 alone, causing an expected rise in mean arterial blood pressure and renal nerve activity at 4 min of 31 ± 8 mmHg and 67 ± 21%, reaching a maximum of 42 ± 11 mmHg and 140 ± 15% after 9 min. Interestingly, although there was larger rise in blood pressure associated with a smaller increase in renal nerve activity, when compared to TC-2559 alone these were not significantly different. There was no significant change in heart rate for the duration of the experiment (Figure 30). Baseline values for MAP and heart rate were 114 ± 14 mmHg and 373 ± 34 beats min^{-1}.

3.1.2.3.4. Effects of PSAB-OFP (α7 nicotinic agonist) in the presence of MLA (i.c.v.).

The effects of PSAB-OFP (3 μmol kg^{-1}, n=5) in the presence of MLA (0.5 μmol kg^{-1}) were blocked (Figure 31). Baseline values for MAP and HR were 103 ± 9 mmHg and 377 ± 43 beats min^{-1}.

Figure 28. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.v.) pre-treated with DhβE (10 μmol kg^{-1}, i.c.v.) and TC-2559 alone (3 μmol kg^{-1}, i.c.v.) and antagonist control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) pre-treated with DhβE compared with antagonist control and changes caused by TC-2559 (#) pre-treated with DhβE compared with TC-2559 alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and **, ## $P<0.01$.

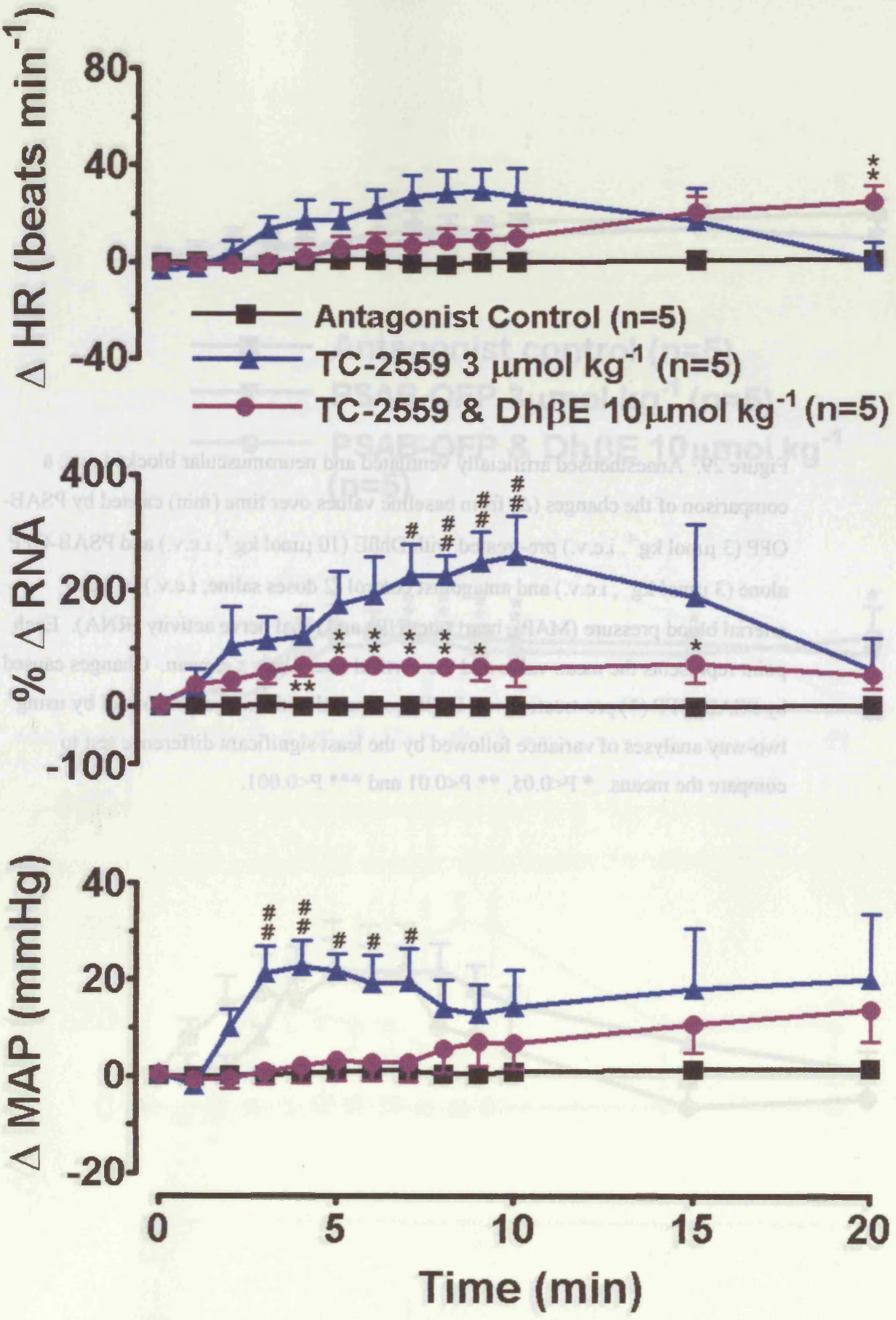

Δ HR (beats min-1)
80
40
0
-40
Antagonist Control (n=5)
TC-2559 3 μmol kg-1 (n=5)
TC-2559 & DhβE 10μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 29. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.v.) pre-treated with DhβE (10 μmol kg^{-1}, i.c.v.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.v.) and antagonist control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) pre-treated with DhβE compared with antagonist control by using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

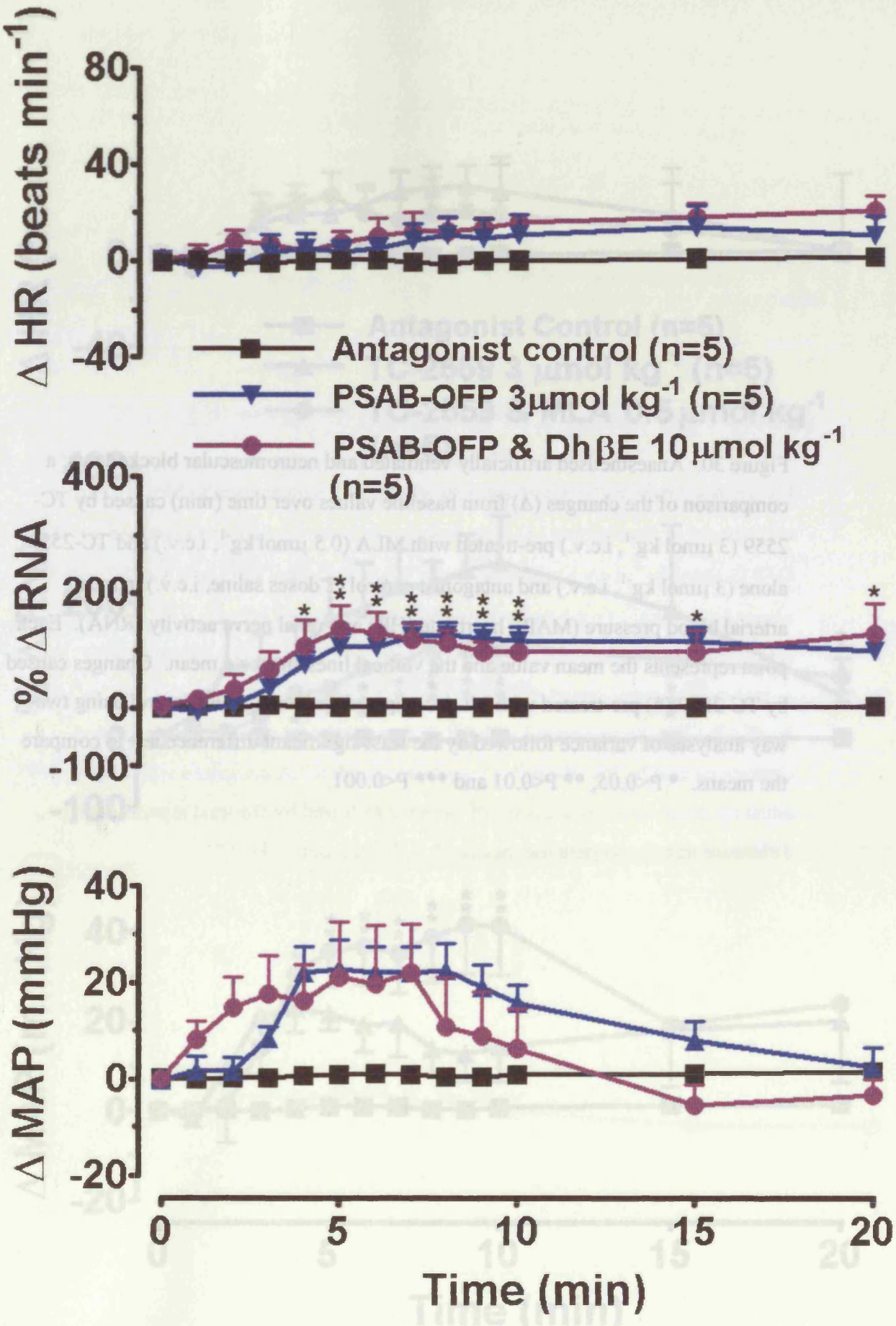

Δ HR (beats min-1)
80
40
0
-40
Antagonist control (n=5)
PSAB-OFP 3μmol kg-1 (n=5)
PSAB-OFP & DhβE 10μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 30. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.v.) pre-treated with MLA (0.5 μmol kg^{-1}, i.c.v.) and TC-2559 alone (3 μmol kg^{-1}, i.c.v.) and antagonist control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) pre-treated with MLA compared with antagonist control using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

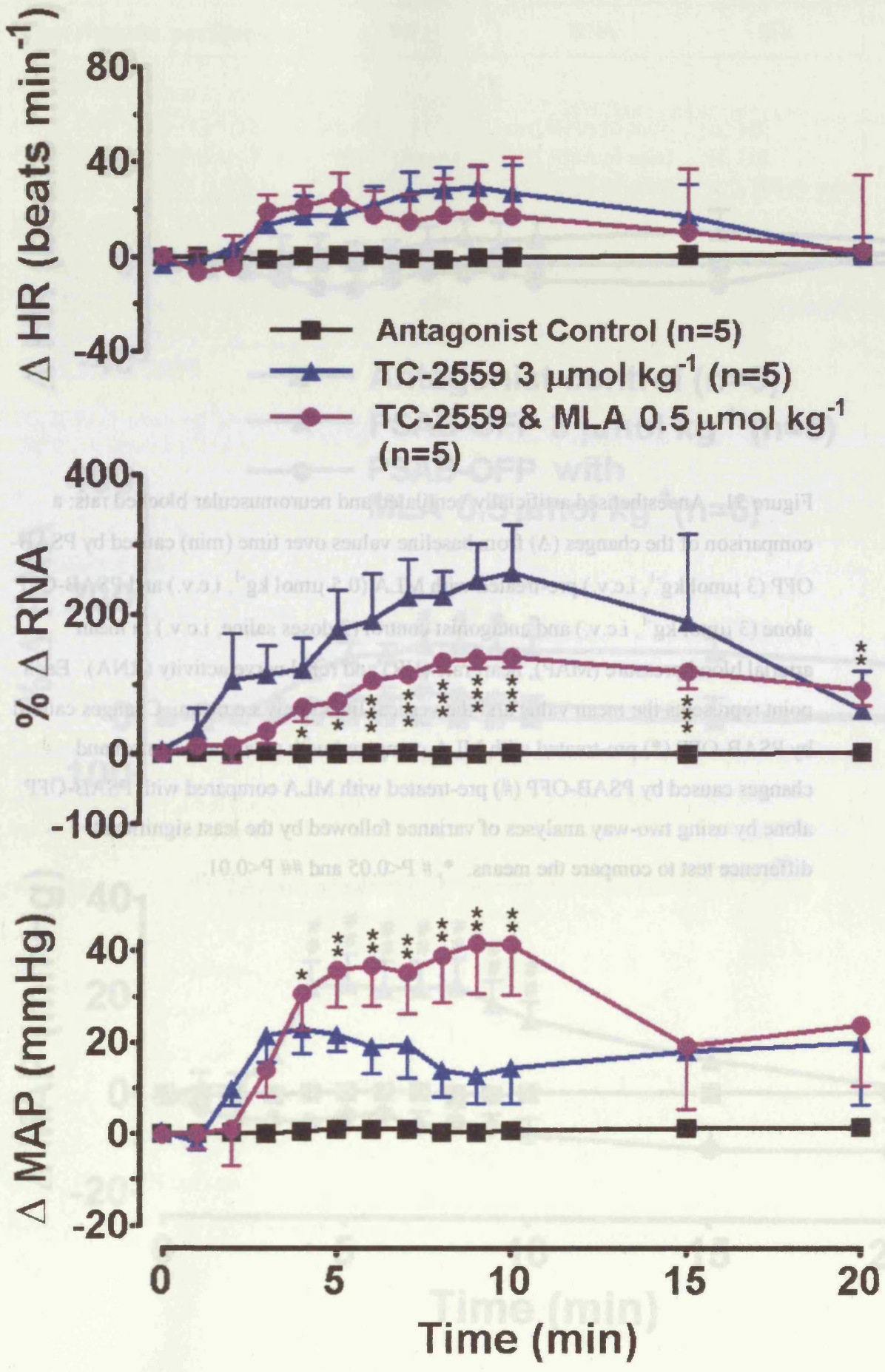
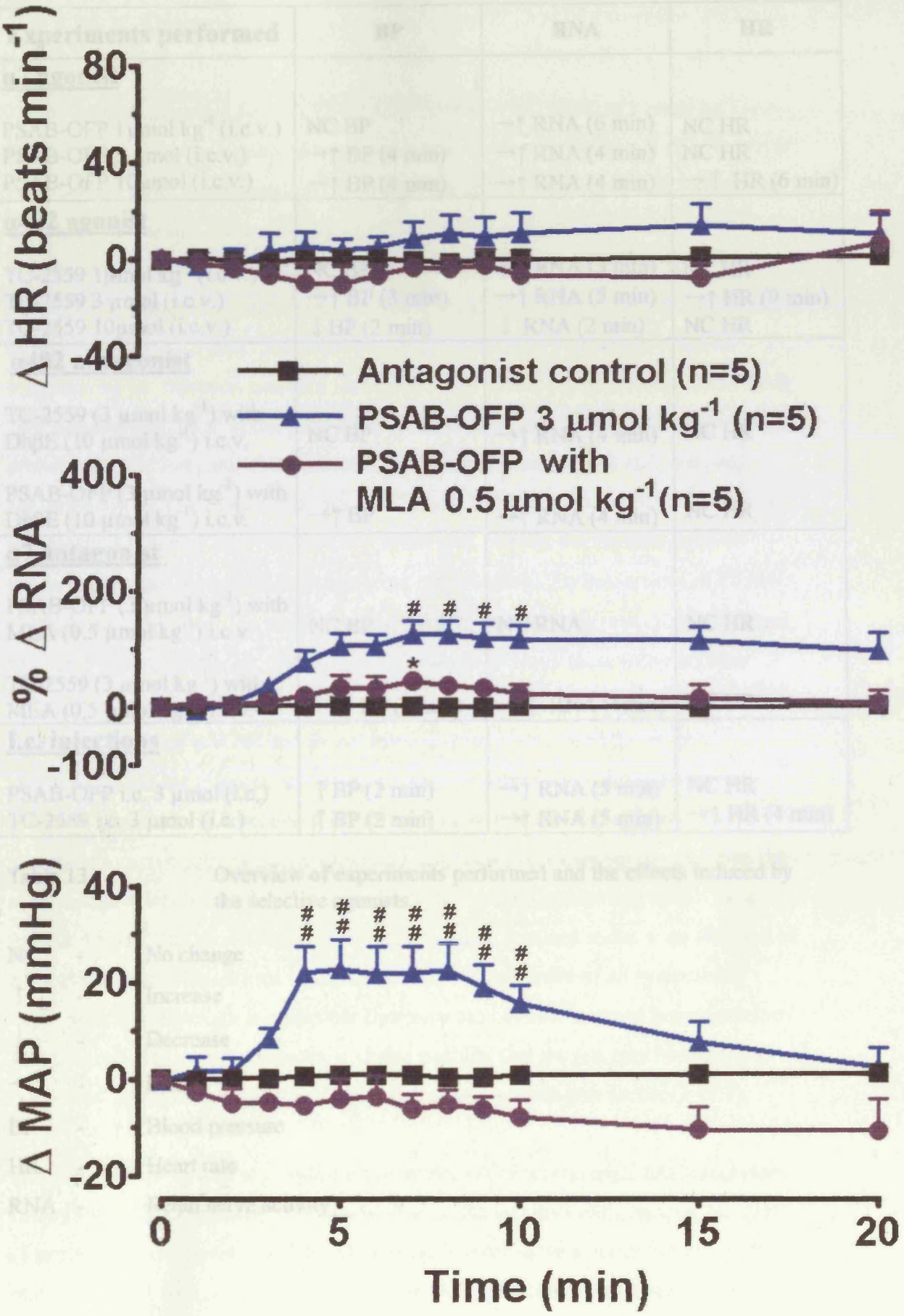

Δ HR (beats min-1)
80
40
0
-40
Antagonist Control (n=5)
TC-2559 3 μmol kg-1 (n=5)
TC-2559 & MLA 0.5 μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 31. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.v.) pre-treated with MLA (0.5 μmol kg^{-1}, i.c.v.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.v.) and antagonist control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) pre-treated with MLA compared with antagonist control and changes caused by PSAB-OFP (#) pre-treated with MLA compared with PSAB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and ## $P<0.01$.


Δ HR (beats min-1)
80
40
0
-40
Antagonist control (n=5)
PSAB-OFP 3 μmol kg-1 (n=5)
PSAB-OFP with
MLA 0.5 μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Experiments performed	BP	RNA	HR
α7 agonist			
PSAB-OFP 1μmol kg^{-1} (i.c.v.)	NC BP	→↑ RNA (6 min)	NC HR
PSAB-OFP 3 μmol (i.c.v.)	→↑ BP (4 min)	→↑ RNA (4 min)	NC HR
PSAB-OFP 10μmol (i.c.v.)	→↑ BP (4 min)	→↑ RNA (4 min)	→ ↑ HR (6 min)
α4β2 agonist			
TC-2559 1μmol kg^{-1} (i.c.v.)	NC BP	→↑ RNA (3 min)	NC HR
TC-2559 3 μmol (i.c.v.)	→↑ BP (3 min)	→↑ RNA (5 min)	→↑ HR (9 min)
TC-2559 10μmol (i.c.v.)	↓ BP (2 min)	↓ RNA (2 min)	NC HR
α4β2 antagonist			
TC-2559 (3 μmol kg^{-1}) with DhβE (10 μmol kg^{-1}) i.c.v.	NC BP	→↑ RNA (4 min)	NC HR
PSAB-OFP (3 μmol kg^{-1}) with DhβE (10 μmol kg^{-1}) i.c.v.	→↑ BP	→↑ RNA (4 min)	NC HR
α7 antagonist			
PSAB-OFP (3 μmol kg^{-1}) with MLA (0.5 μmol kg^{-1}) i.c.v.	NC BP	NC RNA	NC HR
TC-2559 (3 μmol kg^{-1}) with MLA (0.5 μmol kg^{-1}) i.c.v.	→ ↑ BP (4 min)	→ ↑ RNA (4min)	NC HR
I.c. injections			
PSAB-OFP i.c. 3 μmol (i.c.)	↑ BP (2 min)	→↑ RNA (5 min)	NC HR
TC-2559 i.c. 3 μmol (i.c.)	↑ BP (2 min)	→↑ RNA (5 min)	→↑ HR (4 min)

Table 13. Overview of experiments performed and the effects induced by the selective agonists.

NC - No change

↑ - Increase

↓ - Decrease

→ - Delay

BP - Blood pressure

HR - Heart rate

RNA - Renal nerve activity

3.1.3. Discussion.

The α7 nAChR selective agonist PSAB-OFP (Broad *et al.*, 2002) at 1 μmol kg^{-1} i.c.v. was observed to evoke a delayed increase in renal nerve activity from 6 min; however there was no significant change in blood pressure or heart rate. At 3 and 10 μmol kg^{-1}, PSAB-OFP caused a dose-related increase in blood pressure and renal nerve activity, these responses were observed to be delayed occurring after 4 min. Interestingly, there was observed a significant increase in heart rate at the highest dose of PSAB-OFP after 6 min. Administration of PSAB-OFP (3 μmol kg^{-1}) into the hindbrain by i.c. injection caused a faster and larger rise in blood pressure than PSAB-OFP administered i.c.v. at the same dose. Interestingly, the increase in renal nerve activity now did not parallel the increase in blood pressure but was still delayed, occurring after 5 min. Heart rate was unaffected. In the presence of the α7 selective antagonist, MLA (0.5 μmol kg^{-1}, i.c.v.) (Ward *et al.*, 1990), the increase in blood pressure and renal sympathoexcitation were now blocked. Further actions of PSAB-OFP (3 μmol kg^{-1}, i.c.v.) in the presence of α4β2 selective antagonist DhβE (10 μmol kg^{-1}, i.c.v) (Bencherif *et al.*, 2000), at a dose known to block these receptors (see later), were unaffected. These studies confirm that the effect of PSAB-OFP are due to the activation of α7 nAChR and do not involve directly or indirectly the α4β2 receptors. Thus the effect of activating central α7 receptors is to increase blood pressure. For the i.c.v. injection this is associated with a parallel increase in renal sympathetic nerve activity but no change in heart rate. However for the i.c. route the renal sympathoexcitation was delayed compared with the pressor response. Thus overall it is difficult to conclude that the rise in blood pressure is due to an increase in sympathetic nerve activity assuming that RNA is representive of all sympathetic nerve activity. Although it is possible that there has been an increase in sympathetic nerve activity to resistance vessels, it is also possible that the rise may be related to the central release of a pressor hormone such as vasopressin (see section 1.17.1).

The present experiments also indicate that activation of central α4β2 nACh receptors can increase blood pressure. The lowest dose of the selective α4β2 agonist TC-2559 (1 μmol kg^{-1}, i.c.v.) evoked a delayed increase in renal nerve activity occurring after 3 min, associated with no significant change in blood pressure or heart rate. However

the next dose (3 μmol kg^{-1}) did cause a delayed rise in blood pressure associated with an increase in renal nerve activity but heart rate was unaffected. The highest dose of TC-2559 (10 μmol kg^{-1}; i.c.v.) elicited quite oppiste effects from the lower doses there being a delayed fall in blood pressure and renal nerve activity, associated with no overall change in heart rate. However, giving TC-2559 i.c., at the intermediate dose, had a similar effect to i.c.v. administration although the pressor effect had a slightly earlier onset, the increase in renal nerve activity was still delayed but now there was also a delayed tachycardia. The effect of the middle dose of i.c.v. TC-2559 on blood pressure was blocked by i.c.v. α4β2 selective antagonist DhβE and the increase in renal nerve activity was attenuated, confirming that these effects are mainly mediated by central α4β2 receptors. This was confirmed by studies that the effects of TC-2559 i.c.v. were not blocked in the presence of an effective dose (see above) of the α7 antagonist MLA (i.c.v.). However, there was a larger rise in blood pressure which was associated with a smaller increase in renal nerve activity, although these effects were not significant when compared to TC-2559 i.c.v. alone. There were no significant changes in heart rate for the duration of the experiment. Again, as with the action of PSAB-OFP, the rise in blood pressure cannot be clearly attributed to a increase in sympathetic nerve activity to resistance vessels as it is again also possible that the rise may be related to the central release of a pressor hormone such as vasopressin (see section 1.17.1.). However, additional possible explainations for these observed cardiovascular changes may involved a increase in baroreceptor activity due to the increase in BP, which would inhibit renal nerve activity and account for the delayed increase in renal nerve activity. The activation of both receptor subtypes surprisingly has a similar action on the cardiovascular variables. Interestingly the high dose of TC-2559 had a completely opposite effect, which may suggest activation of another type of nAChR. Thus activation of either α7 or α4β2 receptors causes a rise in blood pressure associated with renal sympathoexcitation. When these receptors are activated via the i.c.v. route these effects are delayed whereas via the i.c. route the onset of the rise in blood pressure is earlier, while the rise in renal nerve activity was similarly delayed. Overall the data suggests that these receptors are activating a common central cardiovascular regulatory pathway, although independently.

Sites of Action

The location of α7 nAChR in the human and rat brain is shown in Table 9 & 10. α7 mRNA is found around and close to the area of the lateral 3rd ventricle, with moderate to high α7 mRNA expression in and around the hypothalamus (Wada *et al.*, 1989). Therefore, PSAB-OFP i.c.v. may be mediating its effects at the level of the hypothalamus which has been shown to modulate renal sympathetic outflow, especially the PVN (Coote *et al.*, 1998; Yang & Coote, 1998). However the pressor response and renal sympathoexcitation evoked by PSAB-OFP i.c.v. was not a rapid onset and instead required at least 4 min for a significant increase in blood pressure and renal nerve activity to be observed. Another possible area is the hindbrain which contains major areas involved in cardiovascular regulation such as the NTS and ventrolateral medulla, (see Dampney, 1994; see Sun, 1995). Previous studies have shown α7 expression in both the NTS and RVLM of the rat (Ferreira *et al.*, 2000; 2002; Wada *et al.*, 1989). The faster onset of the effects of PSAB-OFP given i.c. and that they were similar in profile to that of i.c.v. injections suggests that PSAB-OFP has to reach the brainstem to cause its effects. In this respect microinjection of nicotine into the NTS and CVLM results in a depressor response which was blocked by α7 nAChR antagonists MLA and α-BgT (Aberger *et al.*, 2001; Dhar *et al*, 2000; Ferreira *et al.*, 2000; 2002). However, as PSAB-OFP caused a rise in blood pressure this is not consistent with an action at these sites. Another area is the RVLM, which is the site of sympathetic premotor neurons (Amendt *et al.*, 1978; Brown & Guyenet, 1984; Ross *et al.*, 1984). Although a selective agonist has not been applied directly to the RVLM, studies involving microinjection of nicotine demonstrated a large pressor response, similar to PSAB-OFP administered i.c., as well as, a tachycardia (Tseng *et al.*, 1993; 1994). These finding suggest that PSAB-OPF could be acting on the RVLM inducing an increase in sympathetic tone causing vasoconstriction of blood vessels resulting in an increase in blood pressure, which is associated with an increase in renal sympathetic nerve activity. However, although PSAB-OFP i.c. evokes a rapid increase in blood pressure, the onset of renal sympathoexcitation is still delayed. This was not significantly different from the pattern of response when PSAB-OFP was given i.c.v. which has suggested that the increase in blood pressure is independent of the renal sympathoexcitation. These findings, as indicated above, suggest that an increase in sympathetic tone to resistance vessels may not be the

mechanism for the increase in blood pressure and that the increase in renal nerve activity could be a separate action of the nAChR.

A possible mechanism for the PSAB-OFP-evoked pressor effect may be the release of vasopressin. Several studies have indicated that α7 nAChR are found close to and within the PVN and SON of the hypothalamus (Wada *et al.*, 1989; Zaninetti *et al.*, 2000). Axons from the neurons found in the PVN and SON terminate in the posterior pituitary where vasopressin is released into the systemic circulation (see Howl & Wheatley, 1995; see Kombian *et al.*, 2002). Nicotine administered i.c.v. and i.c. causes a significant increase in the release of vasopressin (Bisset *et al*, 1975; Bisset & Feldberg, 1973; Bisset & Chowdrey, 1984). It was observed that nicotine was more effective at inducing vasopressin release when applied to the hindbrain rather than the forebrain (Bisset *et al.*, 1975). In this respect, it has been hypothesised that a nicotinic cholinergic pathway from the NTS, projects to the neurosecretory cells (PVN), which releases vasopressin from the posterior pituitary (Bisset *et al.*, 1975; Bisset & Chowdrey., 1984). Additional pathways have also been suggested from PVN - secretory vasopressin cells to the RVLM which are believed to be involved in cardiovascular regulation (Cheng *et al.*, 2004; Coote *et al.*, 1998; Yang & Coote, 1998).

Administration of vasopressin i.c.v. in conscious and anaesthetised rats was reported to cause a significant increase in blood pressure associated with a significant increase in sympathetic nerve activity (Unger *et al.*, 1984). Furthermore, vasopressin microinjected in to the NTS and RVLM caused a significant increase in blood pressure which was inhibited by a V_{1a} receptor antagonist (Cheng *et al.*, 2004; Unger *et al.*, 1984). Thus it is possible that PSAB-OFP is causing the release of vasopressin both peripherally and/or centrally which results in the increase in blood pressure and renal sympathetic nerve activity.

As TC-2559 causes a similar effect to PSAB-OFP, the arguments for the site of action and mechanism made above for PSAB-OFP would equally apply to TC-2559. Further, autoradiography of nAChRs has indicated the presence of α4β2 nACh receptors at the vasopressin releasing cells PVN and SON, as well as, in the hindbrain

areas such as the ventrolateral medulla and NTS (Dominguez del *et al.*, 1994; Swanson *et al.*, 1987; Wada *et al.*, 1989).

Selectivity of TC-2559

Interestingly, at the highest dose of TC-2559 (10 μmol kg^{-1}, i.c.v.) instead of a dose-related increase in blood pressure, renal nerve activity and heart rate, there was a significant depressor response associated with a significant decrease in renal nerve activity suggesting a different receptor mechanism modulates this response. *In vitro* studies of TC-2559 have shown that although TC-2559 is selective to α4β2 with an EC_{50} = 0.18 μM, the selectivity of TC-2559 changes to α2β4, α3β4, α4β2 and α4β4 at an EC_{50} = 10-30 μM (Chen *et al.*, 2003). Therefore the increased dose of TC-2559 could activate these additional receptors to cause a decrease in blood pressure and sympathoinhibition. Present knowledge dose not enable identification of the site where this action might occur.

Overall, the present interpretation of the data is that activation of α7 and α4β2 nACh receptors are causing a rise in blood pressure probably due to the release of vasopressin from the neurohypophysis by an action mediated via the lower brainstem. The mechanism and site for renal sympathoexcitation remains unclear.

3.2. The role of vasopressin in the cardiovascular action caused by activation of central nAChRs by selective agonists.

3.2.1. Introduction.

Nicotine administered by i.c.v. injections causes the release of vasopressin in rats (Bisset & Chowdrey, 1984; Iitake *et al.*, 1980) and cats (Bisset *et al.*, 1975; Castro de Souza & Silva, 1977), with up to a thirty fold increase in the concentration of vasopressin in the blood collected (Bisset & Feldberg, 1973).

Radioligand binding and immunoreactivity studies have demonstrated that there is a high density of α7 nAChR in the hypothalamus, especially near or on the PVN and SON areas (Wada *et al.*, 1989) involved in vasopressin release.
Intracerebroventricular injections of choline, which has been suggested to be a relatively selective agonist for the α7 nAChR(Albuquerque *et al.*, 1997;Papke *et al.*, 1996), produced a dose-dependent increase in plasma vasopressin in conscious rats (Bisset *et al.*, 1975). This suggests that i.c.v. injections of nicotine are acting on α7 nAChR on or near the PVN and SON, causing the release of vasopressin.
Interestingly, studies have demonstrated that i.c. injections of nicotine have a greater effect than i.c.v. injections on releasing vasopressin in the cat. This was due to nicotine acting at the level of rostral ventrolateral medulla (RVLM) not, as supposed, at the level of the paraventricular nucleus (PVN) to cause release of vasopressin (Bisset *et al.*, 1975). This was also confirmed for the rat, with the i.c. route being more effective than the i.c.v. route (Bisset & Chowdrey, 1984; Souza and Silva Jr, 1977).

Furthermore, central vasopressinergic pathways have also now been identified. For instance, stimulation of the PVN has been shown to cause a five-fold increase in vasopressin in the NTS (Landgraf *et al.*, 1990). Further, vasopressin in pre-synaptic terminals have been shown in the NTS (Buijs & Van Heerikhuize, 1982). In addition,

binding sites for vasopressin have also has been located in the NTS (Dorsa *et al.*, 1983), as the presence of functioning V_1 receptors (Gao *et al.*, 1992). These data suggest that vasopressin input may influence central homoeostatic integrative functioning of NTS neurons. The source of vasopressin input into the NTS has been shown to come from the PVN (Buijs *et al.*, 1978; Nilaver *et al.*, 1980; Sawchenko & Swanson, 1982; Sofroniew & Schrell, 1980; van der *et al.*, 1984). Therefore, this vasopressinergic projection to the NTS is of major importance because it constitutes a pathway by which the PVN could directly modulate baroreceptor function (Michelini, 1994). The NTS, as well as receiving a monosynaptic projection from the PVN, has been shown to send projections to the PVN and SON and cause the release of vasopressin (Buijs *et al.*, 1978; Nilaver *et al.*, 1980; Sawchenko & Swanson, 1982; Sofroniew & Schrell, 1980). This provides a feedback control loop through which the PVN could modulate afferent cardiovascular inputs coming from the periphery to the NTS (see Michelini, 1994).

Attempts have been made to determine the function of vasopressin as a neurotransmitter in the NTS. Microinjections of vasopressin into the NTS has shown to elicit a dose-related increase in blood pressure and heart rate in anaesthetised rats (Matsuguchi *et al.*, 1982; Vallejo *et al.*, 1984). Further vasopressin administration into the NTS in conscious rats also had a pressor effect (King & Pang, 1987). Evidence also exists supporting the suggestion that local vasopressin at the level of the NTS may act to moderate the baroreceptor reflex. Application of a V_1 receptor antagonist within the NTS was demonstrated to attenuate baroreflex mediated decreases in renal nerve sympathetic activity (RNSA) (Hegarty & Felder, 1995) and to impair baroreflex regulation of heart rate in response to large changes in arterial pressure (Michelini & Bonagamba, 1988). Furthermore, studies also found that microinjection of a V_1 receptor antagonist into the commissural NTS caused a small but significant decrease in heart rate (Lowes *et al.*, 1993). Additional studies have shown that these V_1 receptors in the NTS mediate neuronal excitation (Raggenbass *et al.*, 1989). Interesting, studies have shown that around 82% of NTS neurons receiving baroreceptor inputs responded to vasopressin, of these neurons over 60% exhibited excitatory responses, supporting the view that vasopressin acting on these neurons is able to play significant roles in modulating the baroreceptor reflex (Hegarty & Felder, 1997).

The present experiments are carried out to determine the role of vasopressin in the cardiovascular responses to activation of central α7 and α4β2 nAChRs. This was done by investigating the effect of the selective vasopressin V_1 antagonist ([β-Mercapto-β,β-Cyclopentamethylenepropionyl1,O-Me-Tyr2,Arg8]-Vasopressin, (d(CH2)5Tyr(Me)AVP)) given i.c.v., i.c. or i.v. on the responses evoked by central administration (i.c.v. or i.c.) of selective nACh receptor agonists TC-2559 and PSAB-OFP.

3.2.2. Results.

3.2.2.1. Effects of selective nACh receptor agonists given i.c.v. in the presence of the V_1 receptor antagonist (i.v.).

Injection i.v. of the V_1 receptor antagonist d(CH2)5Tyr(Me)AVP (30 μg kg^{-1}; n=5) failed to have any effect on the baseline variables being recorded compared with control (see methods for full protocol).

Saline (5μl, i.c.v.; n=5) in the presence of the V_1 receptor antagonist (30 μg kg^{-1}; i.v., n=5) had no effect on mean arterial blood pressure, heart rate and renal nerve activity for the duration of the experiment. Baseline values for MAP and HR were 106 ± 12 mmHg and 385 ± 20 beats min^{-1}.

3.2.2.1.1. Effects of PSAB-OFP i.c.v. in the presence of the V_1 receptor antagonist (i.v.).

The expected effects evoked by PSAB-OFP (3 μmol kg^{-1}, n=5) in the presence of the V_1 receptor antagonist (30 μg kg^{-1}) were completely blocked, i.e. there was no increase in mean arterial blood pressure, renal nerve activity and heart rate when compared to V_1 receptor antagonist control (Figure 32). Baseline values for MAP and HR were 108 ± 15 mmHg and 397 ± 36 beats min^{-1}.

3.2.2.1.2. Effects of TC-2559 i.c.v. in the presence of the V_1 receptor antagonist (i.v.).

The expected pressor and large delayed renal sympathoexcitation evoked by TC-2559 (3 μmol kg^{-1}, n=5) in the presence of the V_1 receptor antagonist (30 μg kg^{-1}) were also blocked. However, the tendency of renal nerve activity to increase between 2 and 4 min now became significant, reaching a maximum of 30 ± 6% after 3 min (Figure 33). Baseline values for MAP and HR were 108 ± 10 mmHg and 378 ± 32 beats min^{-1}.

3.2.2.2. Effects of selective nACh receptor agonists given i.c. in the presence of the V_1 receptor antagonist (i.v.).

Saline (5μl, i.c.; n=5) in the presence of V_1 antagonist (30 μg kg^{-1}; i.v.) had little or no effect on mean arterial blood pressure, heart rate and renal nerve activity for the duration of the experiment. Baseline values for MAP and HR were 117 ± 5 mmHg and 403 ± 23 beats min^{-1}.

3.2.2.2.1. Effects of PSAB-OFP i.c. in the presence of the V_1 receptor antagonist (i.v.).

Again the expected pressor and sympathoexcitatory response evoked by PSAB-OFP (3 μmol kg^{-1}, n=5) was blocked, however there was now a small but significant fall in blood pressure of -13 ± 3 mmHg after 2 min (Figure 34) and a parallel non-significant decline in heart rate. Baseline values for MAP and HR were 107 ± 27 mmHg and 396 ± 43 beats min^{-1}.

3.2.2.2.2. Effects of TC-2559 i.c. in the presence of the V_1 receptor antagonist (i.v.).

The expected effects evoked by TC-2559 (3 μmol kg^{-1}, n=5) in the presence of the V_1 receptor antagonists (30 μg kg^{-1}) were overall similar in profile. However the initial rise in mean arterial blood pressure was now very variable and did not become significant until after 5 min, reaching 33 ± 10 mmHg at 8 min while the increase in renal nerve activity again was delayed not becoming significant until 6 min reaching 80 ± 38% at 8 min. The expected tachycardia was unaffected (Figure 35). Baseline values for MAP and HR were 109 ± 6 mmHg and 404 ± 41 beats min^{-1}.

Figure 32. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.v.) and control (V_1 antagonist & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (#) pre-treated with V_1 antagonist compared with PSAB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. # $P<0.05$, ## $P<0.01$ and ### $P<0.001$.

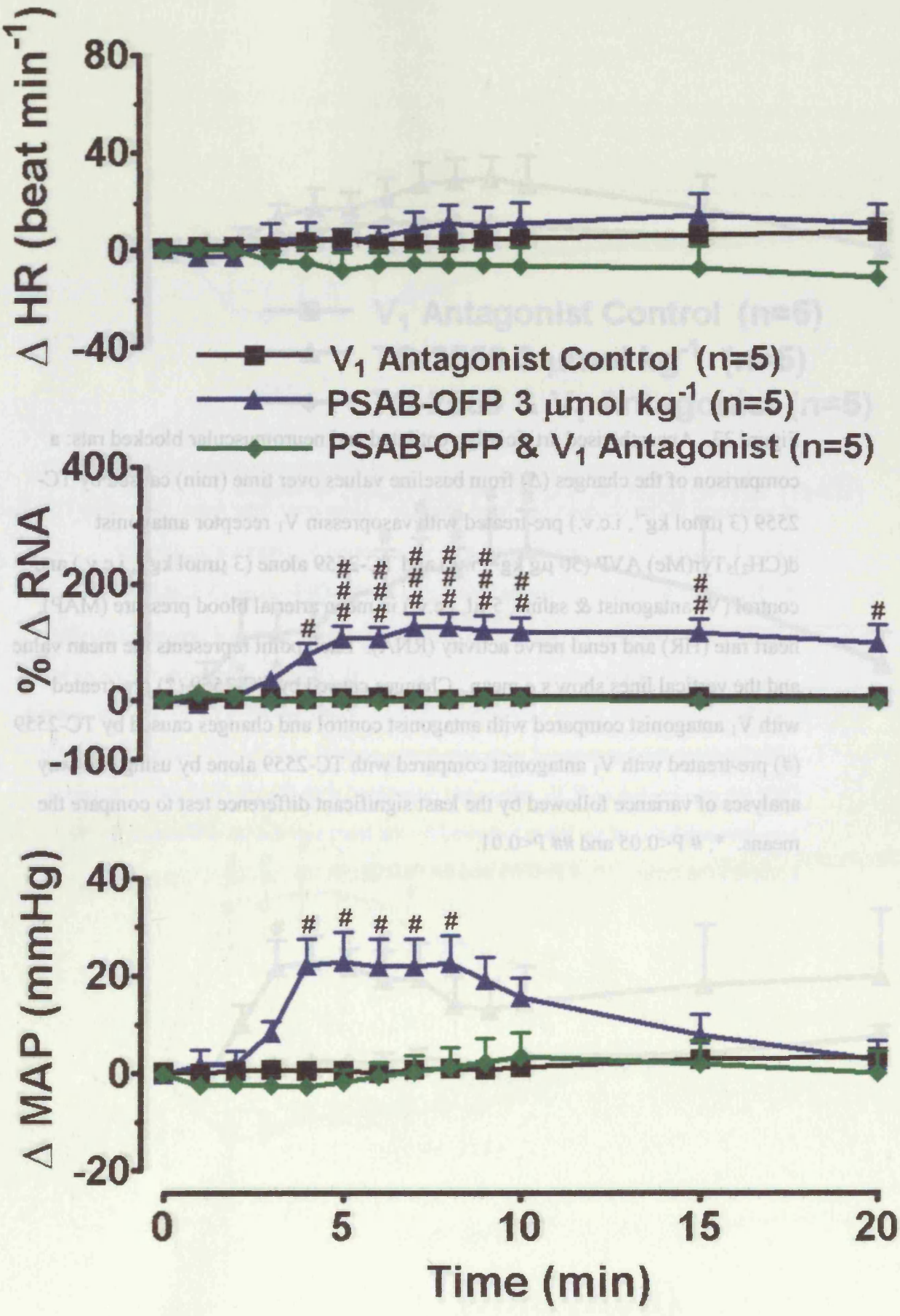

Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
PSAB-OFP 3 μmol kg-1 (n=5)
PSAB-OFP & V1 Antagonist (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 33. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and TC-2559 alone (3 μmol kg^{-1}, i.c.v.) and control (V_1 antagonist & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by TC-2559 (#) pre-treated with V_1 antagonist compared with TC-2559 alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and ## $P<0.01$.

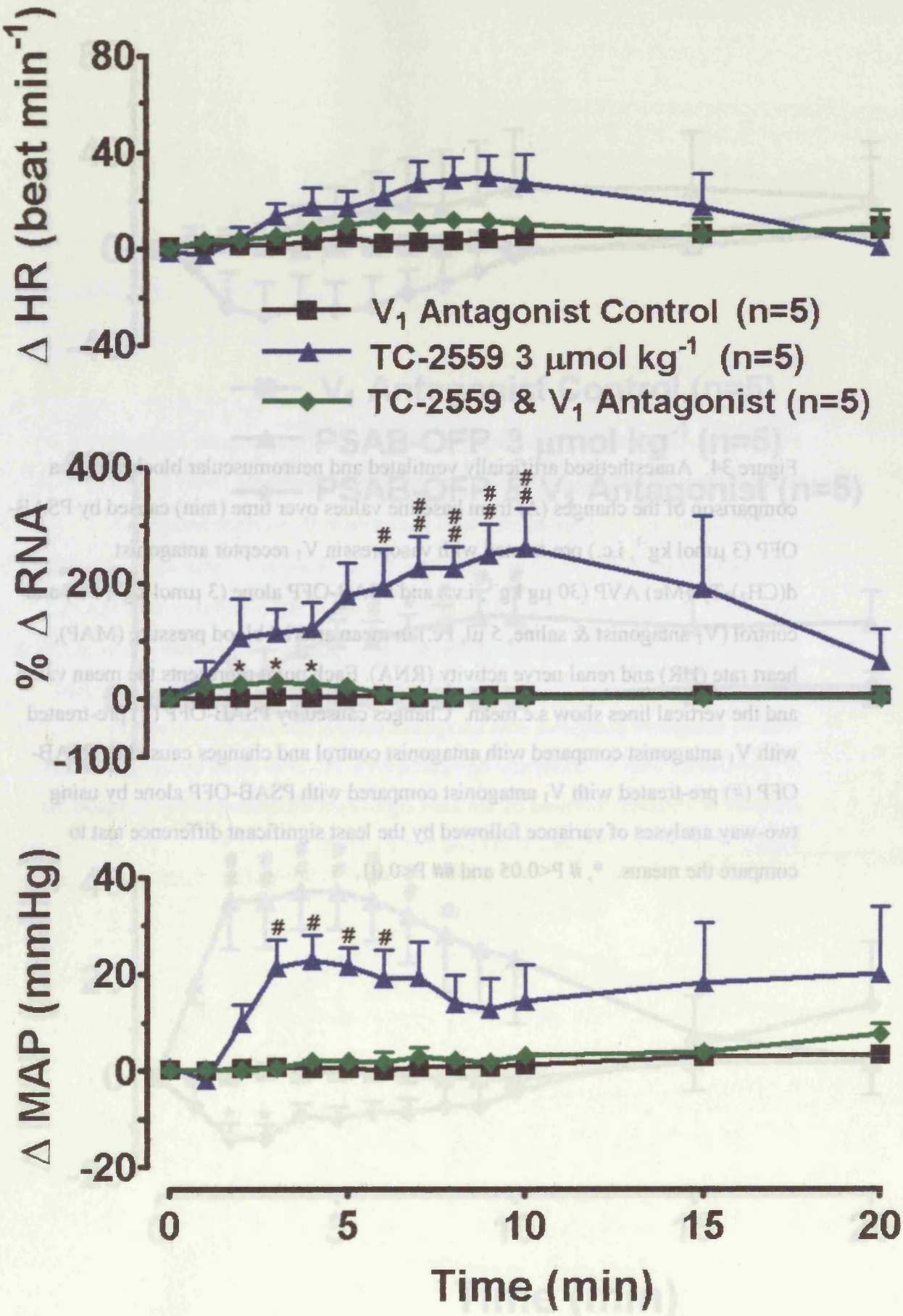

Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
TC-2559 3 μmol kg-1 (n=5)
TC-2559 & V1 Antagonist (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 34. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.) and control (V_1 antagonist & saline, 5 μl, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by PSAB-OFP (#) pre-treated with V_1 antagonist compared with PSAB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and ## $P<0.01$.

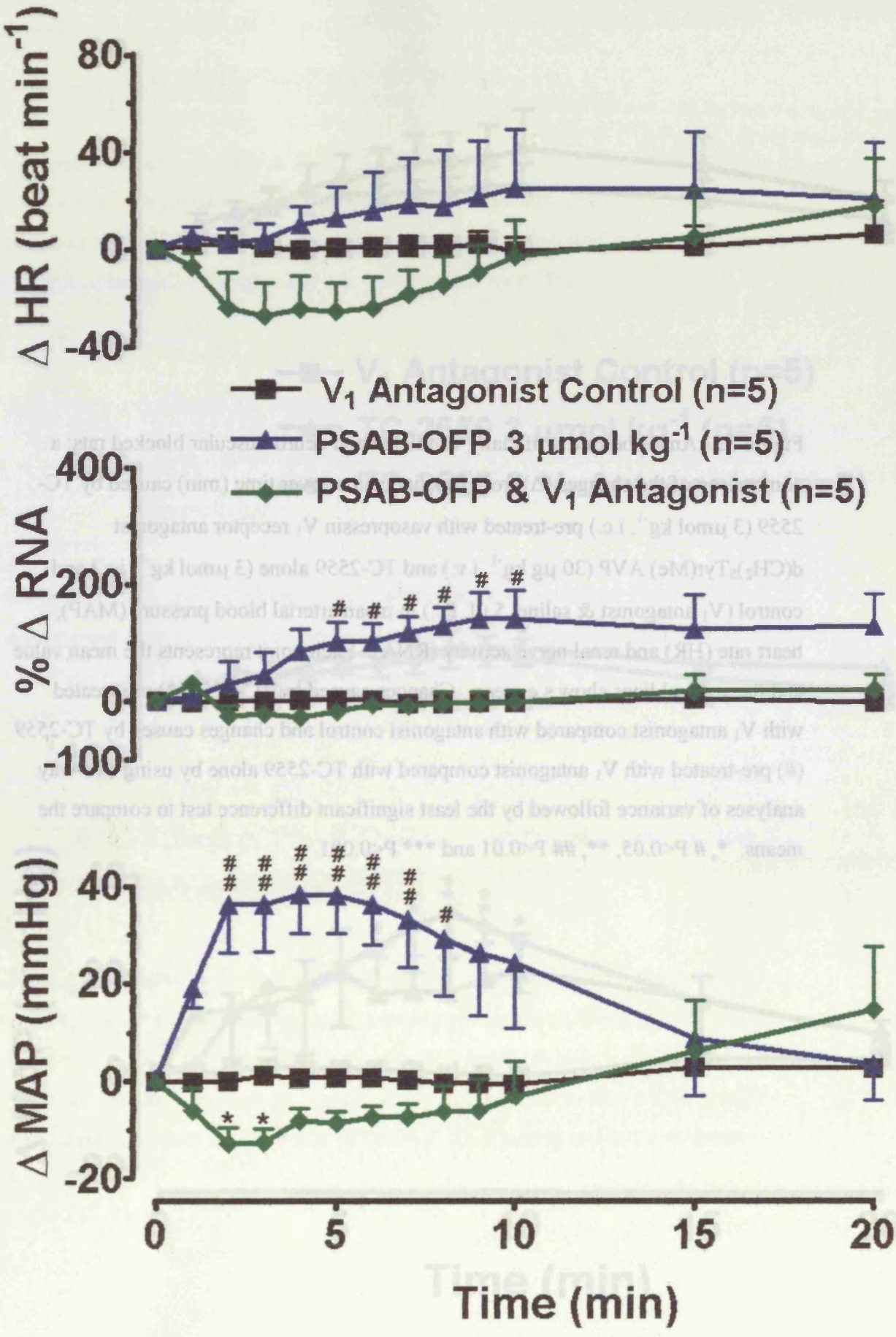
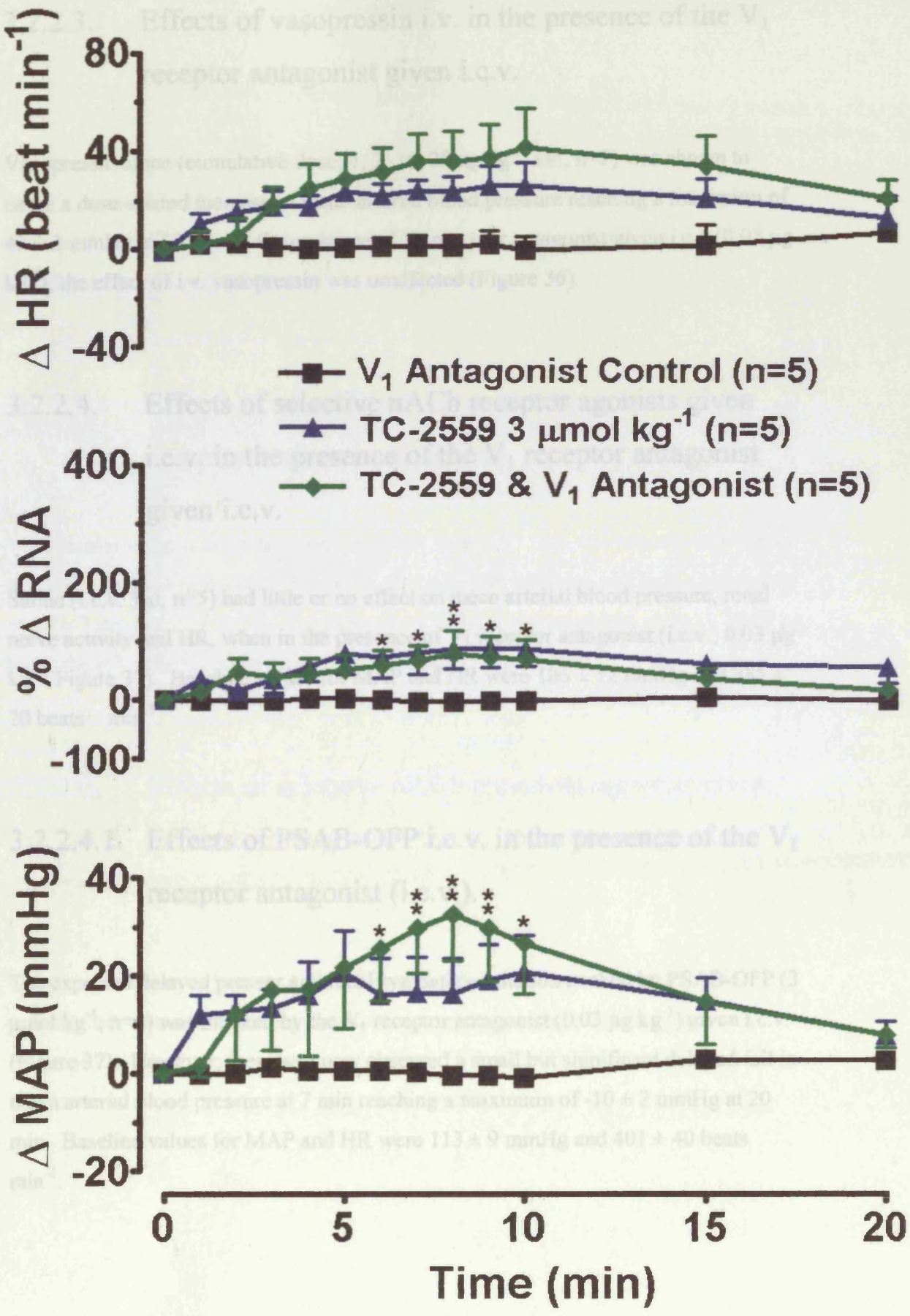

Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
PSAB-OFP 3 μmol kg-1 (n=5)
PSAB-OFP & V1 Antagonist (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 35. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and TC-2559 alone (3 μmol kg^{-1}, i.c.) and control (V_1 antagonist & saline, 5 μl, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by TC-2559 (#) pre-treated with V_1 antagonist compared with TC-2559 alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ and *** $P<0.001$.


Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
TC-2559 3 μmol kg-1 (n=5)
TC-2559 & V1 Antagonist (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.2.2.3. Effects of vasopressin i.v. in the presence of the V_1 receptor antagonist given i.c.v.

Vasopressin alone (cumulative dose; 1, 3, 10, 30 ng kg^{-1}, i.v., n=7) was shown to cause a dose-related increase in mean arterial blood pressure reaching a maximum of 43 ± 3 mmHg at 30 ng. In the presence of V_1 receptor antagonist given i.c.v (0.03 μg kg^{-1}), the effect of i.v. vasopressin was unaffected (Figure 36).

3.2.2.4. Effects of selective nACh receptor agonists given i.c.v. in the presence of the V_1 receptor antagonist given i.c.v.

Saline (i.c.v. 5μl, n=5) had little or no effect on mean arterial blood pressure, renal nerve activity and HR, when in the presence of V_1 receptor antagonist (i.c.v.; 0.03 μg kg^{-1}; Figure 37). Baseline values for MAP and HR were 106 ± 12 mmHg and 385 ± 20 beats min^{-1}.

3.2.2.4.1. Effects of PSAB-OFP i.c.v. in the presence of the V_1 receptor antagonist (i.c.v.).

The expected delayed pressor and renal sympathoexcitation evoked by PSAB-OFP (3 μmol kg^{-1}, n=5) was blocked by the V_1 receptor antagonist (0.03 μg kg^{-1}) given i.c.v. (Figure 37). However, there was now observed a small but significant delayed fall in mean arterial blood pressure at 7 min reaching a maximum of -10 ± 2 mmHg at 20 min. Baseline values for MAP and HR were 113 ± 9 mmHg and 401 ± 40 beats min^{-1}.

3.2.2.4.2. Effects of TC-2559 i.c.v. in the presence of the V_1 receptor antagonist (i.c.v.).

The expected pressor, sympathoexcitation and delayed tachycardia evoked by TC-2559 (3 μmol kg^{-1}, n=5) in the presence of V_1 receptor antagonist (0.03 μg kg^{-1}) given i.c.v. were blocked (Figure 38). Baseline values for MAP and HR were 109 ± 16 mmHg and 387 ± 24 beats min^{-1}.

3.2.2.5. Effects of vasopressin i.v. presence of the V_1 receptor antagonist given i.c.

Vasopressin alone (cumulative dose; 1, 3, 10, 30 ng kg^{-1}, i.v., n=7) in rats was shown to cause a dose-related increase in mean arterial blood pressure, reaching a maximum of 39 ± 7 mmHg at 30 ng. In the presence of V_1 receptor antagonist given i.c (0.03 μg kg^{-1}), the effect of i.v. vasopressin was unaffected (Figure 39).

3.2.2.6. Effects of selective nACh receptors agonists given i.c. in the presence of the V_1 receptor antagonist given i.c.

Saline (i.c., 5μl, n=5) had no effect on mean arterial blood pressure, renal nerve activity and heart rate in the presence of V_1 receptor antagonist (0.03 μg kg^{-1}; i.c., Figure 40 & 41). Baseline values for MAP and HR were 117 ± 5 mmHg and 403 ± 23 beats min^{-1}.

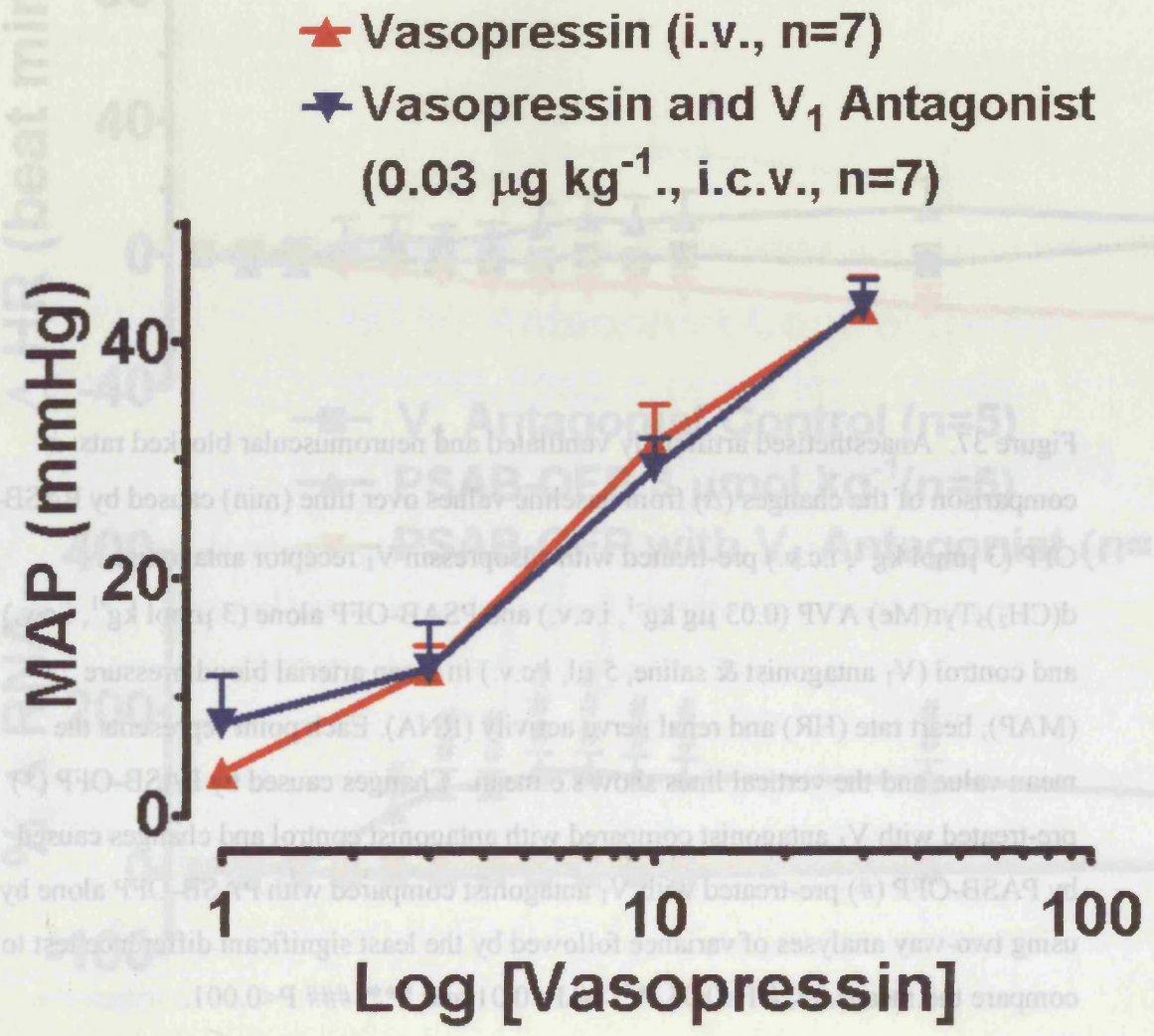


Figure 36. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) caused by vasopressin (cumulative dose; 1, 3, 10 & 30 ng kg^{-1}, i.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.v.) and vasopressin alone (cumulative dose; 1, 3, 10 & 30 ng kg^{-1}, i.v.) on mean arterial blood pressure (MAP). Each point represents the mean value and the vertical lines show s.e.mean.

Figure 37. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PASB-OFP (3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.v.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.v.) and control (V_1 antagonist & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PASB-OFP (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by PASB-OFP (#) pre-treated with V_1 antagonist compared with PASB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ and ***, ### $P<0.001$.

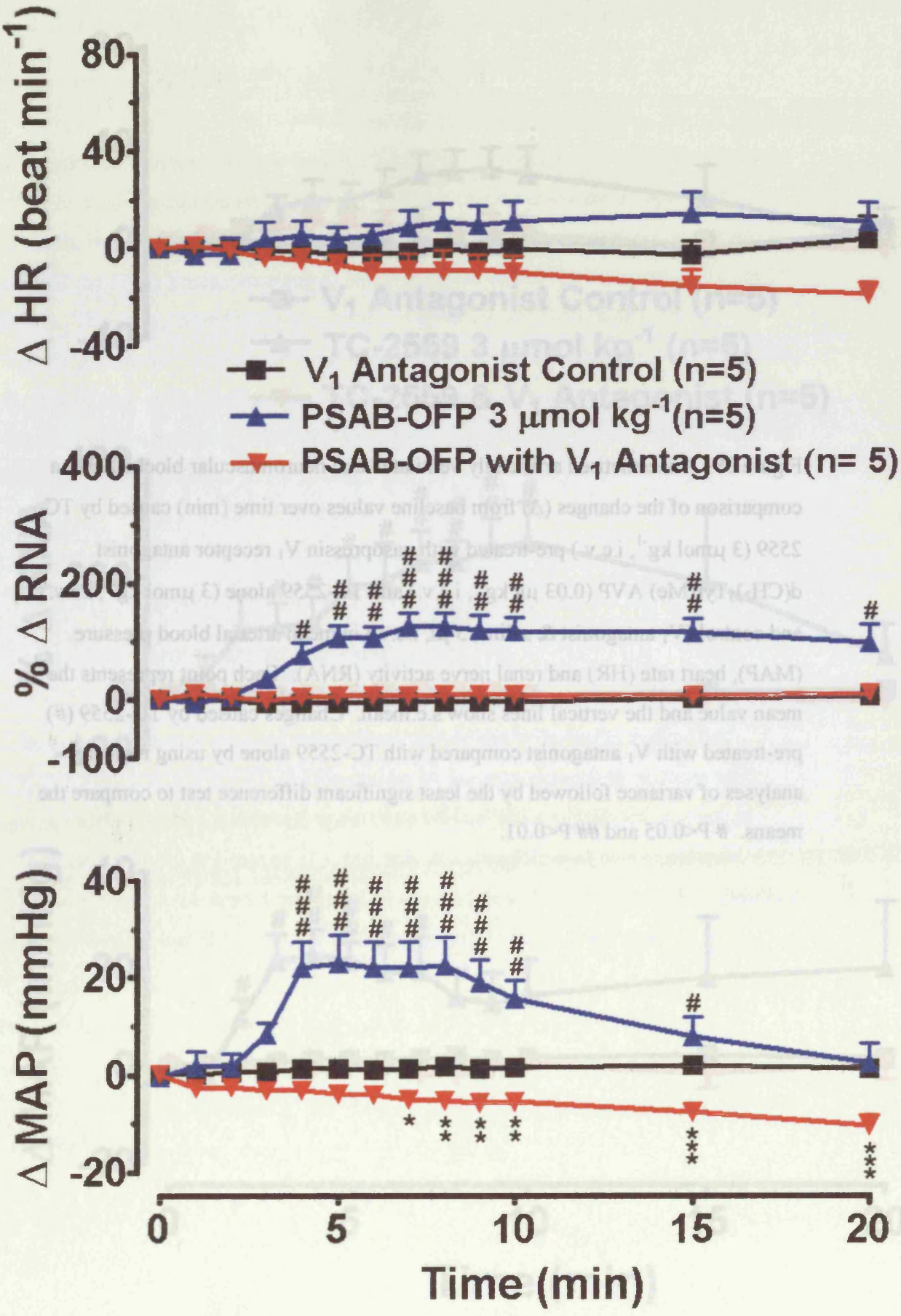
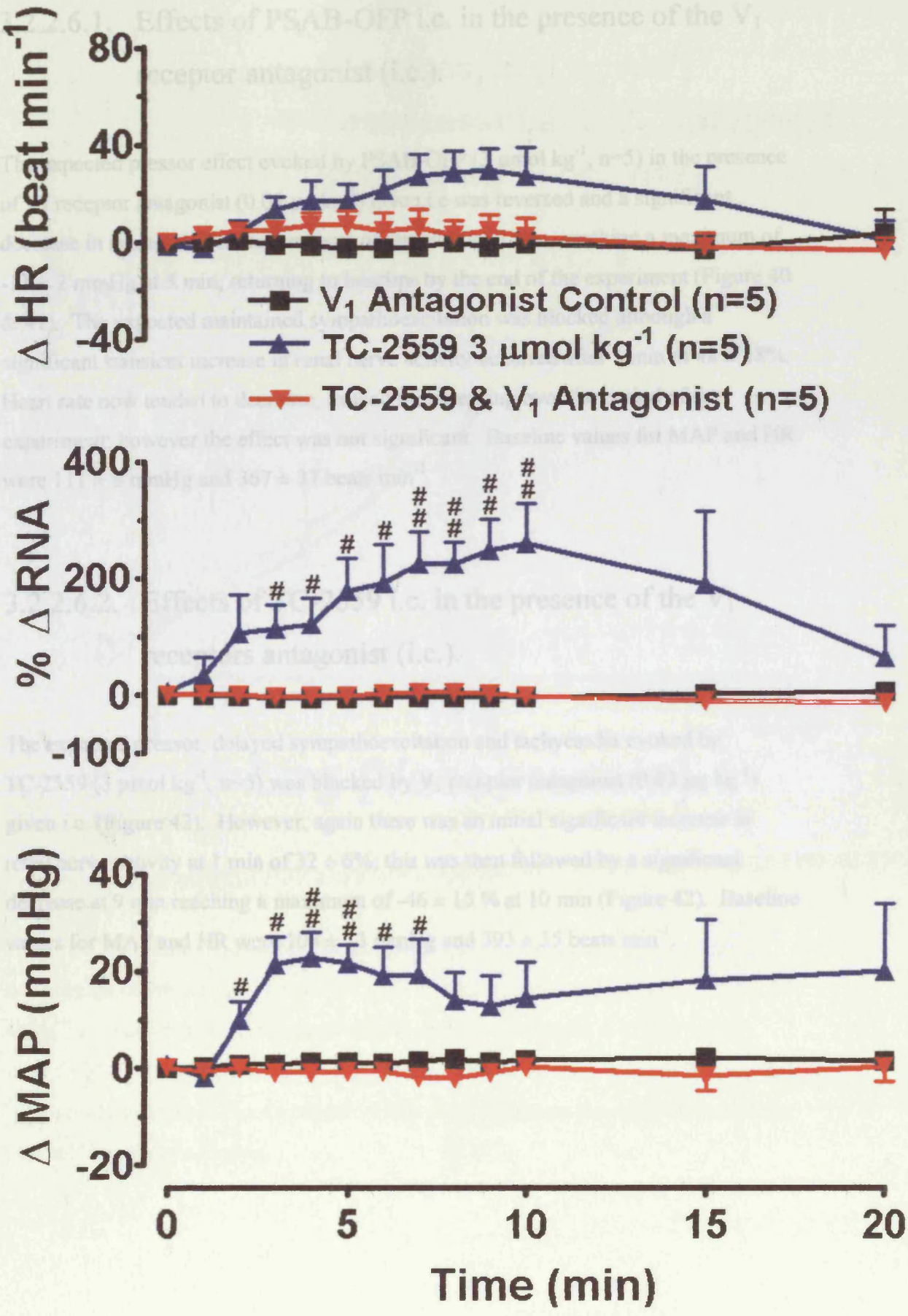

Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
PSAB-OFP 3 μmol kg-1(n=5)
PSAB-OFP with V1 Antagonist (n= 5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 38. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.v.) and TC-2559 alone (3 μmol kg^{-1}, i.c.v.) and control (V_1 antagonist & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (#) pre-treated with V_1 antagonist compared with TC-2559 alone by using two-way analyses of variance followed by the least significant difference test to compare the means. # $P<0.05$ and ## $P<0.01$.


Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
TC-2559 3 μmol kg-1 (n=5)
TC-2559 & V1 Antagonist (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.2.2.6.1. Effects of PSAB-OFP i.c. in the presence of the V_1 receptor antagonist (i.c.).

The expected pressor effect evoked by PSAB-OFP (3 μmol kg^{-1}, n=5) in the presence of V_1 receptor antagonist (0.03 μg kg^{-1}) given i.c was reversed and a significant decrease in mean arterial pressure was observed after 3 min, reaching a maximum of -11 ± 2 mmHg at 5 min, returning to baseline by the end of the experiment (Figure 40 & 41). The expected maintained sympathoexcitation was blocked although a significant transient increase in renal nerve activity occurred after 1 min of 48 ± 18%. Heart rate now tended to decrease, instead of increasing over the period of the experiment; however the effect was not significant. Baseline values for MAP and HR were 111 ± 8 mmHg and 367 ± 37 beats min^{-1}.

3.2.2.6.2. Effects of TC-2559 i.c. in the presence of the V_1 receptors antagonist (i.c.).

The expected pressor, delayed sympathoexcitation and tachycardia evoked by TC-2559 (3 μmol kg^{-1}, n=5) was blocked by V_1 receptor antagonist (0.03 μg kg^{-1}) given i.c. (Figure 42). However, again there was an initial significant increase in renal nerve activity at 1 min of 32 ± 6%; this was then followed by a significant decrease at 9 min reaching a maximum of -46 ± 15 % at 10 min (Figure 42). Baseline values for MAP and HR were 108 ± 13 mmHg and 393 ± 35 beats min^{-1}.

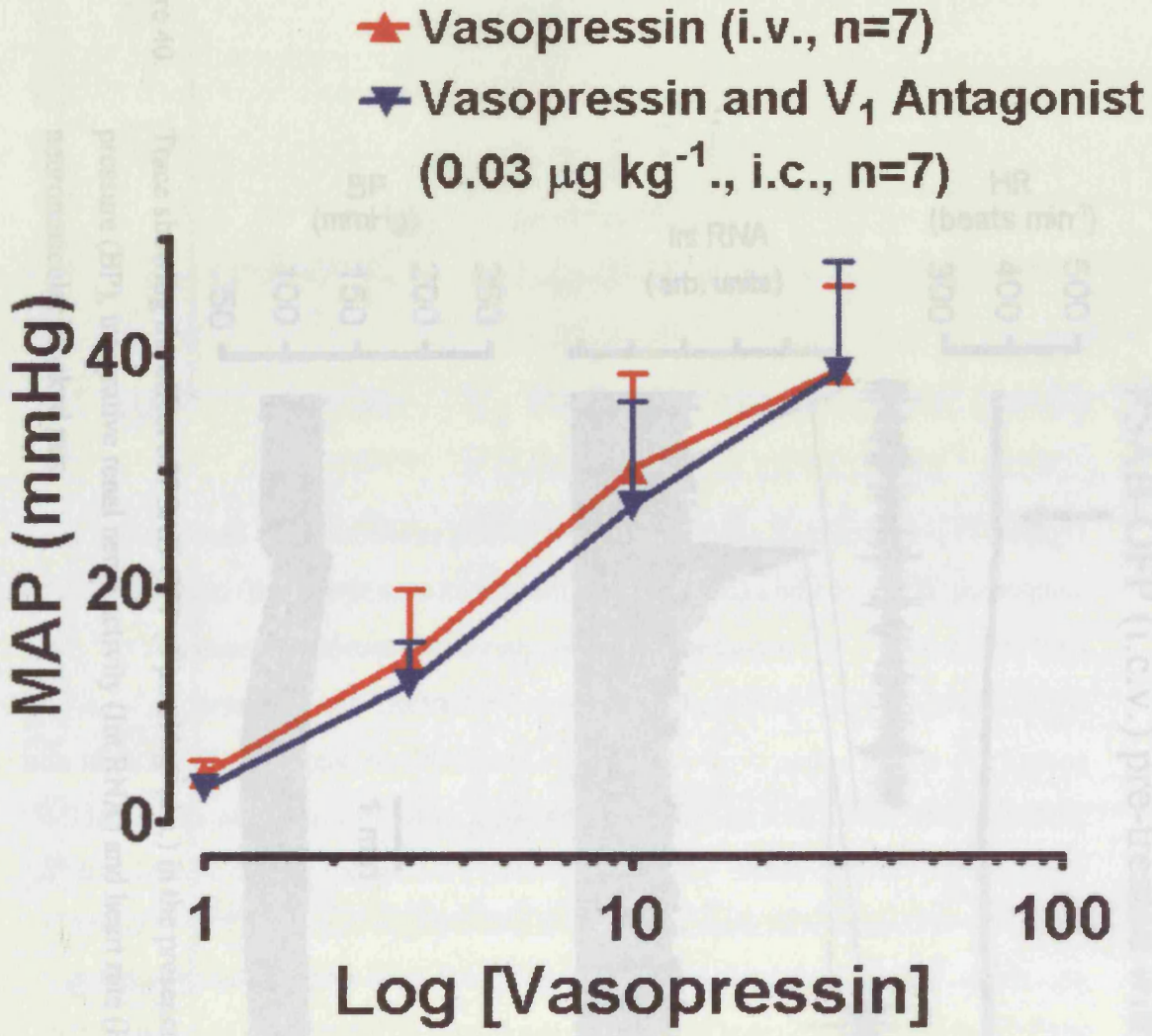


Figure 39. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) caused by vasopressin (cumulative dose; 1, 3, 10 & 30 ng kg^{-1}, i.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.) and vasopressin alone (cumulative dose; 1, 3, 10 & 30 ng kg^{-1}, i.v.) on mean arterial blood pressure. Each point represents the mean value and the vertical lines show s.e.mean.

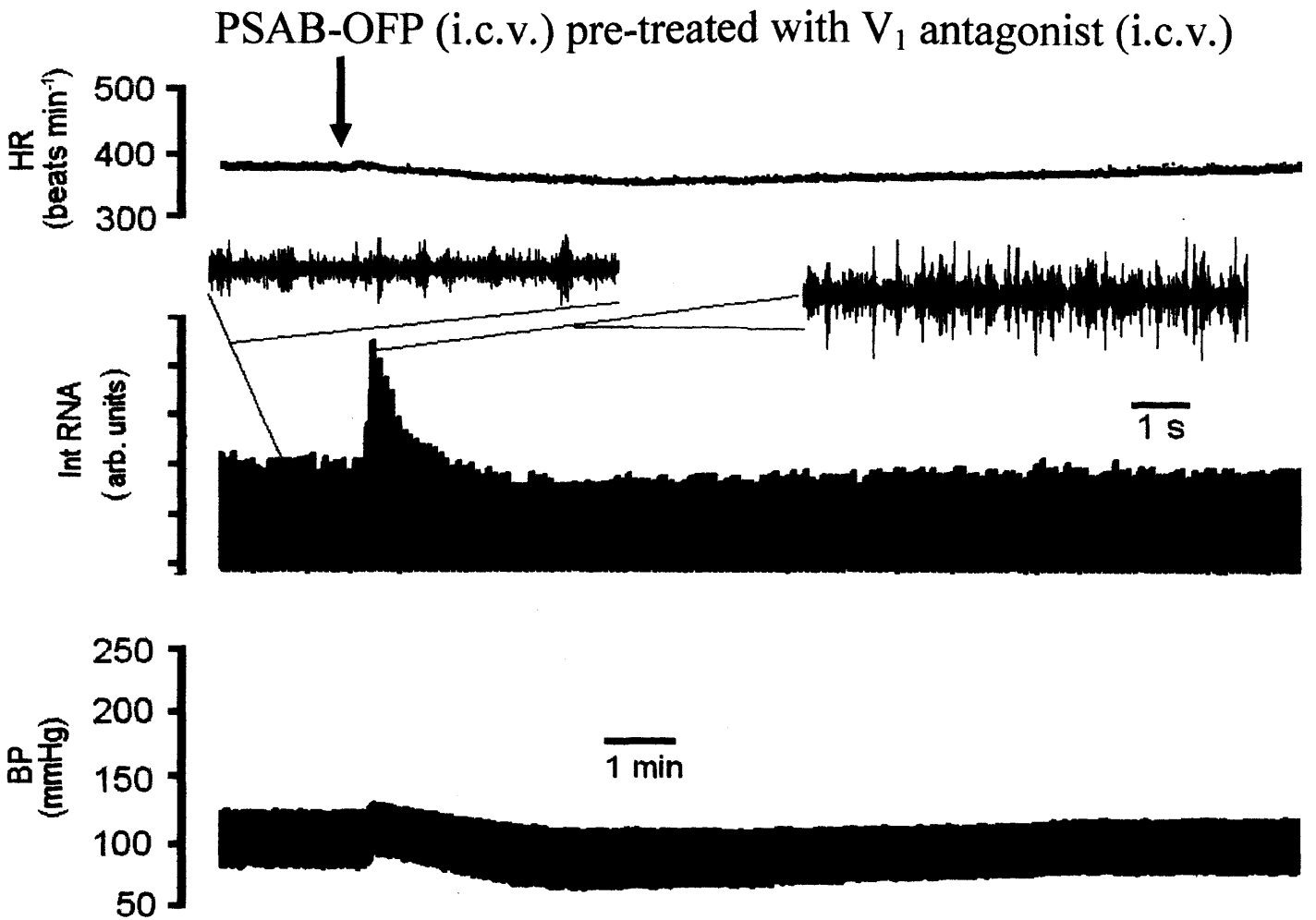


Figure 40. Trace showing the effect of PSAB-OFP (3 μmol kg^{-1}, i.c.) in the presence of V_1 receptor antagonist (0.03 μg kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 41. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.) and control (V_1 anta & saline, 5 μl, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by PSAB-OFP (#) pre-treated with V_1 antagonist compared with PSAB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ and ***, ### $P<0.001$

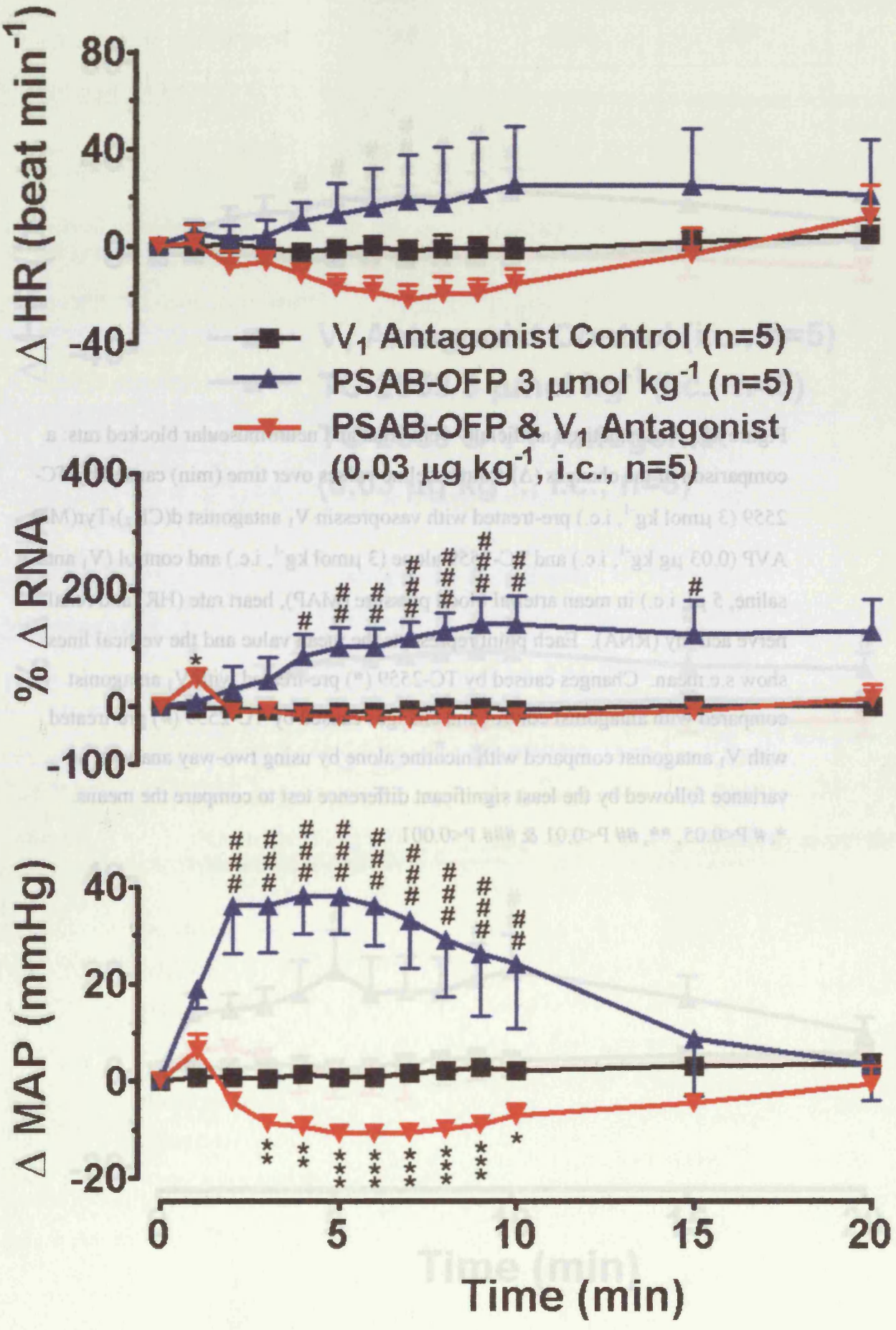
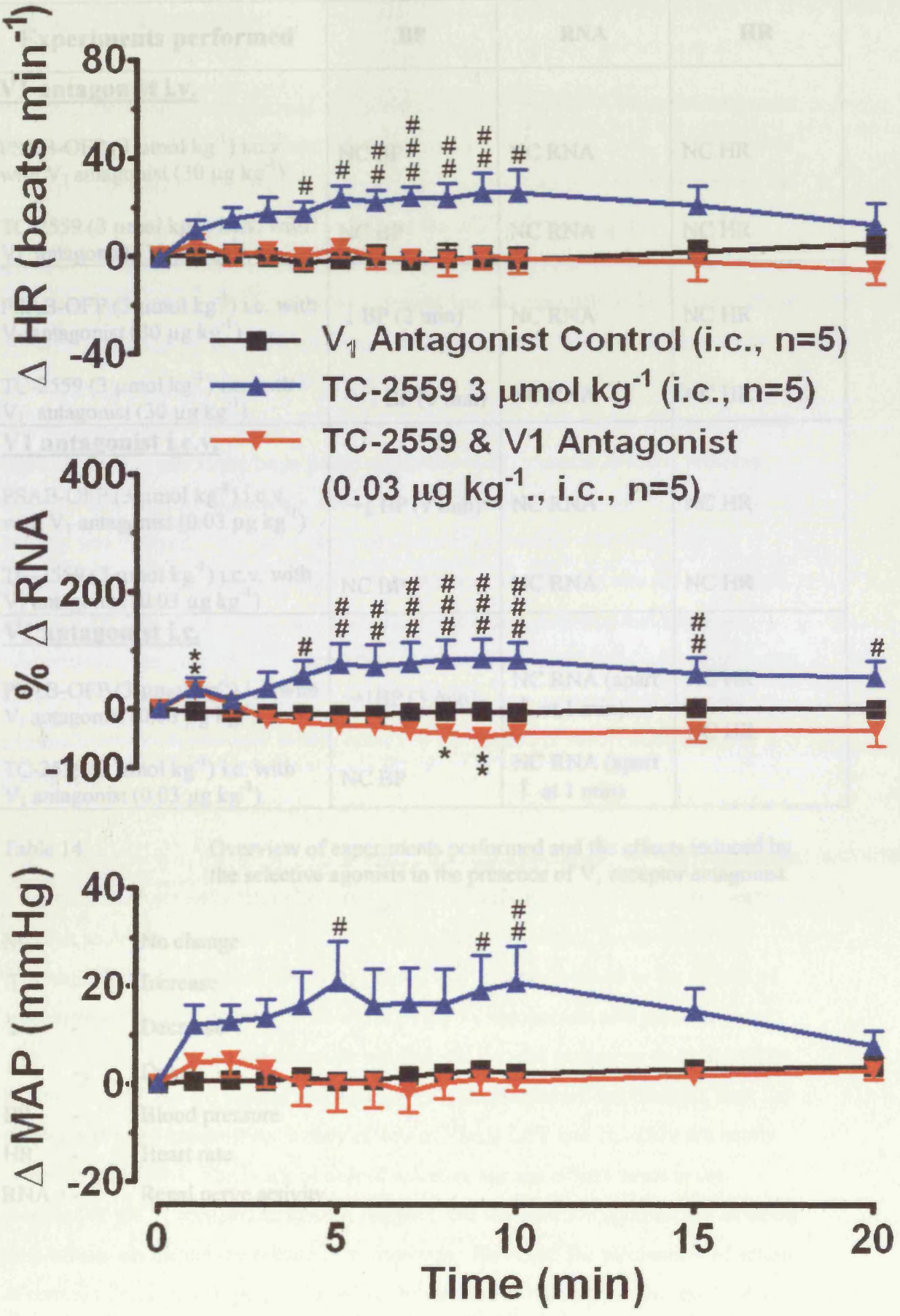

Δ HR (beat min-1)
80
40
0
-40
V1 Antagonist Control (n=5)
PSAB-OFP 3 μmol kg-1 (n=5)
PSAB-OFP & V1 Antagonist
(0.03 μg kg-1, i.c., n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 42. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.c.) pre-treated with vasopressin V_1 antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.) and TC-2559 alone (3 μmol kg^{-1}, i.c.) and control (V_1 anta & saline, 5 μl, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by TC-2559 (#) pre-treated with V_1 antagonist compared with nicotine alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ & ### $P<0.001$


Δ HR (beats min-1)
80
40
0
-40
V1 Antagonist Control (i.c., n=5)
TC-2559 3 μmol kg-1 (i.c., n=5)
TC-2559 & V1 Antagonist
(0.03 μg kg-1., i.c., n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Experiments performed	BP	RNA	HR
V1 antagonist i.v.			
PSAB-OFP (3 μmol kg^{-1}) i.c.v. with V_1 antagonist (30 μg kg^{-1})	NC BP	NC RNA	NC HR
TC-2559 (3 μmol kg^{-1}) i.c.v. with V_1 antagonist (30 μg kg^{-1})	NC BP	NC RNA	NC HR
PSAB-OFP (3 μmol kg^{-1}) i.c. with V_1 antagonist (30 μg kg^{-1})	↓ BP (2 min)	NC RNA	NC HR
TC-2559 (3 μmol kg^{-1}) i.c. with V_1 antagonist (30 μg kg^{-1})	→ ↑ BP (8 min)	NC RNA	NC HR
V1 antagonist i.c.v.			
PSAB-OFP (3 μmol kg^{-1}) i.c.v. with V_1 antagonist (0.03 μg kg^{-1})	→↓ BP (7 min)	NC RNA	NC HR
TC-2559 (3 μmol kg^{-1}) i.c.v. with V_1 antagonist (0.03 μg kg^{-1})	NC BP	NC RNA	NC HR
V1 antagonist i.c.			
PSAB-OFP (3 μmol kg^{-1}) i.c. with V_1 antagonist (0.03 μg kg^{-1})	→↓BP (3 min)	NC RNA (apart ↑ at 1 min)	NC HR
TC-2559 (3 μmol kg^{-1}) i.c. with V_1 antagonist (0.03 μg kg^{-1})	NC BP	NC RNA (apart ↑ at 1 min)	NC HR

Table 14. Overview of experiments performed and the effects induced by the selective agonists in the presence of V_1 receptor antagonist.

NC - No change

↑ - Increase

↓ - Decrease

→ - Delay

BP - Blood pressure

HR - Heart rate

RNA - Renal nerve activity

3.2.3. Discussion.

Present experiments demonstrated that pre-treatment i.v. with a V_1 receptor antagonist at doses known to block the effects of vasopressin i.v. (Anderson *et al.*, 1992) blocked the expected delayed increase in blood pressure and renal nerve activity evoked by the α7 selective receptor agonist PSAB-OFP and the α4β2 selective agonist, TC-2559 given i.c.v. These observations indicate that the cardiovascular effects via the i.c.v. route are mediated by the release of vasopressin into the circulation. However if these agonist were given by the i.c. route in the presence of the V_1 receptor antagonist i.v., although the expected effects of PSAB-OFP i.c. were blocked, PSAB-OFP now caused a fall in blood pressure. While for TC-2559 the profile of the effects observed were similar but due to the large initial variability in the increase in blood pressure this now only reached significant after 5 mins. Similarly the increase in renal nerve activity was delayed. This latter observation is not consistent with view that the effects of i.c. TC-2559 are mediated by the release of vasopressin into the circulation, although the data is consistent for PSAB-OFP and in fact indicates that when the vasopressin released is blocked, activation of α7 nAChRs at the level of brainstem can also cause a fall in blood pressure. Interestingly, this data suggests that the renal sympathoexcitation observed is also caused by the release of vasopressin into the circulation.

The interpretation of the above data is made under the assumption that the vasopressin antagonist does not enter the brain when given i.v., although it can enter areas such as the area postrema, found above the NTS, and subfornical organ, found above the pituitary, (Johnson & Gross, 1993)(Figure 43) and may be involved in the release of vasopressin into the circulation. However, if the V_1 vasopressin antagonist is given centrally (i.c.v. or i.c.) in doses that do not leak out into the periphery, as indicated by the observation that the pressor responses to i.v. vasopressin are not blocked, then the pressor and renal sympathoexcitatory effects of PSAB-OFP and TC-2559 are nearly completely blocked. The block of central selective agonist effects when in the presence of the V_1 receptor antagonist suggests that the selective agonists are inducing their effects via the central release of vasopressin. However, the mechanism of action of central release of vasopressin inducing the increase in blood pressure and RNA is

unclear although it shold be noted that vasopressin may be involved in a central pathway which evokes the release of vasopressin into the circulation to cause the increase in blood pressure, rather than causing an increase in sympathetic drive to resistance vessels or both. Thus the precise mechanism behind the pressor response remains to be determined although it does initially, at least, involve the central release of vasopressin and from the published data on the central administration of nicotine (see section 1.16.) this would imply that these agonists are also causing the release of vasopressin into the peripheral.

Sites of Action.

As previously mentioned in Chapter 3.1, the delayed rise in blood pressure upon i.c.v. injections and the faster and higher rise in blood pressure upon i.c. injections, suggested that the selective agonists are not acting at the forebrain but instead evoking their effects by acting on areas in the hindbrain. Therefore, if the increase in blood pressure is due to vasopressin release, or by the activation of a central vasopressinergic pathway, then this implies that the site at which the selective agonists, PSAB-OFP and TC-2559, act on α7 and α4β2 nAChRs is not at the level of PVN but at hindbrain sites such as the NTS and RVLM. In this respect, vasopressin-containing terminals have been identified in these areas (Buijs & Van Heerikhuize, 1982; Nilaver *et al.*, 1980; Sawchenko & Swanson, 1982; Van der Kooy *et al.*, 1984) as well as, V_1 receptors (Cheng *et al.*, 2004; Dogterom *et al.*, 1978; Phillips *et al.*, 1988a; 1988b; Tribollet *et al.*, 1988). In addition nicotine can also activate neuronal projection from the NTS and RVLM (C1 region) to the PVN and SON (Matta *et al.*, 1993), thus causing vasopressin release into the circulation. Furthermore, studies by Bisset *et al.* (1975) demonstrated that nicotine was more effective at inducing vasopressin release when applied to the hindbrain rather than at the forebrain.

Overall, it is suggested that selective agonists PSAB-OFP and TC-2559 are acting on α7 and α4β2 nAChR at the hindbrain aeas such as the RVLM and NTS. Activation of these cardiovascular regulating areas stimulates the release of vasopressin, via a neuronal projection to the PVN, into the circulation. This pathway from the hindbrain to the PVN is believed to involve vasopressin, possibly acting as a excitatory

neurotransmitter between the synapse of the RVLM to the PVN, as a similar pathway has been observed from the PVN to the RVLM (Yang *et al.*, 2001).

Figures 43 and 44 represent a possible mechanism of action for selective agonists administered in the rat brain. Selective agonists i.c.v. do not act directly on the PVN to cause the release of vasopressin, or if any vasopressin is released it is not stimulating a noted effect. The selective agonists pass to the hindbrain were they bind to α7 or α4β2 nAChRs in the NTS and/or RVLM, stimulating the pathway to the PVN. This results in the release of vasopressin into the periphery inducing an increase in blood pressure (Figure 43). Further, selective agonists administered i.c. are able to cause a faster and greater rise in blood pressure than i.c.v. injections, due to the selective agonists being closer to the RVLM site, therefore more of the agonist is able to bind to nAChRs and stimulate the neurons projecting to the PVN (Figure 44), resulting in a greater amount of vasopressin released into the circulation.

Renal Sympathoexcitation.

The present experiments demonstrate that the renal sympathoexcitation is mediated also by the release of vasopressin. However, is this due to vasopressin released into periphery which feeds back onto brain areas involved in cardiovascular regulation or due to activation of central neuronal pathways? In this respect the renal sympathoexcitatory effect had a similar delay whether the selective nAChR agonists are given i.c. or i.c.v., that of 4 minutes favouring such an interpretation. This feedback could be occurring at the level of area postrema which has been shown to contain V_1 receptors and pathways to cardiovascular regulating areas of the brain such as the nucleus tractus solitarius (Shapiro & Miselis, 1985). Another possible circumventricular organ site that vasopressin is able to feedback into the brain is by acting on the subfornical organ (SFO), possibly via V_{1a} receptors, which is located in the forebrain, lacks a blood brain barrier and projects to the PVN (Ferguson *et al.*, 1984; Li & Ferguson, 1993; Tanaka *et al.*, 1986; Tanaka & Seto, 1988). This is quite an intriguing observation as it implies that vasopressin can affect the kidney directly by action on the collecting ducts and by the sympathetic drive to the kidney which can also be involved in sodium excretion as well as renin release and vasoconstriction.

These hypothesised pathways and feedback mechanisms producing the effects described of the selective nAChR agonists are illustrated in Figures 43 and 44. There are two possible mechanisms of action that may account for the effect of vasopressin on renal nerve activity. 1), Upon vasopressin release into the circulation, by the selective agonists, vasopressin feeds back on to the PVN through the SFO, stimulating the neuronal projection from the PVN to the RVLM. The activation of this pathway stimulates the RVLM which results in an increase in sympathetic tone and an increase in renal sympathetic nerve activity. 2) Instead of the vasopressin feeding back on to the PVN, the site of action is the area postrema, which has a direct innervation of the NTS (Shapiro & Miselis, 1985), and possibly the RVLM, resulting in the increase in renal nerve activity.

The feedback of vasopressin onto the PVN resulting in the increase of renal nerve activity is the most plausible mode of action due to previous studies demonstrating that the PVN is able to induce sympathoexcitation in stress (Badoer, 2001; Blair *et al.*, 1996), as well as, stimulation of the PVN leading to an increase in blood pressure, heart rate and renal nerve activity (Coote *et al.*, 1998). The PVN causes sympathoexcitation both via direct projection to sympathetic pre-ganglionic neurons in the IML, and via collateral projections to neurons of the RVLM that sends excitatory input to the IML (Coote *et al.*, 1998; Shafton *et al.*, 1998). The excitatory input from the PVN to the RVLM may be mediated by vasopressin; acting via V_1 receptors (see Dampney *et al.*, 2003; Yang *et al.*, 2001).

Cardiovascular effects not involving vasopressin.

Interestingly, both agonists when given i.c. in the presence of V_1 receptor antagonist i.c. cause a transient sympathoexcitation after 1 min. This is paralleled by a transient non-significant increase in blood pressure. A possible site for this is the RVLM, as microinjections of nicotine caused pressor responses and a tachycardia (Tseng *et al.*, 1993; 1994). The transient nature is also surprising unless an additional depressor circuit is also being activated to mask this effect and this could be claimed at least for PSAB-OFP where a fall in blood pressure is observed after 2 min and is paralleled by a non-significant decline in heart rate but no change in renal nerve activity.

Interestingly, the delayed decline in blood pressure is also observed with PSAB-OFP given i.c.v. in the presence of the V_1 receptor antagonist also given i.c.v., however no initial renal sympathoexcitation was observed. Again the data suggests that this fall in blood pressure is mediated by α7 nAChRs located in the hindbrain. In this respect, microinjection of nicotine into the NTS has been observed to cause a significant decrease in blood pressure, which was inhibited by α7 nAChR selective antagonist MLA and α-BgT, implying the involvement of α7 nAChR (Dhar *et al.*, 2000; Ferreira *et al.*, 2000; Ferreira, Jr. *et al.*, 2002; Whiteaker *et al.*, 1998).

Thus it is concluded that the major central cardiovascular effects of activating α7 and α4β2 nACh receptors occur at the level of brainstem and is mediated by activation of central pathways which leads to the release of vasopressin into the periphery. This in turn can feedback and cause additional effects such as renal sympathoexcitation. However, there are also other actions but these are overwhelmed by the release of vasopressin centrally and peripherally.

Figure 43. Diagrammatic representation of the pathways involved upon i.c.v. injection of α4β2 selective agonist TC-2559 and α7 selective agonist PSAB-OFP in the rat brain.

1) Selective agonists are injected into the rat brain via i.c.v. injection.
2) The selective agonists do not come into direct contact with the PVN and instead move through the brain until they come into contact with the hindbrain.
3) The selective agonists bind with nAChR found in the RVLM and possibly the NTS.
4) Stimulation of the RVLM by the selective agonists activates the PVN via a neuron projection.
5) The vasopressinergic pathway stimulates the PVN to act on the posterior pituitary and release vasopressin;
6) Release of vasopressin into the circulation, via the blood stream, results in the increase in blood pressure by acting on V_{1a} receptors found on blood vessels causing vasoconstriction.
7) Vasopressin feeds back onto areas of the brain lacking a blood brain barrier, i.e. circumventricular organs, particularly the subfornical organ and area postrema (AP).
8 & 9) Vasopressin feedback onto the PVN stimulates the RVLM and results in an increase in renal nerve activity.
10) Vasopressin may also be acting via the AP, which either stimulates the RVLM directly resulting in a increase in renal nerve activity, or;
11) Via a pathway to the NTS, this relays to the RVLM, resulting in an increase in renal nerve activity.

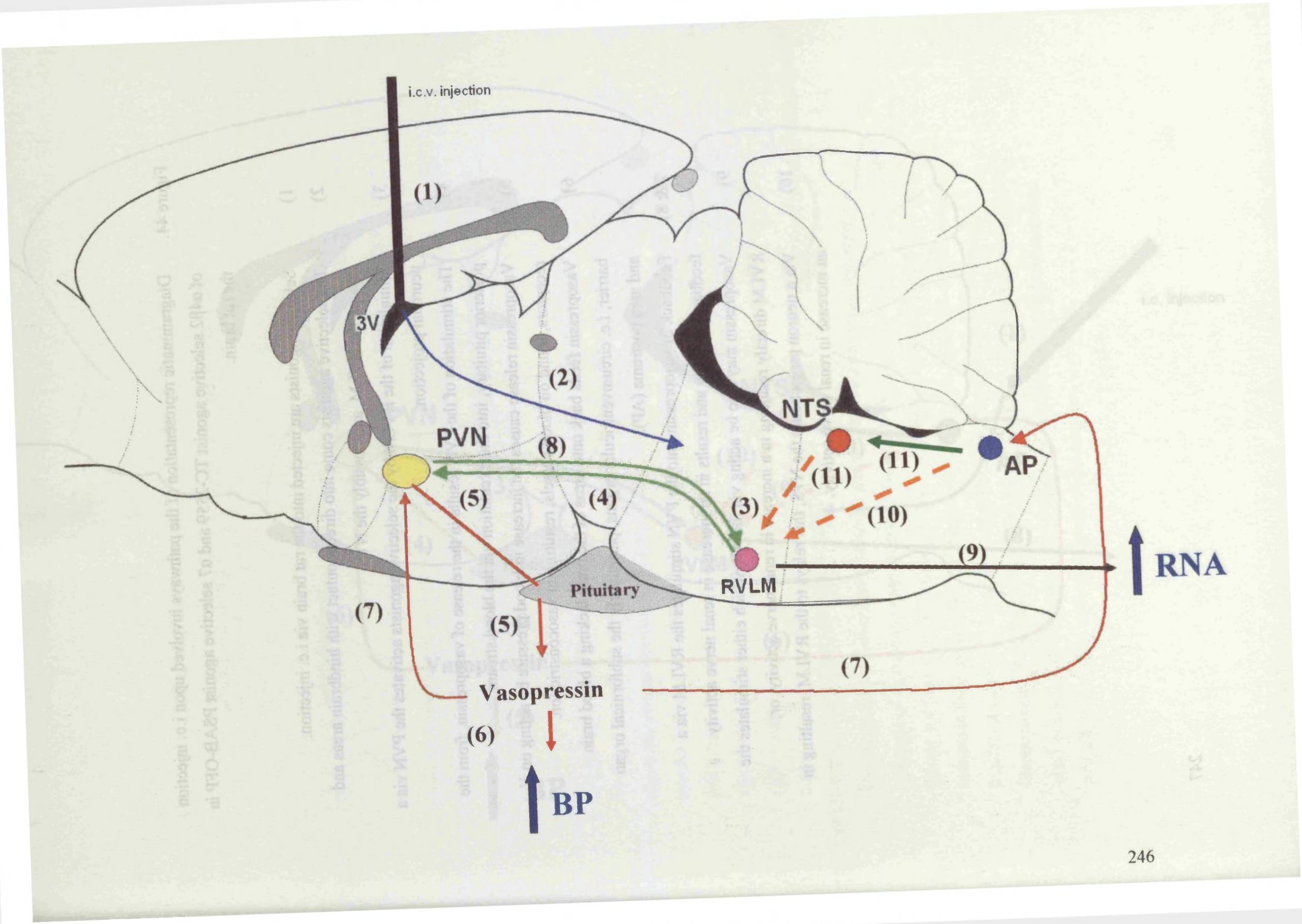
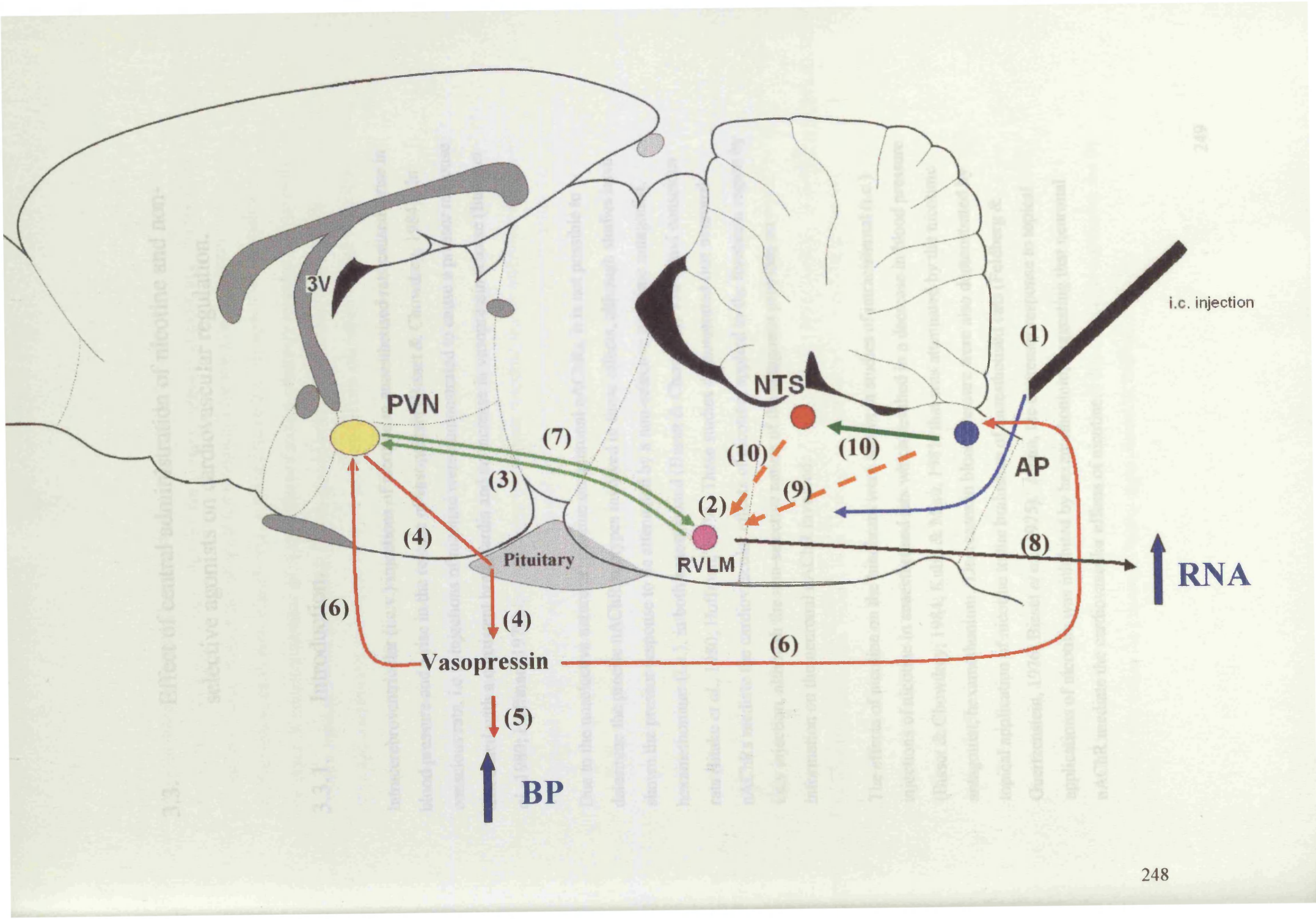

i.c.v. injection
(1)
3V
(2)
NTS
PVN
(8)
AP
(11)
(11)
(5)
(4)
(3)
(10)
(9)
RNA
Pituitary
RVLM
(7)
(5)
(7)
Vasopressin
(6)
BP

Figure 44. Diagrammatic representation of the pathways involved upon i.c. injection of α4β2 selective agonist TC-2559 and α7 selective agonist PSAB-OFP in the rat brain.

1) Selective agonists are injected into the rat brain via i.c. injection.
2) The selective agonists come into direct contact with hindbrain areas and acts on the RVLM, and possibly the NTS.
3) Stimulation of the RVLM by the selective agonists activates the PVN via a neuronal projection.
4) The stimulation of the PVN results in the release of vasopressin, from the posterior pituitary, into the circulation via the blood stream.
5) Vasopressin release causes the increase in blood pressure by acting on V_{1a} receptors, found on blood vessels, resulting in vasoconstriction.
6) Vasopressin feeds back onto areas of the brain lacking a blood brain barrier, i.e. circumventricular organs, particularly the subfornical organ and area postrema (AP).
7 & 8) Feedback of vasopressin onto the PVN stimulates the RVLM via a feedback pathway and results in an increase in renal nerve activity.
9) Vasopressin may also be acting via the AP, which either stimulates the RVLM directly resulting in a increase in renal nerve activity, or;
10) Via a neuron projectionto the NTS, this relays to the RVLM, resulting in an increase in renal nerve activity.


3V
PVN
NTS
AP
i.c. injection
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(10)
Pituitary
RVLM
RNA
Vasopressin
BP

3.3. Effect of central administration of nicotine and non-selective agonists on cardiovascular regulation.

3.3.1. Introduction.

Intracerebroventricular (i.c.v.) injections of nicotine in anaesthetised rats caused a rise in blood pressure and a rise in the release of vasopressin (Bisset & Chowdrey, 1984). In conscious rats, i.c.v. injections of nicotine were demonstrated to cause a pressor response associated with a consistent bradycardia and an increase in vasopressin release (Iitake *et al.*, 1980; Hoffman, 1979).

Due to the unselective nature of nicotine on neuronal nAChRs, it is not possible to determine the precise nAChR subtypes involved in these effects, although studies have shown the pressor response to be attenuated by a non-selective nicotinic antagonist, hexamethonium (i.v.), in both anaesthetised (Bisset & Chowdrey, 1984) and conscious rats (Iitake *et al.*, 1980; Hoffman, 1979). These studies demonstrated that neuronal nAChRs mediate the cardiovascular effects of nicotine, applied to the forebrain region by i.c.v injection, although the non-selective nature of the antagonist provides no information on the neuronal nAChR involved.

The effects of nicotine on the hindbrain were shown in studies of intracisternal (i.c.) injections of nicotine in anaesthetised rats which resulted in a decrease in blood pressure (Bisset & Chowdrey, 1984; Kubo & Misu, 1981) that was attenuated by the nicotinic antagonist, hexamethonium. Decreases in blood pressure were also demonstrated by topical application of nicotine to the brainstem of anaesthetised cats (Feldberg & Guertzenstein, 1976; Bisset *et al.*, 1975). Again, the depressor response to topical applications of nicotine was inhibited by hexamethonium, suggesting that neuronal nAChR mediate the cardiovascular effects of nicotine.

Although there is no published information available for the central actions of selective neuronal nAChR agonists on cardiovascular systems, other than those in Chapters 1 and 3, there are current studies providing some insight to neuronal nACh receptor involvement in cardiovascular effects. Intracerebroventricular injections of choline, a precursor of neurotransmitter ACh and believed to be a relatively selective endogenous receptor agonist for the α7 nAChR (Albuquerque *et al.*, 1997; Papke *et al.*, 1996), produced a dose-related increase in blood pressure in both anaesthetised and conscious rats (Savci *et al.*, 2002; Li & Buccafusco, 2004). The pressor response produced by choline was inhibited by nAChR α7 selective antagonist, MLA. In contrast, α4β2 selective antagonist, Dhβ E, and muscarinic antagonist, atropine, failed to significantly inhibit this choline-evoked pressor response (Li & Buccafusco, 2004). These studies support the notion that choline is causing the pressor response due to activation of α7 nAChR and not α4β2. However, additional studies in conscious rats reported that the pressor response produced by choline is attenuated by mecamylamine, believed to be a relatively selective antagonist for the α3β4 nACh receptor (Aberger *et al.*, 2001), again atropine was observed to have no affect (Savci *et al.*, 2002). This suggests that the α3β4 nAChR may also be involved in the pressor response observed by i.c.v. injection of choline.

Currently there are a number of neuronal nACh receptor agonists, which have been demonstrated to be more selective to specific nAChR subtypes than nicotine. One such agonist is cytisine (see Table 2, 4 & 5), the EC_{50} value for α4β2 is similar to that of nicotine, however studies have demonstrated that cytisine has a high functional potency at the α3β4 nAChR, with an EC_{50} value of 0.9 μM, which is greater than that of nicotine, which has an EC_{50} of 80 μM (Alkondon & Albuquerque, 1993; Luetje & Patrick, 1991). Futher studes demonstrated that cytisine had selective tendencies to α2β4, α3β4 and α4β4 nAChRs suggesting that cytisine may be selective to neuronal nAChR subunits containing a β4 subunit (Alkondon & Albuquerque, 1993; Chavez-Noriega *et al.*, 1997; see Colquhoun & Patrick, 1997). Cytisine administered by i.c.v. injection has been reported to cause the release of vasopressin, suggesting that receptor/s containing the β4 subunit may cause the release of vasopressin (Bisset & Fairhall, 1995).

The present study has been carried out to determine which nAChRs are involved in the central cardiovascular responses to nicotine and other unselective agonists cytisine and DMPP. The agonists will be tested against two neuronal nACh receptor antagonists DhβE, which is selective for the α4β2 receptor (Chavez-Noriega *et al.*, 2000; Gopalakrishnan *et al.*, 1996), and MLA, which is selective for the α7 nACh receptor (Macallan *et al.*, 1988; Turek *et al.*, 1995; Ward *et al.*, 1990). The effective blocking doses for these antagonists have been determined (Chapter 3.1). Additional experiments were carried out using the vasopressin V_1 antagonist ([β-Mercapto-β,β-Cyclopentamethylenepropionyl^1,O-Me-Tyr^2,Arg^8]-Vasopressin, (d$(CH_2)_5$Tyr(Me)AVP)), administered peripherally and centrally by i.v., i.c.v. and i.c. injections in the presence of nicotine, administered by i.c.v. and i.c. injection, to determine if by blocking the effects of vasopressin release, caused by nicotine, other central cardiovascular effects of nicotine can be uncovered.

3.3.2. Results.

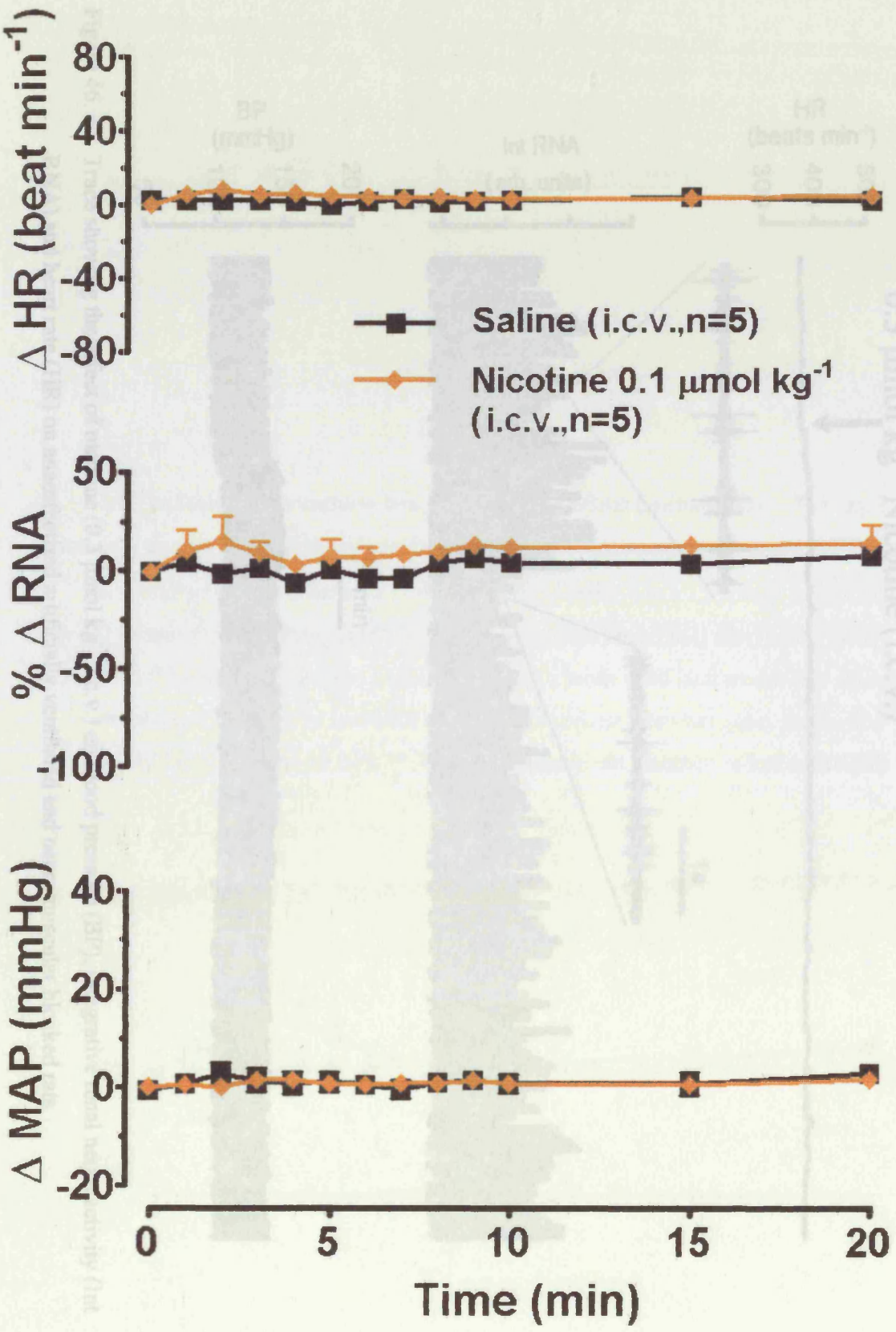
3.3.2.1. Effects of i.c.v. administration of nicotine, DMPP and cytisine.

Saline i.c.v. (5 μl, saline control, n=5) had no effect on mean arterial blood pressure, heart rate and renal nerve activity for the duration of the experiment. Baseline values for MAP and HR were 137 ± 14 mmHg and 380 ± 27 beats min^{-1} (Figures 45 - 48).

3.3.2.1.1. Effects of Nicotine.

Nicotine (0.1 μmol kg^{-1}, n=5) had no significant affect ($P > 0.05$) on mean arterial blood pressure, heart rate and renal nerve activity (Figure 45). Baseline values for MAP and HR were 123 ± 15 mmHg and 361 ± 34 beats min^{-1}. Nicotine (0.3 μmol kg^{-1}, n=5) caused a significant increase in mean arterial blood pressure after 2 min reaching a maximum of 11 ± 2 mmHg at 4 min, this was paralleled by a significant decrease in renal nerve activity at 2 min reaching a maximum of -57 ± 2% at 3 min. Both mean arterial blood pressure and renal nerve activity returned to near baseline after 15 min. There was no significant change in heart rate throughout the duration of the experiment (Figure 46 & 47). Baseline values for MAP and HR were 121 ± 12 mmHg and 388 ± 38 beats min^{-1}. 1 μmol kg^{-1} of nicotine (n=5), again caused, a significant increase in mean arterial blood pressure associated with a significant decrease in renal nerve activity after 1 min, reaching a maximum of 25 ± 9 mmHg and -76 ± 5% after 3 min (Figure 48). Again returning to near baseline after 15 min. Heart rate was unaffected for the duration of the experiment (Figure 48). Baseline values for MAP and HR were 133 ± 29 mmHg and 370 ± 40 beats min^{-1}.

Figure 45. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.1 μmol kg^{-1}, i.c.v.) and saline (5 μl. i.c.v., n=5) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means.


Δ HR (beat min-1)
80
40
0
-40
-80
Saline (i.c.v.,n=5)
Nicotine 0.1 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

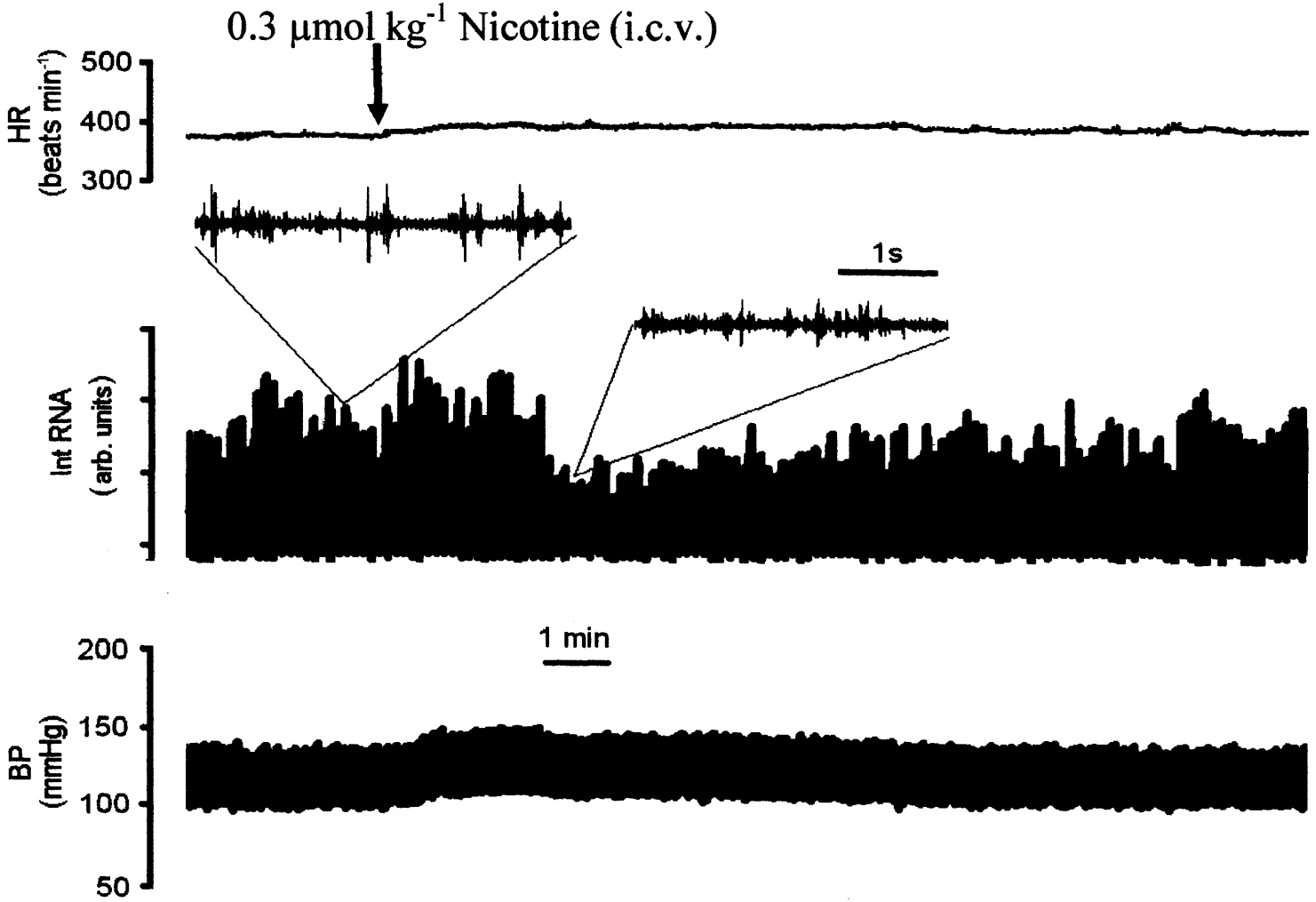


Figure 46. Trace showing the effect of nicotine (0.3 µmol kg^{-1}, i.c.v.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 47. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg-1, i.c.v.) and saline (5 μl. i.c.v., n=5) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

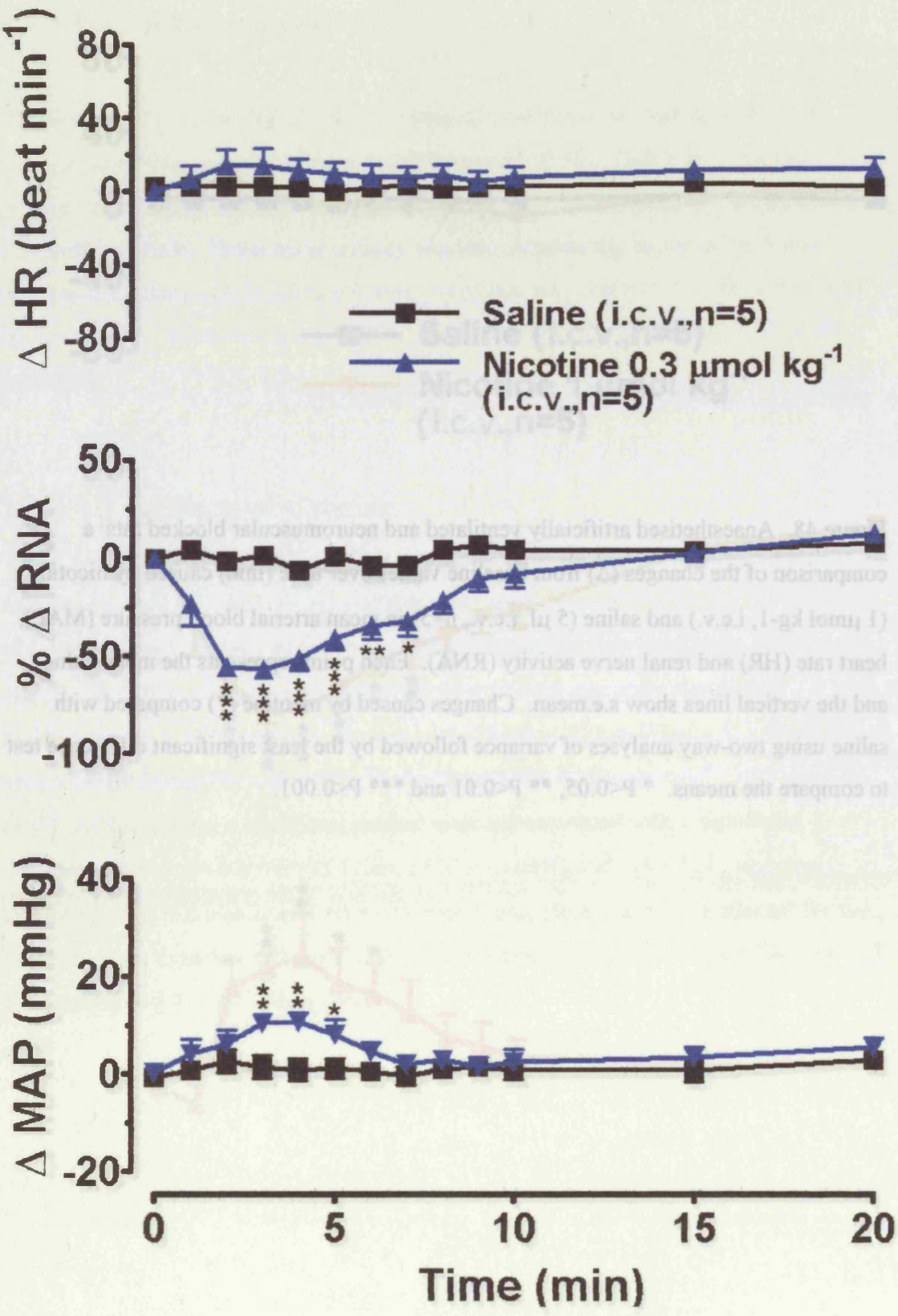
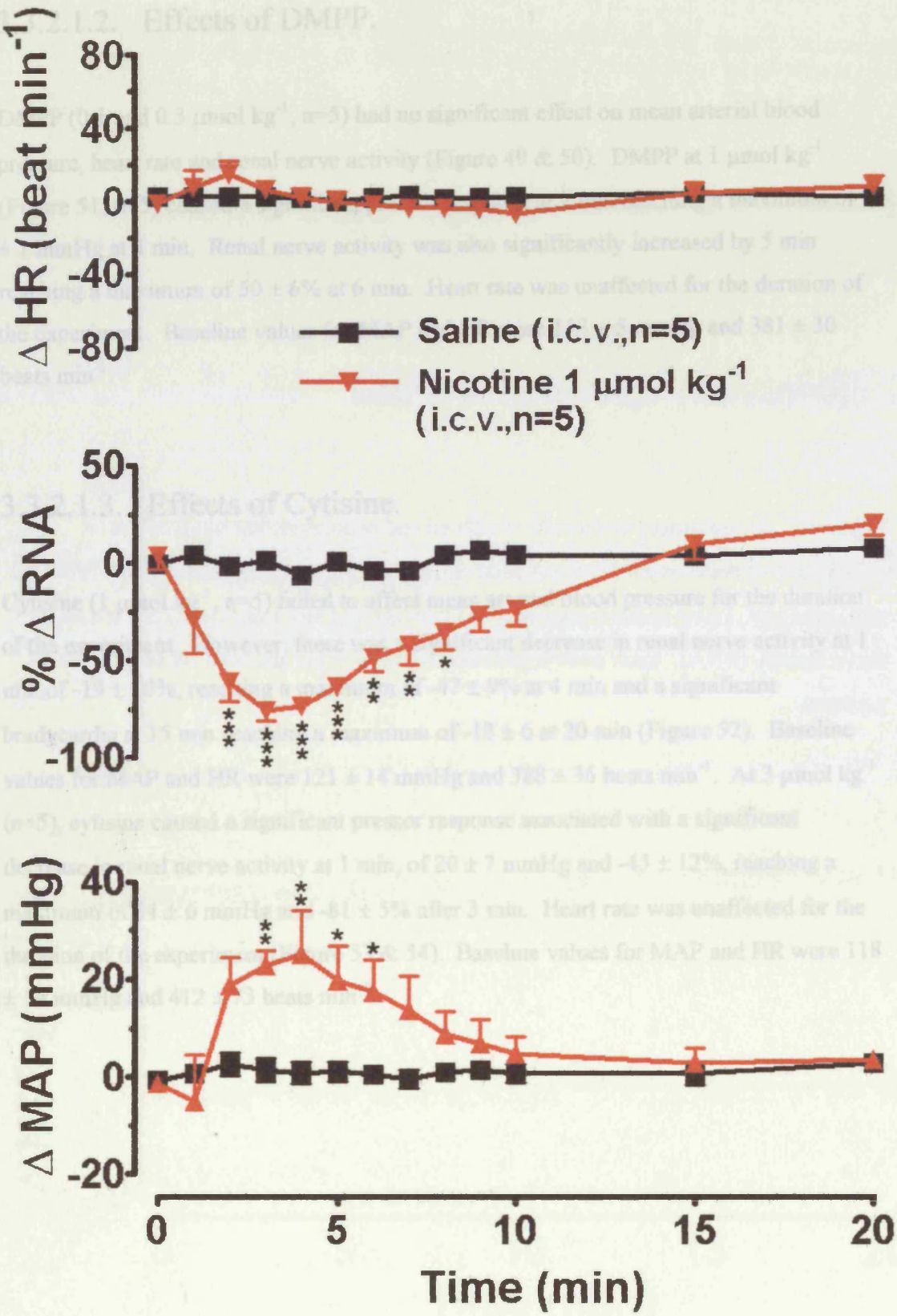

Δ HR (beat min-1)
80
40
0
-40
-80
Saline (i.c.v.,n=5)
Nicotine 0.3 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 48. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (1 μmol kg-1, i.c.v.) and saline (5 μl. i.c.v., n=5) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.


Δ HR (beat min-1)
80
40
0
-40
-80
Saline (i.c.v.,n=5)
Nicotine 1 μmol kg-1
(i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.3.2.1.2. Effects of DMPP.

DMPP (0.1 and 0.3 μmol kg^{-1}, n=5) had no significant effect on mean arterial blood pressure, heart rate and renal nerve activity (Figure 49 & 50). DMPP at 1 μmol kg^{-1} (Figure 51; n=5) caused a significant pressor response at 2 min reaching a maximum of 9 ± 1 mmHg at 4 min. Renal nerve activity was also significantly increased by 5 min reaching a maximum of 50 ± 6% at 6 min. Heart rate was unaffected for the duration of the experiment. Baseline values for MAP and HR were 113 ± 5 mmHg and 381 ± 30 beats min^{-1}.

3.3.2.1.3. Effects of Cytisine.

Cytisine (1 μmol kg^{-1}, n=5) failed to affect mean arterial blood pressure for the duration of the experiment. However, there was a significant decrease in renal nerve activity at 1 min of -19 ± 10%, reaching a maximum of -47 ± 9% at 4 min and a significant bradycardia at 15 min reaching a maximum of -18 ± 6 at 20 min (Figure 52). Baseline values for MAP and HR were 121 ± 14 mmHg and 388 ± 36 beats min^{-1}. At 3 μmol kg^{-1} (n=5), cytisine caused a significant pressor response associated with a significant decrease in renal nerve activity at 1 min, of 20 ± 7 mmHg and -45 ± 12%, reaching a maximum of 34 ± 6 mmHg and -81 ± 5% after 3 min. Heart rate was unaffected for the duration of the experiment (Figure 53 & 54). Baseline values for MAP and HR were 118 ± 14 mmHg and 412 ± 73 beats min^{-1}.

Figure 49. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by DMPP (0.1 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean.

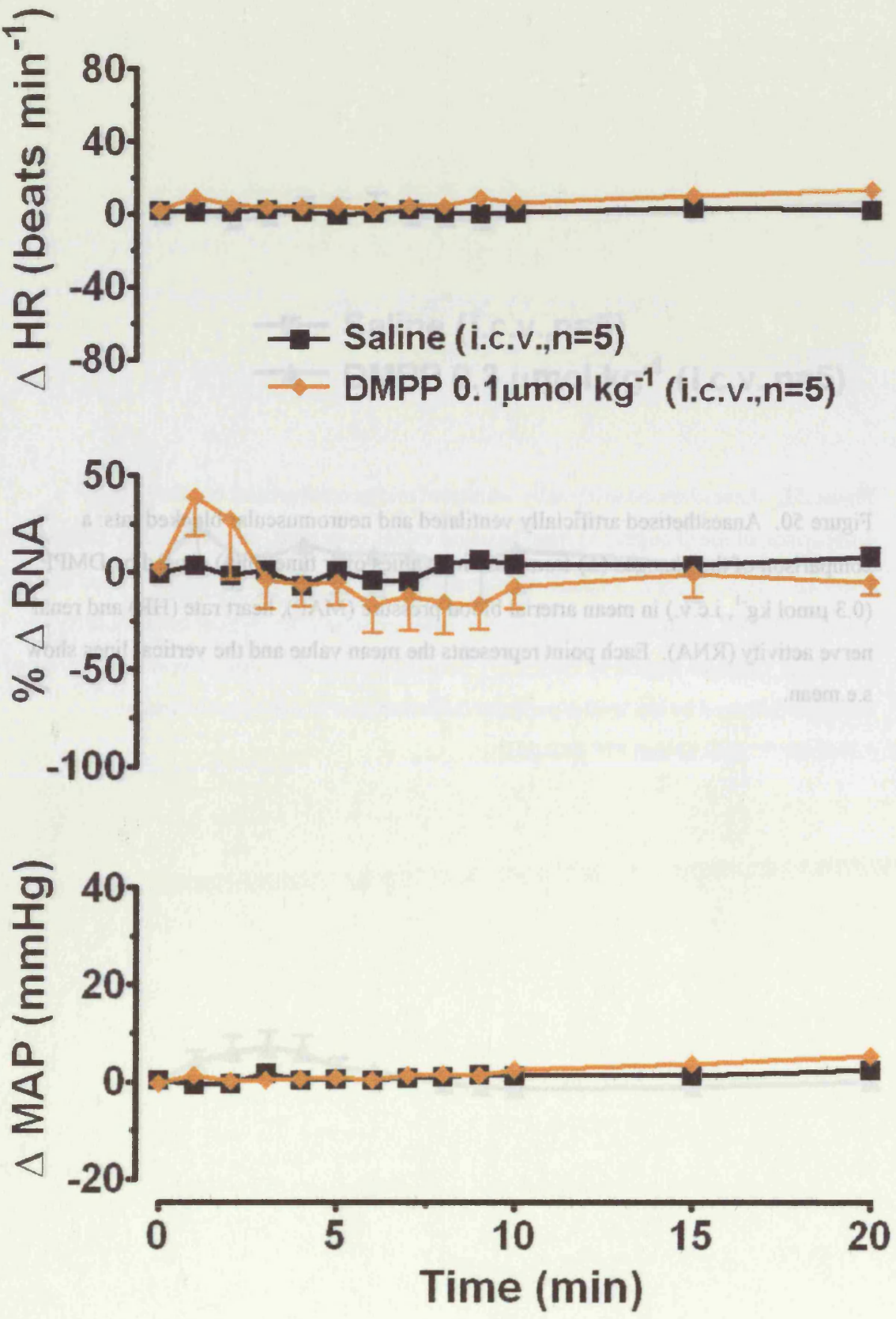

Δ HR (beats min-1)
80
40
0
-40
-80
Saline (i.c.v.,n=5)
DMPP 0.1µmol kg-1 (i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 50. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by DMPP (0.3 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean.

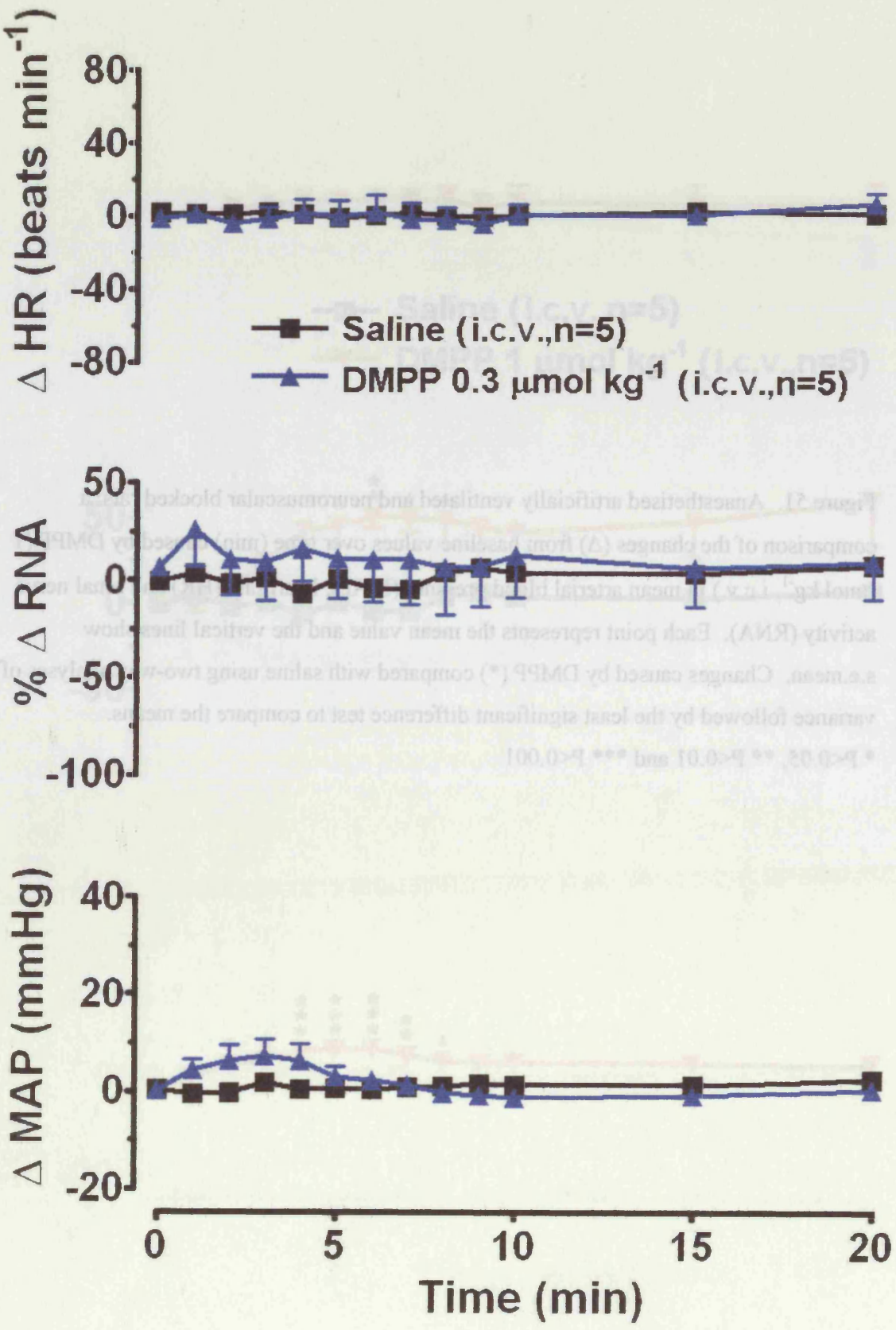

Δ HR (beats min-1)
80
40
0
-40
-80
Saline (i.c.v.,n=5)
DMPP 0.3 μmol kg-1 (i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 51. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by DMPP (1 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by DMPP (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means.
* $P<0.05$, ** $P<0.01$ and *** $P<0.001$

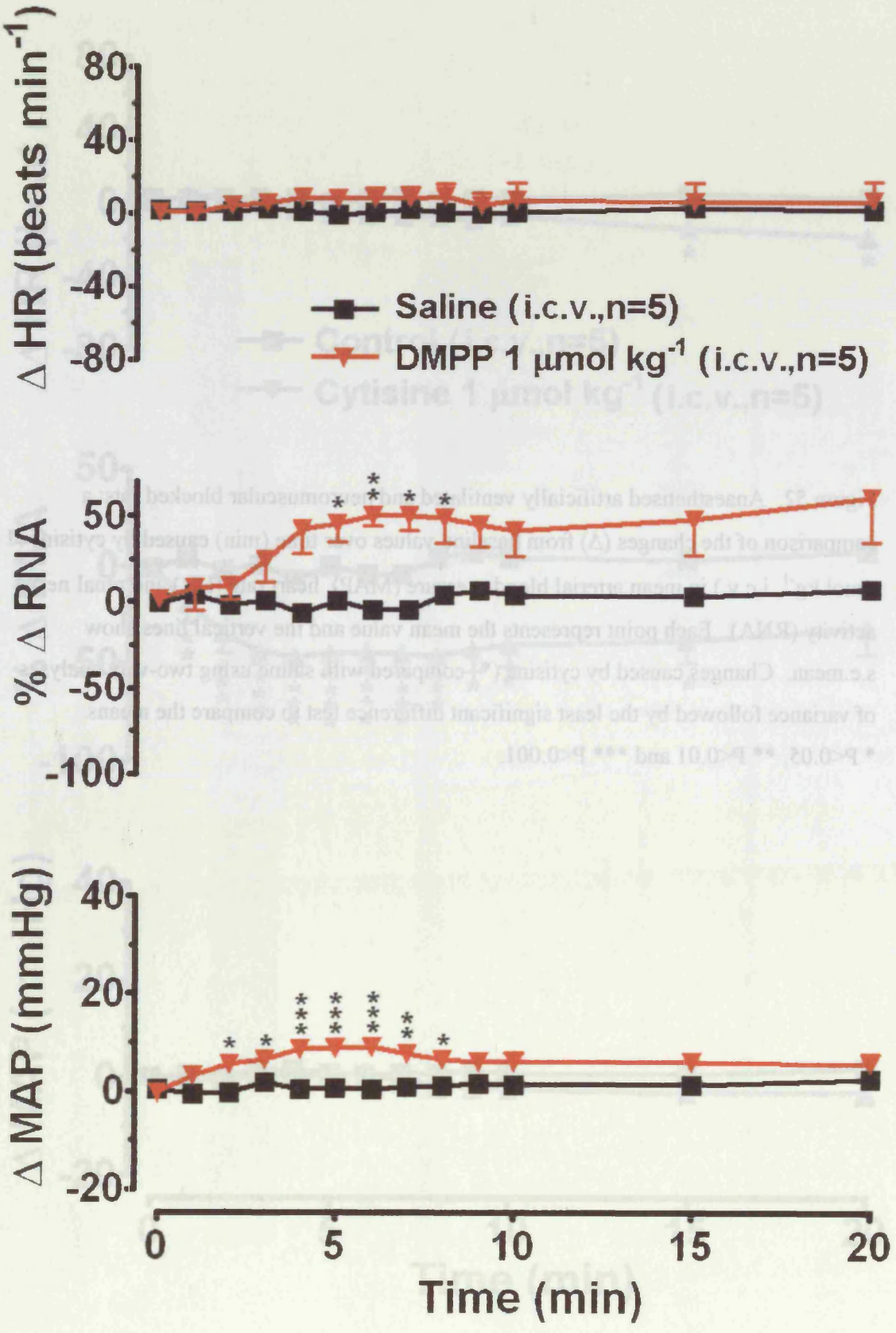
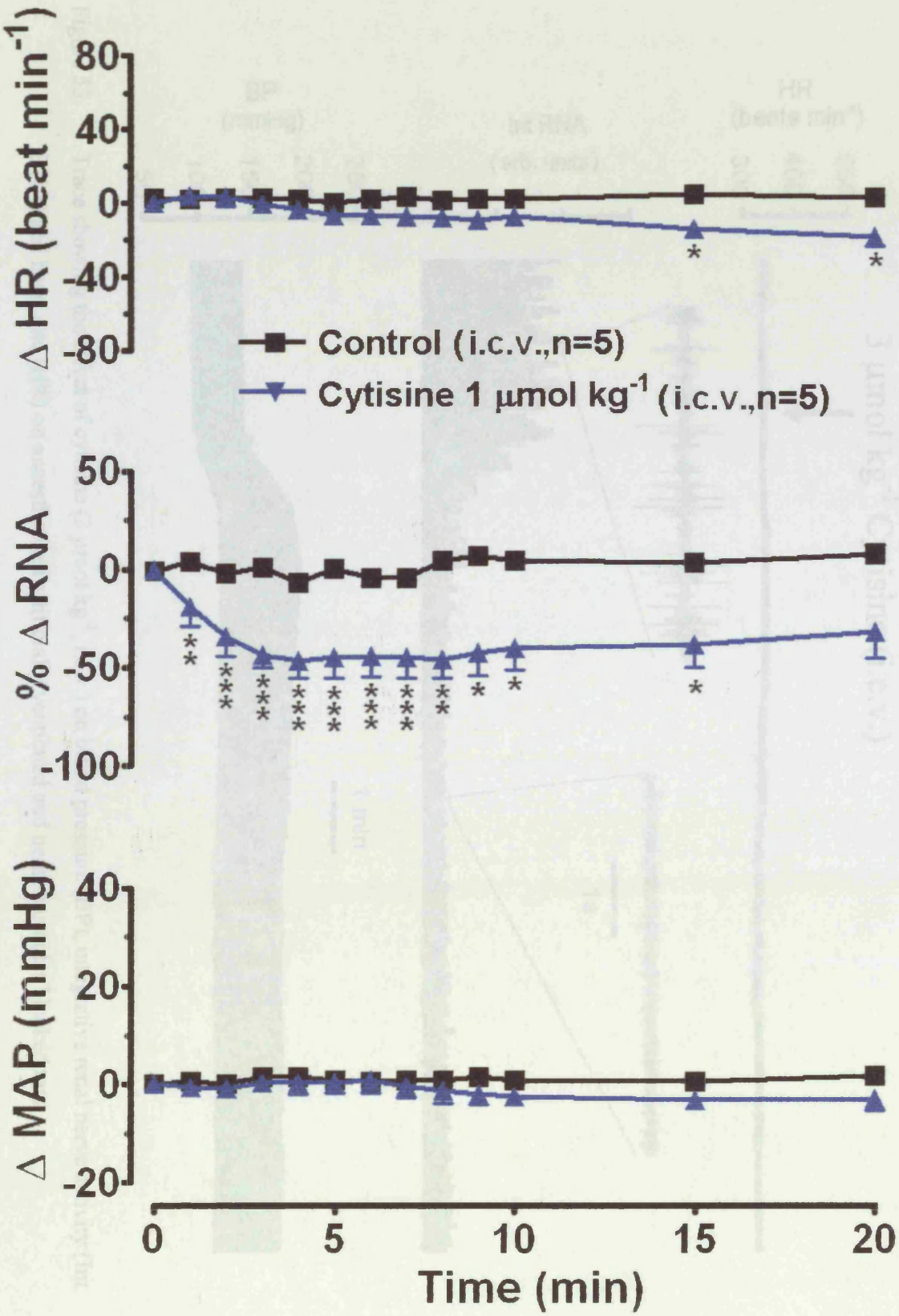

Δ HR (beats min-1)
80
40
0
-40
-80
Saline (i.c.v.,n=5)
DMPP 1 μmol kg-1 (i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 52. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by cytisine (1 μmol kg^{-1}, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by cytisine (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.


Δ HR (beat min-1)
80
40
0
-40
-80
Control (i.c.v.,n=5)
Cytisine 1 μmol kg-1 (i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

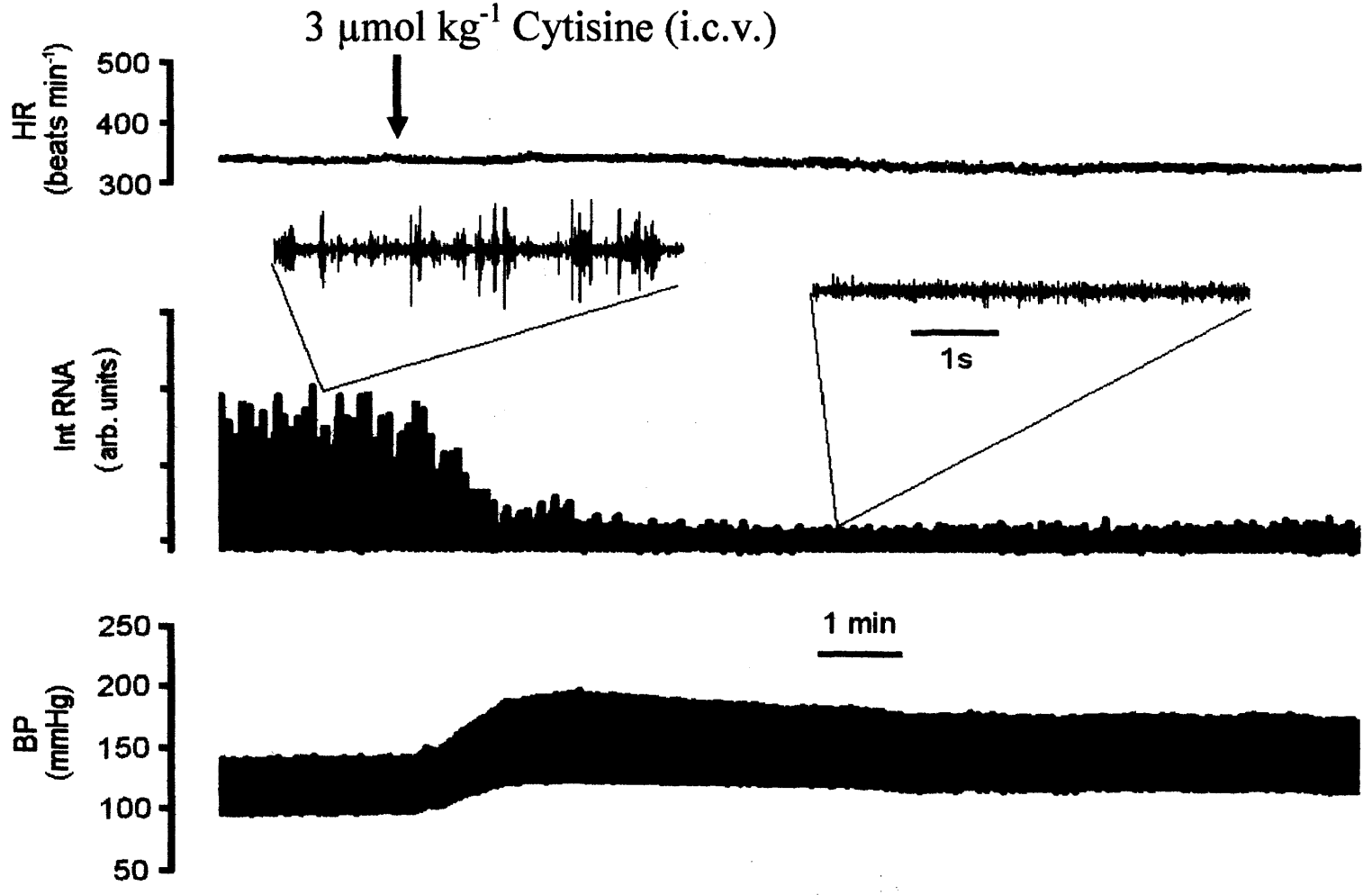
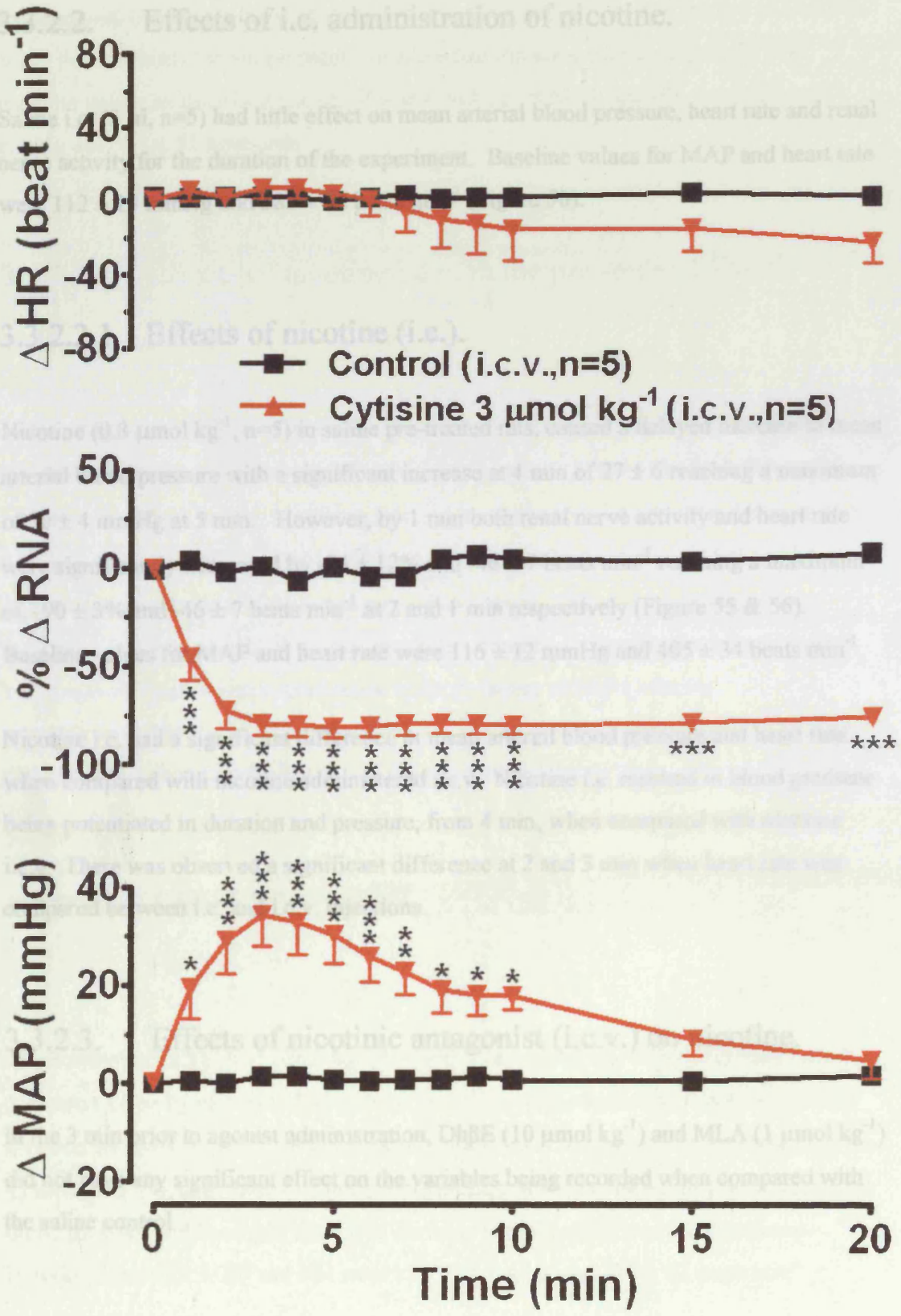


Figure 53. Trace showing the effect of cytisine (3 µmol kg^{-1}, i.c.v.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 54. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by cytisine (3 μmol kg-1, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by cytisine (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.


Δ HR (beat min-1)
80
40
0
-40
-80
Control (i.c.v.,n=5)
Cytisine 3 μmol kg-1 (i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.3.2.2. Effects of i.c. administration of nicotine.

Saline i.c. (5 μl, n=5) had little effect on mean arterial blood pressure, heart rate and renal nerve activity for the duration of the experiment. Baseline values for MAP and heart rate were 112 ± 13 mmHg and 385 ± 32 beats min^{-1} (Figure 56).

3.3.2.2.1. Effects of nicotine (i.c.).

Nicotine (0.3 μmol kg^{-1}, n=5) in saline pre-treated rats, caused a delayed increase in mean arterial blood pressure with a significant increase at 4 min of 27 ± 6 reaching a maximum of 30 ± 4 mmHg at 5 min. However, by 1 min both renal nerve activity and heart rate were significantly decreased by -74 ± 12% and -46 ± 7 beats min^{-1} reaching a maximum of, -90 ± 3% and -46 ± 7 beats min^{-1} at 2 and 1 min respectively (Figure 55 & 56). Baseline values for MAP and heart rate were 116 ± 12 mmHg and 405 ± 34 beats min^{-1}.

Nicotine i.c. had a significant difference in mean arterial blood pressure and heart rate when compared with nicotine administered i.c.v. Nicotine i.c. resulted in blood pressure being potentiated in duration and pressure, from 4 min, when compared with nicotine i.c.v. There was observed a significant difference at 2 and 3 min when heart rate was compared between i.c. and i.c.v. injections.

3.3.2.3. Effects of nicotinic antagonist (i.c.v.) on nicotine.

In the 3 min prior to agonist administration, DhβE (10 μmol kg^{-1}) and MLA (1 μmol kg^{-1}) did not have any significant effect on the variables being recorded when compared with the saline control.

In antagonist control experiments (n=5) where saline was given i.c.v. instead of DhβE or MLA (see methods for full protocol), nicotine had the same effects (Figure 57) on baseline values as described above. Baseline values for MAP and HR were 113 ± 10 mmHg and 393 ± 51 beats min^{-1}.

3.3.2.3.1. Effects of nicotine i.c.v. in the presence of DhβE (i.c.v.).

In the presence of DhβE (10 μmol kg^{-1}) the expected rise in blood pressure and renal sympathoinhibition evoked by nicotine (0.3 μmol kg^{-1}, n=5) were blocked (Figure 58). However there was now a significant increase in renal nerve activity after 2 min of 35 ± 11%, reaching a maximum of 44 ± 7% at 4 min. Again heart rate was unaffected. Nicotine pre-treated with DhβE compared with nicotine alone, showed a significant difference in mean arterial blood pressure from 3 min and a significant change in renal nerve activity from 1 min with nicotine in the presence of DhβE causing sympathoexcitation, unlike nicotine alone which causes sympathoinhibition. The heart rate was unaffected for the duration of the experiment (Figure 58).

3.3.2.3.2. Effects of nicotine i.c.v. in the presence of MLA (i.c.v.).

In the presence of MLA (1 μmol kg^{-1}) the expected evoked rise in mean arterial blood pressure caused by nicotine was blocked. Renal sympathoinhibition was still observed however its onset was delayed significantly, requiring 4 min to cause a decrease of -51 ± 11% (Figure 59), compared to 1 min in the absence of MLA. Interestingly mean arterial blood pressure and heart rate tended to decrease but this effect was not significant. Baseline values for MAP and HR were 117 ± 11 mmHg and 338 ± 83 beats min^{-1}

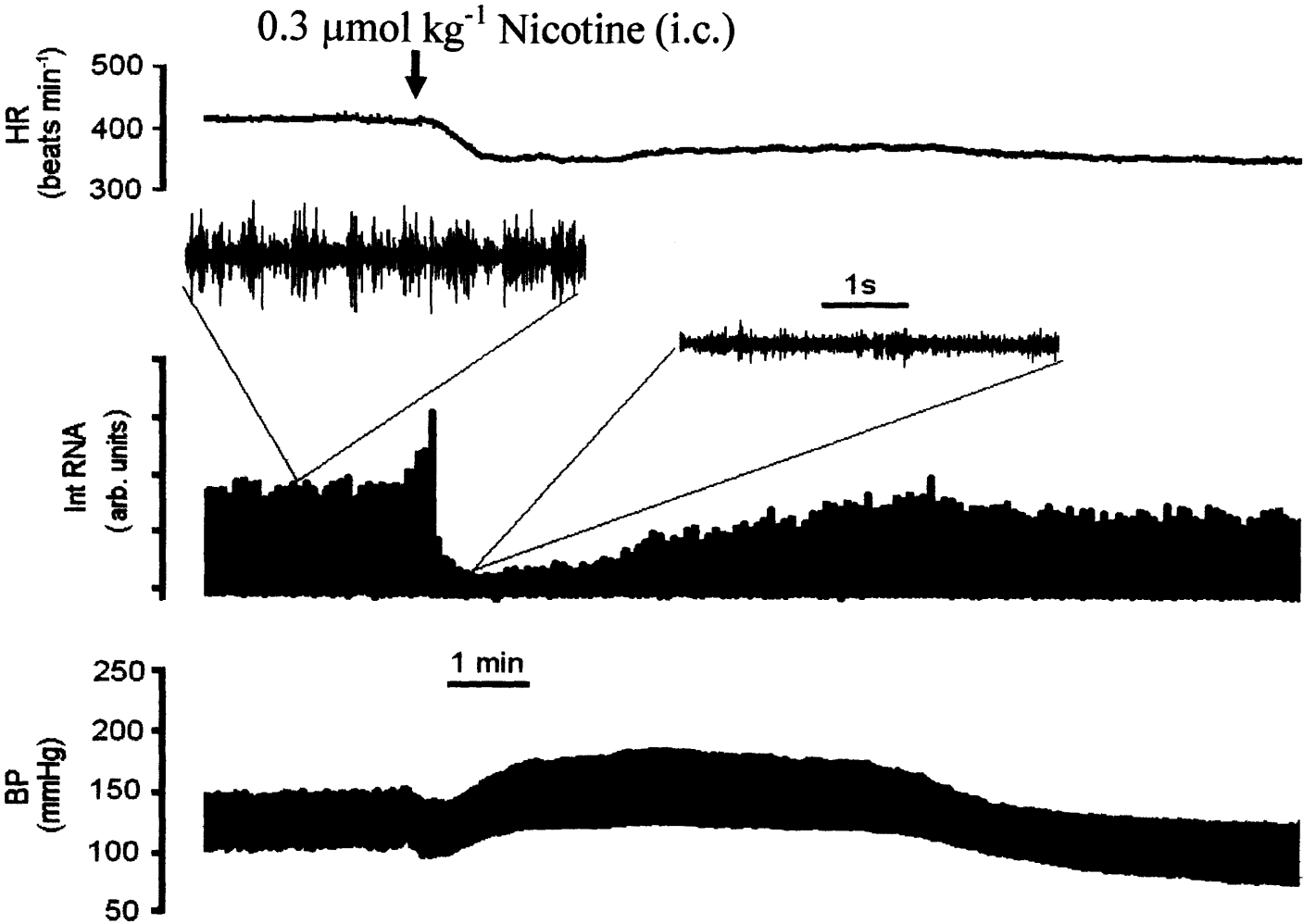


Figure 55. Trace showing the effect of nicotine (0.3 µmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 56. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.) and nicotine (0.3 μmol kg^{-1}, i.c.v.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine i.c. (*) compared with saline and changes caused by nicotine i.c. (#) compared with nicotine i.c.v. using two-way analyses of variance followed by the least significant difference test to compare the means. *, ## P<0.05, **, ## P<0.01 and *** P<0.001.

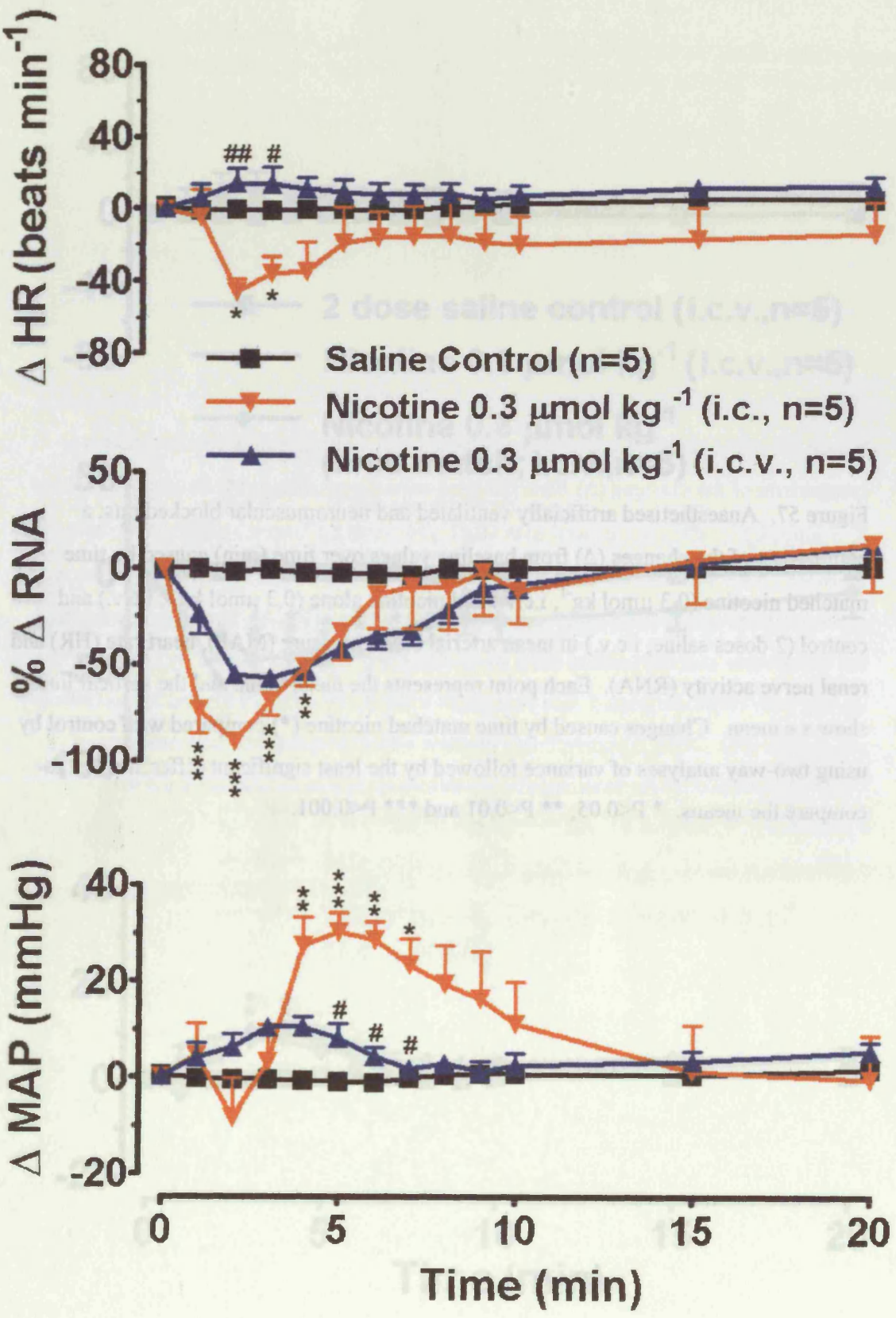

Δ HR (beats min-1)
80
40
0
-40
-80
Saline Control (n=5)
Nicotine 0.3 μmol kg -1 (i.c., n=5)
Nicotine 0.3 μmol kg-1 (i.c.v., n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 57. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by time matched nicotine (0.3 μmol kg^{-1}, i.c.v.) and nicotine alone (0.3 μmol kg^{-1}, i.c.v.) and control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by time matched nicotine (*) compared with control by using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

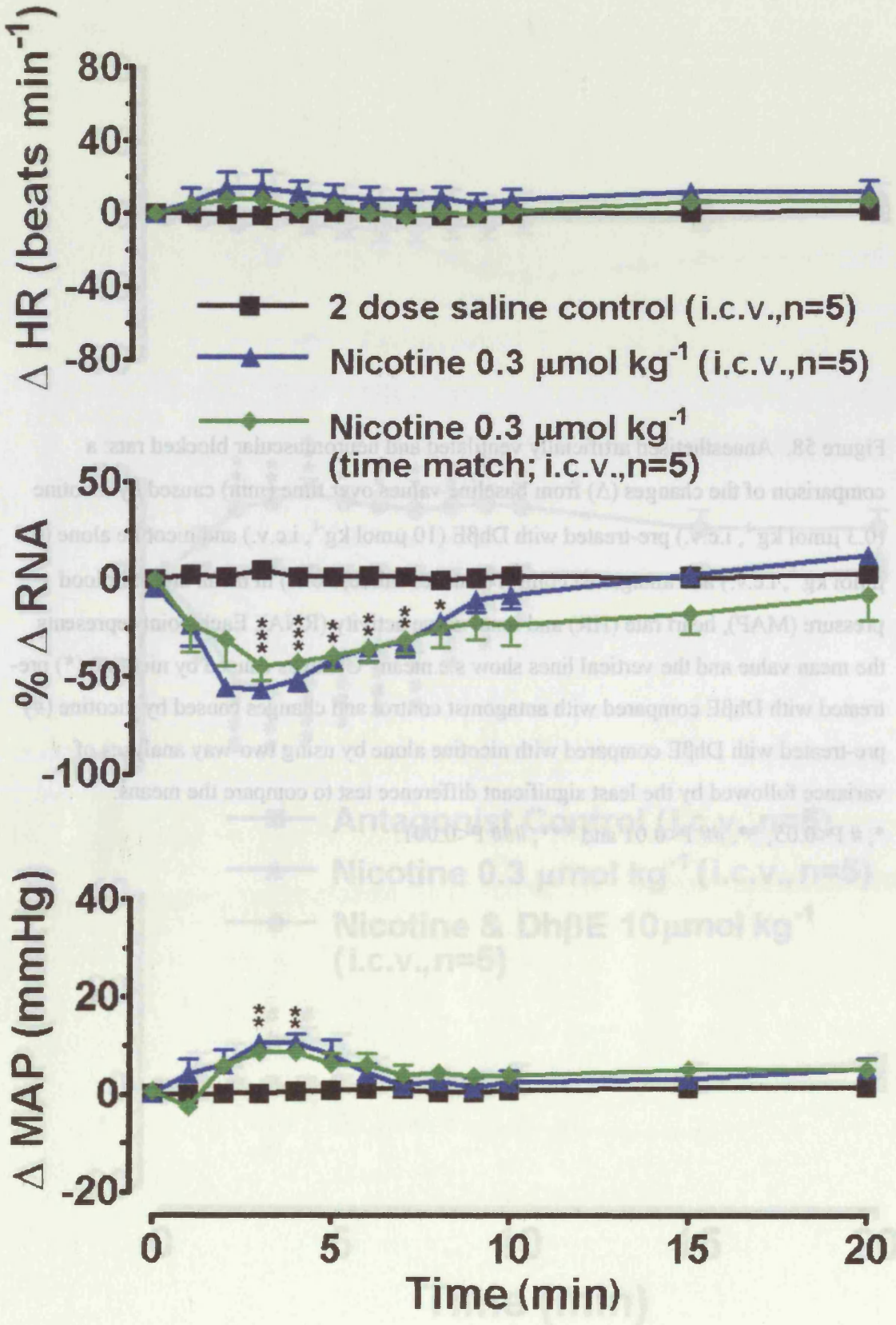

Δ HR (beats min-1)
80
40
0
-40
-80
2 dose saline control (i.c.v.,n=5)
Nicotine 0.3 μmol kg-1 (i.c.v.,n=5)
Nicotine 0.3 μmol kg-1
(time match; i.c.v.,n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 58. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.v.) pre-treated with DhβE (10 μmol kg^{-1}, i.c.v.) and nicotine alone (0.3 μmol kg^{-1}, i.c.v.) and antagonist control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) pre-treated with DhβE compared with antagonist control and changes caused by nicotine (#) pre-treated with DhβE compared with nicotine alone by using two-way analyses of variance followed by the least significant difference test to compare the means.
*, # $P<0.05$, **, ## $P<0.01$ and ***, ### $P<0.001$.

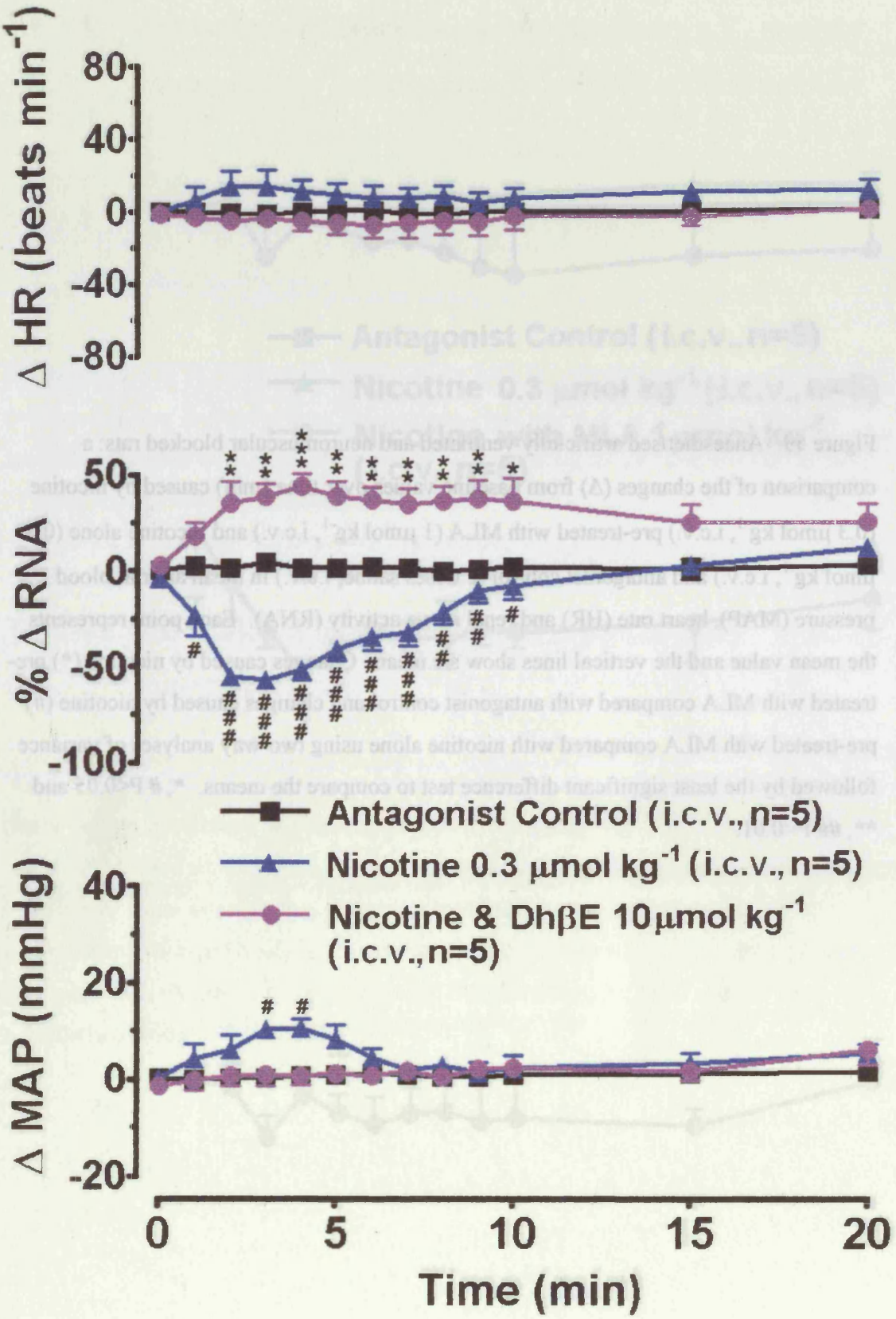
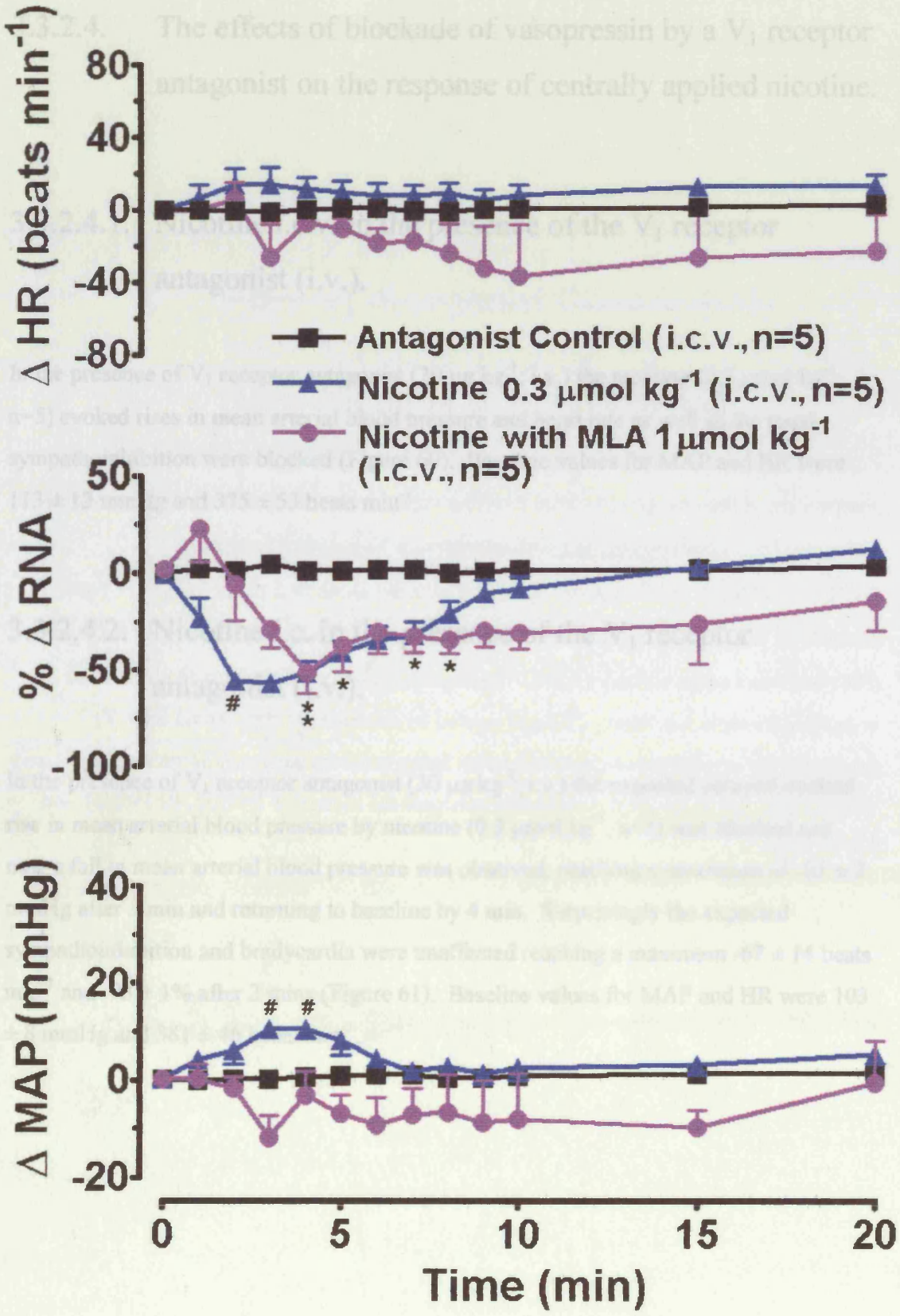

Δ HR (beats min-1)
80
40
0
-40
-80
% Δ RNA
50
0
-50
-100
Antagonist Control (i.c.v., n=5)
Nicotine 0.3 μmol kg-1 (i.c.v., n=5)
Nicotine & DhβE 10μmol kg-1 (i.c.v., n=5)
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 59. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.v.) pre-treated with MLA (1 μmol kg^{-1}, i.c.v.) and nicotine alone (0.3 μmol kg^{-1}, i.c.v.) and antagonist control (2 doses saline, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) pre-treated with MLA compared with antagonist control and changes caused by nicotine (#) pre-treated with MLA compared with nicotine alone using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and **, ## $P<0.01$.


Δ HR (beats min-1)
80
40
0
-40
-80
Antagonist Control (i.c.v., n=5)
Nicotine 0.3 μmol kg-1 (i.c.v., n=5)
Nicotine with MLA 1 μmol kg-1 (i.c.v., n=5)
% Δ RNA
50
0
-50
-100
#
*
*
*
*
Δ MAP (mmHg)
40
20
0
-20
# #
0
5
10
15
20
Time (min)

3.3.2.4. The effects of blockade of vasopressin by a V_1 receptor antagonist on the response of centrally applied nicotine.

3.3.2.4.1. Nicotine i.c.v. in the presence of the V_1 receptor antagonist (i.v.).

In the presence of V_1 receptor antagonist (30 μg kg^{-1}; i.v.) the nicotine (0.3 μmol kg^{-1}, n=5) evoked rises in mean arterial blood pressure and heart rate as well as the renal sympathoinhibition were blocked (Figure 60). Baseline values for MAP and HR were 113 ± 12 mmHg and 375 ± 53 beats min^{-1}.

3.3.2.4.2. Nicotine i.c. in the presence of the V_1 receptor antagonist (i.v.).

In the presence of V_1 receptor antagonist (30 μg kg^{-1}; i.v.) the expected delayed evoked rise in mean arterial blood pressure by nicotine (0.3 μmol kg^{-1}, n=5) was blocked and now a fall in mean arterial blood pressure was observed, reaching a maximum of -25 ± 2 mmHg after 3 min and returning to baseline by 4 min. Surprisingly the expected sympathoinhibition and bradycardia were unaffected reaching a maximum -67 ± 14 beats min^{-1} and -93 ± 1% after 2 mins (Figure 61). Baseline values for MAP and HR were 103 ± 8 mmHg and 381 ± 46 beats min^{-1}.

Figure 60. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and nicotine alone (0.3 μmol kg^{-1}, i.c.v.) and control (V_1 anta & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (#) pre-treated with V_1 antagonist compared with nicotine alone by using two-way analyses of variance followed by the least significant difference test to compare the means. # $P<0.05$, ## $P<0.01$ and ### $P<0.001$.

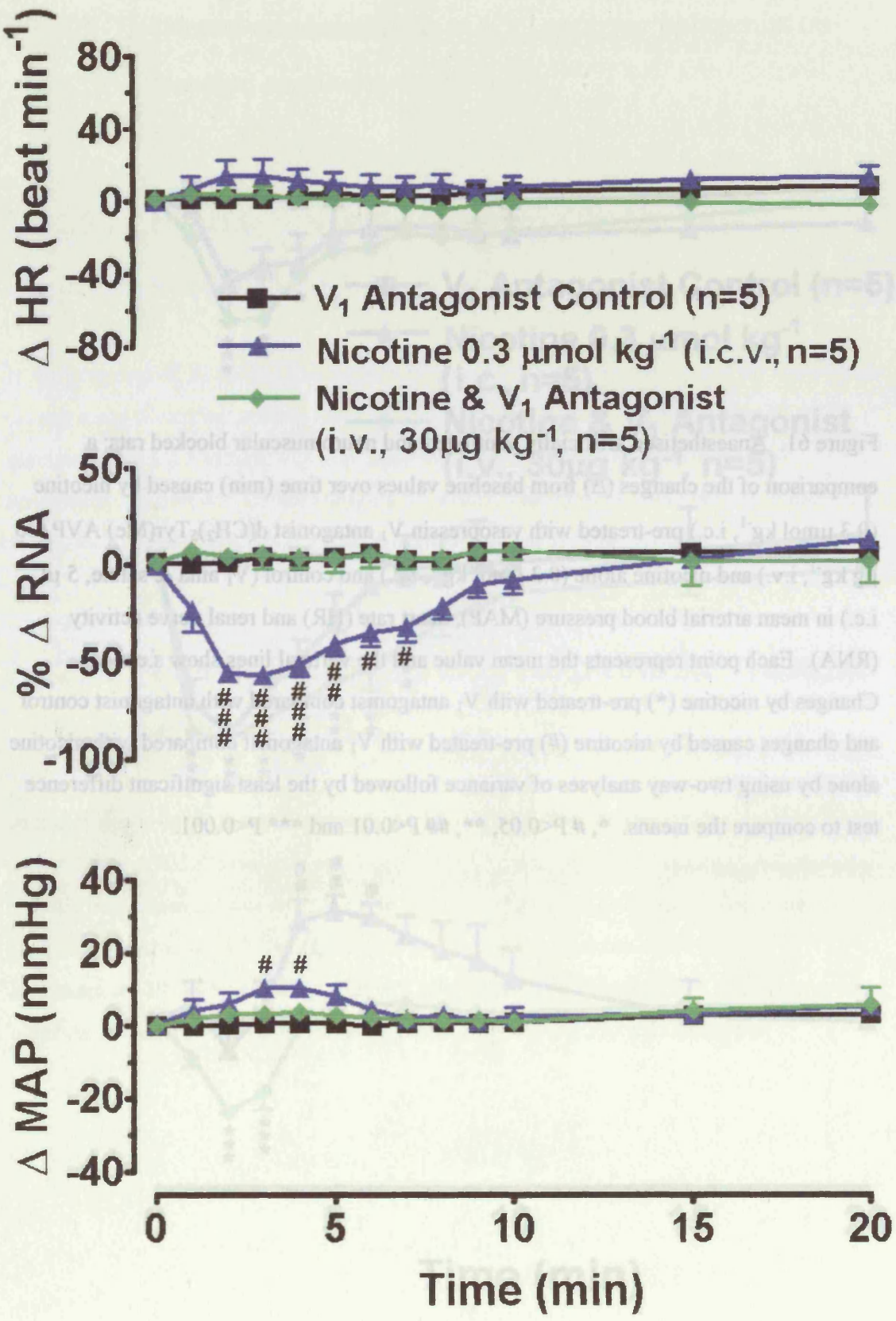
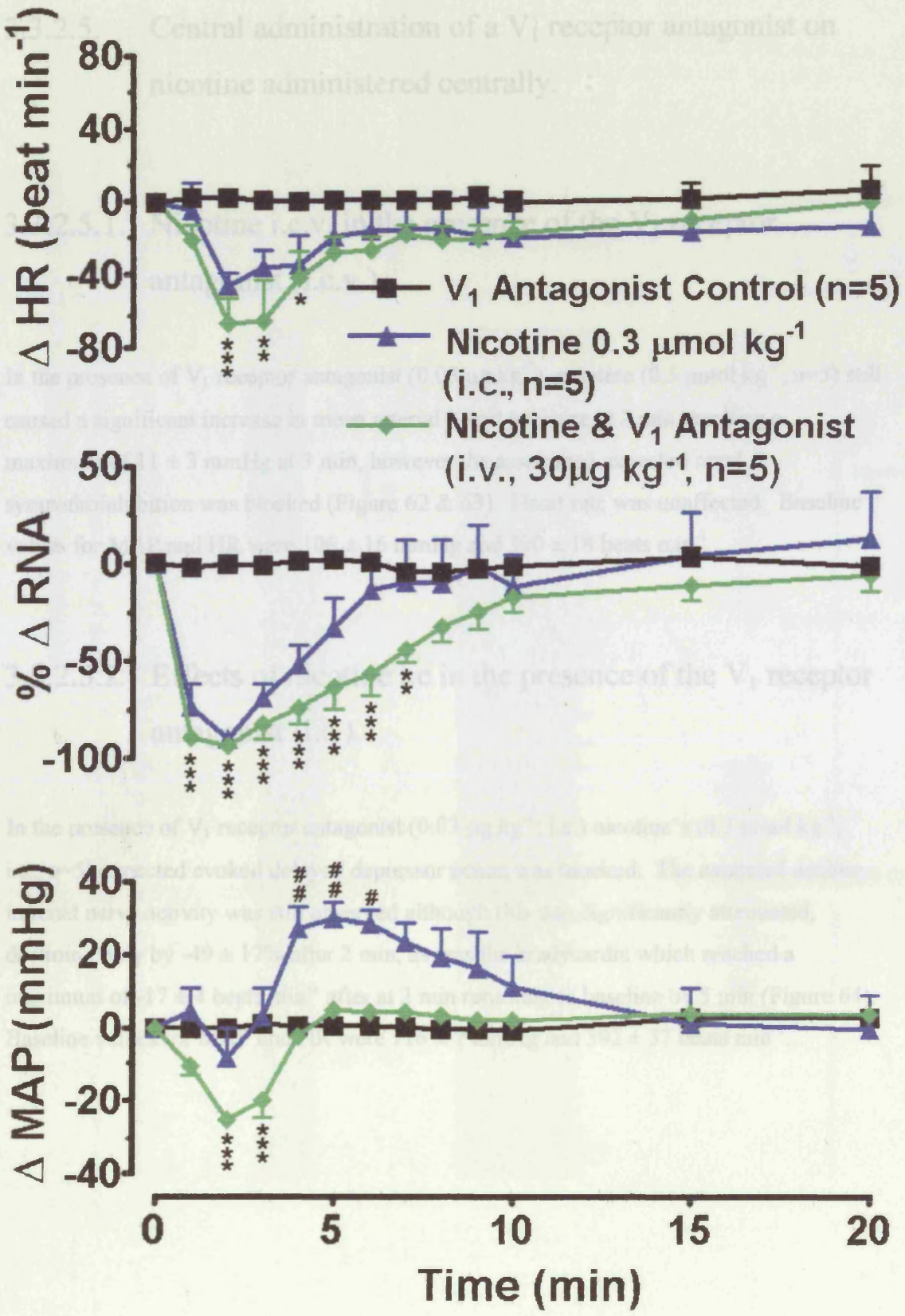

Δ HR (beat min-1)
80
40
0
-40
-80
V1 Antagonist Control (n=5)
Nicotine 0.3 μmol kg-1 (i.c.v., n=5)
Nicotine & V1 Antagonist
(i.v., 30μg kg-1, n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
-40
0
5
10
15
20
Time (min)

Figure 61. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.) pre-treated with vasopressin V_1 antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and nicotine alone (0.3 μmol kg^{-1}, i.c.) and control (V_1 anta & saline, 5 μl, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes by nicotine (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by nicotine (#) pre-treated with V_1 antagonist compared with nicotine alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ and *** $P<0.001$.


Δ HR (beat min-1)
80
40
0
-40
-80
V1 Antagonist Control (n=5)
Nicotine 0.3 μmol kg-1
(i.c., n=5)
Nicotine & V1 Antagonist
(i.v., 30μg kg-1, n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
-40
0
5
10
15
20
Time (min)

3.3.2.5. Central administration of a V_1 receptor antagonist on nicotine administered centrally.

3.3.2.5.1. Nicotine i.c.v. in the presence of the V_1 receptor antagonist (i.c.v.).

In the presence of V_1 receptor antagonist (0.03 μg kg^{-1}), nicotine (0.3 μmol kg^{-1}, n=5) still caused a significant increase in mean arterial blood pressure at 2 min reaching a maximum of 11 ± 3 mmHg at 3 min, however the associated expected renal sympathoinhibition was blocked (Figure 62 & 63). Heart rate was unaffected. Baseline values for MAP and HR were 106 ± 16 mmHg and 390 ± 18 beats min^{-1}

3.3.2.5.2. Effects of nicotine i.c in the presence of the V_1 receptor antagonist (i.c.).

In the presence of V_1 receptor antagonist (0.03 μg kg^{-1}; i.c.) nicotine's (0.3 μmol kg^{-1}, i.c.; n=5) expected evoked delayed depressor action was blocked. The expected decline in renal nerve activity was still observed although this was significantly attenuated, declining only by -49 ± 17% after 2 min, as was the bradycardia which reached a maximum of -17 ± 4 beats min^{-1} after at 2 min returning to baseline by 5 min (Figure 64). Baseline values for MAP and HR were 116 ± 7 mmHg and 392 ± 37 beats min^{-1}.

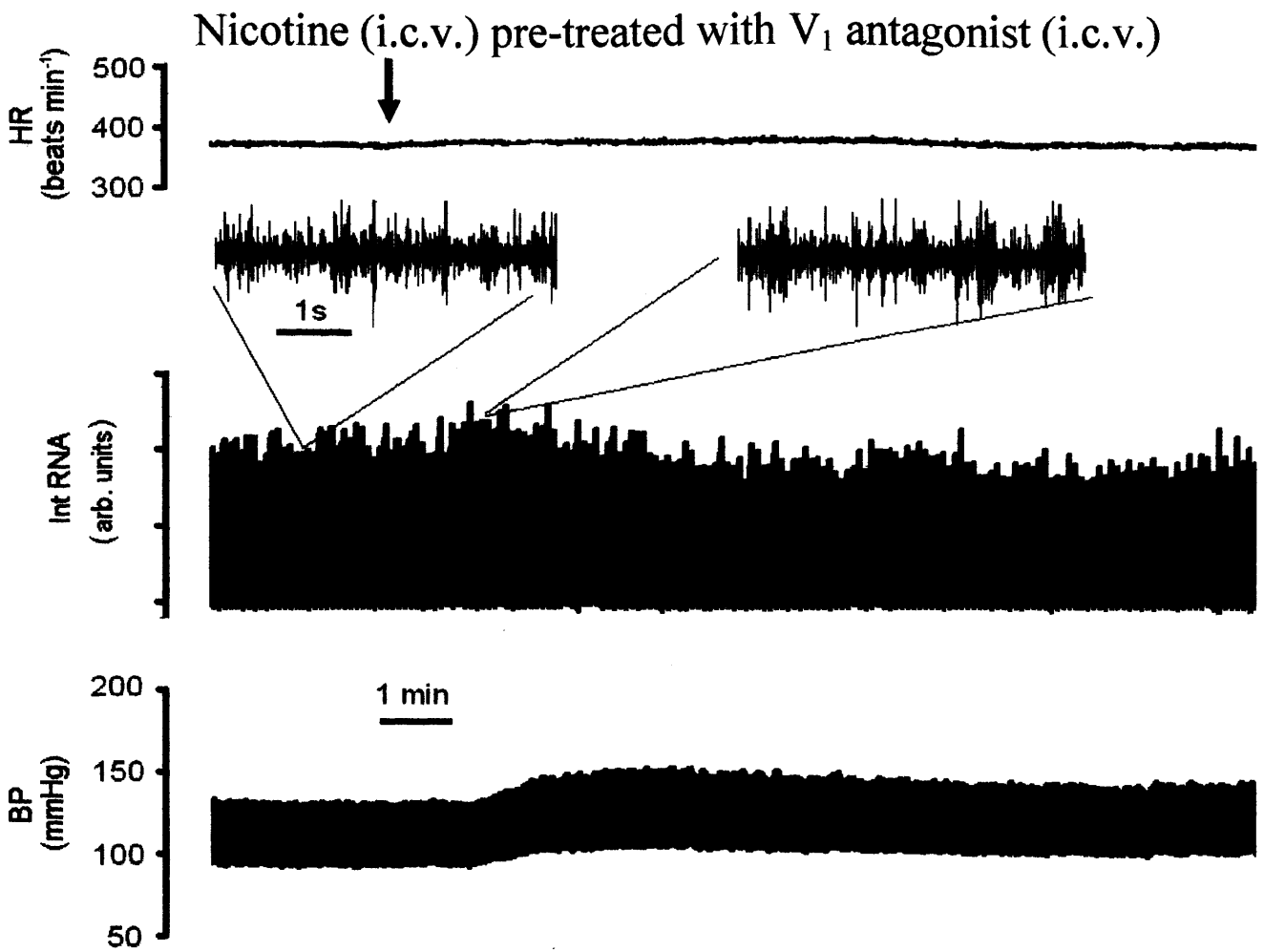


Figure 62. Trace showing the effect of nicotine (0.3 μmol kg^{-1}, i.c.v.) in the presence of V_1 receptor antagonist (0.03 μg kg^{-1}, i.c.v.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 63. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.v.) and nicotine alone (0.3 μmol kg^{-1}, i.c.v.) and control (V_1 anta & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by nicotine (#) pre-treated with V_1 antagonist compared with nicotine alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ and ***, ### $P<0.001$.

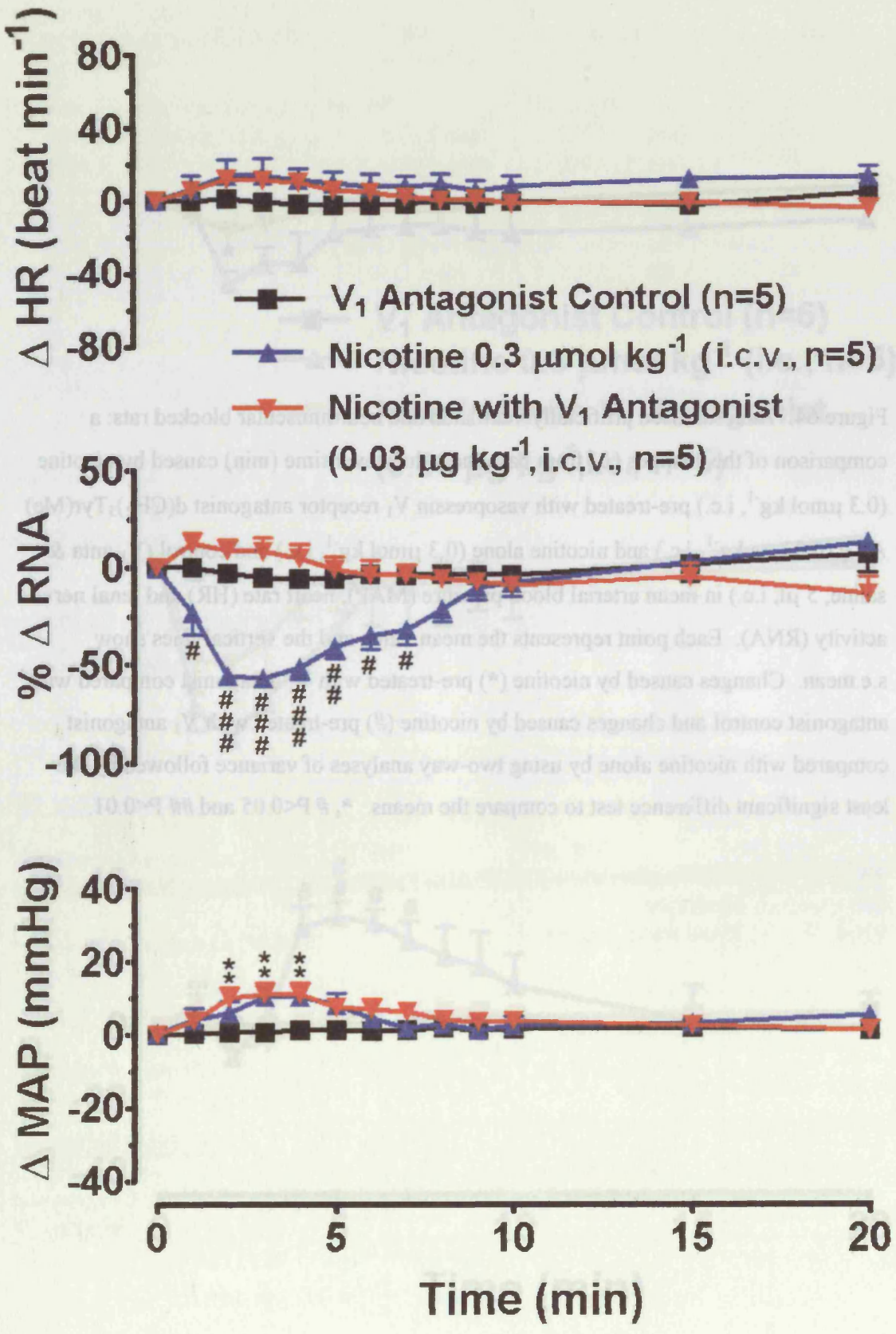

Δ HR (beat min-1)
80
40
0
-40
-80
V1 Antagonist Control (n=5)
Nicotine 0.3 μmol kg-1 (i.c.v., n=5)
Nicotine with V1 Antagonist
(0.03 μg kg-1, i.c.v., n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
-40
0
5
10
15
20
Time (min)

Figure 64. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1}, i.c.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (0.03 μg kg^{-1}, i.c.) and nicotine alone (0.3 μmol kg^{-1}, i.c.) and control (V_1 anta & saline, 5 μl, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine (*) pre-treated with V_1 antagonist compared with antagonist control and changes caused by nicotine (#) pre-treated with V_1 antagonist compared with nicotine alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$ and ## $P<0.01$.

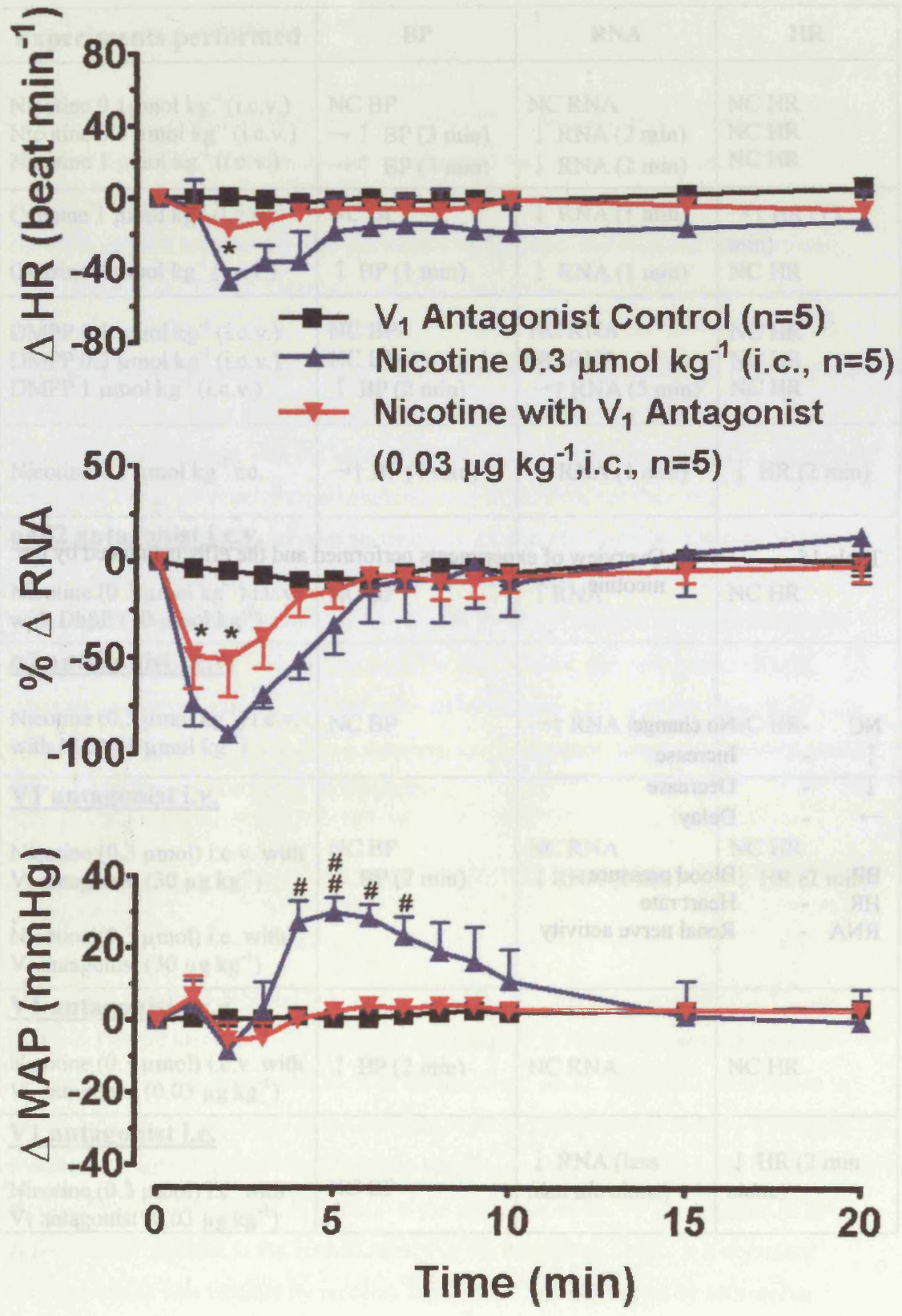

Δ HR (beat min-1)
80
40
0
-40
-80
V1 Antagonist Control (n=5)
Nicotine 0.3 μmol kg-1 (i.c., n=5)
Nicotine with V1 Antagonist
(0.03 μg kg-1,i.c., n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
-40
0
5
10
15
20
Time (min)

Table 15. Overview of experiments performed and the effects induced by the nicotine.

NC - No change
↑ - Increase
↓ - Decrease
→ - Delay

BP - Blood pressure
HR - Heart rate
RNA - Renal nerve activity

Experiments performed	BP	RNA	HR
Nicotine 0.1μmol kg^{-1} (i.c.v.) Nicotine 0.3 μmol kg^{-1} (i.c.v.) Nicotine 1 μmol kg^{-1} (i.c.v.)	NC BP → ↑ BP (3 min) → ↑ BP (3 min)	NC RNA ↓ RNA (2 min) ↓ RNA (2 min)	NC HR NC HR NC HR
Cytisine 1 μmol kg^{-1} (i.c.v.) Cytisine 3 μmol kg^{-1} (i.c.v.)	NC BP ↑ BP (1 min)	↓ RNA (1 min) ↓ RNA (1 min)	→↑ HR (15 min) NC HR
DMPP 0.1 μmol kg^{-1} (i.c.v.) DMPP 0.3 μmol kg^{-1} (i.c.v.) DMPP 1 μmol kg^{-1} (i.c.v.)	NC BP NC BP ↑ BP (2 min)	NC RNA NC RNA →↑ RNA (5 min)	NC HR NC HR NC HR
Nicotine 0.3 μmol kg^{-1} i.c.	→↑ BP (4 min)	↓ RNA (1 min)	↓ HR (2 min)
α4β2 antagonist i.c.v. Nicotine (0.3 μmol kg^{-1}) i.c.v. with DhβE (10 μmol kg^{-1})	NC BP	↑ RNA	NC HR
α7 antagonist i.c.v. Nicotine (0.3 μmol kg^{-1}) i.c.v. with MLA (1 μmol kg^{-1})	NC BP	→↑ RNA (4 min)	NC HR
V1 antagonist i.v. Nicotine (0.3 μmol) i.c.v. with V_1 antagonist (30 μg kg^{-1}) Nicotine (0.3 μmol) i.c. with V_1 antagonist (30 μg kg^{-1})	NC BP ↓ BP (2 min)	NC RNA ↓ RNA (2 min)	NC HR ↓ HR (2 min)
V1 antagonist i.c.v. Nicotine (0.3 μmol) i.c.v. with V_1 antagonist (0.03 μg kg^{-1})	↑ BP (2 min)	NC RNA	NC HR
V1 antagonist i.c. Nicotine (0.3 μmol) i.c. with V_1 antagonist (0.03 μg kg^{-1})	NC BP	↓ RNA (less than nic alone)	↓ HR (2 min alone)

3.3.3. Discussion.

Nicotine i.c.v. caused a dose-related increase in blood pressure as previously observed by Bissett & Chowdry, (1984) and a decrease in renal nerve activity associated with no change in heart rate. Interestingly, in conscious rats, i.c.v. injections of nicotine were shown to cause a pressor response associated with a consistent bradycardia (Hoffman, 1979; Iitake *et al.*, 1980). Given i.c., nicotine also caused a bradycardia, interestingly; the onset of renal sympathoinhibition was immediate while the rise in blood pressure was again delayed but much larger. This was different from that of activation of α7 and α4β2 nACh receptors, in that there was no delayed sympathoexcitation (see Chapters 3.1). However, pre-treatment with the vasopressin V_1 receptor antagonist (i.v., i.c.v. & i.c.) did attenuate / block the renal sympathoinhibition as it had done for the renal sympathoexcitation observed with the selective α7 and α4β2 nAChR agonists, although the V_1 receptor antagonist now had inconsistent effects on nicotine-induced blood pressure responses. The V_1 receptor antagonist given i.v. blocked the pressor response caused by nicotine i.c.v. and i.c., although for the i.c. there was now a fall in blood pressure. However, the rise in blood pressure by nicotine i.c.v. was not blocked by i.c.v. V_1 receptor antagonist, while for i.c. nicotine, i.c. V_1 receptor antagonist pre-treatment, surprisingly blocked the fall in blood pressure.

Sites of Action.

The delayed increase in blood pressure induced by nicotine i.c.v. suggests that the site of action is located away from the forebrain and possibly in brain areas in the hindbrain. Interestingly, nicotine administered into the hindbrain via i.c. injections were shown to induce a biphasic response in blood pressure, with an initial fall followed by a increase which was greater than the blood pressure increase caused by nicotine i.c.v. Studies have recorded that administration of nicotine at the hindbrain, either by i.c. injection or by application of nicotine to the ventral surface of the brainstem, results in a depressor response which was blocked by nicotinic antagonists, but unaffected by muscarinic

antagonist, atropine (Bisset *et al.*, 1975; Bisset & Chowdrey, 1984; Feldberg & Guertzenstein, 1976). Additional studies have shown that nicotine microinjected into the NTS caused a dose-dependent decrease in blood pressure with rapid onset immediately after microinjection (Dhar *et al.*, 2000; Ferreira *et al.*, 2000), therefore it is possible that nicotine i.c. is acting on nAChRs found on the NTS, inducing the initial decrease in blood pressure, possibly via a relay to the CVLM decreasing renal nerve activity. However, the initial decrease in blood pressure is not significant and instead an increase in blood pressure is observed suggesting that the initial decrease in blood pressure is overcome by a increase in blood pressure, possibly mediated by a pressor hormone such as vasopressin. Further studies demonstrated that the increase in blood pressure caused by i.c. nicotine was due to vasopressin release, by inhibition from V_1 antagonist i.v. and i.c. When in the presence of the V_1 antagonist i.v., the initial i.c. nicotine-induced decrease in blood pressure is potentiated, supporting the theory that the initial decrease is overcome by the release of vasopressin into the circulation resulting in a increase in blood pressure. Surprisingly, the fall in blood pressure is blocked when in the presence of the V_1 antagonist centrally. The mechanism of action is currently unknown; however it may be due to the involvement of a central vasopressin pathway.

Interestingly, nicotine i.c.v. in the presence of V_1 antagonist i.c.v. still caused an increase in blood pressure. However, although nicotine is in the presence of a central V_1 antagonist, there is at least 2 min until a significant increase in blood pressure is observed suggesting that nicotine may be acting at an additional site such as the hindbrain, causing a large release of vasopressin (Bisset *et al.*, 1975) which may account for the delayed increase in blood pressure. In addition, the V_1 antagonist may not have completely blocked the V_1 receptors in the hindbrain when injected i.c.v. therefore nicotine was still able to cause an increase in blood pressure. However, it is possible that nicotine i.c.v. is causing the release of vasopressin into the circulation by acting on the PVN directly resulting in the increase in blood pressure, although studies have shown that the release of vasopressin would be low (Bisset *et al.*, 1975), which may also explain the delayed increase in blood pressure. This release of vasopressin was believed to be due to nicotine acting directly on the SON, due to demonstrations that the SON contains neuronal

nAChRs (Hatton & Yang, 2002; Zaninetti *et al.*, 2000) and direct application of nicotine causes an increase in the rate of firing on the supraopticohypophysial tract (Castro de & Rocha E Silva, 1977), as well as, nicotine microinjection into the SON resulting in the increase in vasopressin release (Ota *et al.*, 1992).

Figure 65 suggests possible mechanisms of action for the i.c.v. nicotine-induced increase in blood pressure. 1) involves a direct effect of nicotine on the PVN; resulting in the release of vasopressin into the circulation evoking an increase in blood pressure; 2) nicotine moves from the i.c.v. injection site to sites in the hindbrain, possibly the RVLM, binding to nAChR, stimulating a projection to the PVN which results in the release of vasopressin and the increase in blood pressure. If nicotine i.c.v. induced the release of vasopressin by acting on the hindbrain then it is likely that the release of vasopressin would be caused by a pathway from the hindbrain to the PVN (see Chapter 3.2), therefore the central administration of V_1 antagonist would inhibit this pathway and block the release of vasopressin. However, this was not observed and instead suggests that the release of vasopressin is caused by nicotine acting directly on the PVN. Figure 66 shows a diagrammatic representation of the mechanism of action for nicotine injected i.c. Nicotine comes into contact with cardiovascular regulating areas such as the RVLM and NTS which stimulates the neuron projection to the PVN resulting in a release of vasopressin in to the circulation and an increase in blood pressure. The presence of this pathway is indicated by inhibition by a V_1 antagonist i.c. blocking the pathway to the PVN and preventing the release of vasopressin and increase in blood pressure.

Interestingly, the α7 and α4β2 selective agonists did not cause the release of vasopressin by acting on the PVN directly and instead acted on the hindbrain to evoke its effects (see Chapter 3.2). In this respect, nicotine is not acting on the α7 and α4β2 nAChR in the PVN to induce the release of vasopressin and instead may be acting on an additional nACh receptor subunit found on the PVN, stimulating the release of vasopressin.

Renal sympathinhibition.

Interestingly, due to the rapid nature of the sympathoinhibition, it is suggested that nicotine's site of action is near the i.c.v. injection site. One possible site of action is the hypothalamus which is positioned very closely to the i.c.v. injection site and is believed to contain additional nAChRs to the α4β2 and α7 receptors such as β4-containing nAChRs (Dineley-Miller & Patrick, 1992; Winzer-Serhan & Leslie, 1997). However, although the sympathoinhibition caused by nicotine i.c.v. is fast, the sympathoinhibition response caused by nicotine i.c. is faster suggesting that the areas in the hindbrain are involved in nicotine-induced sympathoinhibition. Further, it may be possible that both the forebrain and hindbrain sites are involved.

A possible mechanism of action is the effect of vasopressin on the area postrema (AP), which has been noted as being a circumventricular organ, contains V1a receptors and shown to be innervated by cardiovascular regulating brain areas such as the NTS, parabrachial nucleus and PVN (see Ferguson, 1991; see Hasser *et al.*, 2000; Hasser & Bishop, 1990; see Johnson & Gross, 1993). Studies have shown that microinjection of vasopressin into the AP cause a decrease in renal nerve activity (see Hasser *et al.*, 2000; Hasser & Bishop, 1990). However the decrease in renal nerve activity observed by central administration of nicotine is not associated with the vasopressin-induced increase in blood pressure evoked by nicotine; therefore suggesting the decrease in renal nerve activity caused by nicotine is independent of vasopressin release. Therefore a possible mechanism of action is the direct affect of nicotine on the CVLM. Previous studies have shown that nicotine microinjected into the CVLM caused a dose-related decrease in blood pressure and heart rate (Aberger *et al.*, 2001) which was suggested to be due to inhibition of the RVLM via GABAnergic neurons form the CVLM, thus resulting in a decrease in sympathetic nerve activity to the heart and blood vessels (Agarwal *et al.*, 1990; Brown & Guyenet, 1984; Cravo *et al.*, 1991; Gordon, 1987). This would account for the rapid decrease in renal sympathetic nerve activity and heart rate upon nicotine administered i.c., due to the i.c. injection site being located close to the CVLM and the delayed decrease in RSNA evoked by nicotine i.c.v. However, the i.c.v. nicotine evoked

renal sympathoinhibition is too fast to be leaking across the brain to evoke an effect, therefore it is suggested that the decrease is caused by forebrain areas such as the PVN innervating cardiovascular regulating hindbrain areas such as the CVLM, NTS and RVLM (see Dampney, 1994; see Sun, 1995). Furthermore Yang & Coote (1998) demonstrated that activation of neurons at the PVN may cause a depressor response via inhibiting the RVLM neurons, therefore it is possible that via a pathway for the PVN to the RVLM nicotine is able to cause a decrease in renal sympathetic nerve activity

Interestingly the nicotine-evoked renal sympathoinhibition is inhibited and attenuated by central V1a receptor antagonist suggesting the involvement of vasopressin centrally possibly acting as a neurotransmitter (Yang *et al.*, 2001) at the synapses between cardiovascular regulating centres.

Nicotine in the presence of selective antagonists.

The present data using antagonists also indicate that nicotine can activate a α4β2 receptor which mediates sympathoinhibition, however this is not observed using the respective selective agonists (see Chapter 3.1). In fact, in the presence of the antagonist DhβE, the renal sympathoinhibition was reversed to a sympathoexcitation associated with no observed changes in blood pressure and heart rate. A possible explanation of this contradiction is that DhβE, at the concentration used, is also blocking another receptor. In this respect it does have a reasonable affinity for the β2 and β4 subunit (Chavez-Noriega *et al.*, 1997; 2000) and receptor/s containing these subunits could mediate this renal sympathoinhibition. In this respect cytisine, which has selective tendencies at neuronal nACh receptors containing the β4 subunit (Alkondon & Albuquerque, 1993; Chavez-Noriega *et al.*, 1997; see Colquhoun & Patrick, 1997)(see section 3.3.1.), was also found to cause profound renal sympathoinhibition and surprisingly this was associated with no change in heart rate although the expected pressor response was still observed. The selectivity of cytisine for nAChR subtypes has been shown *in vitro* to have a low efficacy for nAChRs containing the β2 subunits compared to other agonists such as DMPP and

ACh, but displayed high affinity for α2β4, α3β4 and α4β4 nAChR's (Chavez-Noriega *et al.*, 1997; 2000), indicating that cytisine is selective for the β4-containing nAChR subtypes. Therefore, due to the cardiovascular similarities induced by nicotine and cytisine, it is possible that nicotine is acting on a β4-containing nAChR subtype. However, the receptor involved in the nicotine i.c.v. evoked renal sympathoexcitatory response remains to be determined. Furthermore, in the presence of α7 selective antagonist, MLA, the nicotine-evoked renal sympathoinhibition is not blocked only delayed, although the blood pressure response is blocked. The mechanism for delay is unclear, but presumably it is due to the balance of action of different nAChR which have opposing actions on renal nerve activity. However the ability of both antagonists to block the pressor response remains difficult to explain, although a possible mechanism could be that of blocking one receptor unmasking another's effect, although in the opposite direction i.e. the receptors mediating the fall in pressure observed with TC-2559 at the highest dose. In addition, the nicotinic receptor agonist DMPP, which has been shown to be selective for α2β2, α3β2 and α3β4 nAChR (Chavez-Noriega *et al.*, 1997; Luetje & Patrick, 1991) evoked an increase in blood pressure and renal nerve activity, with no change in heart rate suggesting that a number of neuronal nicotinic receptors upon activation are able to cause an increase in blood pressure and renal nerve activity, most likely via a vasopressinergic pathway and the release of vasopressin.

In conclusion, central administration of nicotine induces an increase in blood pressure via the release of vasopressin in to the circulation, which involves a central pathway for nicotine injected into the hindbrain, and may be involved for nicotine i.c.v. However, nicotine injected into the forebrain may be having a direct effect on the PVN, possibly by acting on β4-containing nAChRs, resulting in the release of vasopressin. Nicotine evoked decrease in renal nerve activity is independent of vasopressin release, making the mechanism of action unknown. Again, the similarities in cardiovascular reponses to β4 selective agonist cytisine suggest that nicotine may be acting on β4-containing subunits to induce the renal sympathoinhibition.

Figure 65. Diagrammatic representation of the pathways involved upon i.c.v. injection of nicotine in the rat brain.

1) Nicotine is administered into the brain via i.c.v. injections.
2) Nicotine comes directly into contact with the PVN and stimulates the release of vasopressin from the posterior pituitary.
3) Vasopressin release causes the increase in blood pressure by acting on V_{1a} receptors, found on blood vessels, resulting in vasoconstriction.
4) An additional mechanism of action involves nicotine not acting directly on the PVN, instead moving through the brain towards the hindbrain.
5) Nicotine binds to nAChRs found on the RVLM and NTS.
6) The RVLM stimulates the PVN via a neuronal projection which results in the release of vasopressin via points 2) and 3).

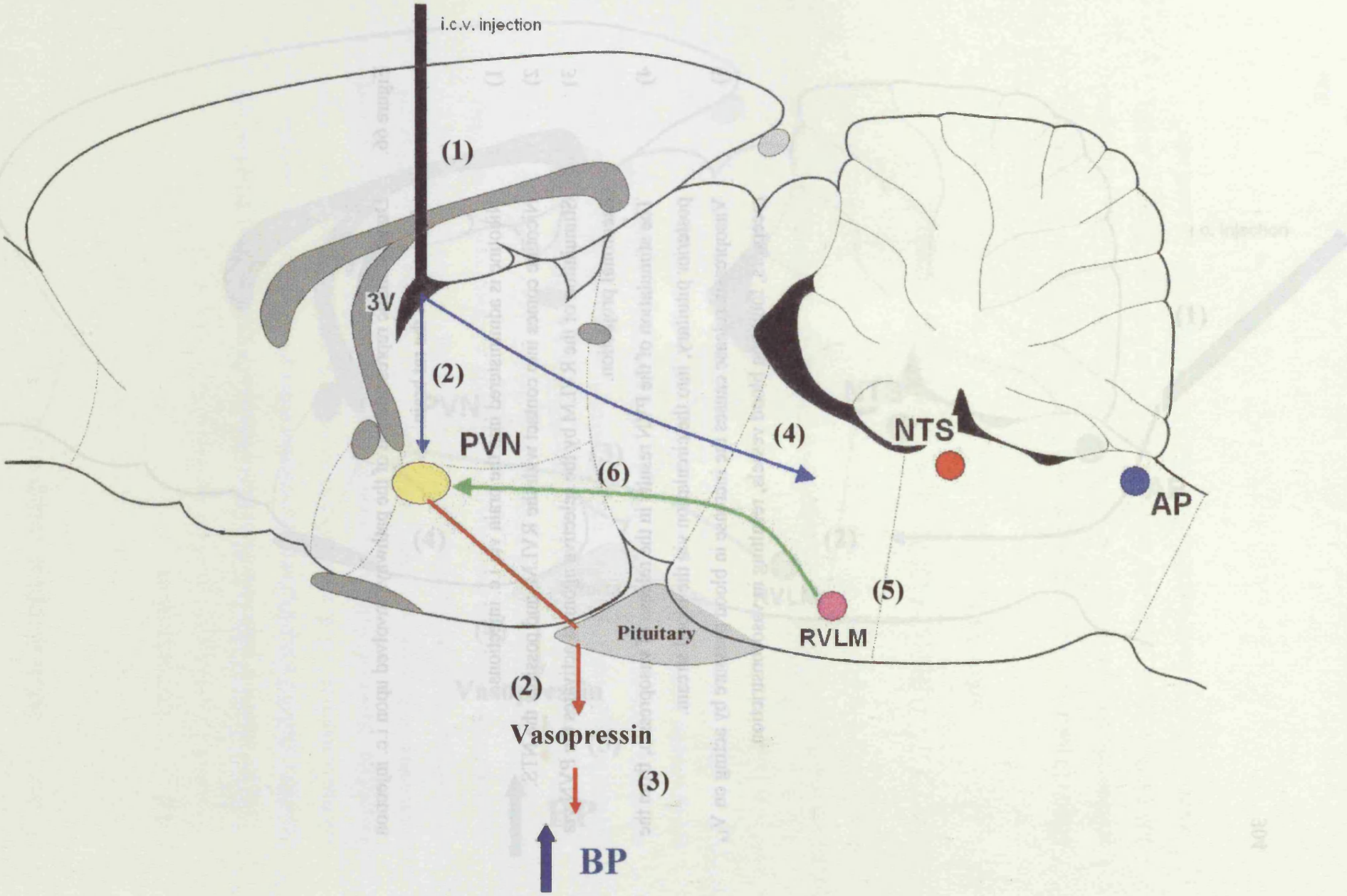
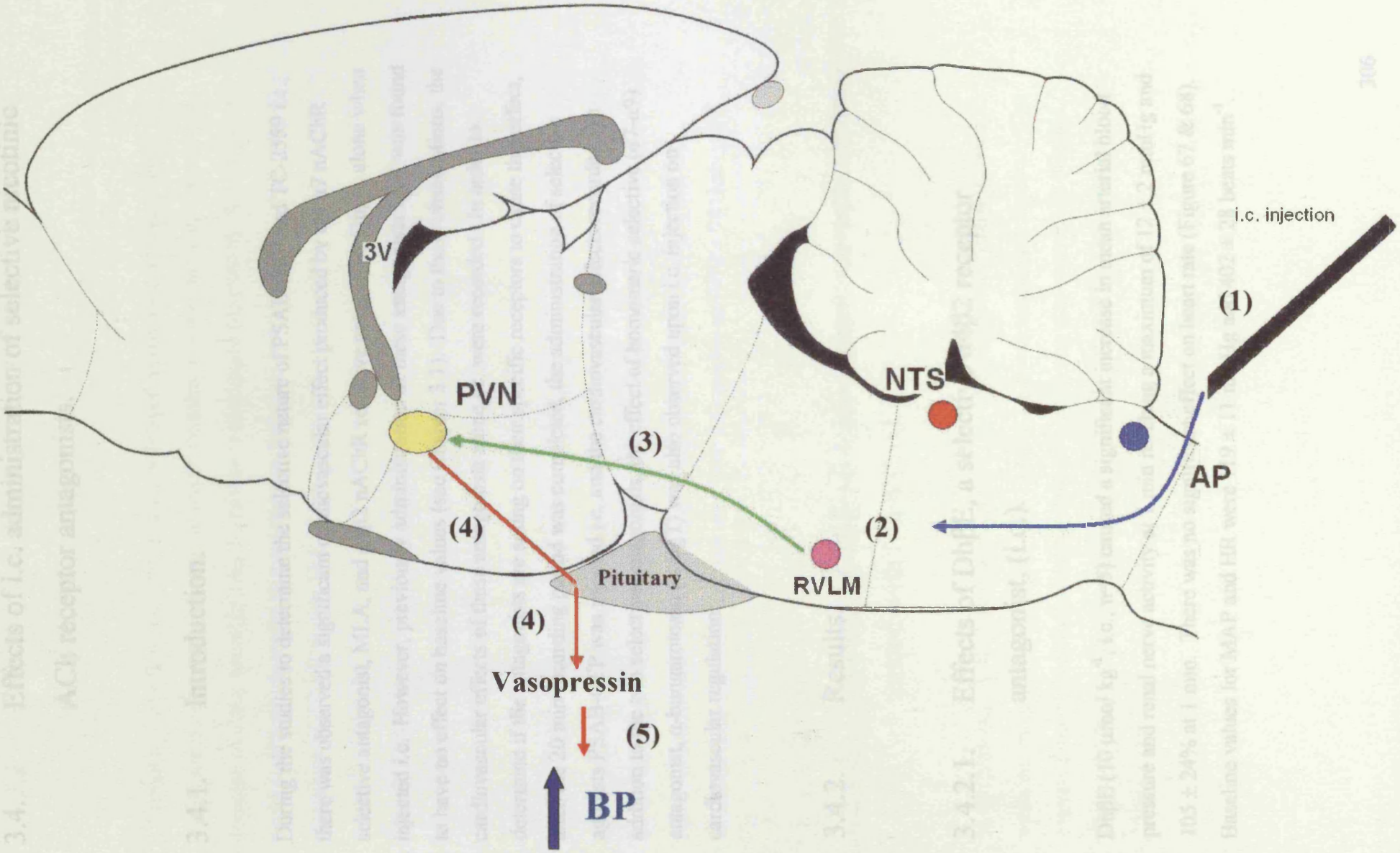

i.c.v. injection
(1)
3V
(2)
PVN
(4)
NTS
(6)
AP
(5)
Pituitary
RVLM
(2)
Vasopressin
(3)
BP

Figure 66. Diagrammatic representation of the pathways involved upon i.c. injection of nicotine in the rat brain.

1) Nicotine is administered into the brain via i.c. injections.
2) Nicotine comes into contact with the RVLM, and possibly the NTS.
3) Stimulation of the RVLM by the selective agonists activates the PVN via aneuronal projection.
4) The stimulation of the PVN results in the release of vasopressin, from the posterior pituitary, into the circulation via the blood stream.
5) Vasopressin release causes the increase in blood pressure by acting on V_{1a} receptors, found on blood vessels, resulting in vasoconstriction.


3V
PVN
NTS
i.c. injection
(1)
AP
(2)
(3)
(4)
(4)
Pituitary
RVLM
Vasopressin
(5)
BP

3.4. Effects of i.c. administration of selective nicotinic ACh receptor antagonists.

3.4.1. Introduction.

During the studies to determine the selective nature of PSAB-OFP and TC-2559 i.c., there was observed a significant cardiovascular effect produced by the α7 nAChR selective antagonist, MLA, and α4β2 nAChR selective antagonist, DhβE, alone when injected i.c. However, previously administration of these antagonists i.c.v. was found to have no effect on baseline values (see Chapter 3.1). Due to these observations, the cardiovascular effects of these antagonists alone i.c. were recorded. In order to determine if the antagonists are acting on their specific receptors to cause this effect, after the 20 min recording period was completed, the administration of selective agonists PSAB-OFP was injected i.c. and the cardiovascular effects recorded. In addition to the two selective antagonists, the effect of homomeric selective (α7-α9) antagonist, α-bungarotoxin (α-BgT) was also observed upon i.c. injection on cardiovascular regulation.

3.4.2. Results.

3.4.2.1. Effects of DhβE, a selective α4β2 receptor antagonist, (i.c.).

DhβE (10 μmol kg^{-1}, i.c., n=5) caused a significant increase in mean arterial blood pressure and renal nerve activity at 1 min reaching a maximum of 12 ± 2 mmHg and 105 ± 24% at 1 min. There was no significant effect on heart rate (Figure 67 & 68). Baseline values for MAP and HR were 119 ± 11 mmHg and 402 ± 28 beats min^{-1}.

3.4.2.2. Effects of MLA, a selective α7 receptor antagonist, (i.c.).

0.1 μmol kg^{-1} of MLA (i.c., n=5) caused a delayed depressor response with a significant decrease in mean arterial blood pressure at 8 min reaching a maximum decrease of -15 ± 4 mmHg after 10 min and returning to baseline by 20 min. There was a significant decrease in renal nerve activity and heart rate after 2 min reaching a maximum of -58 ± 9% and -58 ± 13 beats min^{-1} at 15 min (Figure 69 & 70). Baseline values for MAP and HR were 117 ± 17 mmHg and 413 ± 30 beats min^{-1}. The next dose (0.5 μmol kg^{-1}, n=5) now caused a delayed, after 3 min, significant increase in blood pressure and renal nerve activity, reaching a maximum of 41 ± 8 mmHg and 110 ± 43% at 5 min. Although heart rate tended to decline, it was not significant (Figure 71 & 72). Baseline values for MAP and HR were 94 ± 18 mmHg and 365 ± 46 beats min^{-1}. The highest dose of MLA (1 μmol kg-1, n=5) also caused a significant and similar increase in blood pressure after 3 min reaching a maximum of 37 ± 9 mmHg at 6 min. However, although renal nerve activity increased it was much more variable and was not significant. Heart rate was unaffected (Figure 73). Baseline values for MAP and HR were 115 ± 33 mmHg and 412 ± 78 beats min^{-1}.

3.4.2.3. Effects of PSAB-OFP (α7 receptor agonist) i.c. pre-treated with MLA (i.c.).

In the presence of MLA (0.5 μmol kg^{-1}, i.c., n=5) the expected evoked increase in mean arterial pressure by PSAB-OFP (3 μmol kg^{-1}) was completely blocked. The expected renal sympathoexcitation and tachycardia was also completely blocked (Figure 74 & 75). Baseline values for MAP and HR were 85 ± 13 mmHg and 393 ± 35 beats min^{-1}.

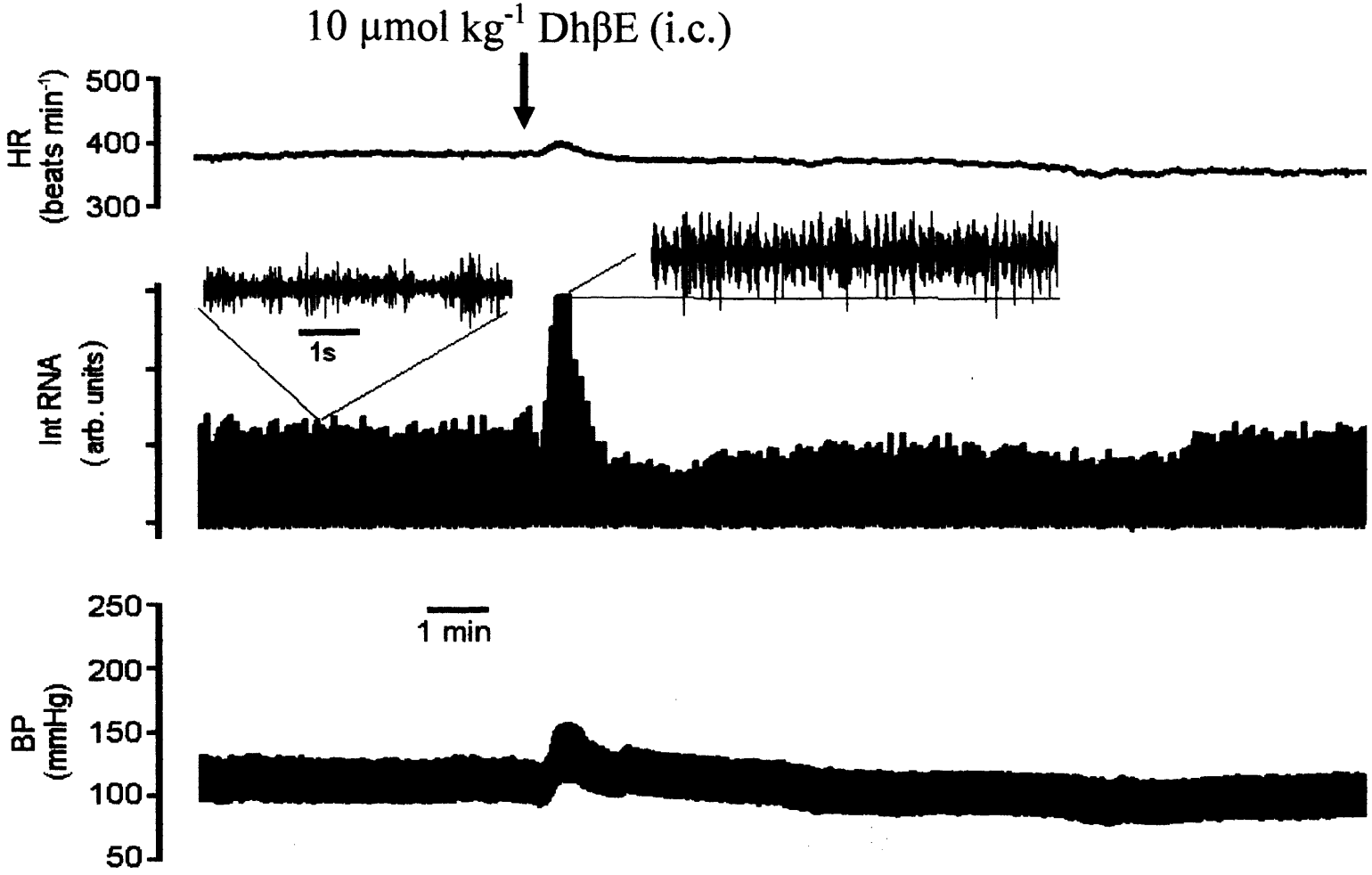
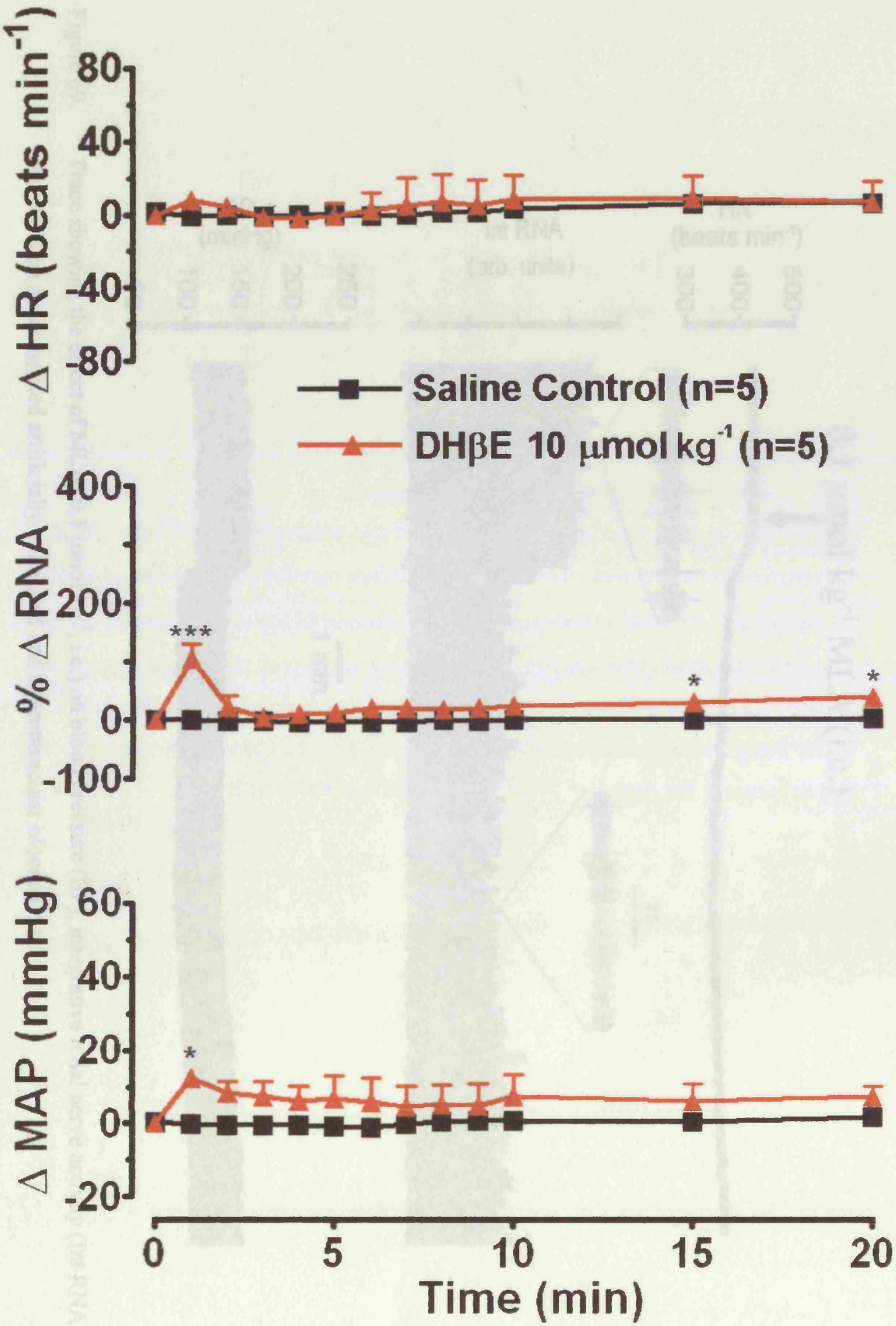


Figure 67. Trace showing the effect of DhβE (10 µmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 68. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by DhβE (10 μmol kg^{-1}, i.c.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by DhβE i.c. (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$ and *** $P<0.001$.


Δ HR (beats min-1)
80
40
0
-40
-80
Saline Control (n=5)
DHβE 10 μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
***
*
*
Δ MAP (mmHg)
60
40
20
0
-20
*
0
5
10
15
20
Time (min)

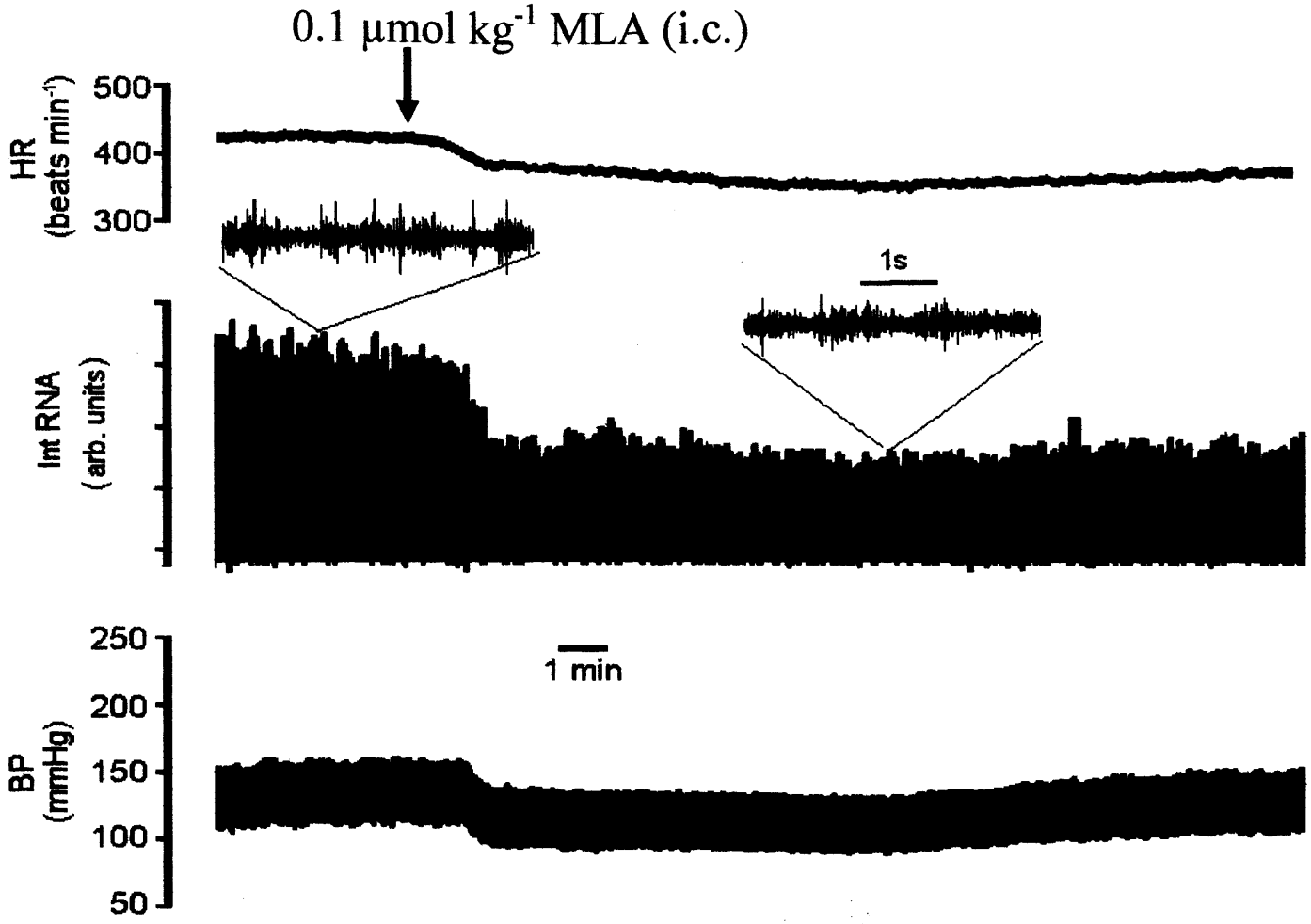
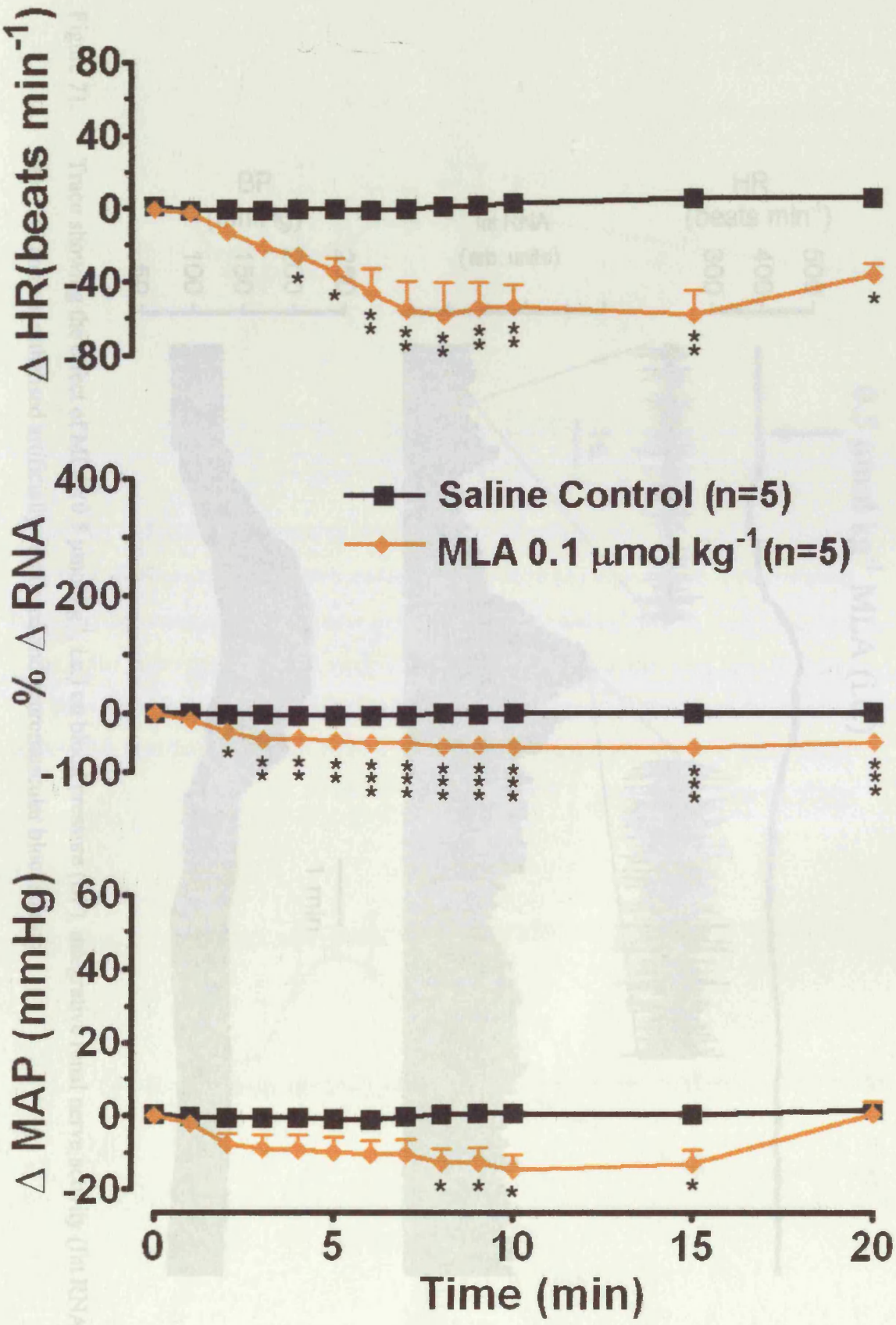


Figure 69. Trace showing the effect of MLA (0.1 µmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 70. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by MLA (0.1 μmol kg^{-1}, i.c.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by MLA i.c. (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.


Δ HR(beats min-1)
80
40
0
-40
-80
% Δ RNA
400
200
0
-100
Saline Control (n=5)
MLA 0.1 μmol kg-1 (n=5)
Δ MAP (mmHg)
60
40
20
0
-20
0
5
10
15
20
Time (min)

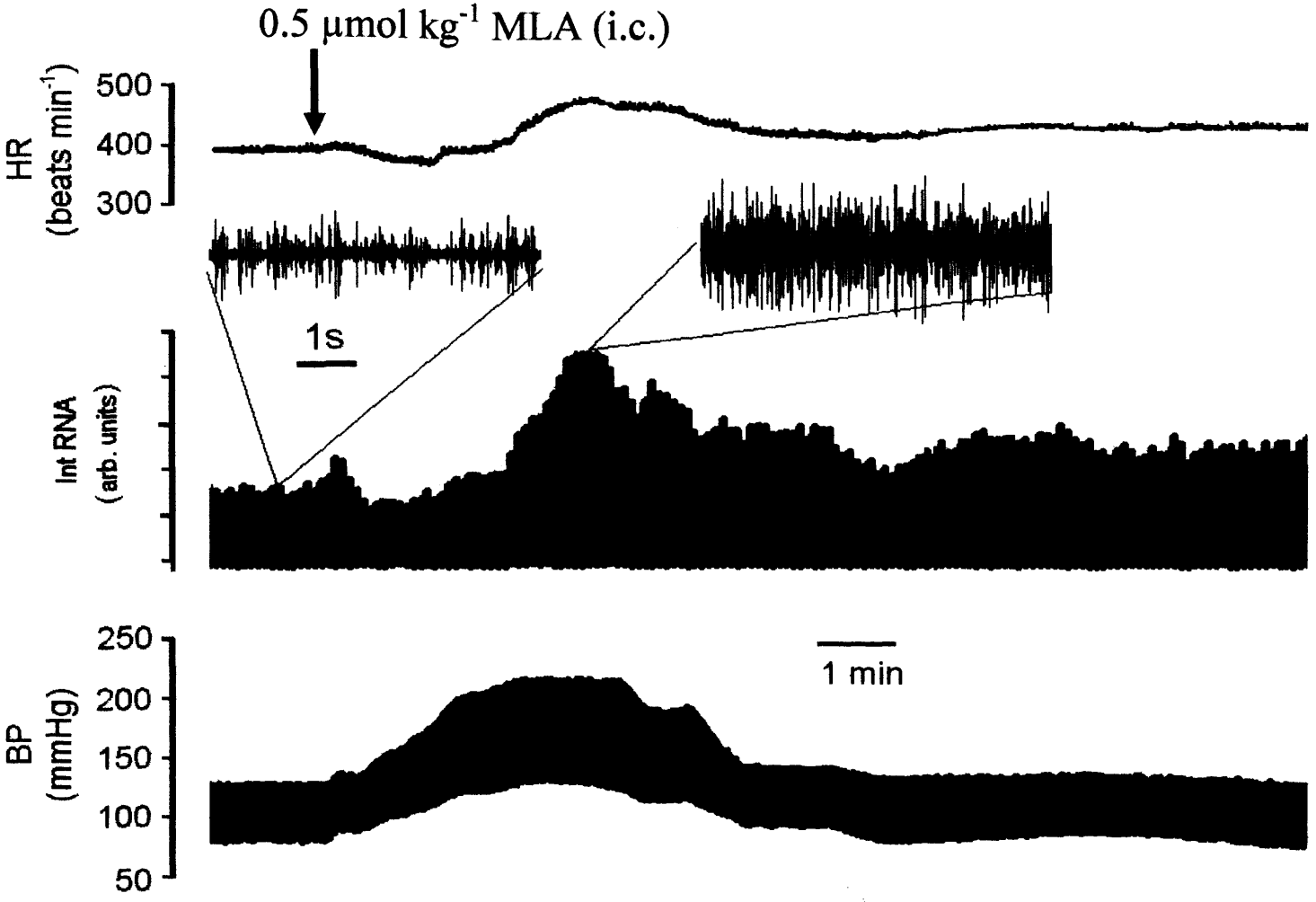


Figure 71. Trace showing the effect of MLA (0.5 µmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 72. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by MLA (0.5 μmol kg^{-1}, i.c.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by MLA i.c. (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$ and ** $P<0.01$.

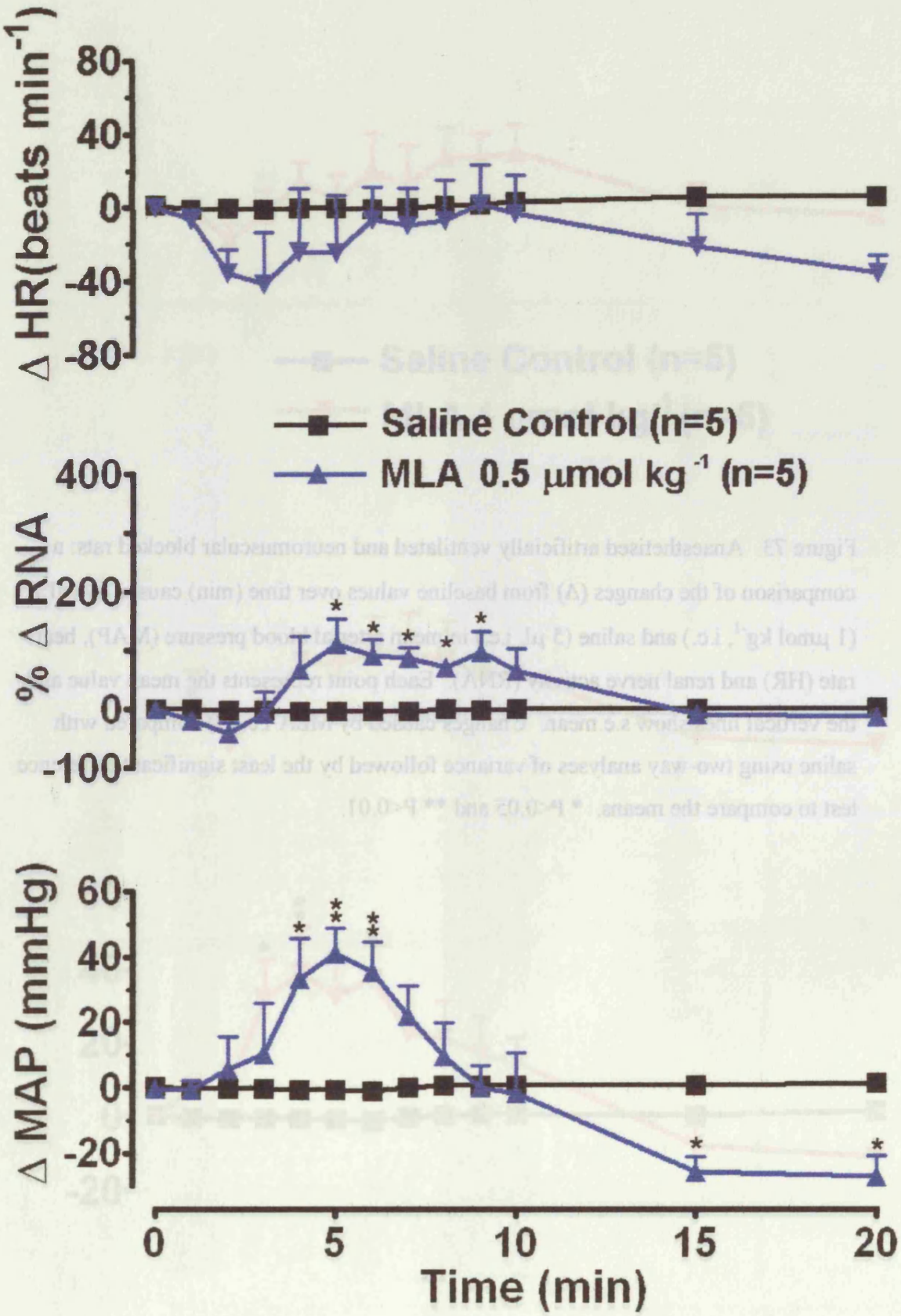
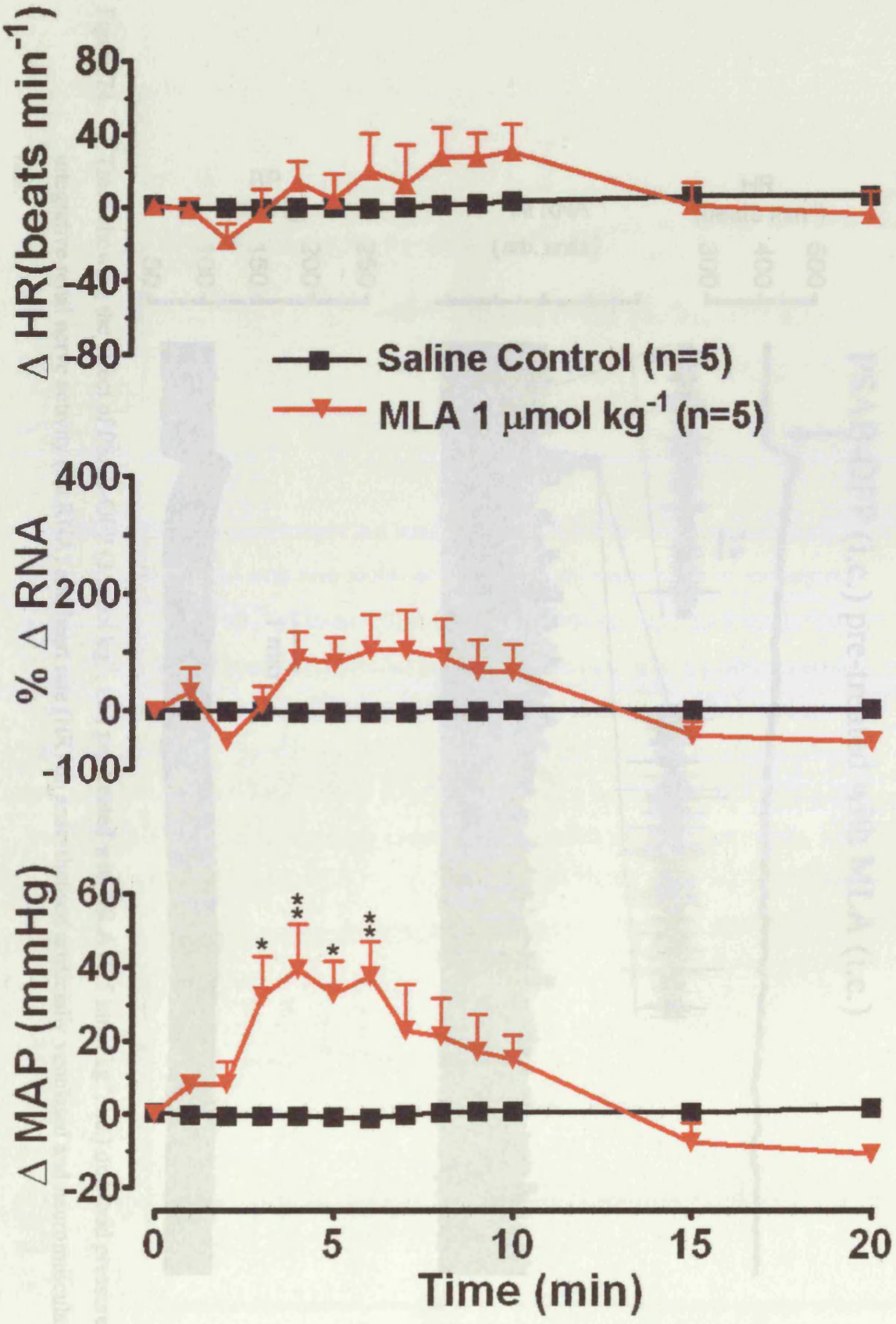

Δ HR(beats min-1)
80
40
0
-40
-80
Saline Control (n=5)
MLA 0.5 μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
60
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 73. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by MLA (1 μmol kg^{-1}, i.c.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by MLA i.c. (*) compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means. * $P<0.05$ and ** $P<0.01$.


Δ HR(beats min-1)
80
40
0
-40
-80
Saline Control (n=5)
MLA 1 μmol kg-1 (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
60
40
20
0
-20
*
**
*
**
0
5
10
15
20
Time (min)

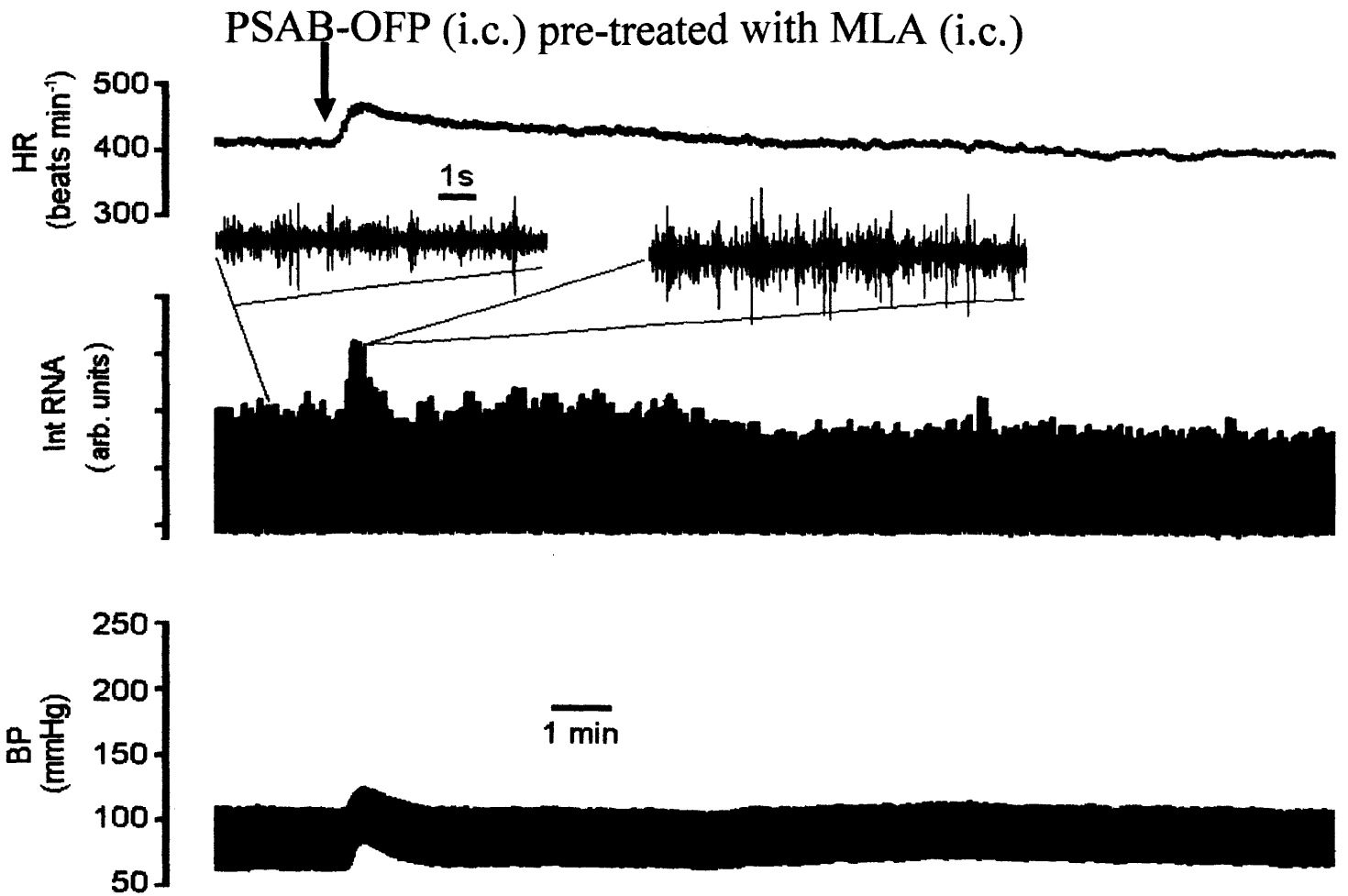
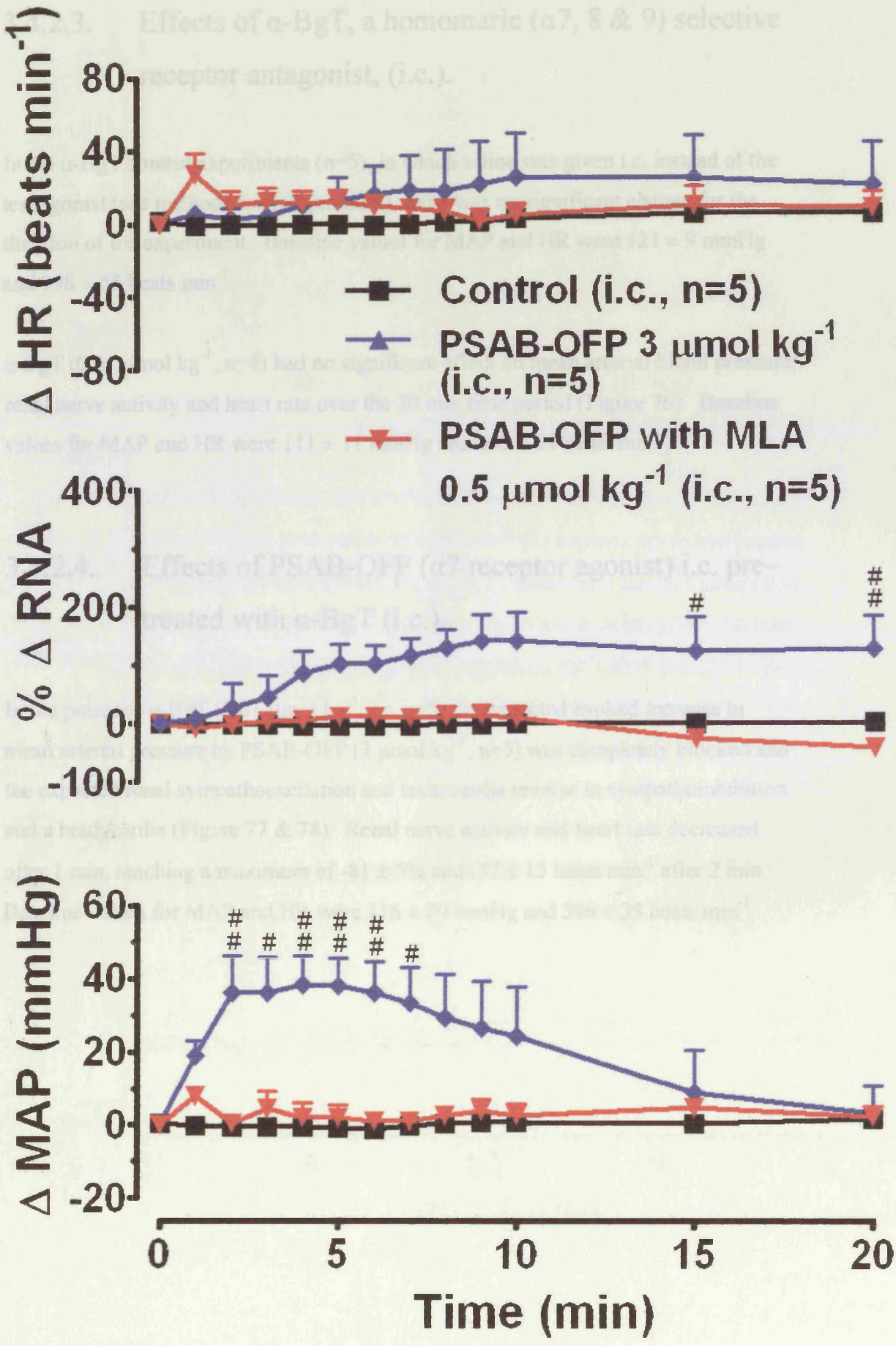


Figure 74. Trace showing the effect of PSAB-OFP (3 μmol kg^{-1}, i.c.) pre-treated with MLA (0.5 μmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats.

Figure 75. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.) pre-treated with MLA (0.5 μmol kg^{-1}, i.c.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.) and control (2 doses saline, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (#) pre-treated with MLA compared with PSAB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. # $P<0.05$ and ## $P<0.01$.


Δ HR (beats min-1)
80
40
0
-40
-80
Control (i.c., n=5)
PSAB-OFP 3 μmol kg-1 (i.c., n=5)
PSAB-OFP with MLA 0.5 μmol kg-1 (i.c., n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
60
40
20
0
-20
0
5
10
15
20
Time (min)

3.4.2.3. Effects of α-BgT, a homomeric (α7, 8 & 9) selective receptor antagonist, (i.c.).

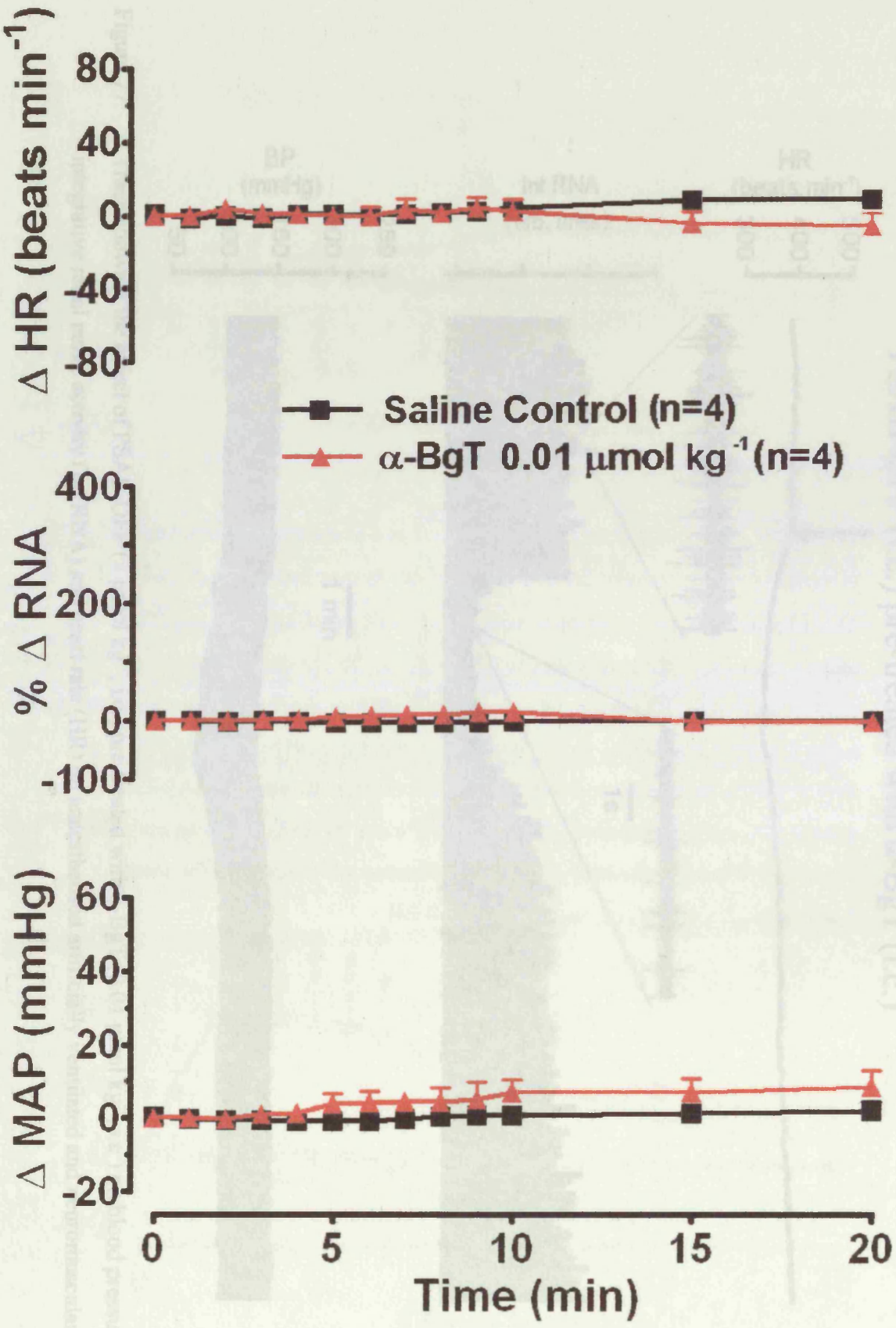
In the α-BgT control experiments (n=5), in which saline was given i.c. instead of the test agonist (see methods for full protocol), there was no significant change for the duration of the experiment. Baseline values for MAP and HR were 121 ± 9 mmHg and 398 ± 55 beats min^{-1}.

α-BgT (0.01 μmol kg^{-1}, n=4) had no significant effect on mean arterial blood pressure, renal nerve activity and heart rate over the 20 min time period (Figure 76). Baseline values for MAP and HR were 111 ± 11 mmHg and 395 ± 37 beats min^{-1}.

3.4.2.4. Effects of PSAB-OFP (α7 receptor agonist) i.c. pre-treated with α-BgT (i.c.).

In the presence α-BgT (0.01 μmol kg^{-1}, i.c. n=5) the expected evoked increase in mean arterial pressure by PSAB-OFP (3 μmol kg^{-1}, n=5) was completely blocked and the expected renal sympathoexcitation and tachycardia reverse to sympathoinhibition and a bradycardia (Figure 77 & 78). Renal nerve activity and heart rate decreased after 1 min, reaching a maximum of -81 ± 5% and -57 ± 15 beats min^{-1} after 2 min. Baseline values for MAP and HR were 116 ± 20 mmHg and 396 ± 35 beats min^{-1}.

Figure 76. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by α-BgT (0.01 μmol kg^{-1}, i.c.) and saline (5 μl. i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by α-BgT i.c. compared with saline using two-way analyses of variance followed by the least significant difference test to compare the means.


Δ HR (beats min-1)
80
40
0
-40
-80
Saline Control (n=4)
α-BgT 0.01 μmol kg-1 (n=4)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
60
40
20
0
-20
0
5
10
15
20
Time (min)

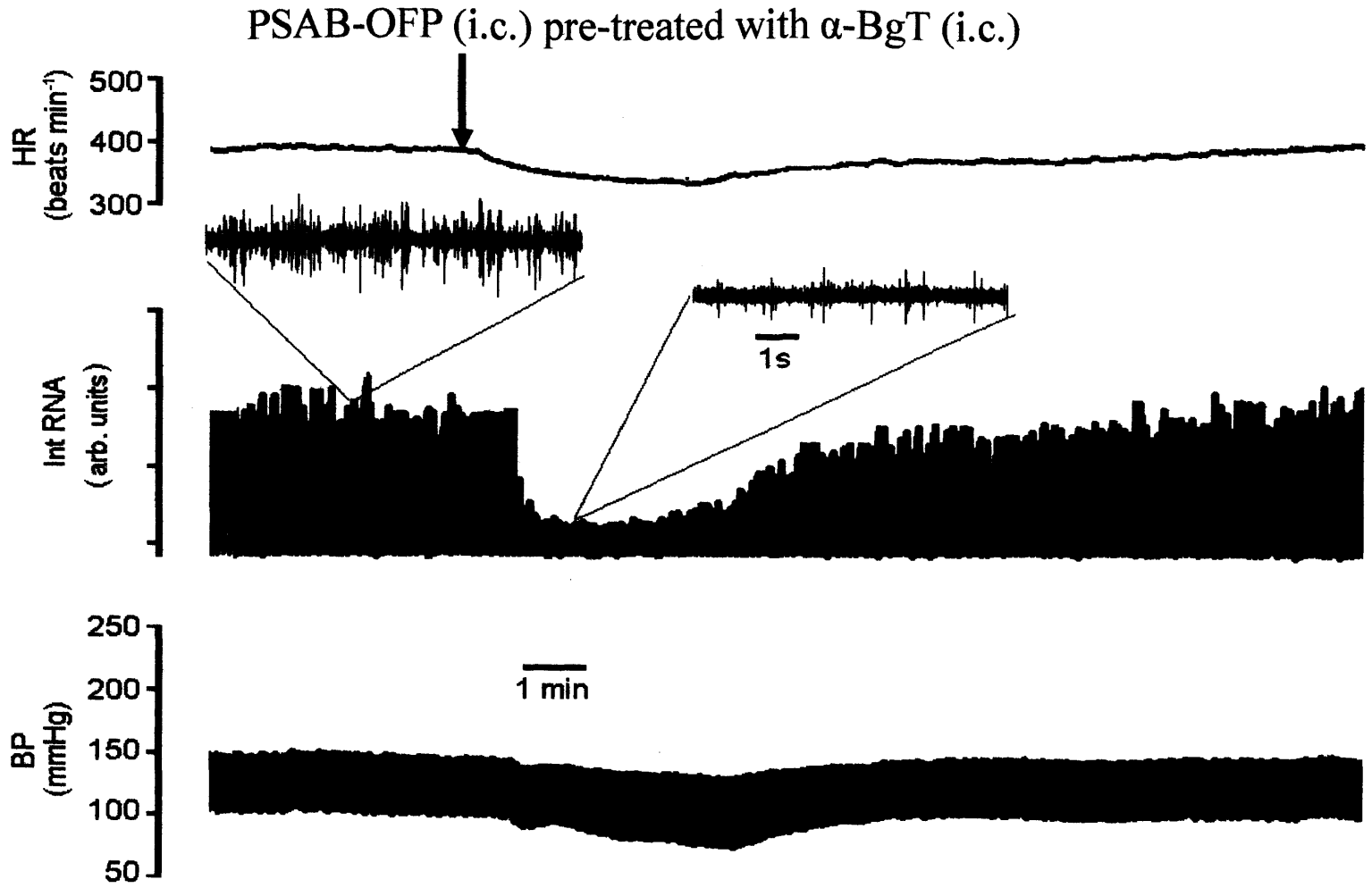
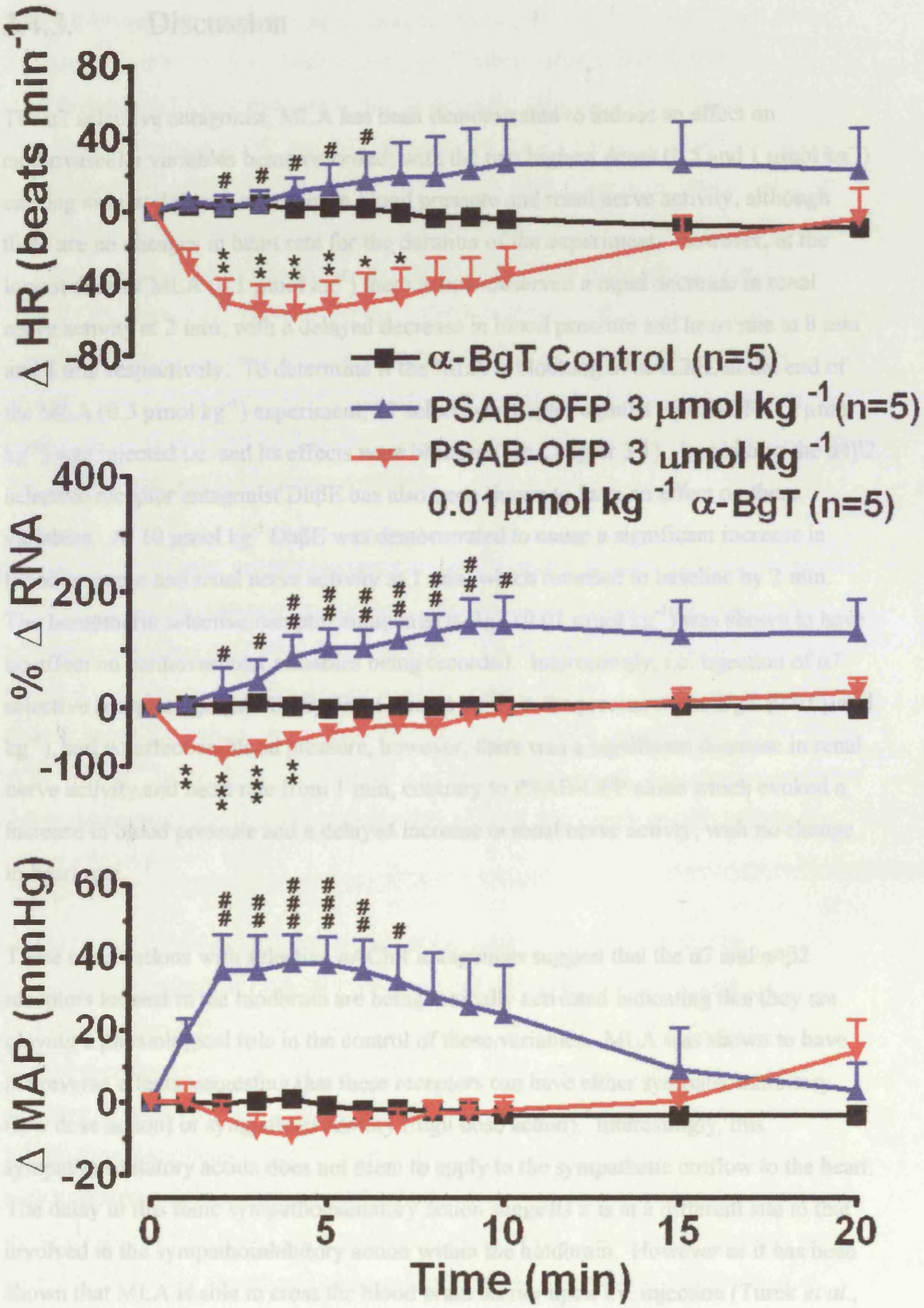


Figure 77. Trace showing the effect of PSAB-OFP (3 μmol kg^{-1}, i.c.) pre-treated with α-BgT (0.01 μmol kg^{-1}, i.c.) on blood pressure (BP), integrative renal nerve activity (Int RNA) and heart rate (HR) on anaesthetised artificially ventilated and neuromuscular blocked rats

Figure 78. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1}, i.c.) pre-treated with α-BgT (0.01 μmol kg^{-1}, i.c.) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.) and control (2 doses saline, i.c.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP (*) pre-treated with α-BgT compared with control and changes caused by PSAB-OFP (#) pre-treated with α-BgT compared with PSAB-OFP alone by using two-way analyses of variance followed by the least significant difference test to compare the means.
*, # $P<0.05$; **, ## $P<0.01$ and ***, ### $P<0.001$.


Δ HR (beats min-1)
80
40
0
-40
-80
α-BgT Control (n=5)
PSAB-OFP 3 μmol kg -1 (n=5)
PSAB-OFP 3 μmol kg -1
0.01 μmol kg -1 α-BgT (n=5)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
60
40
20
0
-20
0
5
10
15
20
Time (min)

3.4.3. Discussion

The α7 selective antagonist, MLA has been demonstrated to induce an effect on cardiovascular variables being recorded; with the two highest doses (0.5 and 1 μmol kg^{-1}) causing similar delayed increases in blood pressure and renal nerve activity, although there are no changes in heart rate for the duration of the experiment. However, at the lowest dose of MLA (0.1 μmol kg^{-1}) there is now observed a rapid decrease in renal nerve activity at 2 min, with a delayed decrease in blood pressure and heart rate at 8 min and 4 min respectively. To determine if the MLA is blocking α7 nAChR, at the end of the MLA (0.5 μmol kg^{-1}) experiment, α7 selective receptor agonist PSAB-OFP (3 μmol kg^{-1}) was injected i.c. and its effects were blocked (see Chapter 3.1). In addition the α4β2 selective receptor antagonist DhβE has also been shown to have an effect on these variables. At 10 μmol kg^{-1} DhβE was demonstrated to cause a significant increase in blood pressure and renal nerve activity at 1 min, which returned to baseline by 2 min. The homomeric selective receptor antagonist α-BgT (0.01 μmol kg^{-1}) was shown to have no effect on cardiovascular variables being recorded. Interestingly, i.c. injection of α7 selective receptor agonist PSAB-OFP (3 μmol kg^{-1}), in the presence of α-BgT (0.01 μmol kg^{-1}), had no affect on blood pressure, however, there was a significant decrease in renal nerve activity and heart rate from 1 min, contrary to PSAB-OFP alone which evoked a increase in blood pressure and a delayed increase in renal nerve activty, with no change in heart rate.

These observations with selective nAChR antagonists suggest that the α7 and α4β2 receptors located in the hindbrain are being tonically activated indicating that they are playing a physiological role in the control of these variables. MLA was shown to have the reverse effects suggesting that these receptors can have either sympathoinhibitory (low dose action) or sympathoexcitatory (high dose action). Interestingly, this sympathoexcitatory action does not seem to apply to the sympathetic outflow to the heart. The delay in this tonic sympathoexcitatory action suggests it is at a different site to that involved in the sympathoinhibitory action within the hindbrain. However as it has been shown that MLA is able to cross the blood brain barrier upon i.v. injection (Turek *et al.*,

1995), it is therefore possible that MLA is not causing these effects at the level of the brainstem but is acting peripherally, although if it is interfering with ganglionic transmission a fall blood pressure would be expected, thus this is very doubtful. MLA, although selective for the α7 nAChRs, it is not specific for the α7 nAChRs over other nAChRs subtypes (Davies *et al.*, 1999; Ward *et al.*, 1990; Yum *et al.*, 1996). MLA is 300X more selective for α7 over muscle and α4β2 nAChRs (Table 4b); however studies have shown that MLA is able to bind to non-α7 nAChRs in avian preparations (Yu & Role, 1998; Yum *et al.*, 1996). Therefore it is possible that MLA is acting on additional receptors to the α7 nAChRs causing an increase in blood pressure and renal nerve activity. Due to the inhibition of α7 selective agonist PSAB-OFP actions, the dose used can be said to be an effective dose for blocking α7 nAChRs, however this does not prove that it is not acting at other receptor subtypes.

Unlike MLA, the α4β2 selective antagonist DhβE produces a rapid transient increase in blood pressure and renal nerve activity at 1 min, however the effects are very short returning to baseline by 2 min. The speed of the DhβE-induced effect suggests that the site of action is close to the i.c. site of injection such as the NTS, which has been shown to contain α4β2 subunits (Dominguez del Toro *et al.*, 1994; Swanson *et al.*, 1987; Wada *et al.*, 1989). The rapid recovery may be due to an additional pathway compensating for the evoked changes, or that the concentration of DhβE is not high enough to keep the receptor blocked.

In addition to the above suggestions, a possible mechanism of action for the cardiovascular effects of the selective antagonists, is that the antagonists are acting on receptors similar to the nicotinic receptor, such as the 5-HT_3 receptor. Previous studies have shown that agonists and antagonists for the 5-HT_3 receptor can act on the neuronal nicotinic receptor (Blanton *et al.*, 2000a). 5-HT_3 receptor antagonist's tropisetron and ondansetron, have been shown to be able to cross over and act on nicotinic receptors, with tropisetron acting as a agonist on α7 nAChRs, whereas ondansetron was shown to act as a antagonist on the α7 receptor (Papke *et al.*, 2004). These two antagonists were also shown to act as antagonists at the α4β2 and α3β4 neuronal nAChR (Papke *et al.*, 2004). Therefore it may be possible that nicotinic antagonists are able to act on the 5-HT_3

receptor, especially as nicotinic antagonist d-tubocurarine has been shown to cross over and act as a 5-HT_3 receptor antagonist (Peters *et al.*, 1993). Although blockade of 5-HT_3 receptor at the level of the brainstem has no effect on resting blood pressure (see Ramage, 2001).

Although α-BgT is a very selective antagonist at α7 nAChRs, with a binding affinity (Ki) of approximately 1 nM (Davis *et al.*, 1999), it had no effect, whereas MLA did (see above). It is possible that the dose of α-BgT used is not high enough to block these receptors, although the expected increase in blood pressure and renal nerve activity induced by α7 selective agonist PSAB-OFP injected i.c. was blocked. A possible explanation for the lack of effect of α-BgT, is due to the very slow binding kinetics of α-BgT, which requires pre-incubation of around 1 hour to cause a complete block *in vitro* (Davies et al., 1999), thus allowing compensatory mechanism to take over. However the data with the α7 selective agonist, PSAB-OFP does not support such an argument. Interestingly, in previous studies nicotine i.c. has been shown to decrease blood pressure and heart rate which was inhibited by α7 selective antagonist MLA, suggesting that α7 nAChRs are also involved in a depressor response. Therefore, it is possible that α-BgT is unable to inhibit all the α7 nAChRs and these may be the ones that have greater tonic drive.

These data imply that central cholinergic pathways, via nAChRs at the level of brainstem, are involved in blood pressure regulation. However, the lack of a dose response curve with MLA and the fact that these antagonists can cause a rise in mean arterial blood pressure as seen with the agonists makes these data difficult to interpret.

3.5. Appendix

Treatment Group	n number	MAP (mmHg)	HR (beats min^{-1})
Saline control (i.c.v.)	5	127 ± 14	380 ± 27
Saline control (i.c.)	5	112 ± 13	385 ± 32
0.1 μmol kg^{-1} Nicotine (i.c.v.)	5	123 ± 15	361 ± 34
0.3 μmol kg^{-1} Nicotine (i.c.v.)	5	121 ± 12	388 ± 38
1 μmol kg^{-1} Nicotine (i.c.v.)	5	123 ± 20	370 ± 40
1 μmol kg^{-1} PSAB-OFP (i.c.v.)	5	122 ± 7	362 ± 44
3 μmol kg^{-1} PSAB-OFP (i.c.v.)	5	124 ± 8	356 ± 23
10 μmol kg^{-1} PSAB-OFP (i.c.v.)	5	126 ± 22	350 ± 23
1 μmol kg^{-1} TC-2559 (i.c.v.)	5	120 ± 17	368 ± 54
3 μmol kg^{-1} TC-2559 (i.c.v.)	5	113 ± 16	366 ± 34
10 μmol kg^{-1} TC-2559 (i.c.v.)	4	125 ± 19	380 ± 14
0.1 μmol kg^{-1} DMPP (i.c.v.)	5	123 ± 23	350 ± 24
0.3 μmol kg^{-1} DMPP (i.c.v.)	5	118 ± 13	265 ± 50
1 μmol kg^{-1} DMPP (i.c.v.)	5	113 ± 5	381 ± 30
1 μmol kg^{-1} Cytisine (i.c.v.)	5	121 ± 14	388 ± 36
3 μmol kg^{-1} Cytisine (i.c.v.)	5	118 ± 14	412 ± 73
0.3 μmol kg^{-1} Nicotine (i.c.)	5	116 ± 12	405 ± 34
3 μmol kg^{-1} PSAB-OFP (i.c.)	5	111 ± 11	387 ± 65
3 μmol kg^{-1} TC-2559 (i.c.)	5	113 ± 14	392 ± 59

Treatment Group	n number	MAP (mmHg)	HR (beats min^{-1})
Antagonist control (2 doses saline, i.c.v.)	5	113 ± 10	393 ± 51
0.3 µmol kg^{-1} Nicotine with 2 doses of saline (i.c.v.)	5	118 ± 23	422 ± 28
0.3 µmol kg^{-1} Nicotine with 10 µmol kg^{-1} DhβE (i.c.v.)	5	114 ± 13	363 ± 37
3 µmol kg^{-1} PSAB-OFP with 10 µmol kg^{-1} DhβE (i.c.v.)	5	114 ± 13	363 ± 33
3 µmol kg^{-1} TC-2559 with 10 µmol kg^{-1} DhβE (i.c.v.)	5	113 ± 5	361 ± 65
0.3 µmol kg^{-1} Nicotine with 0.5 µmol kg^{-1} MLA (i.c.v.)	5	117 ± 11	359 ± 108
3 µmol kg^{-1} PSAB-OFP with 0.5 µmol kg^{-1} MLA (i.c.v.)	5	103 ± 9	377 ± 43
3 µmol kg^{-1} TC-2559 with 0.5 µmol kg^{-1} MLA (i.c.v.)	5	114 ± 14	373 ± 34
0.01 µmol kg^{-1} α-BgT (i.c.v.)	5	114 ± 7	404 ± 41
3 µmol kg^{-1} PSAB-OFP with 0.01 µmol kg^{-1} α-BgT (i.c.v.)	5	120 ± 9	393 ± 54
0.1 µmol kg^{-1} MLA (i.c.)	5	117 ± 17	413 ± 30
0.5 µmol kg^{-1} MLA (i.c.)	5	94 ± 18	365 ± 46
1 µmol kg^{-1} MLA (i.c.)	5	115 ± 13	412 ± 78
0.5 µmol kg^{-1} MLA with 3 µmol kg^{-1} PSAB-OFP 20 min later (i.c.)	5	85 ± 13	393 ± 35
10 µmol kg^{-1} DhβE (i.c.)	5	119 ± 11	402 ± 28
0.01 µmol kg^{-1} α-BgT (i.c.)	5	111 ± 11	395 ± 37
α-BgT control	5	121 ± 9	398 ± 55
3 µmol kg^{-1} PSAB-OFP with 0.01 µmol kg^{-1} α-BgT (i.c.)	5	116 ± 10	396 ± 35

Treatment Group	n number	MAP (mmHg)	HR (beats min^{-1})
V_1 antagonist control (i.v. with i.c.v. agonists)	5	106 ± 12	385 ± 20
V_1 antagonist control (i.v. with i.c. agonists)	5	117 ± 5	403 ± 23
V_1 antagonist control (i.c.v.)	5	117 ± 14	389 ± 40
V_1 antagonist control (i.c.)	5	105 ± 15	381 ± 40
0.3 µmol kg^{-1} Nicotine (i.c.v.) with V_1 antagonist (i.v.)	5	113 ± 12	375 ± 53
3 µmol kg^{-1} PSAB-OFP (i.c.v.) with V_1 antagonist (i.v.)	5	108 ± 15	397 ± 36
3 µmol kg^{-1} TC-2559 (i.c.v.) with V_1 antagonist (i.v.)	5	108 ± 10	378 ± 32
3 µmol kg^{-1} Cytisine (i.c.v.) with V_1 antagonist (i.v.)	5	119 ± 14	419 ± 9
0.3 µmol kg^{-1} DMPP (i.c.v.) with V_1 antagonist (i.v.)	5	102 ± 8	375 ± 23
0.3 µmol kg^{-1} Nicotine (i.c.) with V_1 antagonist (i.v.)	5	103 ± 8	381 ± 46
3 µmol kg^{-1} PSAB-OFP (i.c.) with V_1 antagonist (i.v.)	5	107 ± 17	396 ± 43
3 µmol kg^{-1} TC-2559 (i.c.) with V_1 antagonist (i.v.)	5	109 ± 6	404 ± 41
0.3 µmol kg^{-1} Nicotine (i.c.v.) with V_1 antagonist (i.c.v.)	5	106 ± 16	390 ± 18
3 µmol kg^{-1} PSAB-OFP (i.c.v.) with V_1 antagonist (i.c.v.)	5	112 ± 17	400 ± 49
3 µmol kg^{-1} TC-2559 (i.c.v.) with V_1 antagonist (i.c.v.)	5	190 ± 16	387 ± 24
0.3 µmol kg^{-1} Nicotine (i.c.) with V_1 antagonist (i.c.)	5	116 ± 7	392 ± 37
3 µmol kg^{-1} PSAB-OFP (i.c.) with V_1 antagonist (i.c.)	5	111 ± 8	376 ± 28
3 µmol kg^{-1} TC-2559 (i.c.) with V_1 antagonist (i.c.)	5	109 ± 12	393 ± 35
		1^{st} vasopressin	2^{nd} vasopressin
V_1 antagonist i.c.v. with vasopressin (i.v.)	7	115 ± 22	111 ± 10
V_1 antagonist i.c. with vasopressin (i.v.)	7	116 ± 14	115 ± 10

Treatment Group	n number	MAP (mmHg)	HR (beats min^{-1})
Saline infusion (i.v.)	3	102 ± 7	398 ± 38
0.3 μmol kg^{-1} Nicotine infusion (i.v.)	5	116 ± 19	407 ± 33
3 μmol kg^{-1} PSAB-OFP infusion (i.v.)	3	128 ± 6	403 ± 30
3 μmol kg^{-1} TC-2559 infusion (i.v.)	3	116 ± 5	423 ± 14

Table 16. Overview of experiments performed and the baseline values for the experiments.

3.5.1. Effects of DMPP i.c.v. in the presence of the V_1 receptor antagonist (i.v.).

In the presence of V_1 receptor antagonist (30 μg kg^{-1}; i.v.), the expected evoked rise in mean arterial blood pressure and the delayed renal sympathoexcitation by DMPP (1 μmol kg^{-1}, n=5) was completely blocked (Figure 79). Baseline values for MAP and HR were 102 ± 8 mmHg and 375 ± 23 beats min^{-1}.

3.5.2. Effects of cytisine i.c.v. in the presence of the V_1 receptor antagonist (i.v.).

In the presence of V_1 receptor antagonist (100 μg kg^{-1}; i.v.), the expected evoked rise in mean arterial blood pressure by cytisine (3 μmol kg^{-1}) was significantly attenuated reaching a maximum of 17 ± 3 mmHg compared with 37 ± 14 mmHg. Further expected renal sympathoinhibition was also attenuated and now transient returning to baseline after 4 min (Figure 80). However, again there was a delayed bradycardia with was now significant decrease at 9 min, reaching a maximum of -31 ± 7 beats min^{-1} at 20 min. Baseline values for MAP and HR were 119 ± 14 mmHg and 419 ± 9 beats min^{-1}.

Figure 79. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by DMPP (1 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and DMPP alone (1 μmol kg^{-1}, i.c.v.) and control (V_1 anta & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by DMPP (#) pre-treated with V_1 antagonist compared with DMPP alone by using two-way analyses of variance followed by the least significant difference test to compare the means. # $P<0.05$ and ## $P<0.01$.

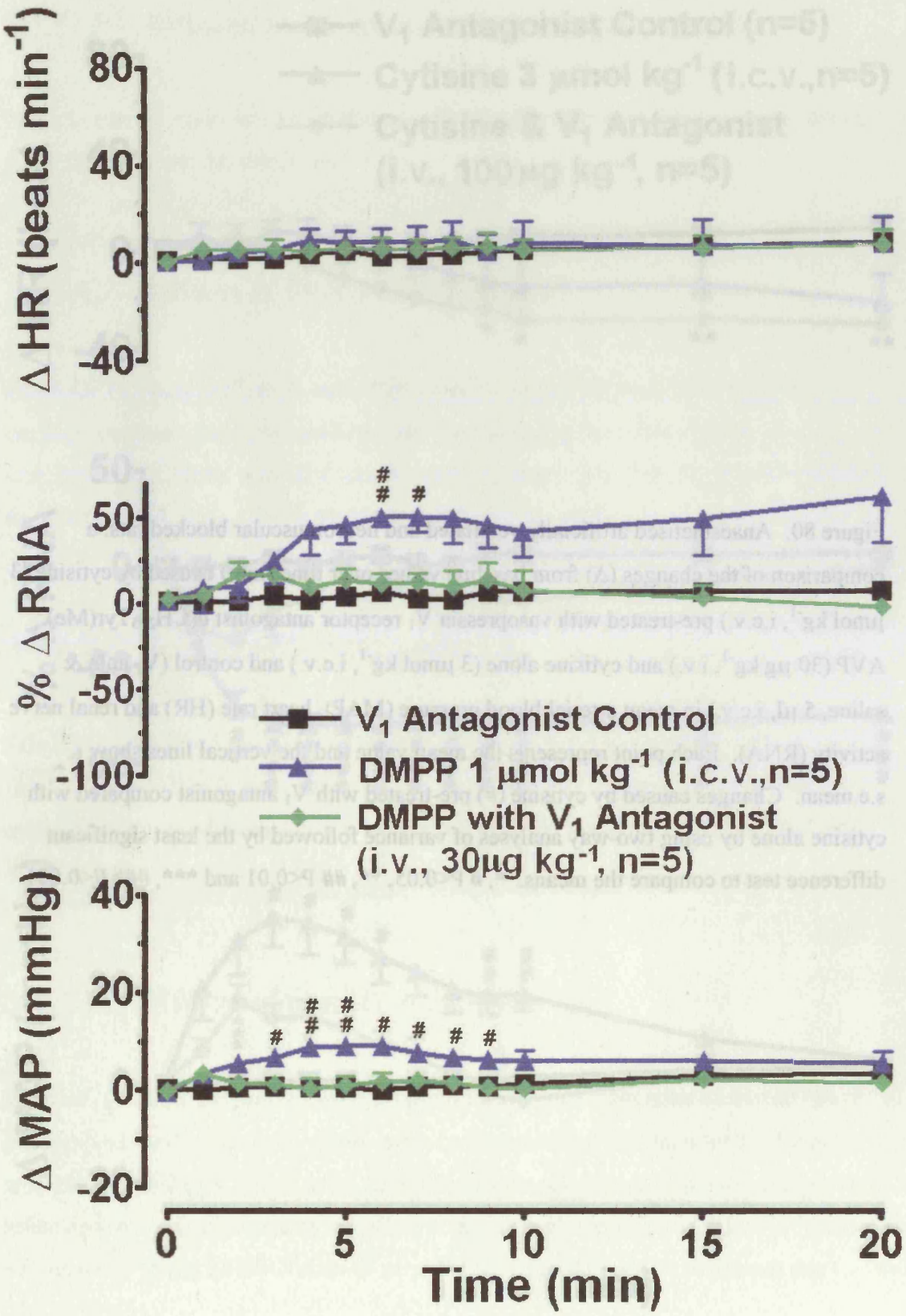
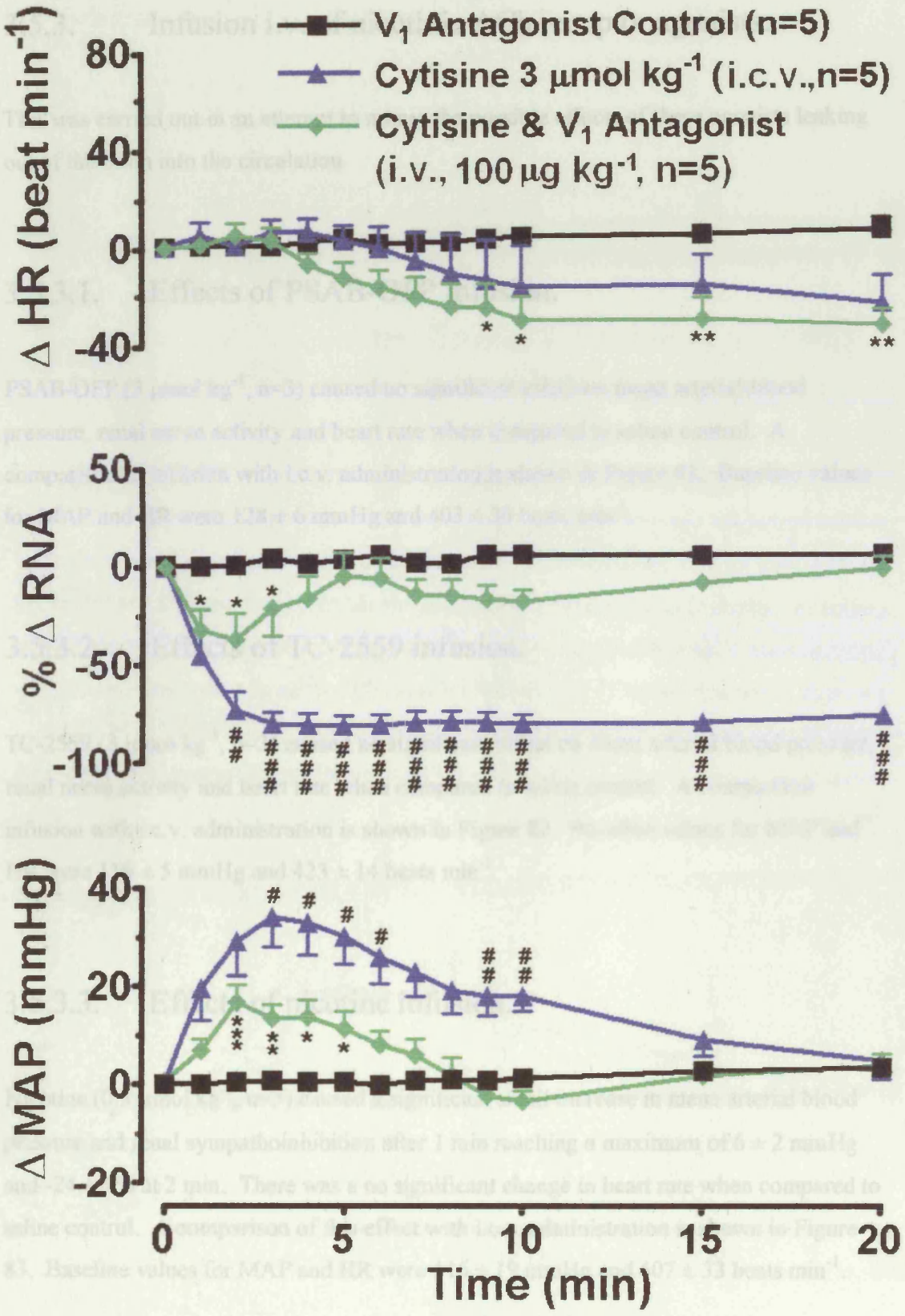

Δ HR (beats min-1)
80
40
0
-40
% Δ RNA
50
0
-50
-100
V1 Antagonist Control
DMPP 1 μmol kg-1 (i.c.v.,n=5)
DMPP with V1 Antagonist
(i.v., 30μg kg-1, n=5)
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 80. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by cytisine (3 μmol kg^{-1}, i.c.v.) pre-treated with vasopressin V_1 receptor antagonist $d(CH_2)_5Tyr(Me)$ AVP (30 μg kg^{-1}, i.v.) and cytisine alone (3 μmol kg^{-1}, i.c.v.) and control (V_1 anta & saline, 5 μl, i.c.v.) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by cytisine (#) pre-treated with V_1 antagonist compared with cytisine alone by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$, **, ## $P<0.01$ and ***, ### $P<0.001$


V1 Antagonist Control (n=5)
Cytisine 3 µmol kg-1 (i.c.v.,n=5)
Cytisine & V1 Antagonist
(i.v., 100 µg kg-1, n=5)
Δ HR (beat min-1)
80
40
0
-40
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.5.3. Infusion i.v. of nicotinic ACh receptor agonists.

This was carried out in an attempt to mimic the possible effects of these agonists leaking out of the brain into the circulation

3.5.3.1. Effects of PSAB-OFP infusion.

PSAB-OFP (3 μmol kg^{-1}, n=3) caused no significant effect on mean arterial blood pressure, renal nerve activity and heart rate when compared to saline control. A comparison of infusion with i.c.v. administration is shown in Figure 81. Baseline values for MAP and HR were 128 ± 6 mmHg and 403 ± 30 beats min^{-1}.

3.5.3.2. Effects of TC-2559 infusion.

TC-2559 (3 μmol kg^{-1}, n=3) caused no significant effect on mean arterial blood pressure, renal nerve activity and heart rate when compared to saline control. A comparison infusion with i.c.v. administration is shown in Figure 82. Baseline values for MAP and HR were 116 ± 5 mmHg and 423 ± 14 beats min^{-1}

3.5.3.3. Effects of nicotine infusion.

Nicotine (0.3 μmol kg^{-1}, n=5) caused a significant small increase in mean arterial blood pressure and renal sympathoinhibition after 1 min reaching a maximum of 6 ± 2 mmHg and -24 ± 4% at 2 min. There was a no significant change in heart rate when compared to saline control. A comparison of this effect with i.c.v. administration is shown in Figure 83. Baseline values for MAP and HR were 116 ± 19 mmHg and 407 ± 33 beats min^{-1}.

Figure 81. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by PSAB-OFP (3 μmol kg^{-1},i.v. infusion) and PSAB-OFP alone (3 μmol kg^{-1}, i.c.v.) and control (saline i.v. infusion) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by PSAB-OFP infusion (*) compared with control and changes caused by PSAB-OFP infusion (#) compared with PSAB-OFP i.c.v. by using two-way analyses of variance followed by the least significant difference test to compare the means.

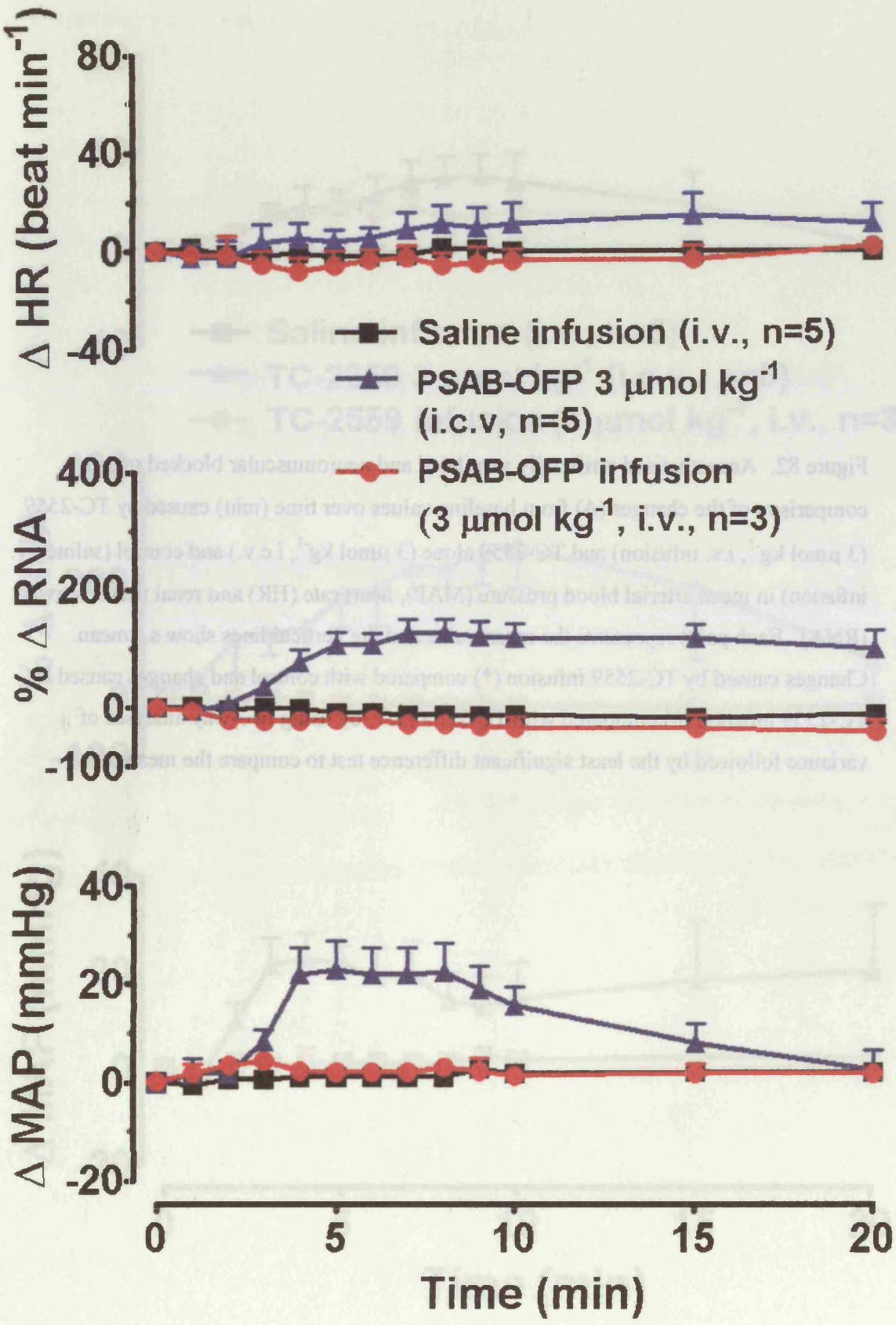

Δ HR (beat min-1)
80
40
0
-40
Saline infusion (i.v., n=5)
PSAB-OFP 3 μmol kg-1
(i.c.v, n=5)
PSAB-OFP infusion
(3 μmol kg-1, i.v., n=3)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 82. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by TC-2559 (3 μmol kg^{-1}, i.v. infusion) and TC-2559 alone (3 μmol kg^{-1}, i.c.v.) and control (saline i.v. infusion) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by TC-2559 infusion (*) compared with control and changes caused by TC-2559 infusion (#) compared with TC-2559 i.c.v. by using two-way analyses of variance followed by the least significant difference test to compare the means.

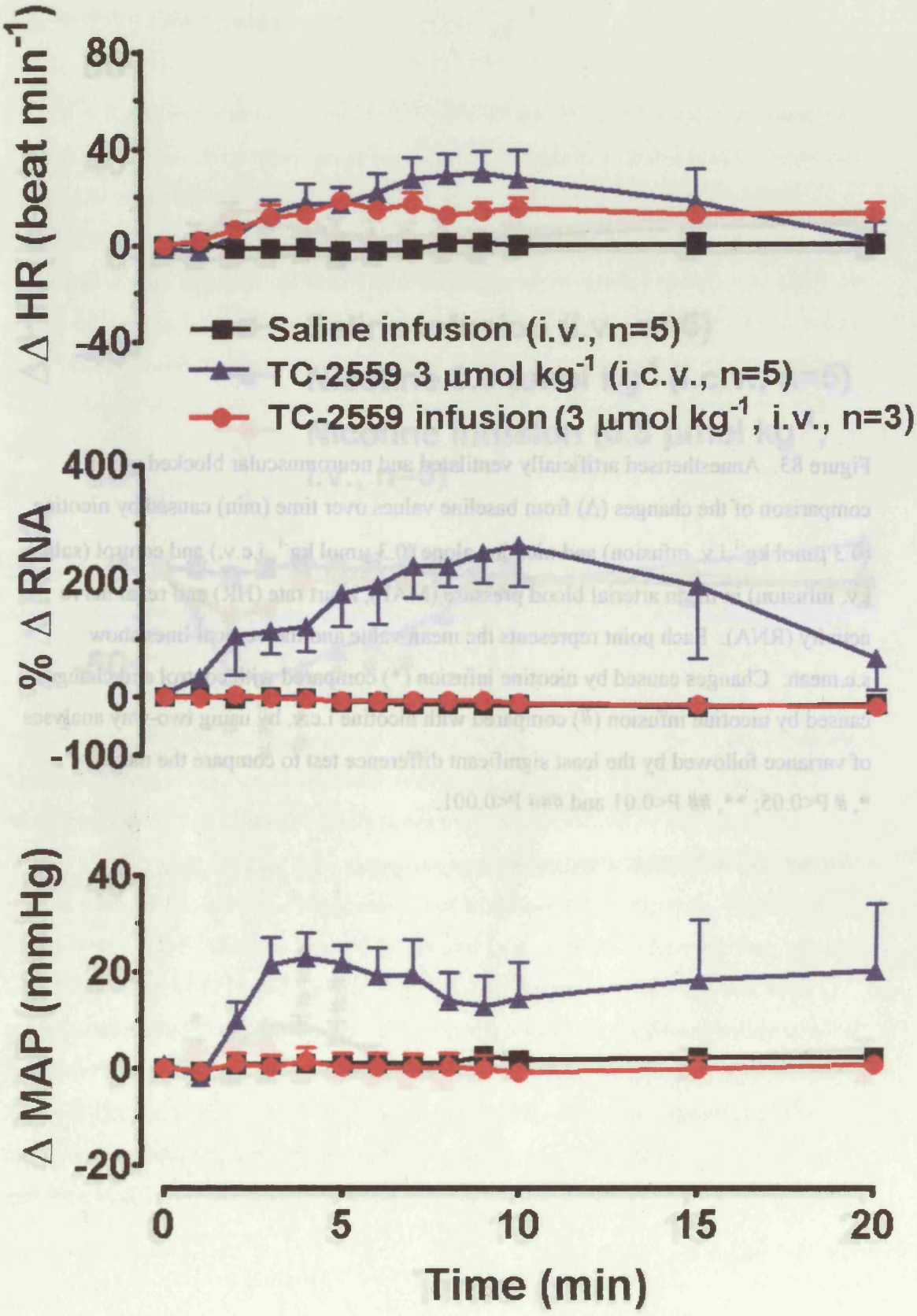
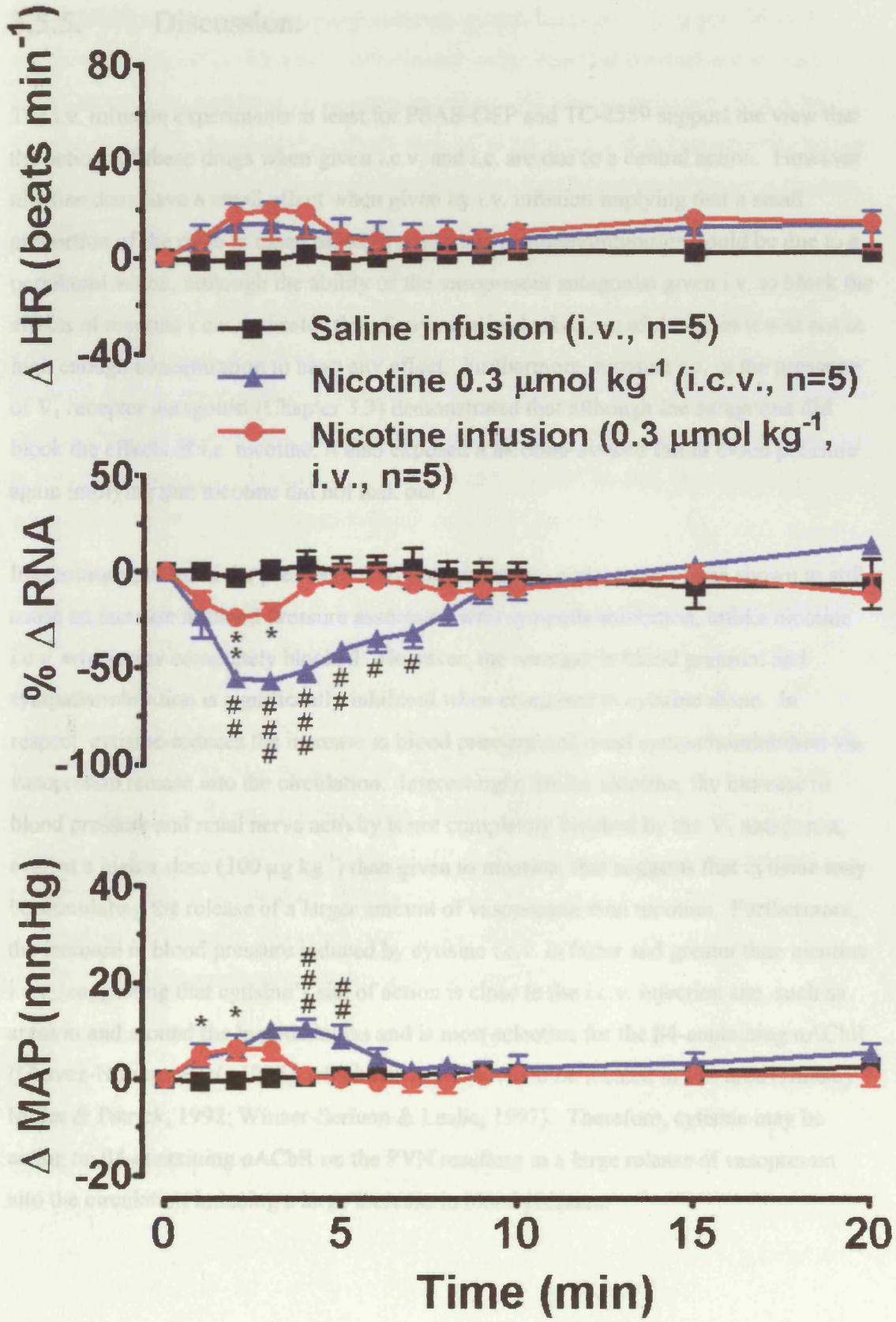

Δ HR (beat min-1)
80
40
0
-40
Saline infusion (i.v., n=5)
TC-2559 3 μmol kg-1 (i.c.v., n=5)
TC-2559 infusion (3 μmol kg-1, i.v., n=3)
% Δ RNA
400
200
0
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

Figure 83. Anaesthetised artificially ventilated and neuromuscular blocked rats: a comparison of the changes (Δ) from baseline values over time (min) caused by nicotine (0.3 μmol kg^{-1},i.v. infusion) and nicotine alone (0.3 μmol kg^{-1}, i.c.v.) and control (saline i.v. infusion) in mean arterial blood pressure (MAP), heart rate (HR) and renal nerve activity (RNA). Each point represents the mean value and the vertical lines show s.e.mean. Changes caused by nicotine infusion (*) compared with control and changes caused by nicotine infusion (#) compared with nicotine i.c.v. by using two-way analyses of variance followed by the least significant difference test to compare the means. *, # $P<0.05$; **, ## $P<0.01$ and ### $P<0.001$.


Δ HR (beats min-1)
80
40
0
-40
Saline infusion (i.v., n=5)
Nicotine 0.3 μmol kg-1 (i.c.v., n=5)
Nicotine infusion (0.3 μmol kg-1, i.v., n=5)
% Δ RNA
50
0
-50
-100
Δ MAP (mmHg)
40
20
0
-20
0
5
10
15
20
Time (min)

3.5.5. Discussion.

The i.v. infusion experiments at least for PSAB-OFP and TC-2559 support the view that the actions of these drugs when given i.c.v. and i.c. are due to a central action. However nicotine does have a small effect when given by i.v. infusion implying that a small proportion of the raise in mean blood pressure and sympathoinhibition could be due to a peripheral action, although the ability of the vasopressin antagonist given i.v. to block the effects of nicotine i.c.v. indicates that if any nicotine leaked out of the brain it was not in high enough concentration to have any effect. Furthermore, nicotine i.c. in the presence of V_1 receptor antagonist (Chapter 3.3) demonstrated that although the antagonist did block the effects of i.c. nicotine, it also exposed a nicotine-evoked fall in blood pressure again implying that nicotine did not leak out.

Interestingly, when in the presence of V_1 antagonist i.v., cytisine i.c.v. was shown to still cause an increase in blood pressure associated with sympathoinhibition, unlike nicotine i.c.v. which was completely blocked. However, the increase in blood pressure and sympathoinhibition is significantly inhibited when compared to cytisine alone. In respect, cytisine-induces the increase in blood pressure and renal sympathoinhibition via vasopressin release into the circulation. Interestingly, unlike nicotine, the increase in blood pressure and renal nerve activity is not completely blocked by the V_1 antagonist, even at a higher dose (100 $\mu g\ kg^{-1}$) than given to nicotine, this suggests that cytisine may be stimulating the release of a larger amount of vasopressin than nicotine. Furthermore, the increase in blood pressure induced by cytisine i.c.v. is faster and greater than nicotine i.c.v., suggesting that cytisine's site of action is close to the i.c.v. injection site, such as areas in and around the hypothalamus and is most selective for the β4-containing nAChR (Chavez-Noriega *et al.*, 1997), which has been shown to be located in this area (Dineley-Miller & Patrick, 1992; Winzer-Serham & Leslie, 1997). Therefore, cytisine may be acting on β4-containing nAChR on the PVN resulting in a large release of vasopressin into the circulation inducing a large increase in blood pressure.

Unlike nicotine, the cytisine-induced decrease in renal nerve activity is parallel to the vasopressin-induced increase in blood pressure, suggesting that the decrease in renal nerve activity may also involve vasopressin release. As already indicated in Chapter 3.2, it is possible for vasopressin to feedback and act on the brain and induce a cardiovascular effect. As previously noted, an area of the brain lacking a blood brain barrier is the area postrema (Hasser & Bishop, 1990), which has been shown to contain V_1 receptors and contain pathways to cardiovascular regulating areas of the brain such as the nucleus tractus solitarius (Shapiro & Miselis, 1985). Infusions of vasopressin i.v. have been demonstrated to cause a significant increase in blood pressure associated with a reflex decrease in renal nerve activity (Hasser & Bishop, 1990). In this respect, cytisine may be causing the release of vasopressin which is feeding back onto the area postrema resulting in a decrease in renal nerve activity. However, until further studies, the mechanism of action for cytisine –induced sympathoinhibition is not known.

The increase in blood pressure and renal nerve activity induced by DMPP was blocked when in the presence of V_1 antagonist i.v. DMPP has been indicated to be selective for $\alpha2\beta2$, $\alpha3\beta2$ and $\alpha3\beta4$ nAChR (Chavez-Noriega *et al.*, 1997; Luetic & Patrick, 1991). The DMPP-induced increase in blood pressure and renal nerve activity is also observed by $\alpha7$ and $\alpha4\beta2$ selective agonists PSAB-OFP and TC-2559 (see Chapter 3.1), similarly they are blocked by V_1 antagonist i.v. (see Chapter 3.2). In this respect, the DMPP-induced increase in blood pressure may be due to the release of vasopressin into the circulation; whereas the increase in renal nerve activity may be due to vasopressin feedback on to the brain. However, until further studies the mechanism of action for DMPP–induced sympathoexcitation is not determined.

CHAPTER 4. GENERAL DISCUSSION

4.1. General Discussion.

The central effects of nicotine on blood pressure has been extensively studied indicating that nicotine administered into the hindbrain of rats, cats and dogs causes a decrease in blood pressure (Bisset *et al.*, 1975; Bisset & Chowdrey, 1984; Feldberg & Guertzenstein, 1976; Kubo & Misu, 1981). Interestingly, application of nicotine into the forebrain areas by injections into the lateral cerebral ventricle caused an increase in blood pressure in rats (Bisset & Chowdrey, 1984). However, due to the unselective nature of nicotine for neuronal nAChRs, the particular nAChRs involved in these responses are unknown. With the recent availability of two selective receptor agonists which have selectivity for the most abundant neuronal nAChRs, α4β2 and α7, and selective antagonists for different neuronal nAChR subtypes it is now possible to investigate the role of specific neuronal nAChRs in central cardiovascular regulation. This was carried out by investigating the effects of these selective receptor agonists, PSAB-OFP and TC-2559 given i.c.v. and i.c. in the presence and absence of selective antagonists, DhβE and MLA, and comparing these effects with that of nicotine and other unselective agonists e.g. cytisine and DMPP. As nicotine is well known to cause vasopressin release, the effects of these agonists were also studied in the presence of a vasopressin V_1 receptor antagonist.

4.1.1. Effect of selective agonists.

Neuronal nAChR α7 and α4β2 selective agonists PSAB-OFP and TC-2559 i.c.v. evoked a delayed increase in blood pressure and renal nerve activity. PSAB-OFP produced a significant increase in heart rate at its highest dose, whereas TC-2559 produced an increase at its intermediate dose. Administered into the hindbrain by i.c. injection caused a faster and greater rise in blood pressure than i.c.v. injections, however the increase in

renal nerve activity was still observed to be delayed requiring up to 5 min. PSAB-OFP evoked no significant change in heart rate, although TC-2559 caused a delayed increase.

The selectivity of PSAB-OFP and TC-2559 for its nACh receptor was demonstrated by the use of α7 selective antagonist, MLA, and α4β2 selective antagonist, DhβE. In the presence of their corresponding antagonist the increase in blood pressure and renal nerve activity was blocked or significantly inhibited, whereas when in the presence of opposite antagonist, there was no change in blood pressure and renal nerve activity increase, demonstrating that the selective agonists, at the doses used, were acting on their individual nACh receptors.

4.1.1.1. Sites of Action.

With studies indicating the presence of α4β2 and α7 nAChR at the hypothalamus, i.c.v. administration of PSAB-OFP and TC-2559 may be mediating their effects at the level of the hypothalamus, which has been shown to modulate renal sympathetic outflow, especially the PVN (Schramm *et al.*, 1993). However the pressor response and renal sympathoexcitation evoked by PSAB-OFP and TC-2559 i.c.v. was not a rapid onset and instead required at least 4 min for a significant increase in blood pressure and renal nerve activity to be observed. In this respect, another possible area of the brain that the selective agonists may be mediating their effects is the hindbrain which contains major areas involved in cardiovascular regulation such as the NTS and ventrolateral medulla, (see Dampney, 1994; see Sun, 1995). With previous studies demonstrating the presence of α4β2 and α7 nAChR at the NTS and RVLM (Ferreira *et al.*, 2000; 2002; Swanson *et al.*, 1987; Wada *et al.*, 1989) and with the faster onset of the effects of PSAB-OFP and TC-2559 given i.c. with similar profiles to that of i.c.v injections, it suggests that PSAB-OFP and TC-2559 have to reach the brainstem to induce their effects.

Although a selective agonist has not been applied directly to the RVLM, studies involving microinjection of nicotine demonstrated a large pressor response, similar to the

selective agonists administered i.c. (Tseng *et al.*, 1993; 1994). These findings suggest that the selective agonists could be acting on the RVLM inducing an increase in sympathetic tone causing vasoconstriction of blood vessels resulting in an increase in blood pressure, which is associated with an increase in renal sympathetic nerve activity. However, although the selective agonists i.c. evoke a rapid increase in blood pressure, the onset of renal sympathoexcitation is still delayed. Upon comparison of i.c. and i.c.v. administration there was observed no significant difference in renal nerve activity delay suggesting that the increase in blood pressure is independent of the renal sympathoexcitation. These finding suggest that an increase in sympathetic tone to resistance vessels to cause the increase in blood pressure may not be the mechanism for the observed increase in blood pressure and that the increase renal nerve activity could be independent to the increase in blood pressure. In this respect, previous studies have demonstrated that nicotine administered i.c.v. and i.c. caused a significant increase in the release of vasopressin (Bisset *et al*, 1975; Bisset & Feldberg, 1973; Bisset & Chowdrey, 1984) indicating that the rise in blood pressure by these selective agonists is probably due to vasopressin release.

4.1.1.2. Vasopressin release.

When in the presence of a V_1 receptor antagonist given i.v., the expected increases in blood pressure and renal nerve activity induced by PSAB-OFP and TC-2559 i.c.v. were blocked. Thus suggesting that the selective agonists effects, via the i.c.v. route, are mediated by the release of vasopressin into the circulation. Now if these agonists were given by the i.c. route in the presence of the V_1 receptor antagonist (i.v.), the expected effects of PSAB-OFP i.c. were blocked. However, for TC-2559, the profile observed was similar due to the large initial variability in the pressor response which now only reached significance after 8 min. Similarly the increase in renal nerve activity was delayed. This latter observation is not consistent with the view that the effects of i.c. TC-2559 are mediated by the release of vasopressin into the circulation, although the data are consistent for PSAB-OFP. Further these data suggested that the renal sympathoexcitation

observed is also caused by the release of vasopressin into the circulation. These findings suggest that the increase in blood pressure caused by central administration of selective agonist is due to the release of vasopressin into the circulation, where vasopressin binds to V_{1a} receptors on blood vessels causing vasoconstriction.

It is possible that the V_1 receptor antagonist could have caused the above effects by entering the brain. Thus a series of experiments was carried out to determine if the central block of V_1 receptors is responsible for some of the effects observed. The doses chosen to give centrally were shown not to leak into the periphery as determined by their failure to block the i.v. evoked pressor response to vasopressin. In the presence of the V_1 vasopressin antagonist given centrally (i.c.v. or i.c.), the pressor and renal sympathoexcitatory effects of PSAB-OFP and TC-2559 were nearly completely blocked, implying that these effects are in fact mediated by central release of vasopressin. However it should be noted that these central vasopressinergic pathways may also be releasing vasopressin into the circulation to cause the rise in blood pressure, rather than causing an increase in sympathetic drive to resistance vessels. Thus the precise mechanism behind the pressor response remains to be determined although it does initially, at least, involve the central release of vasopressin suggesting that these agonists are also causing the release of vasopressin into the periphery. Furthermore, the present data would indicate that this is due to activation of a central vasopressinergic pathway.

As previously noted, the selective agonists are believed to be acting on nAChRs in the hindbrain rather than the forebrain to induce the increase in blood pressure, therefore, if the increase in blood pressure is due to vasopressin release, or by the activation of a central vasopressinergic pathway, then this suggests that the site of action for the selective agonists is not at the forebrain sites such as the PVN but at hindbrain sites such as the NTS and RVLM. In this respect, vasopressin-containing terminals have been identified in these areas (Buijs & Van Heerikhuize, 1982; Nilaver *et al.*, 1980; Sawchenko & Swanson, 1982; Van der Kooy *et al.*, 1984) as well as, V_1 receptors (Cheng *et al.*, 2004; Dogterom *et al.*, 1978; Philips *et al.*, 1988; Tribollet *et al.*, 1988) In addition, nicotine can also activate neuronal projection from the NTS and RVLM (C1 region) to the PVN and SON (Matta *et al.*, 1993), thus causing vasopressin release into

the circulation. Furthermore, studies by Bisset *et al.* (1975) demonstrated that nicotine was more effective at inducing vasopressin release when applied to the hindbrain rather than the forebrain. Overall, it is suggested that selective agonists PSAB-OFP and TC-2559 are acting on α7 and α4β2 nAChR at the hindbrain aeas such as the RVLM and NTS. Activation of these cardiovascular regulating areas stimulates the release of vasopressin, via a neuronal projection to the PVN, into the circulation. This pathway from the hindbrain to the PVN is believed to involve vasopressin, possibly acting as a excitatory neurotransmitter between the synapse of the RVLM to the PVN, as a similar pathway has been observed from the PVN to the RVLM (Yang *et al.*, 2001).
Therefore as it has been suggested that central vasopressin release may be acting as a excitatory neurotransmitter at the synapse of the neuron projecting from the RVLM to the PVN, it is believed that the central administration of V_1 receptor antagonist is blocking V_1 receptors located on the PVN and SON(Ostrowski *et al.*, 1992;Ostrowski *et al.*, 1994), to which the central release of vasopressin bind and stimulate the release of vasopressin into the circulation.

A possible mechanism of action for the PSAB-OFP and TC-2559-induced increase in blood pressure is shown in Figures 47 and 48. Selective agonists i.c.v. do not act directly on the PVN to cause the release of vasopressin, instead pass to the hindbrain were they bind to α7 or α4β2 nAChR's in the RVLM, stimulating a neuronal pathway to the PVN. This results in the release of vasopressin into the circulation inducing an increase in blood pressure (Figure 47). Selective agonists when administered via the i.c. route are closer to the NTS and RVLM, therefore they will have a quicker onset of action and the concentration will be larger at these sites than if administered i.c.v. (Figure 48). Thus resulting in a larger amount of vasopressin being released into the circulation compared to the i.c.v. route.

4.1.1.3. Renal Sympathoexcitation.

The present experiments demonstrate that the renal sympathoexcitation is mediated also by the release of vasopressin. This is believed to be due to the vasopressin released into the circulation feeding back and acting on the brain as renal sympathoexcitation has a similar delay whether the selective nAChR agonists are given i.c. or i.c.v. It is possible that the feedback could be occurring at the area postrema, which has been shown to contain V_1 receptors (Shapiro & Miselis, 1985), as well as acting on the subfornical organ (SFO), possibly via V_{1a} receptors, which is located in the forebrain, both lack a blood brain barrier and project to the PVN (Ferguson *et al.*, 1984; Li & Ferguson, 1993; Tanaka *et al.*, 1986; Tanaka & Seta, 1988). This is quite an intriguing observation as it implies that vasopressin can affect the kidney directly by action on the collecting ducts and indirectly via the sympathetic supply which controls sodium excretion, renin release and local blood vessel tone. The hypothesised pathways and feedback mechanisms producing the effects are illustrated for the selective nAChR receptor agonists in Figures 47 and 48. There are two possible mechanisms of action that may account for the effect of vasopressin on renal nerve activity: - 1) upon the vasopressin release into the circulation vasopressin feeds back on to the PVN via the SFO, activating the neuronal projection from the PVN to the RVLM. The activation of this pathway stimulates the RVLM which results in an increase in sympathetic tone and an increase in renal sympathetic nerve activity. 2) and/or the vasopressin feeds back via the area postrema, which has a direct innervation of the NTS (Shapiro & Miselis, 1985), and possibly the RVLM, resulting in the increase in renal nerve activity.

The feedback of vasopressin onto the PVN resulting in the increase of renal nerve activity is the most plausible mode of action as previous studies have demonstrated that the PVN is able to induce sympathoexcitation in stress (Badoer, 2001; Blair *et al.*, 1996). In addition, studies have shown that the PVN has an excitatory influence on RVLM-spinal vasomotor neurons resulting in increases in blood pressure, heart rate and renal nerve activity (Yang & Coote, 1998), and that electrical stimulation of the PVN results in a threefold increase in vasopressin content in the NTS (Landgraf *et al.*, 1990). The PVN

causes sympathoexcitation both via direct projection to sympathetic preganglionic neurons in the IML and via collateral projections to neurons of the RVLM that sends excitatory input to the IML (Coote *et al.*, 1998; Shafton *et al.*, 1998). The excitatory input from the PVN to the RVLM may be mediated by vasopressin; acting via V_1 receptors (see Dampney, 1994; Yang *et al.*, 2001).

4.1.1.4. Cardiovascular effects not involving vasopressin.

Interestingly, PSAB-OFP given i.c. in the presence of central V_1 receptor antagonist no longer caused an increase in blood pressure, instead there was observed a depressor response after 2 min. There was also observed a delayed decrease in blood pressure with PSAB-OFP given i.c.v. in the presence of the V_1 receptor antagonist also given i.c.v. There was no change in renal nerve activity in response to PSAB-OFP administered at either of these injection sites and suggests that the decrease in blood pressure is due to PSAB-OFP acting at α7 nAChR located in the hindbrain, due to the delay in blood pressure decrease by PSAB-OFP i.c.v. compared to PSAB-OFP administered i.c. In this respect, microinjection of nicotine into the NTS has been observed to cause a significant decrease in blood pressure, which was inhibited by α7 nAChR selective antagonist MLA and α-BgT, implying the involvement of α7 nAChR (Dhar *et al.*, 2000; Ferreira *et al.*, 2000; 2002). Interestingly upon inhibition of vasopressin release, additional affects of nAChR activation are observed which are not overcome by the vasopressin-induced increase in blood pressure.

Interestingly, both agonists when given i.c. in the presence of V_1 receptor antagonist i.c. cause a transient sympathoexcitation after 1 min. This is paralleled by a transient non-significant increase in blood pressure. A possible site for this is the RVLM, as microinjection of nicotine caused pressor response and a tachycardia (Tseng *et al.*, 1993; 1994). The transient nature is also surprising unless an additional depressor circuit is also being activated to mask this effect which is believed to occur for PSAB-OFP.

4.1.2. Effects of nicotine.

Nicotine i.c.v. caused a dose-related increase in blood pressure as previously observed by Bisset & Chowdry (1984) and a decrease in renal nerve activity associated with no change in heart rate. Given i.c., nicotine caused a bradycardia, interestingly; the onset of renal sympathoinhibition was immediate while the rise in blood pressure was again delayed but much larger. This was different from that observed with selective receptor agonists activating α7 and α4β2 nACh receptors, in that there was no observed delayed sympathoexcitation.

4.1.2.1. Sites of Action.

Similar to the selective agonists there is a delayed increase in blood pressure induced by i.c.v. injections suggesting that the site of action is located away from the forebrain and possibly in brain areas in the hindbrain. Interestingly, nicotine administered into the hindbrain via i.c. injections was shown to induce a biphasic response in blood pressure, with an initial fall followed by an increase which was greater than the blood pressure increase caused by nicotine i.c.v. Studies have shown that administration of nicotine into the hindbrain, either by i.c. injection or by application of nicotine to the ventral surface of the brainstem, results in a depressor response which was blocked by nicotinic antagonists, but unaffected by the muscarinic antagonist, atropine (Bisset *et al.*, 1975; Bisset & Chowdrey, 1984; Feldberg & Guertzenstein, 1976). In this respect, it is suggested that nicotine may be acting on areas of the hindbrain inducing a decrease in blood pressure; however nicotine is also acting on additional areas of the hindbrain overcoming the decrease and resulting in an increase in blood pressure. The overall data supports the view that the nicotine-induced increase in blood pressure is due to the release of vasopressin.

4.1.2.2. Vasopressin release.

The increase in blood pressure caused by i.c.v. and i.c. nicotine was blocked / attenuated when in the presence of V_1 receptor antagonist i.v. consistent with the theory that nicotine induces vasopressin release into the circulation. Nicotine i.c. in the presence of the V_1 receptor antagonist given i.v. potentiated the initial decrease in blood pressure, supporting the theory that the initial decrease is overcome by the release of vasopressin into the circulation resulting in an increase in blood pressure. Surprisingly, the fall in blood pressure is blocked in the presence of the V_1 receptor antagonist i.c., suggesting that this is probably due to the involvement of a central vasopressinergic sympathoinhibitory pathway which is not activated by $\alpha 7$ and $\alpha 4\beta 2$ nACh receptors.

Interestingly, nicotine i.c.v. in the presence of V_1 receptor antagonist i.c.v. still caused an increase in blood pressure, suggesting that it is possible that nicotine i.c.v. is causing the release of vasopressin, into the circulation, by directly acting on the PVN. Figure 69 illustrates possible mechanisms involved in the i.c.v. nicotine-evoked increase in blood pressure. 1) Involves a direct effect of nicotine on the PVN; resulting in the release of vasopressin into the circulation. 2) Nicotine moves from the i.c.v. injection site to sites in the hindbrain, possibly the RVLM, activating a pathway to the PVN, which results in the release of vasopressin.

If nicotine i.c.v. induced the release of vasopressin by acting on the hindbrain, then the release of vasopressin would be caused by a neuronal projection from the hindbrain to the PVN (see Chapter 3.2) in which vasopressin was believed to be acting as a excitatory neurotransmitter at the synapse between the RVLM and PVN. Therefore the central administration of a V_1 receptor antagonist would inhibit the V1a receptors found on the PVN and SON (Ostrowski *et al.*, 1992; 1994) and block the stimulation of vasopressin into the circulation. However, this was not observed and instead suggests that the release of vasopressin is caused by nicotine acting directly on the PVN. Figure 70 shows a diagrammatic representation of the mechanism of action for nicotine injected i.c.

Interestingly, the evidence indicates that α7 and α4β2 selective receptor agonists did not cause the release of vasopressin by acting on the PVN directly and instead acted on the hindbrain to evoke this release (see Chapter 3.2). In this respect, it would seem that nicotine is not acting on α7 and α4β2 nAChRs at the level of the PVN but different nACh receptors found in the PVN.

4.1.2.3. Renal sympathoinhibition.

Pre-treatment with the vasopressin V_1 receptor antagonist (i.v., i.c.v. & i.c.) attenuated / blocked the renal sympathoinhibition as it had done for the renal sympathoexcitation observed with the selective α7 and α4β2 nAChR agonists. Interestingly, due to the rapid nature of nicotine i.c.v.-induced sympathoinhibition, it is suggested that nicotine's site of action is near the i.c.v. injection site. However, although the sympathoinhibition caused by nicotine i.c.v. is fast, the sympathoinhibition response caused by nicotine i.c. is faster suggesting that areas in the hindbrain may be involved. Further, it may be possible that both the forebrain and hindbrain sites are involved. A possible mechanism of action is the direct affect of nicotine on the CVLM. Previous studies have shown that nicotine microinjected into the CVLM caused a dose-related decrease in blood pressure and heart rate (Aberger *et al.*, 2001) which was suggested to be due to inhibition of the RVLM via GABAnergic neurons form the CVLM, thus resulting in a decrease in sympathetic nerve activity to the heart and blood vessels (Agarwal *et al.*, 1990; Brown & Guyenet, 1984; Cravo *et al.*, 1991; Gordon, 1987). This would account for the rapid decrease in renal sympathetic nerve activity and heart rate upon nicotine administered i.c., due to the i.c. injection site being located close to the CVLM and the delayed decrease in RSNA evoked by nicotine i.c.v. However, the i.c.v. nicotine evoked renal sympathoinhibition is too fast to be leaking across the brain to evoke an effect, therefore it is suggested that the decrease is caused by forebrain areas such as the PVN innervating cardiovascular regulating hindbrain areas such as the CVLM, NTS and RVLM (see Dampney, 1994; see Sun, 1995). Furthermore Yang & Coote (1998) demonstrated that activation of neurons at the PVN may cause a depressor response via inhibiting the RVLM neurons, therefore it is

possible that via a pathway for the PVN to the RVLM nicotine is able to cause a decrease in renal sympathetic nerve activity

Interestingly the nicotine-evoked renal sympathoinhibition is inhibited and attenuated by central V1a receptor antagonist suggesting the involvement of vasopressin centrally possibly acting as a neurotransmitter (Yang *et al.*, 2001) at the synapses between cardiovascular regulating centres.

4.1.2.4. Nicotine in the presence of selective nAChR antagonists.

To determine which nAChRs are activated by nicotine, the effects of nicotine were studied in the absence and presence of α7 selective receptor antagonist MLA and α4β2 selective antagonist DhβE. The present data indicates that nicotine can activate a α4β2 nACh receptor, which mediates sympathoinhibition, which was not observed using the respective selective receptor agonists (see Chapter 3.1). In fact, in the presence of the receptor antagonist DhβE, the renal sympathoinhibition was reversed to sympathoexcitation and associated with no changes in blood pressure and heart rate. A possible explanation of this contradiction is that DhβE at the concentration used is also blocking another receptor. In this respect, DhβE does have reasonable affinity for the β2 and β4 subunits (Chavez-Noriega *et al.*, 1997; 2000) and receptor/s containing these subunits could mediate this renal sympathoinhibition. Furthermore, cytisine which has a good affinity at receptors containing the β4 subunit (see Introduction) was also found to cause profound renal sympathoinhibition and surprisingly this was associated with no change in heart rate although the expected pressor response was still observed. The selectivity of cytisine for nAChR subtypes has been shown *in vitro* to have a low affinity for nAChRs containing the β2 subunits compared to other agonists such as DMPP and ACh, but displayed high affinity for α2β4, α3β4 and α4β4 nAChR's (Chavez-Noriega *et al.*, 1997; 2000), indicating that cytisine is selective for the β4-containing nAChR subtypes. Therefore, due to the cardiovascular similarities induced by nicotine and

cytisine, it is possible that nicotine is acting on a β4-containing nAChR subtype. However further experiments are required to clarify this issue. Furthermore, in the presence of α7 selective receptor antagonist, MLA the nicotinic-evoked renal sympathoinhibition was not blocked only delayed, although the blood pressure response was blocked. The mechanism for the delay is unclear, but maybe due to the balance of action of different nAChRs which have opposing actions on renal nerve activity. However, the ability of both receptor antagonists to block the pressor response remains difficult to explain, although a possible mechanism could be in one case that of the physiological antagonist again by blocking one receptor unmasking another effect, although in the opposite direction i.e. the receptors mediating the fall in blood pressure observed with TC-2559 at the high dose.

4.1.3. Conclusion.

It is concluded that the major central cardiovascular effects of activating α7 and α4β2 receptors occurs at the level of brainstem by activation of a neuronal projection to the PVN, leading to the release of vasopressin into the circulation. This in turn can feedback and cause additional effects such as renal sympathoexcitation. However, there are also other actions but these are masked by the release of vasopressin centrally and peripherally.

Nicotine (i.c.v. & i.c.) caused the increase in blood pressure by the release of vasopressin into the circulation. It is possible that nicotine i.c.v. differs from the selective receptor agonists in that it is acting on the PVN directly to cause vasopressin release, although the release of vasopressin is low and may account for the delayed increase in blood pressure. However, nicotine is also acting on the hindbrain, causing a greater increase in blood pressure compared to nicotine i.c.v. Nicotine administered into the hindbrain has been shown to release more vasopressin than nicotine i.c.v. therefore, accounting for the larger increase in blood pressure and a faster decrease in renal nerve activity for nicotine i.c. (Figure 69 & 70). Interestingly, the renal sympathoinhibition is independent of the

vasopressin-induced increase in blood pressure, therefore the nicotine evoked decrease in renal nerve activity is independent of vasopressin release, making the mechanism of action possibly involving the inhibition of the RVLM by nicotine acting on nAChR found on the CVLM (Aberger *et al.*, 2001; Agarwal *et al.*, 1990; Brown & Guyenet, 1984).

4.1.4. Future experiments.

In order to clarify and take the data in this thesis further, a number of additional experiments are required.

Firstly, it needs to be determined if the dose of V_1 receptor antagonist given i.v. in these studies is able to get into the brain and act centrally. This will be carried out by studying the effects of i.c.v. and i.c. vasopressin in the presence of the V_1 receptor antagonist given i.v. To determine exclusively if the increase in blood pressure caused by selective receptor agonists and nicotine is induced by the release of vasopressin into the circulation, additional studies could also be preformed. Experiments would involve the central injection of the selective receptor agonists, and nicotine, and at the peak pressor response an injection i.v. of the V_1 receptor antagonist is given. If the increase in blood pressure falls rapidly to baseline then this would indicate that the increase in blood pressure is caused by the release of vasopressin.

Additional experiments would also be preformed to investigate if selective receptor agonists and nicotine are acting on a central angiotensinergic pathway and/or a central 5-HT pathway to cause vasopressin release, as both these pathways are known to be involved (see Ramage, 2001).

Studies have shown that angiotensin II (AT) administered centrally caused an increase in blood pressure by releasing vasopressin (Keil *et al.*, 1975), therefore studies should investigate the effects of these selective receptor agonists and nicotine in the presence of a AT_1 and AT_2 receptor antagonist, administered centrally, to determine if the release of

vasopressin is due to direct action of nAChR activation or activation of central angiotensinergic pathways. Interestingly, circulating angiotensin II could be involved as it has been shown to activate neurons in the subfornical organ to stimulate the PVN. The activation of the PVN can lead to an increase in vasopressin release by acting on magnocellular vasopressin neurons (Ferguson & Wall, 1992), as well as, causing an increase in renal nerve activity due to the pathway from the PVN to the RVLM (Pyner & Coote, 1999; Yang & Coote, 1998).

In addition, due to the similar structure of the neuronal nAChRs and the 5-HT_3 receptors it may be possible that the selective agonists, and especially nicotine, induce their cardiovascular changes by activation of 5-HT_3 receptors. The 5-HT_3 receptor has significant homology to the α7 nAChR, even in the ligand-binding domain (Gurley & Lanthorn, 1998), with previous studies showing that 5-HT is a potent non-competitive antagonist of the neuronal nAChR (Nakazawa *et al.*, 1995). Additional studies of tropisetron and ordansetron indicated that these were shown to not only be an antagonist at the 5-HT_3 receptor but have a crossover effect on nAChR (Mair *et al.*, 1998; Peters *et al.*, 1993). In addition, studies have shown that PSAB-OFP is also a potent agonist for the 5-HT_3 receptor (Broad *et al.*, 2002). Therefore, it may be possible that nicotine and the selective agonists are acting on the 5-HT_3 receptors and inducing some of the cardiovascular effects, therefore the effects of this nicotinic receptor agonist should be observed in the presence of a 5-HT_3 receptor antagonist. However, it has been shown that the 5-HT_3 receptor antagonist have an effect on the nAChR, therefore the results may be variable. It may be interesting to compare the effects of selective 5-HT_3 receptor agonists, on the central cardiovascular effects by administration i.c.v. and i.c. injection, with the effects of selective agonist and nicotine to observe if there are any similarities and possible cross over.

Finally, concerning the effects of nicotine on the cardiovascular system, unlike the activation of the α4β2 and α7 which caused an increase in blood pressure and renal nerve activity, nicotine caused an increase in blood pressure associated with a decrease in renal nerve activity. A possibility is that nicotine is acting on a different receptor to the α4β2 and α7 selective agonists. Due to nicotine's cardiovascular effects being similar to that of

cytisine, which is selective for β4-containing receptors, it suggests that nicotine may be acting on the β4-containing receptor. As the presence of the β4 mRNA has been shown around the forebrain areas such as the hypothalamus (Winzer-Serhan & Leslie, 1997; Dineley-Miller & Patrick, 1992), these receptors may be having a direct effect on nicotine-induced cardiovascular effects. Therefore a future experiment would involve the observation of a β4 selective agonist administered centrally, by i.c.v. and i.c. injection, on central cardiovascular regulation and demonstrate whether these effects were similar to the effects produced by nicotine.

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