The hypothalamic-pituitary-adrenal axis and serotonin metabolism in individual brain nuclei of mice with genetic disruption of the NK1 receptor exposed to acute stress

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Abstract

NK1 receptor antagonists have been reported to have clinical efficacy in the pharmacotherapy of depression. To investigate the possible mechanism of action of NK1 receptor blockade, mice lacking the NK1 receptor (NK1R-/- mice) were used to assess the effects of stress on the hypothalamic-pituitary-adrenocortical (HPA) axis and serotonin (5-HT) turnover in individual brain nuclei. Basal HPA activity and the expression of hypothalamic corticotropin-releasing hormone (CRH) under basal and stress conditions in wild-type (WT) and NK1R-/- mice were identical. Stress-induced increases in plasma ACTH concentration were, however, considerably higher in NK1R-/- mice than in WT mice ($P<0.01$). Genetic deletion of the NK1 receptor increased basal 5-HT turnover in the dorsal raphe- (DRN) and median raphe (MRN) nuclei as evidenced by elevated concentrations of 5-HT and/or 5-hydroxyindolacetic acid (5-HIAA). Stress further magnified the increased 5-HT utilisation in the DRN in NK1R-/- compared with WT mice (5-HT, $P<0.01$; 5-HIAA, $P<0.05$). In the corticolimbic regions critical for the regulation of affective behaviour (medial prefrontal cortex, central nucleus of amygdala and the hippocampal CA1 region), stress increased 5-HT and 5-HIAA concentrations to a similar extent in WT and NK1R-/- mice. 5-HT concentration in the hypothalamic paraventricular nucleus was not affected by stress, but stress induced similar increases in 5-HT and 5-HIAA in the ventromedial and dorsomedial nuclei in both, WT and NK1R-/- mice. Our findings indicate that NK1 receptor activation supresses ACTH release exclusively during acute stress but does not exert sustained, long-lasting inhibition of the HPA axis. Genetic deletion of the NK1 receptor accelerates 5-HT turnover in DRN and MRN under basal and stress conditions. No differences between the responses of serotonergic system to acute stress in WT- and NK1R-/- mice occur in hypothalamic nuclei, corticolimbic regions or individual nuclei of the amygdala, which are implicated in anxiety and depression.

Introduction

A large body of evidence has emerged linking stressful life events with an increased vulnerability for depression and anxiety disorders. Stressful events often precede the onset of depression and the intensity of stress has been associated with the severity of depression (Binder et al. 2010; Heim and Binder, 2012; Aborelius et al. 1999). Stress responses involve activation of the hypothalamic-pituitary-adrenocortical (HPA) axis and autonomic nervous system. There is a strong association between alterations in stress response and dysregulation of the HPA axis with major depression, characterised by impaired glucocorticoid receptor function, an overactive corticotropin-releasing hormone (CRH) system and blunted adrenocorticotropic (ACTH) response to exogenous CRH. Dysfunction of the HPA axis is an endocrine hallmark of major depression (Ströble and Holsboer, 2003; Plotsky et al. 1998).

Impaired serotoninegic neurotransmission in corticolimbic regions comprising the prefrontal cortex, hippocampus and the amygdala has been associated with depression and anxiety. Serotonergic neurones innervating the corticolimbic system are located within the midbrain dorsal raphe (DRN) and median raphe (MRN) nuclei (Wasalues et al. 2011; Lowry, 2002; Asan et al. 2013). Selective serotonin reuptake inhibitors (SSRI), which act to enhance the availability of serotonin (5-HT), are effective in relieving major depression. An increase in extracellular serotonin (5-HT) concentrations in the raphe nuclei after treatment with SSRIs has been well documented. However, conflicting findings have been reported on the effects of acute or repeated administration of SSRI on 5-HT release in the cortical and limbic brain regions (Invernizzi et al. 1992; Malagié et al. 1995; Bosker et al. 1995; Dawson et al. 2002). Besides its inferred involvement in the pathophysiology of depression, 5-HT is also a key regulator of central stress reactions and a robust activator of the HPA axis (Owens and Nemeroff, 1991; Chaouloff 1993; Carrasco and Van de Kar, 2005). Serotonergic projections to the PVN, where CRH neurones are localised, arise from the DRN and MRN (Sawchenko et al. 1983; Lowry, 2002). In turn, the CRH input to serotonergic neurones situated in the caudal part of the DRN may be critical for the activation of the corticolimbic serotonergic system by anxiogenic stimuli and inescapable shock (Lowry et al. 2000; Hammack et al. 2002).

Substance P (SP) - preferring NK1 receptors are also highly expressed in the mesolimbic system and the antidepressant efficacy of several NK1 receptor antagonists has been reported in preclinical studies and in 5 independent randomized, double-blind, placebo controlled clinical trials (Kramer et al. 1998; Rupniak, 2002; Ratti et al. 2011; Rupniak and Kramer, 2017). SP also plays an important role in the control of affective behaviour and the initiation and regulation of neuroendocrine responses to stress (Rupniak and Kramer, 1999). For instance, SP increases the

excitability of neurones in the hypothalamic paraventricular nucleus (PVN) controlling the sympathoadrenal system and elicits a cardiovascular defence reaction (Unger et al. 1988; Womack et al. 2007; Feetham and Barret-Jolley, 2014). The involvement of SP and NK1 receptors in the control of CRH expression and ACTH release is less clear (Culman et al. 2010; Ebner et al. 2008 and 2009; Jessop et al. 1992, Larsen et al. 1993). SP is co-localised with 5-HT and NK1 receptors are robustly expressed on DRN neurones. SP, CRH and other neurotransmitters, such as glutamate and GABA, regulate the neuronal activity of the DRN and responsiveness of serotonergic neurones to aversive stimuli (Conley et al. 2002; Valentino et al, 2003; Commons et al. 2003; Lacoste et al. 2006 and 2009).

Alterations in serotonergic function have been suggested to contribute to the antidepressant efficacy of NK1 receptor antagonists. For instance, the effect of an SSRI on 5-HT outflow in cortical region was potentiated in NK1R-/- mice or by sustained pharmacological blockade of NK1 receptors (Froger et al. 2001; Guiard et al. 2005; Guiard et al. 2006 for review). Pharmacological blockade or deletion of the NK1 receptor also increased the firing rate of DRN neurons and caused desensitisation of 5-HT1A autoreceptors, an effect resembling that seen with SSRIs (Froger et al. 2001; Santarelli et al. 2001; Conley et al, 2002). Collectively, these findings might indicate that NK1 receptor antagonists alleviate major depression by enhancing 5-HT neurotransmission in the forebrain, associated with desensitisation of 5-HT1A receptors (Haddjeri and Blier, 2001; Conley et al, 2002). The role of NK1 receptors in the modulation of 5-HT neurotransmission has been so far studied in the raphe nuclei and large forebrain regions, but not in the distinct forebrain regions linked to the regulation of mood, anxiety and neuroendocrine stress responses, such as the medial prefrontal cortex, amygdaloid - and hypothalamic nuclei. The present experiments used NK1R-/- mice to further investigate the effects of stress on CRH expression in the hypothalamus, activation of the HPA axis and on 5-HT turnover in the DRN, MRN and corticolimbic brain regions processing physiological responses to stress and which are implicated in the pathogenesis of depression.

Materials and Methods

Animals

Adult male wild-type (WT) and NK1R-/- mice aged 6-10 weeks, weighing 25-30 g, were obtained from Professor S. P. Hunt, University College London, London, United Kingdom. Mice were derived from a 129/Sv x C57BL/6 genetic background which were crossed an outbred MF1

strain (De Felipe et al. 1998). All mice were allowed free access to food and water and maintained on a 12 h light/dark cycle at room temperature 21 °C.
Immobilization (Kvetnansky and Mikulaj, 1970) has been widely employed to assess the utilisation of serotonin in relation to the regulation of neuroendocrine responses to stress. Animals were rapidly killed by cervical dislocation immediately after the termination of stress. All experimental protocols were approved by the Governmental Committee for the Ethical Use of Experimental Animals in the German Federal State of Schleswig-Holstein.

Measurement of plasma ACTH and corticosterone

Trunk blood was collected in ice-cold tubes containing 20 µl of 10 % EDTA following decapitation. Plasma was separated by centrifugation and used for determination of ACTH and corticosterone. ACTH was determined using an Allégro HS-ACTH kit (Nichols Institute Diagnostics, Ca, USA). Aliquots of plasma for corticosterone determination were extracted with dichlormethane and followed by paper chromatography. Corticosterone was determined by radioimmunoassay (Culman et al. 1997).

Northern Blot analysis of CRH mRNA in the hypothalamus

RNA was extracted using Trizol Reagent (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer`s instructions. Equal amounts of RNA per sample were separated on 1.5 % agarose-formaldehyde gels. Subsequently, gels were blotted onto nylon membranes (GE Healthcare Limited, Buckinghamshire, UK) overnight. Blots were dried and the RNA cross-linked with UV (1,5 J/cm^2). Gels were stained with ethidium bromide and photographed. After 20 min of pre-hybridization at 42 °C in RapidHyb buffer (Sigma-Aldrich, Taufkirchen, Germany) with herring sperm DNA, hybridization was carried out using [α^{32}P]-labelled antisense CRH probes (mCRH 48mer: CCA gCT CCg TgC TgC TgT CgA gCg ggC gCT gTg gCA TCT gCA gCT gCT) over night at 42 °C. Blots were washed with 1% SDS + 0.1% 20xSSPE and exposed at -80 °C using Kodak XAR film and x-ray intensifying screens. Autoradiographs as well as photographs of the ethidium bromide-stained gels were scanned with an LKB densitometer. Amounts of mRNA measured in the autoradiographs were corrected on the basis of the amount of 28S and 18S rRNA present in the gels.

Microdissection of brain nuclei and regions

Brains were rapidly removed from the skull and immediately frozen on dry ice and stored at -80°C. For the isolation of brain nuclei, brains were cut into slices of 200 µm thickness in a Leica

cryostat at –15°C. The slices were thaw-frozen on glass slides and rapidly frozen on dry ice. The brain nuclei and regions were isolated by punching out of cylinders of tissue using hollow metal needles with inner diameters of 200, 300 and 500 µm (Comparative Atlas of Mouse Brain) (Hof et al. 2000).

HPLC with electrochemical detection

Individual brain nuclei and regions were homogenised in 0.1 M HCl (90 or 120 µl depending on the area) containing internal standard 3,4-dihydroxybenzylamine hydrobromide. Five to 12 µl of the homogenate in triplicates were removed for protein determination (Lowry et al. 1951). The remaining homogenate was centrifuged at 15 000 g for 10 min at 4 °C and the supernatant was stored at – 80°C until the probes were analysed for 5-HT and 5-HIAA using modified HPLC methods with electrochemical detection (Chiueh et al. 1983). The tissue samples were thawed and aliquots of the supernatant (25 – 40 µl depending on the analysed area) were injected using a Waters, WISP 710B autosampler. Chromatographic separation was accomplished using the C_{18} reverse radial pack, 4 mm inside diameter x 20 cm (ET 200/4 Nucleosil 100-5 C_{18} column; Macherey-Nagel, Düren, Germany). The mobile phase consisted of 1.4 g heptansulfonic acid, 0.1 g EDTA, 8.5 ml trimethylamine, 6.5 ml orthophosphoric acid (85 %) and 60 ml acetonitrile dissolved in 1 l of ultra-pure water. The electrochemical detection was accomplished using Waters 460 electrochemical detector, the potential was set at 0.75 V. The results were recorded by a data module (Waters 730) and the areas of indole peaks were integrated for the calculation of concentration from the mean peak areas of four chromatographic analyses of the respective standards (50 or 100 pg). The concentrations of 5-HT and, 5-HIAA are expressed as pg per microgram of protein.

Effects of immobilization stress on plasma ACTH and corticosterone concentrations and CRH expression in the hypothalamus

WT and NK1R-/- mice were immobilized for 15 or 120 min (n = 7/group). Non-stressed wild-type- and NK1R-/- mice (n = 7 /group) served as controls. The trunk blood was collected and the hypothalami (n = 6) were isolated for assessment of CRH expression immediately after immobilization. One group of WT mice (n = 7) and NK1R-/- mice (n = 7) were immobilized for 120 min and then returned to their home cages for 120 min prior to blood collection and isolation of the hypothalami (see above).

Effects of immobilization stress on 5-HT utilisation in the raphe nuclei and individual forebrain nuclei

WT and NK1R-/-mice (n = 7 – 8/group) either remained in their home cages or were immobilized for 15 min. Brains from the control mice and mice exposed to immobilization stress were quickly removed from the skull, frozen on dry ice and stored at –80°C. The brains were then cut in a cryostat and processed as above. The individual brain nuclei were used for quantification of 5-HT and 5-HIAA.

Chemicals

5-hydroxytryptamine hydrochloride, 5-hydroxyindolacetic acid, 3,4-dihydroxybenzylamine hydrobromide, heptansulfonic acid, trimethylamine were obtained from Sigma – Aldrich (Taufkirchen, Germany). NSD 1015 was dissolved in distilled water (50 mg/ml). All other chemicals were purchased from Merck (Darmstadt, Germany).

Statistical Analyses

The values are expressed as the means ± SEM. The distribution of the data was analysed by Kolmogorov-Smirnov test and Bartlett´s test was used to test the homogeneity of variances. The statistical evaluation of changes in ACTH and corticosterone in plasma was carried out by two-way analysis of variances (ANOVA) with repeated measures (within factor time and between factor group, WT versus NK1R-/- mice), followed by post-hoc multiple pairwise comparisons (Hochberg adjusted). Statistical analysis of the data on 5-HT and 5-HIAA in individual brain regions was carried out by one-way ANOVA followed by a post-hoc Bonferroni test for pairwise comparisons. Statistical significance was accepted at $P<0.05$.

Results

Effects of immobilization stress on plasma ACTH and corticosterone concentrations and CRH expression in the hypothalamus

Basal plasma ACTH concentrations in WT and NK1R-/- mice were indistinguishable. As expected, immobilization stress induced an immediate and sustained increase in plasma ACTH concentrations, which was markedly higher in NK1R-/- mice than in WT mice (time effects: $P<0.001$; interaction: $P = 0.002$; group effect: $P=0.003$). Compared to WT mice, the ACTH response in NK1R-/- mice was 2.6- fold higher after 15 min- and 4-fold higher after 120 min of immobilization (Fig. 1, upper panel). After the termination of immobilization stress, ACTH

concentrations in plasma declined to baseline values over the following 2 h when animals were returned to their home cages (Fig. 1, upper panel).

Plasma corticosterone concentrations increased to the same extent in WT and NK1R-/- mice and remained elevated for 2 h after returning animals to their home cage(time effect: $P<0.0001$; interaction: $P=0.705$; group effect: $P=0.182$) (Fig.1, middle panel). The identical corticosterone responses to acute stress in WT and NK1R-/- mice are consistent with a previous report (Delgado-Morales et al. 2012). Surprisingly, only slight alterations in CRH expression in the hypothalamus were detected after stress. CRH mRNA appeared to increase in both WT- and NK1R-/- mice exposed to 2 h of immobilization followed by a 2 h rest period, however this was not significant compared with basal CRH mRNA levels.

Effects of immobilization stress on 5-HT utilisation in the raphe nuclei

Basal 5-HT utilisation in the DRN was elevated in NK1R-/- compared with WT mice as evidenced by increased concentrations of 5-HT (ANOVA $F_{3,24}= 12.961$; $P<0.001$) and 5-HIAA (ANOVA $F_{3,24}= 10.222$; $P<0.001$) (Fig. 2, upper panel). Stress did not alter 5-HT concentration in the DRN of WT mice but further increased the 5-HT concentration in NK1R-/- mice. Immobilization resulted in significantly elevated 5-HIAA concentrations in both WT and NK1R-/- mice. In NK1R-/- mice, stress was associated with an additional rise in 5-HIAA concentrations (Fig. 2, upper panel). Non-significant changes were observed in 5-HIAA/5-HT ratios, with parallel changes in the concentrations of both indoles (WT basal: 0.95 ± 0.06; WT stress: 1.07 ± 0.09; NK1R-/-basal: 0.89 ± 0.06; NK1R-/- stress: 0.79 ± 0.03). Stress had no effect on 5-HT concentrations in the MRN (ANOVA $F_{3,24}= 1.486$, $P>0.05$). Both basal and stress-induced 5-HIAA concentrations in NK1R-/- mice were significantly higher than those in WT mice (ANOVA $F_{3,24}= 5.383$; $P<0.01$) (Fig. 2, middle panel). Immobilization of WT mice resulted in a higher 5-HIAA/5-HT ratio in WT mice (ANOVA $F_{3,24}=4.742$; $P<0.01$; WT basal: 1.09 ± 0.07; WT stress: 1.49 ± 0.11, $P<0.05$). Minor changes in 5-HT concentrations associated with a marked rise in 5-HIAA resulted in relatively higher 5-HIAA/5-HT ratios under basal and stress conditions in NK1R-/- mice, the difference was not statistically significant (NK1R-/- basal: 1.42 ± 0.12; NK1R-/- stress: 1.54 ± 0.03). No significant alterations in 5-HT turnover under stress were detected in the nucleus raphe magnus of either, WT or NK1-/- mice (Fig. 2, lower panel).

Effects of immobilization stress on 5-HT utilisation in corticolimbic and hypothalamic nuclei

Compared with basal levels, immobilization produced a marked increase in 5-HT utilization in the frontoparietal cortex (FPC), medial prefrontal cortex (mPFC) and central nucleus of the

amygdala (CeA). The concentrations of 5-HT and 5-HIAA rose markedly and similarly in WT and NK1R-/- mice (FPC: 5-HT, ANOVA F_{324}=15.024, P<0.001; 5-HIAA, $F_{3,24}$=17.831, P<0.001; mPFC: 5-HT, $F_{3,24}$=8.697, P<0.001; 5-HIAA, $F_{3,24}$=14.016, P<0.001; CeA: 5-HT, $F_{3,24}$= 8.737, P<0.001; 5-HIAA, $F_{3,24}$= 6.428, P<0.01) (Table 1). Immobilization did not alter 5-HT or 5-HIAA concentrations in the medial nucleus of the amygdala (MeA). However, a small decrease in 5-HT concentration and parallel non-significant elevation of 5-HIAA in stressed WT mice resulted in a significant rise in the 5-HIAA/5-HT ratio. Stress failed to affect 5-HT turnover in the CA3 region, but elevated 5-HIAA concentrations were observed in the CA1 region in WT and NK1R-/- mice exposed to stress (Table 1). The data in Table 2 illustrates that stress did not change 5-HT and 5-HIAA concentrations in the PVN (5-HT, ANOVA $F_{3,24}$= 2.174, P = 0.118; 5-HIAA, $F_{3,24}$= 2.53, P = 0.133) or in the anterior hypothalamic nucleus (AHN) (5-HT, $F_{3,24}$= 2.797, P = 0.06; 5-HIAA, $F_{3,24}$= 1.673, P = 0.19), nor did it alter the ratios 5-HIAA/5-HT. In both WT and NK1R-/- mice, stress produced similar and marked increases in 5-HT and 5-HIAA concentrations in the ventromedial hypothalamic nucleus (VMN) (5-HT, ANOVA $F_{3,24}$= 16.288, P<0.001; 5-HIAA, $F_{3,24}$= 19.220, P<0.001) and dorsomedial hypothalamic nucleus (DMN) (5-HT, ANOVA $F_{3,24}$= 10,621, P<0.001; 5-HIAA, $F_{3,24}$= 28.06, P<0.001). No significant changes in the ratio 5-HIAA/5-HT were observed as the increases in 5-HT and 5-HIAA concentrations were almost identical (Table 2).

Discussion

Genetic deletion of the NK1 receptor dramatically augmented the ACTH response to stress, which was associated with minor changes in CRH expression in the hypothalamus. Absence of the NK1 receptor enhanced basal 5-HT turnover only in the DRN and stress further augmented the increased 5-HT utilisation in this region. Exposure of WT- and NK1R-/- mice to stress caused similar increases 5-HT turnover in the MRN, VMN and DMN of the hypothalamus, the CeA, hippocampal CA1, and cortical regions.

Our present data strongly indicates that activation of NK1 receptors in the brain inhibits ACTH release during acute stress, and this is consistent with previous evidence for an inhibitory role of SP in the regulation of the HPA axis. (Unger et al. 1988, Chowdrey et al. 1990; Larsen et al. 1993; Jessop 1999 for review). We report here that the basal and the post-stress concentrations of ACTH in plasma, and, even more importantly, the basal expression of CRH in WT and NK1R-/- mice are identical. These results clearly demonstrate that the inhibitory action of the SP on the HPA axis only operates during stress exposure and indicates that SP does not exert a sustained,

long-lasting inhibition of the HPA axis. A direct inhibitory action of SP on CRH neurones is unlikely since SP is an excitatory neurotransmitter, and hence an inhibitory neurotransmitter such as GABA may mediate inhibition of the stress-related activation of CRH neurones. It is notable that CRH neurons receive more than 2/3rds of all the GABAergic synaptic input to the parvocellular part of the PVN (Boudaba et al. 1996; Miklos and Kovacs, 2002; Cullinan et al. 2008 for review). Accordingly, we propose that SP exerts its inhibitory effects on CRF neurones via activation of the inhibitory GABAergic neurones located in the close vicinity of the PVN or adjacent hypothalamic areas. In contrast to the present findings, there is other evidence pointing to a stimulatory role of SP in the regulation of the HPA axis (Ebner et al. 2008; Culman et al. 2010). Analysis of the available data suggests that SP in the hypothalamic neuronal circuits robustly inhibits the immobilization stress-induced activation of the HPA axis, while SP acting within neuronal systems in the septal area and brainstem, which initiate and control central responses to stress, instead provides an excitatory input to CRH neurones. The interplay between the excitatory and inhibitory actions of SP in different neuronal systems involved in stress responses results in a complex modulation of HPA activity depending on the nature, duration and severity of exposure to stressful stimuli. It is noteworthy that chronic treatment with the SSRI, fluoxetine, did not alter the HPA axis response to acute stress (Stout et al. 2002), The latter finding indicates that serotoninergic system is unlikely to play a role in the exaggerated HPA axis response to stress observed in NK1R-/- mice.

Serotoninergic fibres innervating cortical and limbic areas and the hypothalamus originate in the DRN and MRN, containing high concentrations of 5-HT in intracellular vesicles and in the extracellular space (Sawchenko et al. 1983; Lowry, 2002; Adell et al. 2002). Compared to WT mice, higher rates of 5-HT synthesis and degradation were detected in NK1R-/- mice under basal conditions and stress in the DRN, as evidenced by increased 5-HT and 5-HIAA, and also in the MRN, where only 5-HIAA was elevated. Genetic deletion or pharmacological blockade of NK1 receptors resulted in a desensitisation of inhibitory 5-HT_{1A} autoreceptors, thereby allowing an increase in the basal firing rate of 5-HT neurones (Froger et al, 2001; Valentino et al. 2003; Valentino and Commons, 2005; Blier et al. 2004: Guiard et al. 2007). The accelerated 5-HT synthesis and degradation in non-stressed NK1-/- mice also appears to be consistent with the enhancement of serotoninergic neurotransmission and firing of DRN neurones previously reported in NK1R-/-mice and animals treated with NK1 receptor antagonists(Santarelli et al. 2001; Conley et al, 2002). These findings indicate that endogenous SP mediates an inhibitory effect on serotoninergic neurons in the DRN. As stress further enhanced the already increased 5-HT turnover in the DRN and, to some extent, in the MRN of NK1R-/- mice, it is reasonable to

assume that NK1 receptor deletion reduces the inhibitory activity of neurons in the DRN that are activated under stress, such as GABAergic interneurones (Commons and Valentino, 2002). In addition, other neurotransmitters, e.g. noradrenaline acting on α1 adrenoceptors in the DRN, may contribute to the stress-induced enhancement of 5-HT turnover in NK1-/- mice. Experimental data suggests that chronic blockade of NK1 receptors increases burst firing of neurones in the locus coeruleus, the source of the noradrenergic input to the DRN, and noradrenaline release from nerve terminals (Maubach et al. 2002; Guiard et al. 2004).

To our knowledge, the effects of stress on 5-HT metabolism in individual brain nuclei have not previously been studied in mice. The determination of 5-HT and 5-HIAA concentrations ex vivo and the ratio of 5-HIAA/5-HT provides a good index of the overall turnover rate and release of 5-HT (Smythe et al. 1983; Broadhurst and Briley, 1988). These measurements can provide an estimate on 5-HT release especially in small brain regions, e.g. individual hypothalamic or amygdaloid nuclei in which the 5-HT overflow cannot be assessed using microdialysis. Deletion of NK1 receptors had no effect on basal 5-HT metabolism in any of the areas studied. In line with previous findings, acute stress resulted in a significant rise of 5-HT and/or 5-HIAA concentrations in cortical regions and certain hypothalamic nuclei. In previous studies, accelerated utilisation of 5-HT was observed in terminal fields of 5-HT projections derived from distinct subdivisions of DRN and MRN activated by stress (Lowry, 2002; Waselus et al. 2011). It is believed that the neuronal activity and firing of the DRN and MRN neurones controls the release of 5-HT in the forebrain. In the present study, although considerably higher rates of 5-HT synthesis and degradation were detected in the DRN and MRN of NK1R-/- mice, this was not associated with a corresponding acceleration of 5-HT turnover in any forebrain region studied. Presumably 5-HT transmission in the forebrain need not explicitly depend on the firing rate of DRN neurones. Alternatively, deletion of the NK1 receptor may cause activation of local neuronal circuits preventing an exaggerated 5-HT release in forebrain areas under stress.

The present findings of the accelerated 5-HT utilisation together with increases in the extracellular tissue levels of 5-HT efflux in the mPFC and the CeA induced by stress (Mo et al. 2008; Mahar et al. 2014 for review) suggest a role for 5-HT in the integration of behavioural, emotional and adrenocortical components of stress responses in the corticolimbic areas (Dalley et al. 2004; Morgane et al. 2005; Goldstein et al. 1995). Although SP in the MeA was reported to modulate anxiety-related behaviour in response to stress, we have not observed any alteration in 5-HT utilisation in this area (Ebner et al. 2004). The accelerated 5-HT utilisation observed in the CA1 region is compatible with the increased activity of the raphe-hippocampal serotonergic system resulting from a transient rise in glucocorticoid concentration elicited by stress (Meijer

and de Kloet, 1998). Interestingly, antidepressant treatment or electroconvulsive shock also activates serotonergic neurotransmission in the hippocampus (Dremencov et al. 2003).
Activation of 5-HT neurotransmission in the hypothalamus characterised by increased rates of 5-HT synthesis, release and degradation upon stress has been repeatedly reported (Culman et al. 1980 and 1984; Lowry et al.2003; Shimizu et al. 1992). Consistent with previous studies, stress enhanced 5-HT and 5-HIAA concentrations in the VMN and DMN (Culman et al. 1980; Lowry et al.). Both nuclei have been implicated in neuroendocrine and autonomic homeostasis and in a broad array of behavioural functions, including affective behavior and ingestive and sexual behaviours (Canteras et al. 1994: Bernardis and Bellinger, 1998). SP in the DMN provides an excitatory input to the PVN mediated by NK1 receptors (Womack and Barret-Jolley, 2007).
In the present study, immobilization stress did not alter 5-HT neurotransmission in the PVN. This somewhat surprising finding was not entirely unexpected. In our previous studies in rats, we have never observed any stress-responsive alterations in 5-HT metabolism in the PVN (Culman et al. 1980; Culman et al. 1984). Hence, the stress-induced alterations in 5-HT release and utilisation may only occur in the dorsal medial parvocellular part of the PVN, where CRF neurones projecting to the median eminence are located (Swanson 1987). The isolation technique and the analytical method used to measure 5-HT and 5-HIAA concentrations did not allow determination of indoles in the subdivisions of the PVN, but only in the whole nucleus comprising both the parvocellular and magnocellular parts.
A few lines of evidence suggests that the antidepressant actions of NK1 receptor antagonists may, at least in part, result from increased 5-HT neurotransmission. First, blockade or deletion of NK1 receptors increase firing rates and neuronal activity of DRN in a manner resembling the effects of SSRI treatment. Second, blockade or genetic deletion of NK1 receptors has been demonstrated to potentiate the effects of SSRIs on 5-HT overflow in forebrain regions, suggesting that combining SSRI and NK1 receptor antagonists may be synergistic (Haddjeri and Blier, 2001; Santarelli et al. 2001; Froger et al. 2001; Guiard et al. 2004, 2005; Gobert et al. 2009). However, a key question needs to be answered whether or not NK1 receptor antagonists alone, similarly to SSRI, increase 5-HT neurotransmission. To our knowledge, neither selective blockade nor genetic deletion of NK1 receptors alter *basal extracellular levels of 5-HT* in forebrain regions (David et al. 2004; Gobert et al. 2009; Guiard et al. 2006 for review). We demonstrate here that genetic deletion of the NK1 receptor had no impact on the basal or stress – related 5-HT metabolism in corticolimbic regions implicated in pathophysiology of depression. In our view, these findings do not support the notion that the antidepressant and anxiolytic

actions of NK1 receptor antagonists are dependent on increasing serotoninergic neurotransmission.

Methodological considerations

Genetic deletion of the NK1 receptor may result in compensatory events, such as altered neuronal activity of other neurotransmitter systems and circuits. Secondly, a detailed description of DRN-forebrain collaterals in term of stress-related circuitry has been established and functional subsets of serotonergic neurons in the DRN controlling the HPA axis have been defined (Waselus et al. 2011; Lowry 2002). The microdissection technique used in the present study did not allow isolation of individual subdivision of the DRN and MRN. Moreover, the concentrations of 5-HT and 5-HIAA in individual subsets of the DRN and MRN would have been under the detection limit of the used analytical method. The same considerations apply to the PVN (see above).

In summary, genetic disruption of the NK1 receptor remarkably augmented the ACTH response to stress. Our data provides indirect evidence that SP activates neuronal circuits in the forebrain which inhibit CRF release in acute immobilization stress. SP acting on NK1 receptors does not exert a sustained, long-lasting inhibition over the HPA axis; the inhibitory actions are more likely operating only during the stress exposure. Genetic deletion of the NK1 receptor enhanced the basal 5-HT turnover only in the DRN and MRN. Exposure of NK1R-/- mice to stress augmented the accelerated 5-HT utilisation the serotonergic cell groups, but not in the amygdala, hippocampus and cortical regions linked to the pathogenesis of anxiety and depression. Our present findings do not support the assumption that NK1 receptor antagonists alleviate depression by enhancing 5-HT neurotransmission in forebrain regions.

Legends to the figures:

Fig. 1. ACTH- (upper panel), corticosterone (middle panel) concentrations in plasma of wild-type (NK1R+/+) mice (empty circles) and NK1R-knock-out (NK1R-/-, WT) mice (solid circles) exposed to acute stress. Values are presented as the means ± SEM. * $P<0.05$; ** $P<0.01$ and ***$P<0.001$, statistical comparison to the basal value within the same group. † $P<0.05$ and †† $P<0.01$, statistical comparison to the control, wild-type mice at the respective time point, calculated by two-way analysis of variances (ANOVA) for repeated measurements followed by post-hoc multiple pairwise comparisons (Hochberg adjusted). Lower panel: CRH mRNA in the hypothalamus of NK1R+/+ (control) mice (empty columns) and NK1R-/- mice (solid columns). CRH mRNA levels in both, WT- and NK1R-/- mice exposed to immobilization were not significant compared with basal CRH mRNA levels.

Fig. 2. Effect of acute stress on serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) concentrations in the dorsal raphe nucleus (DRN) (upper panel), median raphe nucleus (MRN) (middle panel) and nucleus raphe magnus (NRM) (lower panel) in wild-type (NK1R+/+) mice and NK1R knock-out (NK1R-/-) mice. Values are presented as the means ± SEM. *$P<0.05$ and ** $P<0.01$, statistical comparison to the appropriate value detected in control, non-stressed NK1R+/+ and NK1R-/- mice, respectively. † $P<0.05$ and †† $P<0.01$, statistical comparison to the appropriate value detected in non-stressed or stressed NK1R+/+ mice, calculated with one-way ANOVA followed by a post hoc-Bonferroni test.