THE ROLE OF THE CHEMOKINE SDF-1 AND ITS RECEPTORS CXCR4 AND CXCR7 IN THE MIGRATION OF GnRH NEURONS

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I, Fani Memi, confirm that the work presented here in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Reproduction in mammals is initiated and maintained by a small population of cells called Gonadotropin-releasing hormone (GnRH) neurons, scattered throughout the preoptic area and anterior hypothalamus. These neurons originate in the nasal placode, and migrate across the nasal compartment in association with olfactory, vomeronasal and terminal nerves to reach their targets in the hypothalamus. In humans, defective GnRH neuron migration results in gonadal dysfunction and subsequent infertility. As mutated genes identified so far in patients account for only 30% of the cases, many unknown genes involved in GnRH neuron development still need to be discovered.

One of the molecules required for their early migration is the chemokine SDF-1 which is expressed in the embryonic nasal mesenchyme in an increasing rostral to caudal gradient, presumably guiding CXCR4-expressing GnRH neurons towards the forebrain. Mice lacking CXCR4, the receptor for SDF-1, exhibit defective GnRH neuron migration along with a significant reduction in number. This thesis focuses on the role of the more recently identified second SDF-1 receptor, CXCR7, in GnRH neuron development. A detailed analysis of the expression pattern of CXCR7 in the nasal region and comparison to that of its agonist (SDF-1) as well as CXCR4, was elucidated for the first time. CXCR7 was found to be expressed along the migratory path of GnRH neurons in the nasal region, but not by GnRH neurons or their guiding axons. The role of CXCR7 in GnRH neuron migration *in vivo* was assessed using transgenic mice deficient for this receptor. This analysis revealed that in these mice, many GnRH cells

remained in the nasal compartment, clustering or found ectopically in the olfactory epithelium. Interestingly, CXCR4 was downregulated in CXCR7 defective mice, suggesting that CXCR7 affects GnRH migration indirectly, by regulating CXCR4 in a non-cell autonomous manner.

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Abbreviations

µg	Microgram
µm	Micrometer
µM	Micromolar
Ab	Antibody
AZ	Apical zone
BAC	Bacterial artificial chromosome
BSA	Bovine serum albumen
BV	Blood vessel
BZ	Basal zone
cAMP	cyclic adenosine monophosphate
CB	Cavernous body
CC	Cartilaginous capsule
CP	Cribriform plate
CXCL12	Chemokine C-X-C motif ligand 12
CXCR4	Chemokine C-X-C motif receptor 4
CXCR7	Chemokine C-X-C motif receptor 7
D	Dorsal
DAB	diaminobenzidinetetrachloride
DAPI	4',6-diamido-2phenylindole
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
E	Embryonic day

EGFP	Enhanced green fluorescent protein
Epith	Epithelium
FB	Forebrain
FSH	Follicle stimulating hormone
GAP	GnRH-associated peptide
GBC	Globose basal cell
GnIH	Gonadotropin-inhibitory releasing hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GS-IB4	Griffonia Simplicifolia isolectin type B4
HBC	Horizontal basal cell
HET	Heterozygote
HH	Hypogonadotropic Hypogonadism
HPG	Hypothalamic pituitary gonadal
IHH	Idiopathic hypogonadotropic hypogonadism
III	Third ventricle
ISH	*In situ* hybridization
IZ	Intermediate zone
KO	Knockout
KS	Kallmann's syndrome
LH	Luteinizing hormone
M	Medial
ME	Median eminence
MPOA	Medial preoptic area

NC	Neural crest
NFJ	Nasal/forebrain junction
NGS	Normal goat serum
NM	nasal mesenchyme
NP	Nasal placode
OB	Olfactory bulb
OE	Olfactory epithelium
Olf	Olfactory
ORN	Olfactory receptor neuron
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Triton
PFA	Paraformaldehyde
RT	Room temperature
SDF-1	Stromal cell-derived factor 1
SEM	Standard error mean
Sus	Sustentacular
SV40	Simian virus
UTR	Untranslated region
VNN	Vomeronasal nerve
VNO	Vomeronasal organ
WT	Wild-type

Chapter 1. Introduction

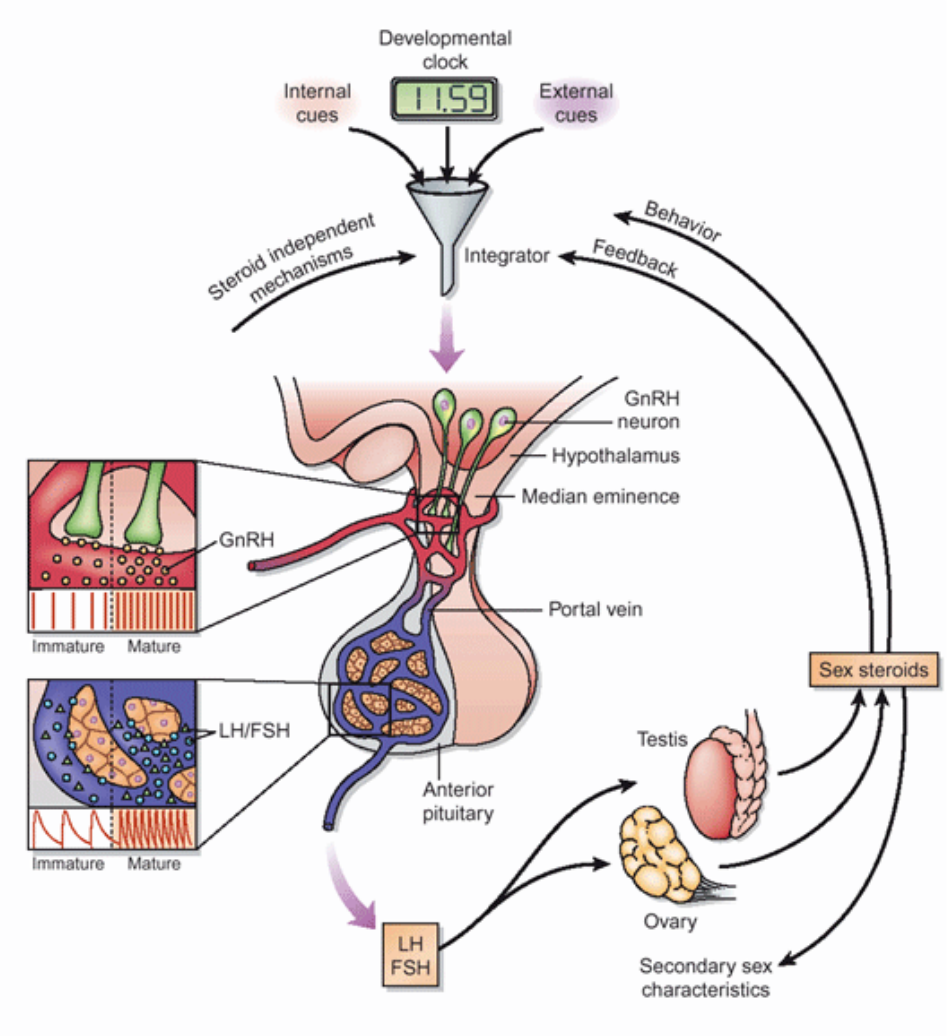
1.1 General concepts

The journey from childhood to adulthood requires crossing the Rubicon of puberty. Puberty comprises the startling physiological and behavioural transformations that occur during the transition from juvenile life into sexual maturation and reproductive competence (McCarthy 2013). The physical process, which takes years in humans and few days in rodents, is the outcome of a finely orchestrated hormonal milieu that induces growth and completes programmes of sexual differentiation begun long before *in utero*. The onset of puberty in mammals is usually determined as the activation (or in some species, reactivation) of the neuroendocrine reproductive axis, which typically occurs earlier in females than in males; despite its physiological importance, the onset of puberty in mammals is characterized by a poorly understood mix of genetics and environment (Kauffman, 2010). Studies in the past four decades have established that puberty is a product of increased activity of the hypothalamic-pituitary-gonadal axis leading to production of gonadal steroids and other growth-associated hormones (such as prolactin). Contrary to popular belief, however, puberty is not a simple developmental progression of maturation of the reproductive axis, as this system is fully mature long before puberty and, in fact, must be suppressed during a period called the juvenile hiatus (McCarthy, 2013). The crucial component of the axis is the gonadotropin-releasing hormone (GnRH) neuron. Without these neurons, there is no vertebrate reproduction. GnRH neurons reside almost exclusively in

the preoptic area and hypothalamus, and project to the median eminence, where they release GnRH in a pulsatile manner that promotes luteinizing hormone release from the anterior pituitary; luteinizing hormone in turn reaches the gonad and promotes steroidogenesis (Figure 1.1) (Colledge et al., 2010). All of these components— the GnRH neurons, the pituitary and the gonads— are fully mature very early in life and even, in the case of male, capable of steroidogenesis *in utero* (McCarthy, 2013).

Figure 1.1 Regulation and action of the Hypothalamic-Pituitary-Gonadal axis

GnRH neurons projecting to the median eminence direct pituitary gonadotropin and gonadal steroid hormone secretion. Sex steroids promote secondary sex characteristics in peripheral tissues, regulate GnRH neurons via a neuroendocrine feedback loop and facilitate social behaviours by acting on central neural circuits. The pubertal increase in GnRH neuronal activity and episodic gonadotropic secretion is grossly timed by a developmental clock and fine-tuned by the neural integration of multiple permissive internal and external signals. At the onset of puberty, steroid feedback and steroid-independent neural mechanisms are engaged to disinhibit and excite GnRH neurons. (Adopted by Sisk and Foster 2004)


Developmental clock
11.59
Internal cues
External cues
Steroid independent mechanisms
Integrator
Behavior
Feedback
GnRH neuron
Hypothalamus
Median eminence
GnRH
Portal vein
Immature
Mature
LH/FSH
Immature
Mature
Anterior pituitary
Sex steroids
Testis
Ovary
LH
FSH
Secondary sex characteristics

1.2 Regulation and action of the Hypothalamic-Pituitary-Gonadal axis

The Hypothalamic-Pituitary-Gonadal axis is composed of three major hierarchical components: GnRH from the hypothalamus, the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary, and the sex steroids and other hormone products of the gonads (Colledge et al., 2010). Puberty is initiated by neuroendocrine events that activate the pulsatile release of GnRH into the hypophyseal portal blood system. After release from the hypothalamus, GnRH acts on the pituitary to stimulate the release of LH and FSH, which then induce steroid production and gametogenesis in the gonad (Figure 1.1).

Following its release in the portal vessels, the decapeptide binds to GnRH receptors present on the pituitary gonadotropes and initiates gene expression of α- and β-chains of LH and FSH, and promotes their secretion by inducing inositol-1,4,5,-triphosphate (IP3) and diacylglycerol (DAG) generation, resulting in the mobilization of intracellular calcium and protein kinase C (PKC), respectively (Fink et al., 2012)

LH binds to specific G-protein coupled receptors on the surface of Leydig cells of the testis and the thecal cells of the ovary, activating adenyl cyclase and generating intracellular second messenger cAMP which, acting via protein kinase A (PKA), leads to gonadal steroid biosynthesis. LH influences the conversion of cholesterol to pregnenolone, the most critical step in testosterone biosynthesis (Fink et al., 2012).

FSH binds to specific receptors on Sertoli cells in the testis and granulosa cells in the ovary to stimulate folliculogenesis. FSH stimulates the activity of aromatase, an enzyme catalyzing the conversion of androgens to estrogens. Moreover, the hormones inhibin B and activin are formed in Sertoli and granulosa cells in an FSH-dependent manner. On one hand, inhibin B exerts a specific negative feedback inhibition on pituitary FSH secretion. On the other hand, both activins A and B stimulate FSH secretion. Testosterone and estradiol inhibit FSH either directly or through GnRH suppression (Fink et al., 2012)

Recently, another peptide, the gonadotropin-inhibitory releasing hormone (GnIH) has been reported to inhibit gonadal function at multiple levels, including the hypothalamus, pituitary and gonads (Bentley et al., 2009). Moreover, studies trying to elucidate the mechanisms that control GnRH neuronal activity brought to light a group of neurons that express kisspeptin, an essential molecule that activates GnRH secretion (Kauffman 2010). A most recent study involves, for the first time, epigenetic mechanisms in regulation of the puberty onset (Lomniczi et al., 2013).

1.3 GnRH secretion deficiency results in hypogonadism

The significance of the GnRH system in human reproduction is evident by the severity of the condition caused by defects in proper secretion of the decapeptide. The most common syndrome caused by GnRH deficiency is Hypogonadotropic Hypogonadism (HH), a disorder of the HPG axis, characterized by gonadal failure and absent or delayed pubertal sexual maturation, resulting from absent or severely reduced circulating levels of LH

and FSH. Clinical signs also include micropenis and cryptorchidism in the male, and amenorrhoea in the female. Hypogonadotropic Hypogonadism may either be congenital or acquired, secondary to hypothalamic or pituitary lesions. Congenital HH may be isolated, or occur in association with craniofacial midline defects and in certain disorders such as the Prader-Willi syndrome (Fink et al., 2012). Congenital abnormalities leading to HH may result from diminished GnRH secretion, occurring in isolation either with normosmia (idiopathic HH) or in association with anosmia/hyposmia (Kallmann's Syndrome; KS). Most cases of isolated HH are idiopathic, with defects identified to date in more than 10 separate genetic loci, accounting for about 30-40% of the reported presentations. Multiple modes of inheritance have been described in familial cases, including autosomal dominant, autosomal recessive or X-linked recessive (Table 1.1). More recently, further evidence for a genetic basis of normosmic of IHH has come to light through homozygosity mapping in consanguineous families, including the identification of a key role for kisspeptin in triggering GnRH release and of a critical role for neurokinin B (NKB) in normal sexual maturation.

The application of conventional linkage studies to investigate the genetic basis of HH has proven difficult, both because of its rarity and the infrequency of familial transmission; more importantly, pedigrees tend to be small since the majority of patients without treatment remain infertile. Three modes of inherited KS are described: X-linked (*KAL1*, OMIM:308700), autosomal dominant (*KAL2*, OMIM:147950) or autosomal recessive (*KAL3*, OMIM:244200), with only *KAL1* and *KAL2* cloned (Balasubramanian et al., 2010). Other genes

found mutated in cases with HH are *GnRHR1*, *FGF8* and *FGFR*, the gonadotropin β-subunits *(LH*β and *FSH*β*)*, *NELF*, pituitary transcription factors *(HESX1*, *LHX3*, and *PROP1)*, *DAX1*, leptin and its receptor *(LEP* and *LEPR)*, prohormone convertase 1 *(PC1)*, *GPR54*, and *KiSS1* (Table 1.1) (Balasubramanian et al., 2010, Bianco and Kaiser, 2009).

Table 1 | The genetic basis of idiopathic hypogonadotropic hypogonadism

Genes related to IHH	Gene product	Mode of inheritance	Cases for this specific form of IHH (%)	Cases of IHH overall (%)
Kallmann syndrome ~60% of total				
KAL1	Anosmin 1	X-linked	5–10	3–6
FGFR1	Fibroblast growth factor receptor 1	Autosomal dominant	10	6
FGF8	Fibroblast growth factor 8	Autosomal dominant	<5	<2
PROK2, *PROKR2*	Prokineticin 2, prokineticin receptor 2	Autosomal recessive	5–10	3–6
CHD7	Chromodomain-helicase-DNA-binding protein 7	NR	10	6
Unidentified	NA	NA	60–75	NR
Normosmic IHH ~40% of total				
GNRHR	Gonadotropin-releasing hormone receptor	Autosomal recessive	16–40	6.5–16
KISS, *KISS1R*	Kisspeptin 1, KiSS-1 receptor	Autosomal recessive	5	2
TAC3, *TACR3*	Neurokinin B, Neurokinin B receptor	Autosomal recessive	NR	NR
LEP, *LEPR*	Leptin, leptin receptor	Autosomal recessive	<5	<2
PCSK1	Neuroendocrine convertase 1	Autosomal recessive	<5	<2
Unidentified	NA	NA	~50	NR

IHH incidence in the population is 1–10 cases in 100,000 births. Patients with Kallmann syndrome have partial or total loss of sense of smell, whereas in normosmic IHH, sense of smell is not affected. In the unidentified group, the genetic basis of the IHH phenotype has not been identified. Abbreviations: IHH, idiopathic hypogonadotropic hypogonadism; NA, not applicable; NR, not reported.

Table 1.1 was adopted from Bianco and Kaiser,2009

1.4 The evolution of the GnRH gene

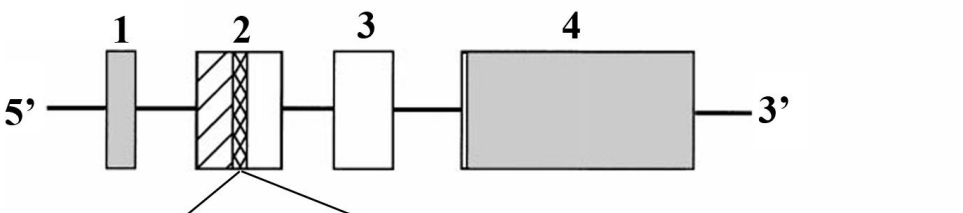
The first *GnRH* to be identified was the mammalian *GnRH* or *GnRH1*, with primary sequence invariant across mammals, amphibians and bony fish that evolved before the teleost fishes. The amino-acid residue sequence of the mammalian form of the decapeptide is: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 and is initially produced as a 92-amino acid pre-pro-GnRH, encoded by the *GnRH* gene. The GnRH preprohormone mRNA encodes GnRH and the GnRH-

associated peptide (GAP), separated by a canonical cleavage site. The preprohormone mRNAs are encoded by four exons (Figure 1.2). Exon 1 encodes the 5'-UTR exclusively. Exon 2 encodes the signal peptide, GnRH decapeptide, the proteolytic cleavage site, and the N-terminus of GAP. Exon 3 encodes the central portion of GAP, and exon 4 encodes the C terminus of GAP along with the 3'-UTR (Fernald and White, 1999). Post-translational cleavage by prohormone convertase-1 (PC1) of the leading 23 amino acids (AAs) and the supporting gonadotropin associated peptide (GAP) 56 AAs results in the decapeptide GnRH that is released into the hypophysial portal blood (Fernald and White, 1999, Sheward et al., 1985, Roch et al., 2011).

The only region of the GnRH prohormone that appears highly conserved across species is the GnRH decapeptide. The selective conservation of the GnRH decapeptide probably reflects its functional significance and thus, low tolerance for structural alterations during evolution. Characteristics that define vertebrate GnRH peptides include length, an N-terminal pyroglutamate, high conservation of the first four and final two amino acids, and an amidated amino acid at the C-terminus.

Figure 1.2 Schematic diagram showing the organization of GnRH gene.

Single hatched bars, signal sequence coding region; cross-hatched bars, GnRH-coding region; open bars, GnRH-associated peptide (GAP) coding region. Horizontal lines adjacent to exons represent introns. (Adapted from Fernald and White, 1999).



GnRH: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2

Once GnRHs were identified in non-mammalian vertebrates, sequence variations were discovered. GnRH peptides were classified into four types based on their sequence and location of expression. GnRH1 peptides are expressed primarily in the preoptic-hypothalamic area of vertebrates, whereas GnRH2 is consistently found in nuclei of the midbrain tegmentum (Fernald and White, 1999, Guilgur et al., 2006, Zohar et al., 2010). To date, 15 different vertebrate GnRH sequences have been identified; 9 are GnRH1s, 3 are GnRH2s, 1 is a single invariant GnRH3 and 2 are GnRH4 sequences. Most vertebrates express two or three of the different types of GnRHs. Mammals, birds, reptiles and amphibians express several distinct GnRH1 peptides and a common GnRH2s, whereas teleosts express GnRH2 as well as GnRH1 and GnRH3. In fish that express both GnRH1 and 3, the individual peptides are detected in distinct populations of neurons in the forebrain, extending from the olfactory bulbs to the hypothalamic region (Zohar et al., 2010, Guilgur et al., 2006). Both identified GnRH4s have been found only in the lamprey, an ancient jawless fish. The dominant function of GnRH in vertebrates, the activation of reproduction through the stimulation of pituitary gonadotropin secretion, is a function primarily of GnRH1 (referred in this thesis as GnRH). GnRH3 is the prime mediator in some teleosts, such as salmon and zebrafish (Roch et al., 2011).

There are relatively few GnRH cells (1200-1500) in the brain, and they do not form a tight grouping as seen for other neuroendocrine cells (Silverman et al., 1988, Wray and Hoffman, 1986a). In rodents, the vast majority of GnRH cells are found in the ventral preoptic area of the brain, with a smaller number scattered throughout the hypothalamus (Clarke, 1987). Given this position, the

question arose as to the relative importance in the control of reproduction. Original studies by two groups attempted to answer this question in non-human primates by electrolytic ablation of the neuronal population in the preoptic area, which caused loss of reproductive function that was subsequently restored (Clarke and Cummins, 1987, Norman et al., 1976).

1.5 Embryonic origins of GnRH cells

Despite their physiological importance, the location of the progenitors of the GnRH cells has not yet been clearly defined. In 1989, two laboratories demonstrated independently that GnRH cells of the mouse forebrain appeared to arise from the olfactory placode in the nose and migrate into the brain during embryonic development (Schwanzel-Fukuda and Pfaff, 1989, Wray et al., 1989a). Moreover, the number of GnRH cells found in the nose during prenatal development equals the number of GnRH cells found in brain postnatally. As development progresses, the number of GnRH cells in the nose reduces as the number of GnRH cells in brain concomitantly increases. Thus, it was proposed that the olfactory placode generated not only olfactory sensory neurons and support cells, but also GnRH neurons.

However, observations on transgenic mice and transplantation experiments on chick and zebrafish have suggested the olfactory placode, the anterior pituitary and neural crest (NC) cells as possible GnRH neuron generators (Daikoku and Koide, 1998, Daikoku-Ishido et al., 1990, Dellovade et al., 1998, Metz and Wray, 2010, Murakami and Arai, 1994, Murakami et al., 1992, Mulrenin et al., 1999, Suter et al., 2000, Palevitch et al., 2007, Whitlock et

al., 2003, Whitlock et al., 2006, Whitlock, 2004, Forni et al., 2011b, el Amraoui and Dubois, 1993, elAmraoui and Dubois, 1993). Recent studies excluded the adenohypophyseal lineage, as mice lacking the anterior pituitary display normal GnRH cell number (Metz and Wray, 2010), supporting the hypothesis that, in mammals, these neuroendocrine cells associate with the nasal placode.

Since during early development the nasal placode and cranial NC cells share a common border, originating from ectoderm near the neural plate, a mixture of NC and olfactory placode cells has been suggested (Couly and Le Douarin, 1985, Whitlock, 2004, Schlosser, 2010), implying a NC origin for GnRH neurons. Indeed, a most recent study in mouse revealed that GnRH neurons are derived from two different lineages, with the majority originating from ectodermal cells and the remaining fraction from the NC (Forni et al., 2011b). These data may explain the heterogeneity that has been described within GnRH-1 expressing neurons at both the morphological and molecular levels (Wray and Hoffman, 1986b, Jasoni et al., 2005, Cottrell et al., 2006, Constantin et al., 2009, Jasoni et al., 2009, Klenke et al., 2010). Furthermore, a number of genetic mutations in mouse selectively affect only ~30% of the GnRH neurons, providing further support for the heterogeneous nature of the population (Kramer et al., 2000, Kruger et al., 2004, Givens et al., 2005, Tsai et al., 2005, Giacobini et al., 2007, Cogliati et al., 2007, Miller et al., 2009, Forni et al., 2011a).

1.6 Development of GnRH neurons

In mice, the olfactory placodes develop as thickenings of the ectoderm on the ventrolateral sides of the head around E9.5. Following a series of invaginations,

the olfactory pits form, that later will give rise to the vomeronasal organ (medial olfactory pit) and the olfactory epithelium (dorsolateral olfactory pit). Accordingly, the vomeronasal nerve and GnRH neurons emerge from the vomeronasal organ and, from the olfactory epithelium, olfactory nerves develop. The time of origin of GnRH neurons was estimated within a narrow time frame, day 10 (not day 9 or 11) of embryogenesis (Schwanzel-Fukuda and Pfaff, 1989, Wray et al., 1989b). *In situ* hybridization studies (Wray et al., 1989a) revealed that the time of detection of GnRH mRNA, which makes these neurons distinguishable from their precursors, coincided with the immunohistochemical localization of GnRH at E11 (considering detection of a sperm plug as day of gestation). At about E11.5, the GnRH cells emerge from the epithelium, begin to migrate, organized in cords, in association with olfactory/vomeronasal nerve axons across the nasal septum (Schwanzel-Fukuda and Pfaff, 1989). On days 12 and 13, most of GnRH cells are present on the nasal septum (Figure 1.3). By E14, the majority of these cells are found crossing the cribriform plate and migrating into the forebrain, with migration virtually complete by E18 (Figure 1.4).

Figure 1.3 Sagittal section of an E14.5 mouse embryo head stained for GnRH.

GnRH-1 cells can be seen migrating from the VNO through the nasal forebrain junction (NFJ) into the forebrain. Asterisk shows location of inset. OB, olfactory bulb; NFJ, nasal/forebrain junction; VNO, vomeronasal organ; OE; olfactory epithelium; III, third ventricle (adapted from Wray, 2010).

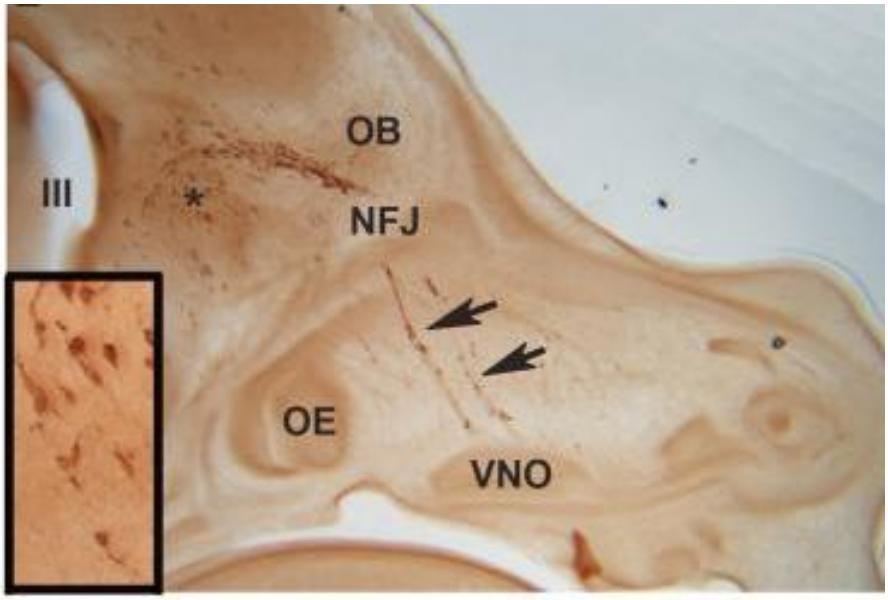
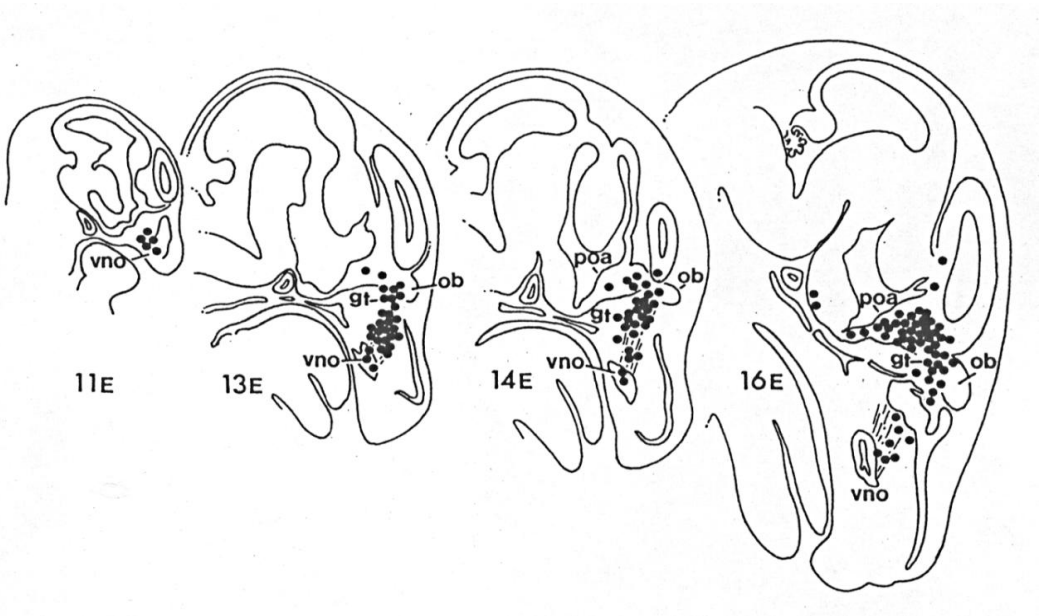

OB
III
NFJ
OE
VNO

Figure 1.4 Migratory route of GnRH neurons in the embryonic mouse brain.

GnRH neurons (black dots) originate in the medial wall of the olfactory placode, migrate along the olfactory axons and enter the brain by perforating the cribriform plate. They then migrate to reach their final destination in the preoptic area (poa) of the hypothalamus. At E11, these cells are seen in the vomeronasal organ (vno) and medial wall of the olfactory placode. In the 16-day old fetal mouse brain, most of the GnRH neurons have reached the hypothalamus. Gt, Ganglion terminale; ob, olfactory bulb. (Adopted from Schwanzel-Fukuda et al., 1989).


vno
11E
gt
ob
vno
13E
poa
ob
gt
vno
14E
poa
gt
ob
vno
16E

1.7 GnRH neuron migration

The underlying mechanisms that control the migration of GnRH neurons are not completely understood. However, recent studies have identified a plethora of factors that regulate different steps along their migratory route, revealing the high complexity of this system. Apart from molecules acting directly on GnRH neurons, many have been shown to influence their migration indirectly by altering the pace or targeting their guides, the olfactory axons. The migratory route of GnRH neurons can be divided into three distinct compartments:

i. After their birth in the olfactory placode, neurons migrate across the nasal mesenchyme along with peripherin-immunopositive olfactory and vomeronasal axons
ii. Cells cross the cribriform plate and enter the brain
iii. Cells continue their migration guided by a branch of vomeronasal nerve that turns caudally into the basal forebrain

Once they reach their appropriate destinations in the developing hypothalamus, they stop migrating, detach from their axonal guides and extend their axons to the median eminence, where they secrete the decapeptide.

A number of molecules have been found to control GnRH neuron migration in each compartment (Cariboni et al., 2007, Wierman et al., 2011) (Figure 1.5)

- Factors involved in the initiation of GnRH neuron migration include: a) adhesion molecules that enable them to associate with olfactory axons (Anosmin, Psa-NCAM); b) guidance molecules (EphA5, NELF); c)

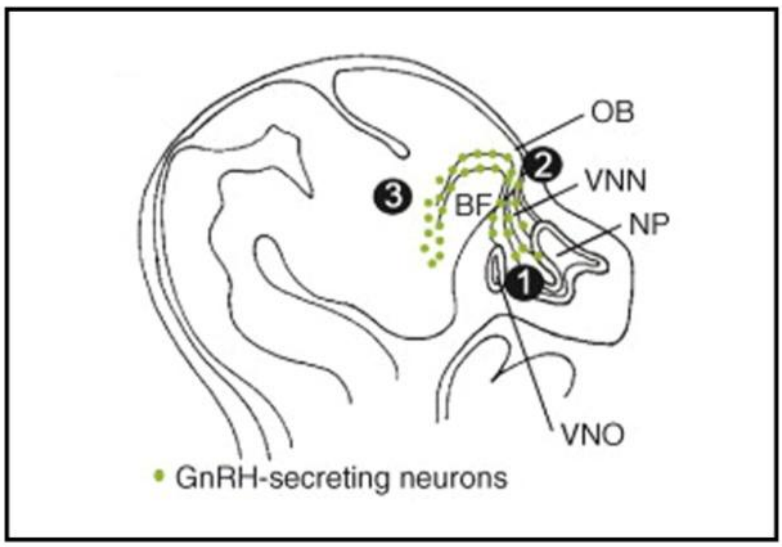
neurotransmitters (GABA); d) growth factors (FGF8); e) transcription factors (Ebf2); and f) chemokine SDF-1 and its receptor CXCR4.

- Factors involved in guiding vomeronasal nerve and GnRH neurons through the nasal compartment and towards the forebrain: Netrin-1 and its receptor DCC, lactosamine, Semaphorins and their receptors Neuropilins and Plexins, and Reelin.
- Factors that regulate GnRH neuron migration through the cribriform plate and onto the hypothalamus: Hepatocyte growth factor (HGF)/cMET, transcription factor Nhlh2.
- Factors that influence the cessation of migration, the extension of GnRH axons to the median eminence, and the establishment of neurosecretory contacts for hormone secretion: the recently identified kisspeptin and FGFR1.

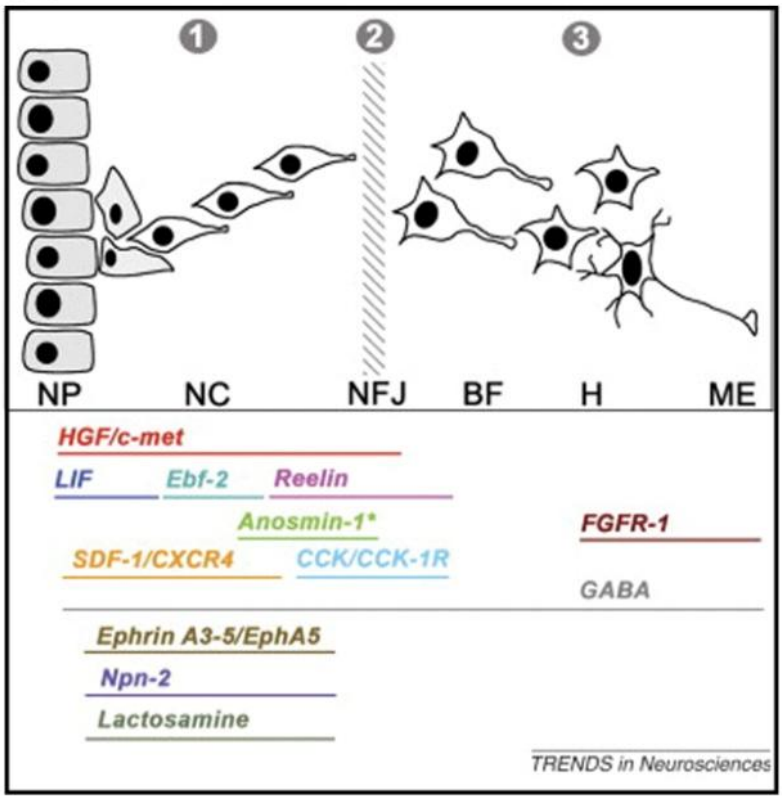
Figure 1.5 Factors that affect the migration of GnRH neurons through the three compartments.

Cartoon illustrating the movement of GnRH neurons from their origin in the nasal placode (NP), through the nasal compartment (NC), and their deflection at the level of the nasal-forebrain junction (NFJ) as they progress towards the basal forebrain (BF). Their migration finally terminates in the hypothalamus (H) from where they project to the median eminence (ME).

(a)


OB
VNN
BF
NP
VNO
GnRH-secreting neurons

(b)


NP
NC
NFJ
BF
H
ME
HGF/c-met
LIF
Ebf-2
Reelin
Anosmin-1*
FGFR-1
SDF-1/CXCR4
CCK/CCK-1R
GABA
Ephrin A3-5/EphA5
Npn-2
Lactosamine
TRENDS in Neurosciences

Included is a list of factors that have been shown to affect GnRH neurons at different stages of their journey. Anosmin-1 is the only human factor included in this list (*) (Adopted from Cariboni et al. 2007).

Since the initial descriptions of GnRH neuronal migration in mice, this process has been described in a number of different species. These include rats (Daikoku-Ishido et al., 1990), opossums (Cummings and Brunjes, 1995), chickens (Murakami et al., 1991, Norgren and Brackenbury, 1993, Sullivan and Silverman, 1993), fish (Chiba et al., 1994, Parhar et al., 1996), amphibians (Muske and Moore, 1988, Murakami et al., 1992) and primates (Ronnekleiv and Resko, 1990, Terasawa et al., 1993), including humans (Schwanzel-Fukuda et al., 1996, Boehm et al., 1994).

1. 8 Tools for GnRH neuron migration studies

GnRH neurons represent a small population of around a thousand cells dispersed widely from the nasal area to the forebrain, thus hampering their isolation. Additionally, the lack of naturally occurring tumors has made it difficult to establish immortalized GnRH cell lines to use as models. However, recent advances in molecular biology technologies together with the development of transgenic mice expressing oncogenes, allowed the creation of GnRH cell lines with different properties. Specifically, Mellon and colleagues (Mellon et al., 1990) created a transgenic mouse using the 5' flanking DNA of the rat GnRH gene to target expression of SV40 T-antigen in GnRH neurons. Two of these transgenic mouse lines developed anterior hypothalamic tumours which, when isolated for cell culture, resulted in the GT-1 cell population. One of the

three homogenous cell populations acquired, GT1-7, which secrete GnRH when depolarized, became one of the most utilised neuronal cell models for studies not only of GnRH cells, but also of basic neuronal functions. In a similar way, GN11 cells were developed from T-Ag driven tumours, but this time tumours developed in the olfactory placode and before cells migrate to the hypothalamus; thus, these cells are considered to be immature GnRH neurons (Radovick et al., 1991). Two cell lines were derived from these tumours, NLT and GN11 cells, exhibiting distinct phenotypic differences, as the former expressed GnRH at levels 10-fold higher than the latter, and are considered of a maturation stage between that of GN11 and GT1-7 cells.

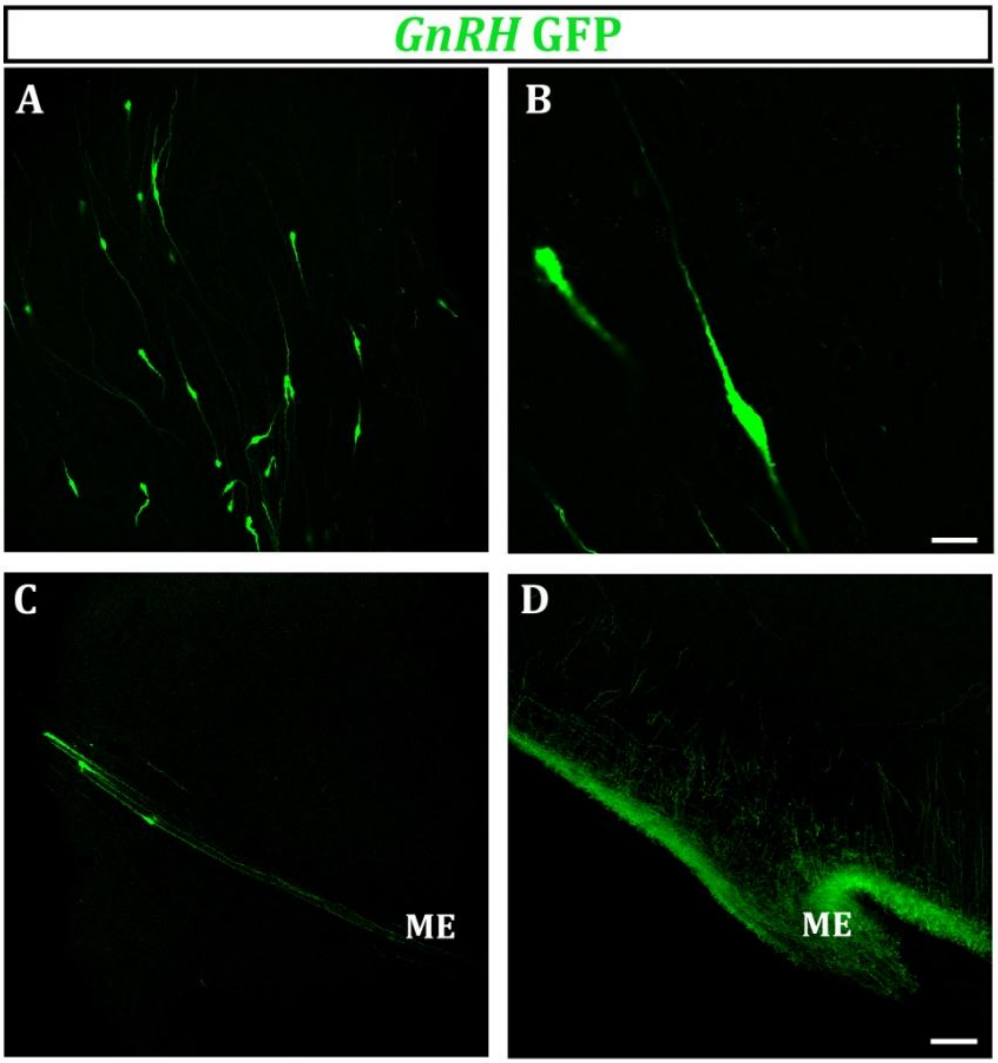
These cell lines are a very useful tool for the study of GnRH neurons as they represent unlimited homogeneous populations of specific neuronal cell types with the advantage of limiting the uncontrolled variables compared to the *in vivo* approaches, where cells receive input from numerous other neurons. As well, little is known about the molecular mechanisms involved in intracellular signalling, promoter regulation, regulation of gene transcription, or regulation of secretion in native neurons due to the complexity and difficulty of studying molecular events *in vivo*. The use of cell lines provides a simpler model to begin these investigations. Since few studies have been performed *in vivo*, researchers are unable to state whether neuronal cell lines function in a similar way to native neurons. For this reason, caution must be taken when extrapolating findings from the cell lines to the *in vivo* model. As well, cell lines lack the complexity and integrated network of neuronal connections and signalling. Despite these limitations, studies with cell lines can be used to understand the

in vivo model by pointing to lines of investigation, as well as confirming molecular events. So far, studies of hormonal regulation of gene expression or receptor activation suggest that cell lines reliably replicate existing *in vivo* studies (Mayer et al., 2009). *In vitro* models other than cell lines have been developed in order to study GnRH neuron migration in a more controlled experimental environment, but being more close to the *in vivo* situation than cell lines. These include nasal explants (Fueshko and Wray, 1994) and slice preparations from embryonic nasal compartment and brain (Tobet et al., 1996).

Transgenic mice with GnRH neurons labelled with a green fluorescent protein (GFP) (Figure 1.6) have been invaluable in allowing identification of these neurons, thus enabling the study of their morphology as well as the localization of molecules with dual-label immunofluorescence (Spergel et al., 1999, Suter et al., 2000). Furthermore, isolation of these GFP-labelled GnRH neurons permitted the study of their gene expression profiles (Jasoni et al., 2005).

Figure 1.6 GFP labelled GnRH neurons in adult rat brain sections.

A: GnRH neurons dispersed in the hypothalamus. B: Two of these neurons at higher magnification. C: GnRH neurons projecting to median eminence (ME). D: GnRH neuron axons in the ME. Scale bars: A,C,D, 100 μm; B: 10 μm.


GnRH GFP
A
B
C
ME
D
ME

1.9 SDF-1 in CNS development

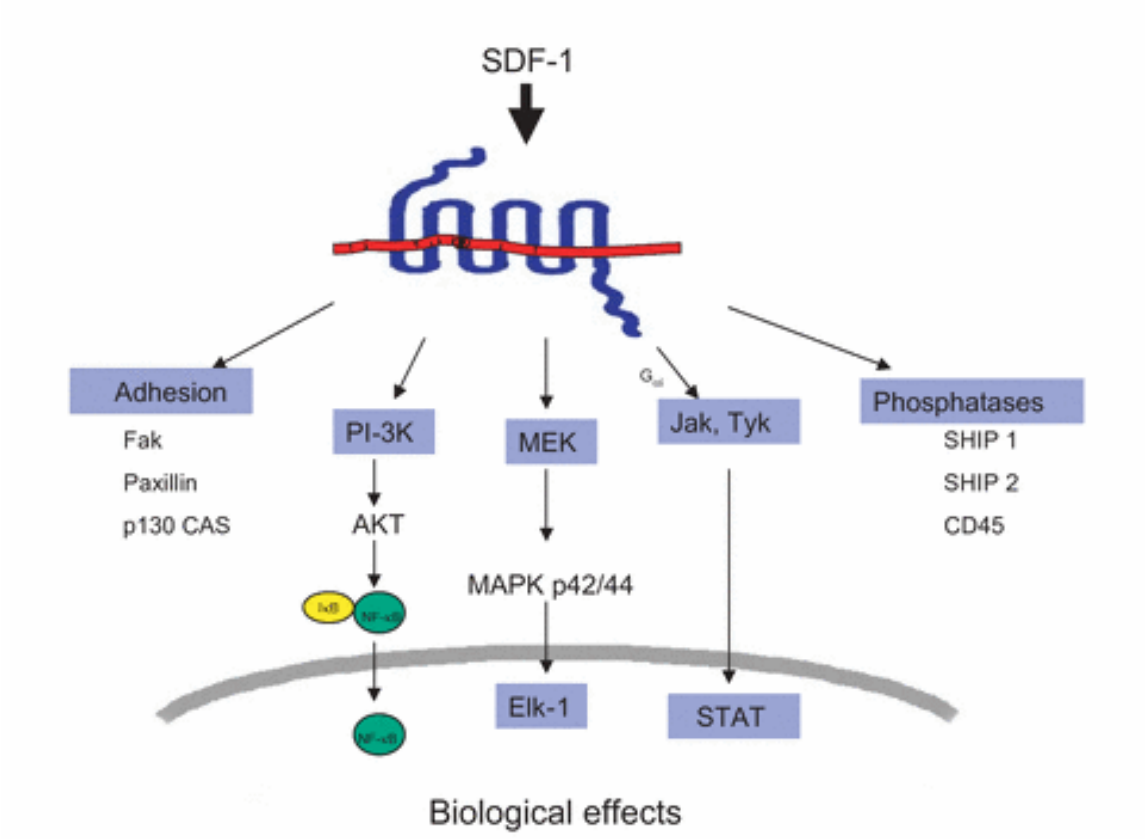
Guided cell migration holds a key role in brain development. Even slight alterations in the molecular regulation of the migration process lead to severe changes in brain morphology, connectivity and function, as exemplified by a variety of genetic diseases affecting brain structure and function in humans (Marin and Rubenstein, 2003). Over the past years, a variety of molecules that regulate the correct migration of neurons by acting as repellents or attractants and providing positional information have been identified. These include, for example, slit/robo, neuregulin/ErbB, GDNF/GFRalpha1 as well as semaphorin/neuropilin signalling complexes (Marin and Rubenstein, 2003, Tiveron and Cremer, 2008). Furthermore, G-protein-coupled receptor signalling activated by chemotactic cytokines (chemokines) is also employed for directed migration of neurons from specialized neurogenic regions to their final destinations (Stumm and Hollt, 2007, Borrell and Marin, 2006).

Chemokines are a superfamily of small, highly conserved, secreted molecules that were originally shown to play a crucial role in the control of leukocyte trafficking during inflammatory response and immune surveillance (Rot and von Andrian, 2004, Moser et al., 2004, Rossi and Zlotnik, 2000). However, it is now considered that they are involved in a wide range of processes including implantation of the embryo, development, cancer metastasis and pain regulation. They exert their function by binding to specific seven-span transmembrane receptors coupled to G-protein (GPCRs). Upon activation of such receptor, GTP replaces GDP in the α-subunit of the G-protein heterotrimer (Ga, β and γ) which, in turn, causes dissociation of this complex

from the receptor that is free now to trigger downstream signalling. Following binding of the chemokine to its receptor, desensitization and downregulation of the receptor occurs in many cases, suggesting a potential mechanism of regulation of chemokine signalling (Mithal et al., 2012). Specifically, the ligand/receptor complex can undergo endocytosis (Thelen, 2001, Neel et al., 2005, Borroni et al., 2010), with the ligand being led to degradation (Borroni et al., 2010, Jones et al., 2006), whereas the receptor can recycle to the plasma membrane. Endocytosis and recycling can, therefore, regulate the availability of chemokine receptors at the cell surface (Hanyaloglu and von Zastrow, 2008, Neel et al., 2005, Borroni et al., 2010). There are more than 50 different chemokines and 20 different chemokine receptors. Most of the chemokines bind to multiple receptors, and the same receptor usually binds more than one chemokine (Kucia et al., 2004). However, one chemokine does not fit this general description. Stromal cell-derived factor-1 (SDF-1, also called CxCl12) was believed to bind only to CXCR4, and CXCR4 only to SDF-1 (Horuk, 2001). Many studies have shown the ability of this chemokine to induce motility, chemotactic responses, and adhesion by activation of several signalling pathways, leading to phosphorylation of MAPK p42/44, activation of PI-3K-AKT-NF-κB axis, and calcium efflux (Figure 1.7) (Kucia et al., 2004). SDF-1 has also been implicated in proliferation and cell survival (Bagri et al., 2002).

Figure 1.7 Signalling pathways activated by SDF1-CXCR4 axis.

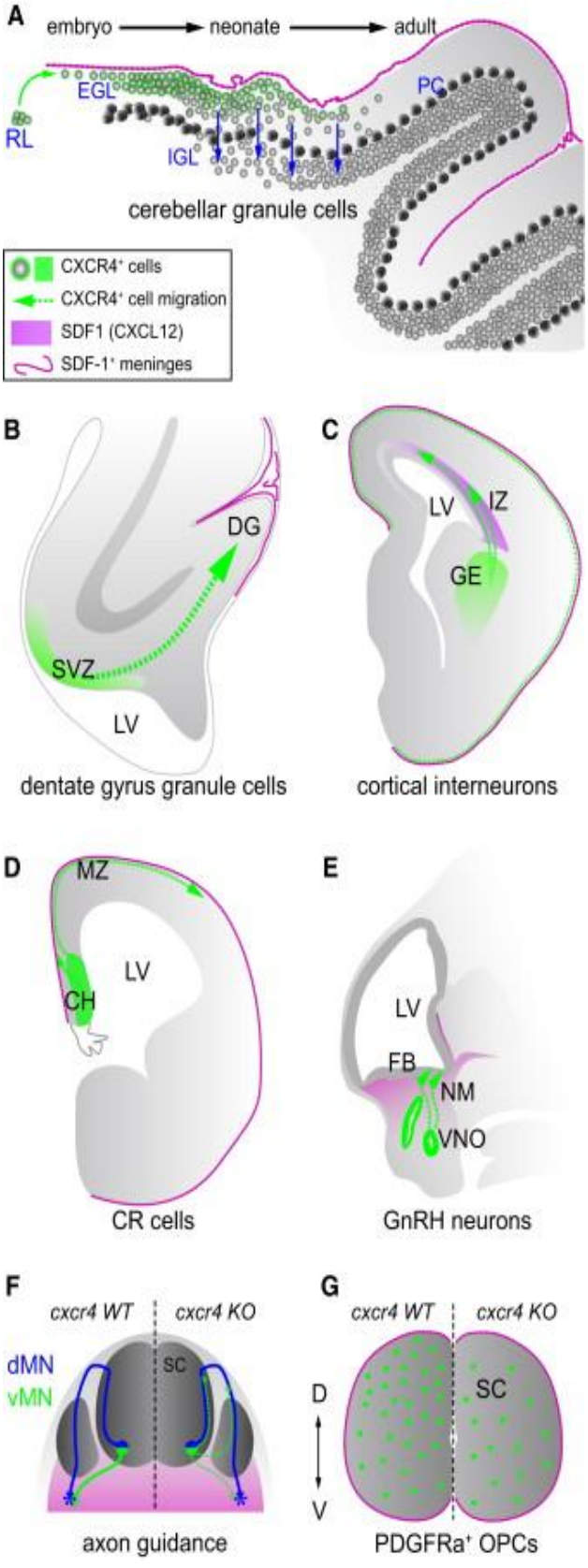
Interaction of SDF-1 with G coupled seven transmembrane receptor activates different pathways in different types of cells. Activation of these pathways regulates locomotion, chemotaxis, adhesion and secretion of CXCR4-expressing cells (Adopted by Kucia et al., 2004)


SDF-1
Adhesion
Fak
Paxillin
p130 CAS
PI-3K
AKT
MEK
MAPK p42/44
Jak, Tyk
Phosphatases
SHIP 1
SHIP 2
CD45
Elk-1
STAT
Biological effects

SDF-1 is the most extensively studied chemokine due to its wide expression pattern during development, and the gross developmental defects in almost every system exhibited in mice deficient for SDF-1/CXCR4 (Li and Ransohoff, 2008). These mice die perinatally because of hematopoetic and cardiac defects, showing aberrant cerebellar development and neuronal proliferation (Ma et al., 1998, Zou et al., 1998). Based on recent findings, SDF-1 has emerged as an indispensable regulator for targeted migration of neurons in the cerebellum, dentate gyrus, cerebral cortex, dorsal root ganglia, and nuclei in the brainstem in rodents (Figure 1.8) (Borrell and Marin, 2006, Ma et al., 1998, Bagri et al., 2002, Klein et al., 2001, Belmadani et al., 2005, Lu et al., 2002, Odemis et al., 2005, Vilz et al., 2005, Stumm et al., 2003). Upon reaching their destinations, neurons extend dendrites to characteristic regions and grow axonal processes to selective target tissues and cells. Evidence that CXCR4 regulates axonal guidance has been provided in motor neurons, retinal ganglion cells, primary afferents, and sympathetic neurons (Lieberam et al., 2005, Chalasani et al., 2003). Thus, the SDF-1/CXCR4 system continues to influence the development of neurons during and after the migratory process. In addition to neuronal development, CXCR4 also influences the migration and survival of oligodendrocytes (Dziembowska et al., 2005).

Figure 1.8 Signalling by the chemokine SDF-1 and its receptor CXCR4 mediates numerous developmental events.

Panels show schematic diagrams of embryonic and postnatal rodent brain and spinal cord with SDF-1 expression shown in purple and cells expressing CXCR4 shown in green. (A) Granule cell migration during embryonic, neonatal, and adult stages is depicted from left to right. SDF-1 is secreted by the meninges (purple dotted lines), which attracts rhombic lip (RL)-derived, CXCR4-expressing granule cells that migrate tangentially along the cerebellar surface and proliferate, forming the external granule cell layer (EGL). Postnatally, granule cells cease to proliferate, downregulate CXCR4, and migrate through the Purkinje cell (PC) layer to form the internal granule cell layer (IGL). (B) In the hippocampus, SDF-1, secreted by the adjacent meninges, attracts $CXCR4^+$ granule cells, which migrate from their SVZ germinal zone to form the dentate gyrus (DG) during late fetal/early postnatal development. (C) SDF-1, expressed by cortical cells within the intermediate zone (IZ), acts as an attractant for $GABA^+$ interneurons derived from the ganglionic eminence (GE). (D) CXCR4-expressing Cajal-Retzius (CR) cells migrate tangentially within the marginal zone (MZ) from the cortical hem (CH). Meningeal SDF-1 attracts the CH-derived CR cells and maintains their superficial position within the MZ. (E) Gonadotropin-releasing hormone (GnRH) neurons migrate from the vomeronasal organ (VNO) into the basal forebrain on a gradient of SDF-1 produced by the nasal mesenchyme. (F) SDF-1/CXCR4 affects axon pathfinding by modulating the responsiveness to repellents and/or attractants. CXCR4 is expressed on spinal cord (SC) ventral motor neuron (vMN) axons (green), and


A
embryo
neonate
adult
EGL
PC
RL
IGL
cerebellar granule cells
CXCR4⁺ cells
CXCR4⁺ cell migration
SDF1 (CXCL12)
SDF-1⁺ meninges
B
DG
SVZ
LV
dentate gyrus granule cells
C
LV
IZ
GE
cortical interneurons
D
MZ
LV
CH
CR cells
E
LV
FB
NM
VNO
GnRH neurons
F
cxcr4 WT
cxcr4 KO
dMN
vMN
SC
axon guidance
G
cxcr4 WT
cxcr4 KO
D
V
SC
PDGFRa⁺ OPCs

SDF-1 (purple) is expressed within the surrounding mesenchyme. In this model, SDF-1 renders these axons insensitive to ventral repellant cues. Dorsal motor neurons (dMNs) (blue), which do not express CXCR4 on their axons, are sensitive to the repellent cues, and exit the neural tube dorsally. In CXCR4 KO embryos, vMNs axons are more sensitive to repellents and often project dorsally (dotted green lines). (Adapted from Lieberam et al., 2005). (G) The number of PDGFRa+ OPCs in the mouse E14 SC is reduced in the CXCR4 KO (right) as compared to the WT (left). The reduction is more obvious dorsally. (Adopted from Deverman and Patterson, 2009).

During olfactory system development, SDF-1 is expressed by mesenchymal cells in a rostral to caudal gradient, being more intense in the nasal/forebrain junction, whereas expression of its receptor CXCR4 is detected in neurons of the olfactory epithelium (OE) (Tissir et al., 2004, Schwarting et al., 2006b, Toba et al., 2008a). GnRH neurons express the chemokine receptor early in their development (E12.5), when they leave the VNO to migrate to the brain. Based on this expression pattern, it is fair to assume that SDF-1/CXCR4 signalling could be important in early GnRH system development. Indeed, analysis of CXCR4 deficient mice revealed impaired GnRH neuron migration together with a decrease (45%) in the total number of these cells. Specifically, in CXCR4 null mice, GnRH neurons failed to exit the VNO at E12 and start their migration to the forebrain and, at E13, when half of the population is supposed to have migrated into the forebrain, only 3% had crossed the cribriform plate, and none had migrated ventrally or caudally towards the hypothalamus. This suggests an important role of SDF-1/CXCR4 in the early stages of GnRH neuronal migration (Toba et al., 2008a, Schwarting et al., 2006b). Thus, *SDF-1* and *CXCR4* represent novel candidate genes potentially involved in the clinical and neuroendocrine phenotype of KS.

Given the large size (89 amino acids for the unprocessed mouse SDF-1 precursor), susceptibility to enzymatic degradation, and binding to the extracellular matrix of the SDF-1 protein (McQuibban et al., 2001), it is assumed that SDF-1-secreting cells influence CXCR4-expressing neuronal structures in a spatially confined paracrine manner. Consistently, SDF-1 is highly expressed along the migration pathways of CXCR4-expressing neurons (Klein et al., 2001,

Lu et al., 2002, Lieberam et al., 2005, Bagri et al., 2002, Chalasani et al., 2003, Belmadani et al., 2005, Vilz et al., 2005). Since immune cells which are attracted by low concentrations of SDF-1 can be repulsed by high concentrations of SDF-1 (Poznansky et al., 2000), a high SDF-1 content along a neuronal migration route might cause attraction or repulsion depending on the local SDF-1 concentration gradient. In non-neuronal cells, rapid agonist-promoted internalization, lysosomal sorting, ubiquitination, and degradation of CXCR4 have been shown (Marchese and Benovic, 2001). If a similar mechanism existed in migrating neuronal cells as suggested (Baudouin et al., 2006), persistent SDF-1/CXCR4 signalling would require high-level mRNA expression and synthesis of CXCR4 to prevent rapid CXCR4 desensitization and downregulation. Conversely, a reduction of *CXCR4* gene expression would result in rapid loss of responsiveness to SDF-1. Recently, a new mechanism for regulation of CXCR4 was proposed that involves a newly identified SDF-1 receptor, CXCR7.

1.10 A new player, CXCR7

SDF-1 and CXCR4 defective mice exhibited similar phenotypes, and it was believed that CXCR4 is the only receptor of SDF-1 until a few years ago, when the previously orphan G protein RDC1 (renamed CXCR7) was shown to bind SDF-1 with a higher affinity (Balabanian et al., 2005a, Burns et al., 2006). The high sequence conservation of CXCR7 across mammalian species implies its functional importance, and indeed two independent CXCR7-deficient mouse lines showed defects in heart development and lethality soon after birth (Sierro et al., 2007, Gerrits et al., 2008). The addition of a third member to SDF-1

chemokine system predicted a higher level of complexity in the mechanisms underlying SDF-1 functions.

CXCR7 belongs to a subgroup of "atypical" chemokine receptors that includes DARC, D6 and CCXCKR, whose properties are somewhat unusual for GPCRs because, even though they bind chemokines, they do not actually activate G proteins due to lack of the amino acid motif that has been typically associated with the activation of G proteins by chemokine receptor (Graham, 2009, Borroni et al., 2008). In the case of CXCR7, it has been shown that although this receptor shares sequence homology with 7-transmembrane GPCRs, the “DRYLAIV” amino acid motif in the second intracellular loop, which is highly conserved among chemokine receptors and considered to be necessary for G protein coupling (Colvin et al., 2004) is altered to “DRYLSIT” in CXCR7 (Lagane et al., 2005, Colvin et al., 2004, Zhu and Murakami, 2012). In line with this sequence alternation, typical chemokine responses such as calcium mobilization, chemotaxis, and activation of PI3K/AKT pathways, often fail to be detected upon ligand stimulation of CXCR7 (Burns et al., 2006, Zhu and Murakami, 2012, Mazzinghi et al., 2008, Boldajipour et al., 2008). These “oddities” set CXCR7 apart from classical chemokine receptors like CXCR4, suggesting that the two are unlikely to be functionally redundant.

Despite the fact that these "atypical" receptors fail to activate G-proteins, they were reported to associate with non-G protein-related functions such as the activation of β-arrestin mediated signalling (Rajagopal et al., 2010a, Rajagopal et al., 2010b). Another notion put forward is that these molecules primarily function as “decoy” receptors. That is to say, they can bind

chemokines and remove them from the external environment through receptor-mediated endocytosis. Once internalized by a decoy receptor, a particular chemokine may be degraded or even perhaps re-released intact from another part of the cell, a process known as transcytosis (Borroni et al., 2008).

1.11 Cellular and molecular functions of CXCR7

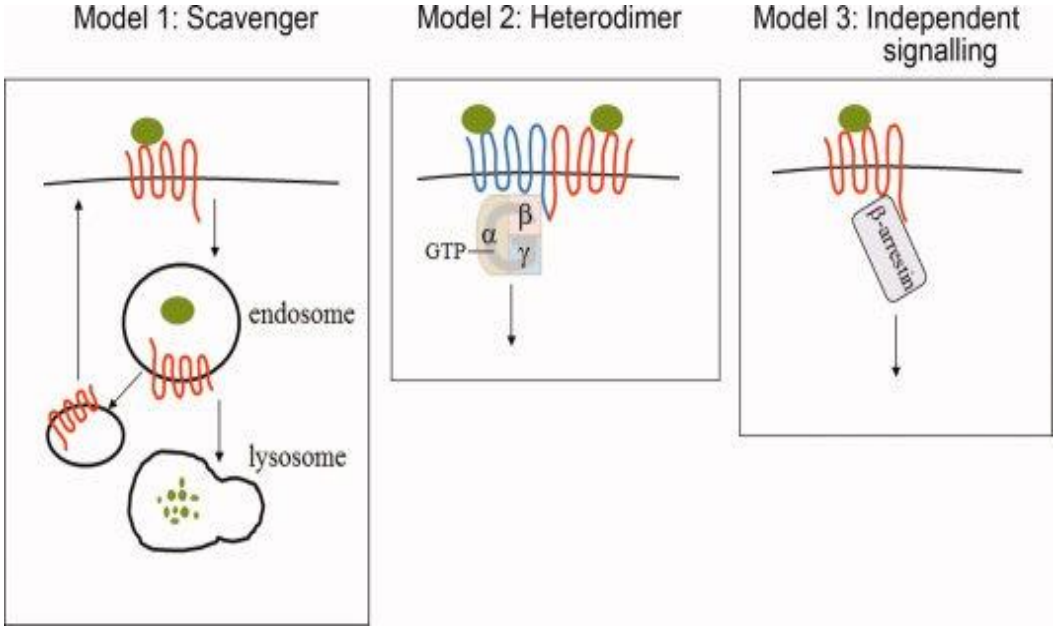
Most chemokine receptors are expressed on the cell surface and exhibit only a limited tendency to spontaneously internalize. CXCR7, however, rapidly cycles between the plasma membrane and endosomes, where is preferentially localized (Canals et al., 2012, Ray et al., 2012). The fact that CXCR7 display ligand-dependent and -independent cycling that grants the ability to internalize chemokine without becoming saturated, as well as the finding that it has higher binding affinity for SDF-1 than CXCR4 (its cognate receptor) led to the hypothesis that CXCR7 acts as a scavenger or "decoy" receptor. This model proposes that CXCR7 functions as a scavenger receptor that mediates SDF-1 internalization and subjects it to degradation in lysosomes (Figure 1.9) (Ray et al., 2012, Dambly-Chaudiere et al., 2007, Boldajipour et al., 2008, Luker et al., 2010, Naumann et al., 2010). This ability of CXCR7 to clear excess SDF-1 from the extracellular space could contribute to keeping the concentration of the chemokine within an optimal range, and/or shape the required gradient. Strong *in vivo* support of this model comes from an elegant study in zebrafish on the migration of primordial germ cells (PGCs) (Boldajipour et al., 2008). PGCs (expressing CXCR4b) migrate along an SDF-1a gradient from their site of origin to the position where the gonad develops and differentiate into gametes

(Doitsidou et al., 2002). Boldajipour and colleagues (Boldajipour et al., 2008) provided evidence that CXCR7 is required for proper migration of PGCs in a non-cell autonomous manner. Expressed in the somatic tissue and in PGCs, CXCR7 was shown to mediate SDF-1 endocytosis, whereby clearing SDF-1 protein from non-target areas and sharpening the localized SDF-1 protein distribution necessary for precise PGCs guidance. A second model proposed for CXCR7's function suggests that it can form heterodimers with CXCR4 when co-expressed in the same cell and, as a result, modify CXCR4 downstream signalling (Figure 1.9) (Sierro et al., 2007, Levoye et al., 2009, Decaillot et al., 2011). While some reports have shown that the co-expression of CXCR4 and CXCR7 in cell lines enhances calcium mobilization (Sierro et al., 2007) and chemotaxis (Decaillot et al., 2011) others have demonstrated compromised CXCR4 signalling (Levoye et al., 2009). Finally, a third model suggests that CXCR7 can mediate signalling independently from CXCR4 in response to SDF-1 (Figure 1.9) (Balabanian et al., 2005a, Zabel et al., 2009, Odemis et al., 2010, Odemis et al., 2012, Rajagopal et al., 2010b). Most GPCRs can signal via both G proteins and β-arrestins in a rather balanced manner. Coupling with β-arrestins triggers receptor desensitization and endocytosis, and also activates downstream ERK1/2 pathway (Luttrell and Lefkowitz, 2002). Whereas CXCR7 is considered incapable of signalling via G proteins, studies recently have provided direct evidence that it can recruit β-arrestin 2 and activate ERK1/2 pathway (Rajagopal et al., 2010a, Zabel et al., 2009), putting CXCR7 into the category of β-arrestin-biased GPCRs. More recently, one report showed quite surprisingly that CXCR7 could even signal via G protein and trigger calcium mobilization in *CXCR4*-deficient primary astrocytes upon SDF-1 binding

(Odemis et al., 2012). However, caution should be exercised as it is still unclear in this case whether CXCR7 signals by itself or by heterodimerizing with another as-of-yet unknown receptor. One implication from models (2) and (3) is that CXCR7 alone or the CXCR7/CXCR4 heterodimer can mediate different downstream signalling and, thus, lead to different cellular responses compared with CXCR4 alone (Zhu and Murakami, 2012). Indeed, several lines of evidence suggest that while CXCR4 predominantly controls chemotaxis, CXCR7 is more important for regulating cell-cell adhesion (Burns et al., 2006, Hartmann et al., 2008, Mazzinghi et al., 2008). In summary, each of the above three models has the support of a fair amount of experimental evidence and none are mutually exclusive. For example, coupling to β-arrestin 2 would enable CXCR7 to trigger SDF-1 internalization, as well as β-arrestin 2-mediated ERK1/2 activation simultaneously in the same cell. The differential expression patterns of CXCR7 and CXCR4 showing co-expression in some cells and non-overlapping in others, and the fact that *CXCR7* and *SDF-1* are often co-expressed in somatic tissues (Schonemeier et al., 2008, Tiveron et al., 2010) suggest that all three models might apply *in vivo* dependent on the cellular and tissue context (Zhu and Murakami, 2012).

Figure 1.9 Three major models that have been proposed for the cellular and molecular functions of CXCR7.

Green, SDF-1; orange, CXCR7; blue, CXCR4. (Adopted from Zhu and Murakami, 2012).


Model 1: Scavenger
Model 2: Heterodimer
Model 3: Independent signalling
endosome
lysosome
GTP
α
β
γ
β-arrestin

Signal transduction via cell surface receptors requires the presence of receptor protein at the plasma membrane. As it happens for all receptors, the magnitude of the cellular response elicited by a ligand binding to a GPCR is dictated by the level of receptor protein at the plasma membrane, which results from a fine balance between two pathways, one that delivers properly folded receptor to the cell surface and a second that removes the receptor by endocytosis (Canals et al., 2012, Neel et al., 2005). One of the most common events for receptor desensitization and internalization involves the recruitment of the β-arrestin protein, which binds to the activated and phosphorylated receptor. This uncouples the receptor from its G protein and scaffolds the binding of proteins involved in formation of clathrin-coated pits and receptor endocytosis (Hanyaloglu and von Zastrow, 2008, Jean-Alphonse and Hanyaloglu, 2011). Once internalized in early endosomes, some GPCRs are dephosphorylated and subsequently recycled back to the plasma membrane where they can again respond to agonists, a process termed re-sensitization. Alternatively, a subclass of GPCRs enter the degradation pathway, where they are targeted to lysosomes for proteolysis (Jean-Alphonse and Hanyaloglu, 2011). For many GPCRs, chronic treatment with agonist results in a significant decrease in the cellular receptor levels by a process termed downregulation, primarily ascribed to lysosomal receptor degradation.

Apart from receptor phosphorylation, reversible ubiquitination constitutes a key regulatory mechanism for GPCRs (Shenoy et al., 2001, Shenoy et al., 2007). This post-translational modification results in the covalent addition of the small protein ubiquitin to the intracellular lysine side chains of

GPCRs, with profound consequences for endocytic cycles of GPCRs (Shenoy et al., 2007) In particular, CXCR4 has been shown to undergo SDF-1-induced ubiquitination resulting in lysosomal degradation of the receptor. CXCR4 ubiquitination occurs after receptor internalization, and is mediated by the E3 ubiquitin ligase AIP4 via its interaction with β-arrestin (Bhandari et al., 2007) highlighting a novel function of β-arrestins in endosomal sorting of GPCRs.

1.12 CXCR7 structure

Recent work established that the two SDF-1 receptors (CXCR4 and CXCR7) are fundamentally different in their functions. CXCR4 represents a G protein-coupled chemoattractant receptor showing rapid phosphorylation at the C-terminal domain and β-arrestin-dependent endocytosis in response to SDF-1 stimulation (Orsini et al., 1999, Busillo et al., 2010) Sustained stimulation causes CXCR4 degradation (Marchese and Benovic, 2001) a mechanism expected to desensitize the SDF-1/CXCR4 pathway once migratory cells reach SDF-1-rich environments. On the other hand, CXCR7 functions as a scavenger receptor by constitutively internalizing and recycling to the cell membrane, maintaining nearly constant levels of cell surface receptors. The scavenger activity of CXCR7 controls levels of chemokine ligands available for signalling through CXCR4. By removing chemokine ligands from the extracellular space and transporting these molecules to lysosomes for degradation (Naumann et al., 2010, Luker et al., 2010, Zabel et al., 2009), CXCR7 establishes gradients of this molecule necessary for CXCR4-depedent chemotaxis (Boldajipour et al., 2008, Torisawa et al., 2010). CXCR7 predominantly localizes to intracellular

compartments, including early and late endosomes, lysosomes and potentially the endoplasmic reticulum (Luker et al., 2010). CXCR7 constitutively internalizes and recycles from the cell membrane to scavenge and degrade chemokine ligands. Concomitant with redistribution to the cell membrane, deletion of the intracellular tail of CXCR7 decreased constitutive internalization of the receptor. An earlier study (Zabel et al., 2009) established the intracellular tail of CXCR7 as the structural determinant of localization, trafficking, chemokine scavenging and signalling properties of this receptor.

What is the structural basis of this difference between the two receptors? Little is known about structural determinants that uncouple CXCR7 from G proteins, cause ligand-independent internalization, and permit it to act as a decoy receptor. Ray and colleagues (Ray et al., 2012) showed that the intracellular tail of CXCR7 is the critical determinant of receptor trafficking, chemokine scavenging and signalling. In detail, they showed that the CXCR7 C-terminus permits fast endocytosis of ligand-free and SDF-1-loaded receptors. Constitutively internalizing receptors bearing the CXCR7 C terminus were rapidly degraded in the absence of SDF-1. Although SDF-1 caused degradation of CXCR4, it did not accelerate degradation of CXCR7.

A recent study (Hoffmann F, 2012), using exchange of the C-terminus domain between CXCR4 and CXCR7, shed light on the structural elements underlying CXCR7 behaviour. Specifically, replacement of the C-tail of CXCR7 with the one of CXCR4, abrogate ligand-independent internalization of CXCR7. Conversely, CXCR4 bearing C-terminal domain of CXCR7 triggers its rapid ligand-independent internalization. Moreover, this spontaneously internalizing

CXCR4–7 tail mutant did not activate any G protein signalling, whereas ligand uptake was accelerated. It, therefore, seems likely that the CXCR7 C terminus is sufficient to prevent efficient receptor/G protein coupling and to permit rapid receptor-mediated SDF-1 sequestration. However, the C terminus seems not to be the only CXCR7 domain that prevents effective G protein signalling, because the CXCR7–4 tail mutant, which did not internalize in the absence of ligand, failed to signal through the G proteins tested (Hoffmann et al., 2012). Furthermore, the cluster of Ser-350, Thr-352, and Ser-355 was shown to be of particular relevance for internalization of SDF-1-CXCR7 complexes (Ray et al., 2012).

Direct comparison of the CXCR4 and CXCR7 wild type receptors with their tail-swap mutants under experimental conditions producing similar total protein levels clearly revealed that the CXCR7 C-terminus is responsible for rapid receptor-mediated SDF-1 uptake and degradation. Given that CXCR4 becomes degraded after long term SDF-1 exposure, and that the steady state expression of CXCR7 is not influenced by SDF-1, it is conceivable that CXCR7 is the more efficient SDF-1 scavenger under physiological conditions (Hoffmann et al., 2012). As a consequence of its scavenger function, CXCR7 prevents SDF-1-induced CXCR4 degradation and balances the SDF-1/CXCR4 pathway (Sanchez-Alcaniz et al., 2011).

1.13 The role of CXCR7 function in the developing CNS

Like CXCR4, CXCR7 is expressed in the developing brain (Stumm and Hollt, 2007, Tiveron and Cremer, 2008) and may be involved in the regulation of

neuronal cell migration. Despite the fact that CXCR7 is highly expressed in the prenatal cortex and postnatal dentate gyrus (Schonemeier et al., 2008, Tiveron et al., 2010) an initial analysis of the developing CNS including the cerebellum, dentate gyrus and spinal cord, all of which are noted for showing defects in CXCR4- or SDF-1-deficient mice, did not show obvious defects in CXCR7 mutants (Sierro et al., 2007)

Two recent studies on the role of CXCR7 in cortical interneuron migration revealed that these cells in CXCR7-deficient mice failed to remain the MZ and SVZ/IZ migratory streams and entered the cortical plate prematurely, a defect very similar to that exhibited by CXCR4-deficient mice. In more detail, Wang and colleagues showed that both CXCR4 and CXCR7 are essential for responding to SDF-1 and for regulating interneuron migration, demonstrating that CXCR4 cannot compensate for the loss of CXCR7. They also provided evidence that, in immature cortical interneurons, CXCR7, but not CXCR4, strongly promotes MAP kinase signalling. The second group (Sanchez-Alcaniz et al., 2011) further demonstrated with a series of elegant experiments that CXCR7 is not essential for the synthesis or transport of CXCR4 to the plasma membrane, but it modulates the levels of CXCR4 receptors by titrating the concentration of the available SDF-1. In this context, loss of CXCR7 -which is constantly involved in binding and internalizing SDF-1- leads to accumulation of SDF-1 extracellularly, hypereractivation of CXCR4 and, as a result, to the loss of CXCR4 as well (Marchese and Benovic, 2001, Alsayed et al., 2007, Kolodziej et al., 2008). Moreover, transplantation experiments showed that CXCR7 plays a cell-autonomous role *in vivo.*

The role of CXCR7 in the developing nervous system is bound to extend beyond the migrating cortical interneurons. Zhu and colleagues (Zhu and Murakami, 2012) have found that precerebellar neuronal migration is affected in CXCR7-deficient mice and the defect differs from that in CXCR4 mutants. It is almost certain that the list will grow and new roles will be assigned to this second receptor of SDF-1. The key issue, however, is to have a clear and thorough understanding of the intricate mechanisms highlighted by the interplay between SDF-1 and its two receptors in a complex and dynamic tissue environment such as the developing brain.

1.14 Project aims

Reproduction in mammals is initiated and maintained by a small population of neurons, scattered throughout the preoptic area and anterior hypothalamus in the brain, which release the decapeptide known as GnRH. The release of GnRH governs the secretion of two pituitary gonadotropic hormones, luitenizing and follicle-stimulating hormones which, in turn, control gametogenesis in both sexes. Surprisingly, GnRH neurons originate in the nasal placode and migrate to their destinations in the brain by following the olfactory/vomeronasal nerves as they pass through varying molecular environments. In humans, failure of GnRH neuron development leads to clinical syndromes, such as hypogonadotropic hypogonadism and Kallmann syndrome, characterized by gonadal dysfunction and absence or delayed puberty. The etiology of these disorders is largely unknown. The aim of my PhD project is to study the molecular mechanisms that control GnRH neuron migration with the ultimate aim to identify candidate genes associated with these reproductive disorders.

One of the molecules shown to be required during the early steps of GnRH neuron migration is CXCR4, which was considered the only receptor for the chemokine SDF-1, until recent studies provided evidence that another receptor, CXCR7, can bind to SDF-1 with even higher affinity that CXCR4. Considering the already known role for SDF1/CXCR4 signalling in the migration of GnRH neurons, it is plausible to hypothesize that the newly characterized CxCr7 might also be important in the GnRH neuronal system.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Mice

For expression studies, wild-type mice of C57Bl/6J background, obtained from Charles River Ltd, were used. Embryos of transgenic *CXCR7*-EGFP and *CXCR4*-EGFP mice (The Gene Expression Nervous System Atlas project GENSAT; http://www.gensat.org/index.html), bred on CD1 background, were maintained at the laboratory of Professor R Stumm (Jena). *CXCR7*, *SDF-1* and *CXCR4* knockout embryos were obtained by mating heterozygous mice (Zou et al., 1998, Sierro et al., 2007) which were on C57BL/6 background. Noon of the day after mating was defined as embryonic day (E) 0.5. All animal procedures were performed in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, German and EU guidelines.

2.1.2 Antibodies

ANTIGEN	HOST	DILUTION	SUPPLIER
GnRH	rabbit	1:1000 (DAB) 1:200 (Fluo)	ImmunoStar
Peripherin	rabbit	1:1000 (DAB) 1:500 (fluo)	Millipore
GFP	chicken	1:500 1:1500 (enhancement)	Aves
βIII-tubulin	mouse	1:500	Sigma
CXCR4 (UMB-2)	rabbit	1:1000 (enhancement)	Kindly provided by Prof Stumm
CXCR4 (2B11)	rat	1:500 (enhancement), 1:100	BD Biosciences
HuC/D	mouse	1:500	Molecular Probes
Pax6	rabbit	1:500	Millipore
BLBP	rabbit	1:300	Millipore
GS-IB4 (Griffonia Simplicifolia)	biotinylated	1:200	Vector

Table 2.1 Primary antibodies

ANTIBODY	HOST	CONJUGATED WITH	DILUTION	SUPPLIER
Anti-rabbit IgG	goat	Biotin	1:200	Vector
Anti-chicken IgG	goat	Biotin	1:200	Vector
Anti-rat IgG	goat	Biotin	1:200	Vector
Anti-mouse IgG	goat	AF 488/568	1:200	Invitrogen
Anti-rabbit IgG	goat	AF 488/568	1:200	Invitrogen
Streptavidin	-------	AF 488/555	1:200	Invitrogen

Table 2.2 Secondary antibodies. AF: Alexa Fluor

Probe	Enzyme for linearization	Polymerase	Supplier
CXCR7	HindIII	SP6	R.Stumm
CXCR4	SphI	SP6	R.Stumm
SDF-1	SaI	T3	R.Stumm

Table 2.3 *In situ* probes

2.2 Methods

2.2.1 In situ hybridization

2.2.1.1 Preparation of digoxygenin-labelled probes

20 µg of plasmid DNA was linearised with the correct restriction enzyme, extracted twice with equal volumes of phenol-chloroform, precipitated with 2.5 volumes of ethanol and 1/25 of 2 M NaCl, washed with 70% ethanol, re-suspended in 20 µl DEPC-ddH_2O.

In vitro transcription reactions were composed of: 1 µg linear template DNA, 1X transcription buffer (Promega), 100 µM DTT; 1X digoxigenin (DIG) RNA labelling mix (10 mM each of ATP,CTP,GTP,UTP,DIG-UTP; Roche), 20 units RNasin (Promega), 20 units of the appropriate RNA polymerase in 20 µl of DEPC-ddH_2O. The mixture was incubated at 37° C for 2 hours. The RNA was precipitated with 2.5 volumes of ethanol and 1/25 of 2 M NaCl, washed with 70% ethanol, re-suspended in 20 µl DEPC-ddH_2O and stored at -80° C.

2.2.1.2 Method

In situ hybridization was performed as described previously (Faux et al., 2010). Briefly, embryonic brains were dissected in PBS and fixed in 4% paraformaldehyde (PFA) made in phosphate buffer saline (PBS; pH 7.4) overnight, followed by cryoprotection in 30% sucrose, and treated with diethyl pyrocarbonate (DEPC) made in PBS at 4°C for one day. Brains were frozen in Tissue-Tek OCT and sectioned coronally at 20 µm. Sections were dried at room temperature (RT) for 2 hours, before overnight incubation at 65°C in

hybridization buffer 1× DEPC-treated “salts” (200 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 5 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM Na_2HPO_4; Sigma-Aldrich), 50% deionized formamide (Ambion), 0.1 mg/ml RNase-free yeast tRNA (Invitrogen), 1× Denhardts (RNase/DNase free; Invitrogen), 10% dextran sulfate (Sigma-Aldrich) containing 100–500 ng/ml digoxigenin (DIG)-labelled RNA probes. After hybridization, sections were washed three times in a solution containing 50% formamide 1× SSC (Ambion) and 0.1% Tween 20 (Sigma-Aldrich) at 65°C, and two times at RT in 1× MABT (20 mM maleic acid, 30 mM NaCl, 0.1% Tween 20; Sigma-Aldrich) before incubating in a solution containing 2% blocking reagent (Roche) and 10% NGS in MABT, followed by overnight incubation in alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:1500; Roche). Nitrobluetetrazolium chloride (NBT) (Roche)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche) diluted 1:1000 in MABT with 5% polyvinyl alcohol (VWR International) was used for colorimetric detection for 2-8 hours. Finally, sections were washed in H_2O, dehydrated through series of ethanol and mounted with DPX (VWR).

2.2.2 Immunohistochemistry

2.2.2.1 Immunoperoxidase

Firstly, sections were treated with 0.3% H_2O_2 in 1X PBS for 20 minutes at RT, followed by 3 washes with 0.2% PBST. They were subsequently incubated in blocking solution (10% Normal Goat Serum in PBT) for 1 hour and then, overnight at RT with the primary antibody diluted in 1% NGS/PBT. The following day, sections were washed three times with PBT and incubated with

the secondary antibody for 2 hours at RT. They were then treated with avidin-biotin-peroxidase complex (ABC-HRP kit, Vector) at RT for 2 hours. The activity of the peroxidase was revealed using DAB (0.005% 3,3 diaminobenzidinetetrachloride in H_2O, Sigma-Aldrich) as chromogen and 0.005% H_2O_2 as substrate. The reaction was stopped with 1X PBS and sections were dehydrated through ascending ethanol concentrations (70, 80, 95 and 100% for 1 minute, each) and mounted in DPX (VWR).

2.2.2.2 Immunofluorescence

Mouse embryos were fixed in Bouin/Hollande fixative for 16 hours (E12.5-E14.5) or 24 hours (E16.5) and subjected to routine paraffin embedding (Stumm et al., 2001). Sections were cut at 8 µm for immunohistochemistry, and labelling was performed as described previously (Stumm et al., 2007). Briefly, endogenous peroxidase was blocked with methanol/H_2O_2 after deparaffinization. For antigen retrieval, sections were bathed for 25 minutes in 0.01 M citrate buffer (pH 6.0) at 95°C. Sections were then blocked in 50 mM PBS, containing 5% bovine serum albumin (BSA), and incubated overnight with primary antibody in 50 mM PBS containing 1% BSA. The following day, sections were washed in 1X PBT and incubated with fluorescent conjugated secondary antibodies at RT for 2 hours. The secondary antibody was washed out with several washes with 1X PBS, and tissues were treated with DAPI for 5 minutes. Sections were mounted in Mowiol (Sigma-Aldrich).

TSA enhancement (Vector ABC kit; Vector Laboratories) was performed for UMB-2 prior to detection with AF555 and AF488 (Invitrogen). Double

fluorescence immunohistochemistry for UMB-2 and GnRH (both rabbit) was performed by staining first for UMB-2 as aforementioned and, following washes in a series of ethanol (50% to 100% and back to 50%) and PBS, sections were blocked again in PBS/BSA for an hour and incubated overnight in anti-GnRH antibody (1:100). Subsequent detection of UMB-2 was performed using streptavidin AF488 (1:200), and localization of GnRH with Alexa 568 (1:200, Invitrogen). Controls were used to ensure that secondary antibodies (Alexa 568) did not bind to UMB-2. Nuclei were counterstained with DAPI (Sigma-Aldrich).

2.2.3 Double in situ hybridization/immunohistochemistry

When necessary, immunohistochemistry followed *in situ* hybridization. In these cases, instead of mounting sections after the i*n situ* hybridization protocol, they were fixed with 4% PFA for 30 minutes, washed with PBST, followed by H_2O_2 incubation, and taken through the immunohistochemistry procedure.

2.2.4 Quantification of GnRH-positive cells

For each genotype, we analyzed at least three embryos at each age, and all GnRH-positive cells were counted in every section of each head under a 40× microscope objective. For all experiments, data were expressed as mean ± standard error of the mean (SEM). To determine statistical significance, we used unpaired Students *t*-test, with $p<0.05$ considered to be statistically significant (*p<0.05;**p<0.01; ***p<0.001).

Chapter 3. CXCR7 expression in the developing nasal area

3.1. Introduction

Since GnRH neurons begin their migration in the developing nasal compartment, a short introduction on the development of this area follows.

During early embryonic development (E10), olfactory placodes (of cranial non-neural ectodermal origin) begin to invaginate to form the nasal/olfactory pit which, in the following two days, will give rise to two separate cavities, the main olfactory cavity/epithelium and the vomeronasal cavity/epithelium. Concurrently, subsets of epithelial cells delaminate and enter the mesenchyme of the medial nasal process, adjacent to the presumptive olfactory epithelium. The earliest migratory cells express neuroepithelial markers, and a later migratory population expresses GnRH as well as odorant receptors (Schwarzenbacher et al., 2004, Schwanzel-Fukuda and Pfaff, 1989, Fornaro et al., 2001). In parallel with early neural differentiation, cartilage condensation and chondrogenesis establishes the nasal septum, resulting in a single nasal prominence with two nares at the craniofacial midline.

The OE is pseudostratified neuroepithelium that comprises multiple cell types. In addition to mature and immature olfactory receptor neurons[1] (ORNs), which reside in the middle/intermediate pseudolayer, there are two

[1] Bipolar neurons with a single dendrite that projects to the apical surface of the epithelium ending in a knob-like swelling from which emanate 10-30 fine cilia. On the basal pole of each ORN, a thin unmyelinated axon projects to the main olfactory bulb.

subpopulations of basal cells, the horizontal basal cells (HBCs) and the globose basal cells (GBCs), as well as supporting or sustentacular (SUS) cells[2] that reside in the apical pseudolayer. These cell types differentiate after placodal induction and are thought to arise from a common progenitor (Nicolay et al., 2006). Interestingly, at the very earliest stages of epithelial development, neurogenesis follows a slightly different scheme than later in embryogenesis, postnatal and adult stages. At early embryonic stages (e.g., E11-E14), the majority of dividing cells are found apically in the developing OE while, later in development, proliferation is found predominantly near the base of the OE (Smart, 1971, Carson et al., 2006).

3.2. Comparative expression patterns of CXCR7, SDF-1 and CXCR4 in the developing nasal area

Previous studies, using predominantly *in situ* hybridization (Tissir et al., 2004, Schwarting et al., 2006b), reported expression of *SDF-1* and *CXCR4* in the developing accessory olfactory system,. In detail, Schwarting et al. (2006) showed that *SDF-1* is expressed by mesenchymal cells, forming a rostral to caudal gradient, presumably attracting CXCR4 expressing GnRH neurons towards the brain.

The identification of a second receptor for SDF-1, namely CXCR7, suggested a higher complexity of regulation of SDF-1 signalling. This prompted

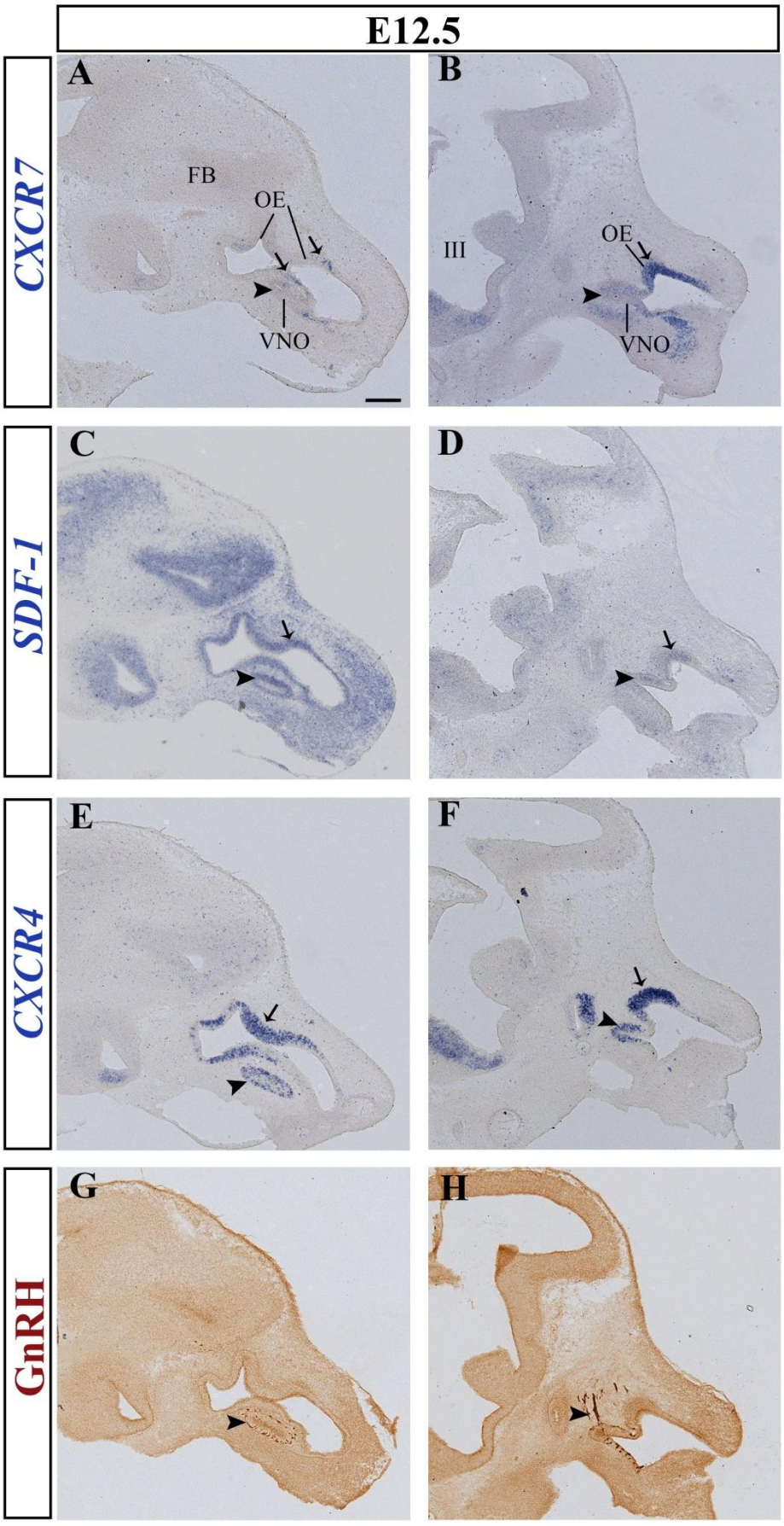
[2] Columnar epithelial cells that span the entire thickness of the OE from the airway surface to the basal lamina. Oval nuclei of supporting/SUS cells align in a single row along the apical aspect of OE, the most apically located epithelial cells/nuclei within the mammalian OE.

us to investigate whether this second receptor plays a role in the development of the GnRH system. For this, a detailed description of the spatiotemporal expression profile of *CXCR7* was first undertaken, especially since no information was available about its expression in the developing nasal area, where GnRH neurons begin their migration. Further, the *CXCR7* expression pattern was compared to that of its agonist *SDF-1* and the receptor *CXCR4*, since its function is tightly associated with these molecules.

For the expression analysis, *in situ* hybridization was performed to establish the general distribution of *CXCR7*, *SDF-1* and *CXCR4* transcripts at 3 different developmental stages (E12.5, E14.5 and E16.5), covering the migration timeline of GnRH neurons in the nasal compartment. Between E12.5 and E14.5, only minor changes were observed in the overall expression patterns of the three genes in the nasal area. As depicted in Figure 3.1, *in situ* hybridization on sagittal head sections, revealed expression of *CXCR7* as early as E12.5. Specifically, *CXCR7* transcripts were detected in the area surrounding the VNO (Figure 3.1A,B, arrowheads), where GnRH neurons emerge from (Figure 3.1G,H), and in the OE (Figure 3.1A,B, arrows). Moreover, its expression pattern was complementary to that of *SDF-1* in the VNO and overlapping in the OE. *CXCR4* expression was also detected in the developing OE and VNO (Figure 3.1E,F)

Figure 3.1 Comparison of CXCR7, SDF-1 and CXCR4 expression patterns in sagittal head sections of mice at E12.5 processed for *in situ* hybridization.

A,**B**: *In situ* hybridization with CXCR7 probe revealed expression in the VNO (arrowheads) and OE (arrows). **C**,**D**: SDF-1 was also expressed in the VNO (arrowheads) and OE (arrows). **E**,**F**: CXCR4 transcripts were detected in cells of the OE (arrows) and VNO (arrowheads). **G**,**H**: Immunohistochemistry for GnRH in sections at the same level showed GnRH neurons (arrowheads) migrating from the VNO in the nasal area towards the brain. Abbreviations: VNO, vomeronasal organ; OE, olfactory epithelium. Scale bar: 200 µm


E12.5
CXCR7
SDF-1
CXCR4
GnRH
A
B
C
D
E
F
G
H
FB
OE
VNO
III

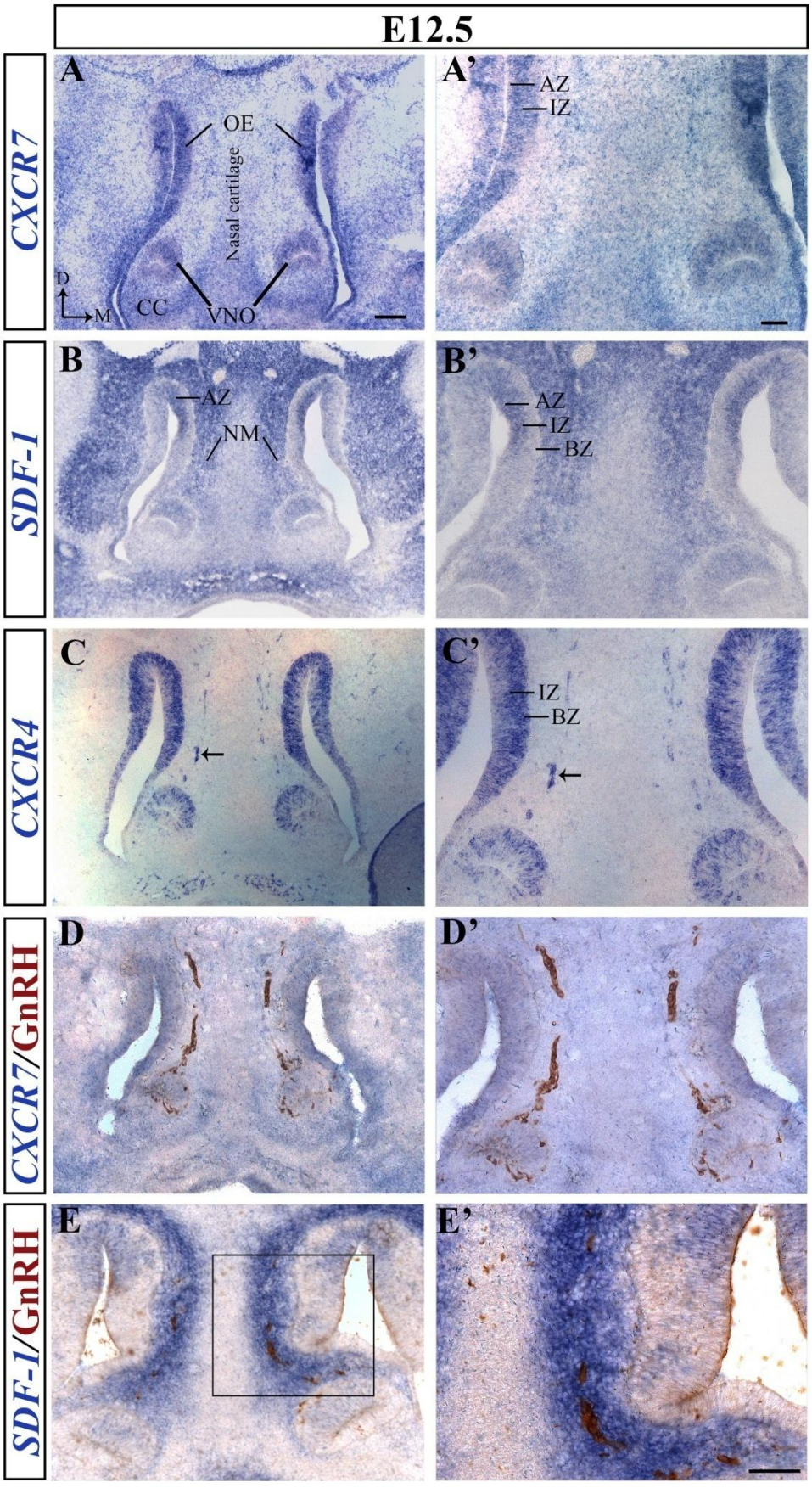
Looking in more detail in coronal sections (Figure 3.2A,A'), *CXCR7* transcripts were detected mainly in the sensory epithelium of the VNO and the surrounding area, as well as in the OE. *CXCR7* was also present in the surrounding cartilaginous capsule adjacent to VNO. Interestingly, *SDF-1* expression was found to overlap with *CXCR7* inside the VNO and in the dorsally located mesenchyme, whereas the two were expressed in a complementary fashion in the tissue around the VNO, with *SDF-1* being present mostly in the lamina propria adjacent to the basal zone of VNO. At the same time, *CXCR4* was prominently expressed in the VNO, dominating all zones. Migrating cells in the nasal area, resembling GnRH neurons, were also *CXCR4*-positive (Figure 3.2C,C').

In the developing OE, which is a highly dynamic structure with multilayered neurogenic regions containing different classes of progenitor cells that give rise to ORNs (Treloar et al., 2010, Balmer and LaMantia, 2005), *CXCR7* signal was predominantly expressed throughout the area. Strikingly, *SDF-1* and *CXCR7* expressions highly overlapped in the apical layer of the OE, while CXCR4 was localized mostly in the basal and intermediate layers. As the OE has not yet adopted its mature pattern at this stage (E12.5), identification of the cell types expressing *SDF-1* and its receptors based on location was not possible. In the nasal mesenchyme, *SDF-1* expression formed a gradient along the route of GnRH neuron migration (Figure 3.2E,E'), confirming previous findings (Schwarting et al., 2006b). *CXCR7* was detected mainly in the ventral nasal mesenchyme around the VNO and nasal septum, with a very faint signal present

in a narrow corridor along the septum (all the way to the brain, Figure 3.2D') that coincided with the migratory route of GnRH neurons.

Figure 3.2 Comparative expression patterns of CXCR7, SDF-1 and CXCR4 in the developing nasal area of mice at E12.5.

A,**A'**: Low and higher magnification of a coronal head section at the level of the nose shows CXCR7 expression in the VNO and surrounding area as well as in the OE. **B**,**B'**: SDF-1 was expressed in the VNO and NM in a gradient. SDF-1 transcripts were also detected in the AZ of the OE. **C**,**C'**: CXCR4 expression in the nose was detected in cells in the VNO and the BZ and IZ of the OE, as well as in migrating GnRH neurons (arrows). **D**,**D'**: *In situ* hybridization for *CXCR7* and immunohistochemistry showed GnRH neurons emerging from the CXCR7-expressing VNO and migrating in the NM. **E**,**E'**: *In situ* hybridization for *SDF-1* and immunohistochemistry showed GnRH neurons migrating on *SDF-1* expressing substrate in the NM. Abbreviations: OE, olfactory epithelium; VNO, vomeronasal organ; NM, nasal mesenchyme; AZ, apical zone; IZ, intermediate zone; BZ, basal zone; CC, cartilaginous capsule. Scale bars: A-D : 100 µm;, A',B',C',D',E,E': 50 µm


E12.5
CXCR7
A
OE
Nasal cartilage
D
M
CC
VNO
A'
AZ
IZ
SDF-1
B
AZ
NM
B'
AZ
IZ
BZ
CXCR4
C
C'
IZ
BZ
CXCR7/GnRH
D
D'
SDF-1/GnRH
E
E'

At E14.5, *CXCR7* expression pattern in the area surrounding the VNO and in the ventral mesenchyme was similar to E12.5, although *CXCR7* signal became restricted to the intermediate and apical zones of the sensory epithelium of the VNO (Figure 3.3A, arrowhead), where vomeronasal sensory neurons (pheromone receptor neurons) and supporting cells (also known as sustentacular) reside (Treloar et al., 2010). *CXCR7* was also present in the surrounding cartilagenous capsule and the cavernous body (a structure containing blood vessels, venus sinus and extrinsic nerves) adjacent to the VNO (Figure 3.3A,A'). *SDF-1* expression was found to overlap with *CXCR7* in the VNO (Figure 3.3A',B', arrowheads, AZ) and in the medially located mesenchyme (Figure 3.3A',B', arrowheads). However, these genes were also expressed in a complementary fashion in the nasal mesenchyme (Figure 3.3A',B', arrows, NM) and in the tissue around the VNO, with *SDF-1* being present mostly in the lamina propria adjacent to the basal zone of the VNO (Figure 3.3B', arrow). *CXCR4* was robustly expressed in the basal zone of the VNO (Figure 3.3C'), where progenitor and immature sensory neurons reside, and to a lesser extent in the intermediate zone. Cells in the nasal area that appeared to be migrating and resembling GnRH neurons were also *CXCR4*-positive (Figure 3.3C,C', arrows).

At this stage, the OE shows mature organization and neurogenesis is initiated. Specifically, supporting cell nuclei become organized as a single apical layer, where they begin to self-renew, while neurogenesis takes place primarily in the basal layer (Carson et al., 2006), with stem cell marker expression polarized to the basal and apical layers of OE. At this time in development, *CXCR7* was detected mainly in the apical zone (supporting cells) of the OE and,

to a lesser extent, in the intermediate zone, with a faint signal also detected in the basal layer (Figure 3.3A''). *SDF-1* was restricted again in the dorsomedial apical part of OE, overlapping with *CXCR7*. *CXCR4,* on the other hand, was primarily expressed in the basal and intermediate layers, in complementary patterns to *CXCR7*. In agreement with a previous report (Schwarting et al., 2006a), we also detected *SDF-1* along the route of migrating GnRH neurons in the nasal mesenchyme (Fig. 3D-D'', arrows). *CXCR7* was also detected in the mesenchyme, showing a decreasing ventral to dorsal gradient. Finally, by E16.5, *CXCR7* transcription appeared downregulated in the area, especially in the OE, retaining the same spatial expression pattern, whereas in the VNO, it maintained the same levels (in the apical zone). *SDF-1* and *CXCR4* gene expression remained similar, but were more well-defined. *CXCR4* appeared restricted in the intermediate zone of OE, blood vessels and in the basal and intermediate layers of the VNO.

Figure 3.3 Expression of *SDF-1* and its receptors, *CXCR7* and *CXCR4*, in the nasal area of E14.5 mice as revealed by *in situ* hybridization.

A: Low magnification image of a coronal head section at the level of the nose reveals *CXCR7* expression in the VNO and NM. **A'**: Higher magnification of the VNO area shows expression of *CXCR7* in the IZ and AZ as well as in the CB and CC. **A''**: High magnification of the OE, where *CXCR7* mRNA is localized in the IZ and AZ. **B**: *SDF-1* was expressed in the VNO and NM along the migratory route of GnRH neurons. Arrowheads in **A** and **B** indicate overlapping expression of *CXCR7* and *SDF-1* in the AZ of the VNO. **B'**: Higher magnification of the VNO area shows expression of *SDF-1* in the AZ and in the surrounding mesenchyme. Overlapping and complementary expression of *CXCR7* and *SDF-1* are indicated with arrowheads and arrows, respectively. **B''**: Higher magnification of the OE area where *SDF-1* is highly expressed in the NM and the AZ. **C**: *CXCR4* expression in the nose area was localized in cells in the OE and VNO as well as in migrating GnRH neurons (arrows). **C',C''**: Higher magnification images illustrating the expression of *CXCR4* in the BZ and IZ of the VNO (C') and OE (C''). **D,D'**: *In situ* hybridization for *SDF-1* and immunohistochemistry demonstrated migrating GnRH neurons (arrows) on *SDF-1* expressing substrate in the NM. These neurons are shown in higher magnification in the VNO area and NM next to the OE (**D''**). Abbreviations: OE, olfactory epithelium; VNO, vomeronasal organ; NM, nasal mesenchyme; AZ, apical zone; IZ, intermediate zone; BZ, basal zone; CB, cavernous body; CC, cartilaginous capsule. Scale bars: A-D: 200 µm; A'-D´: 100 µm.

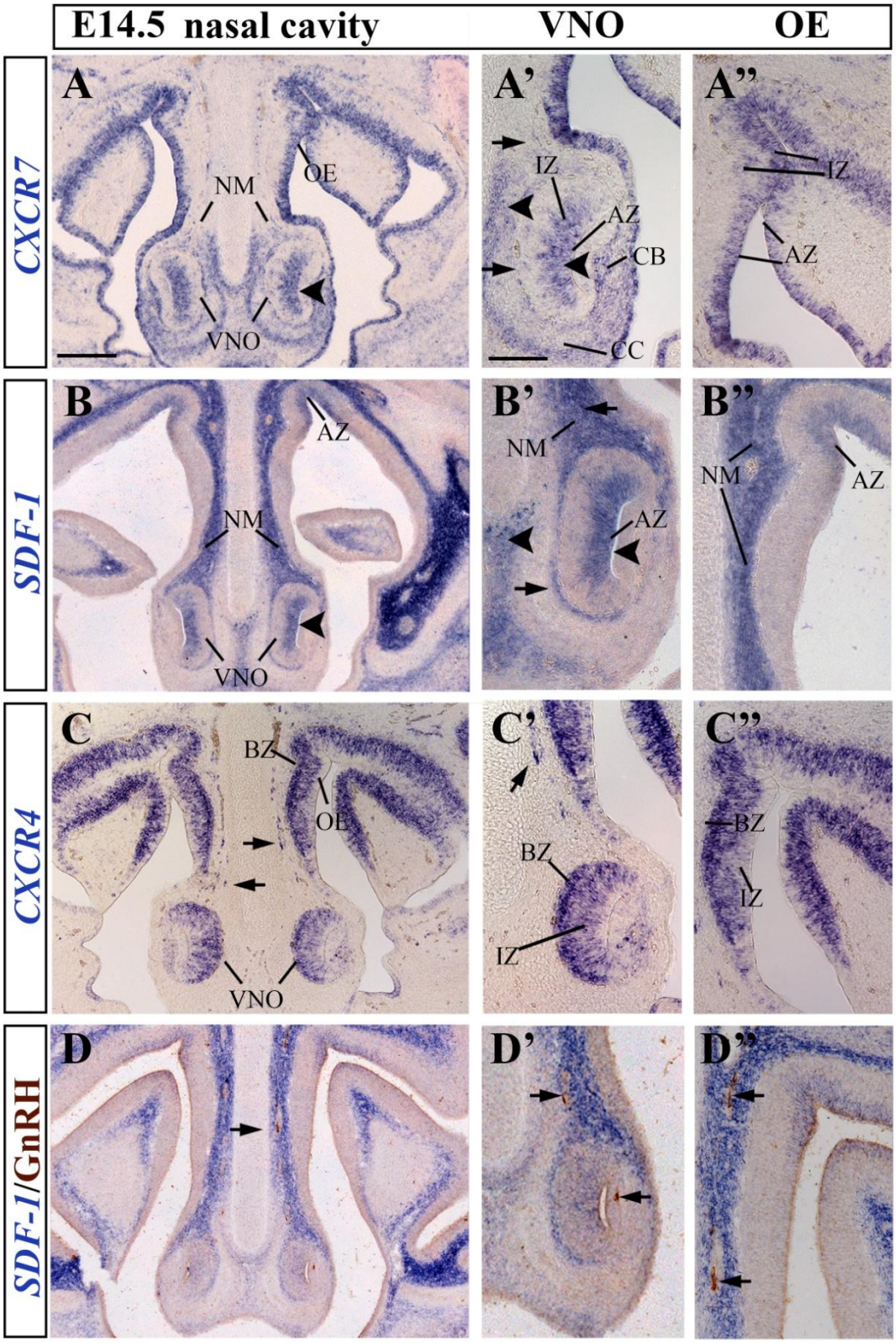
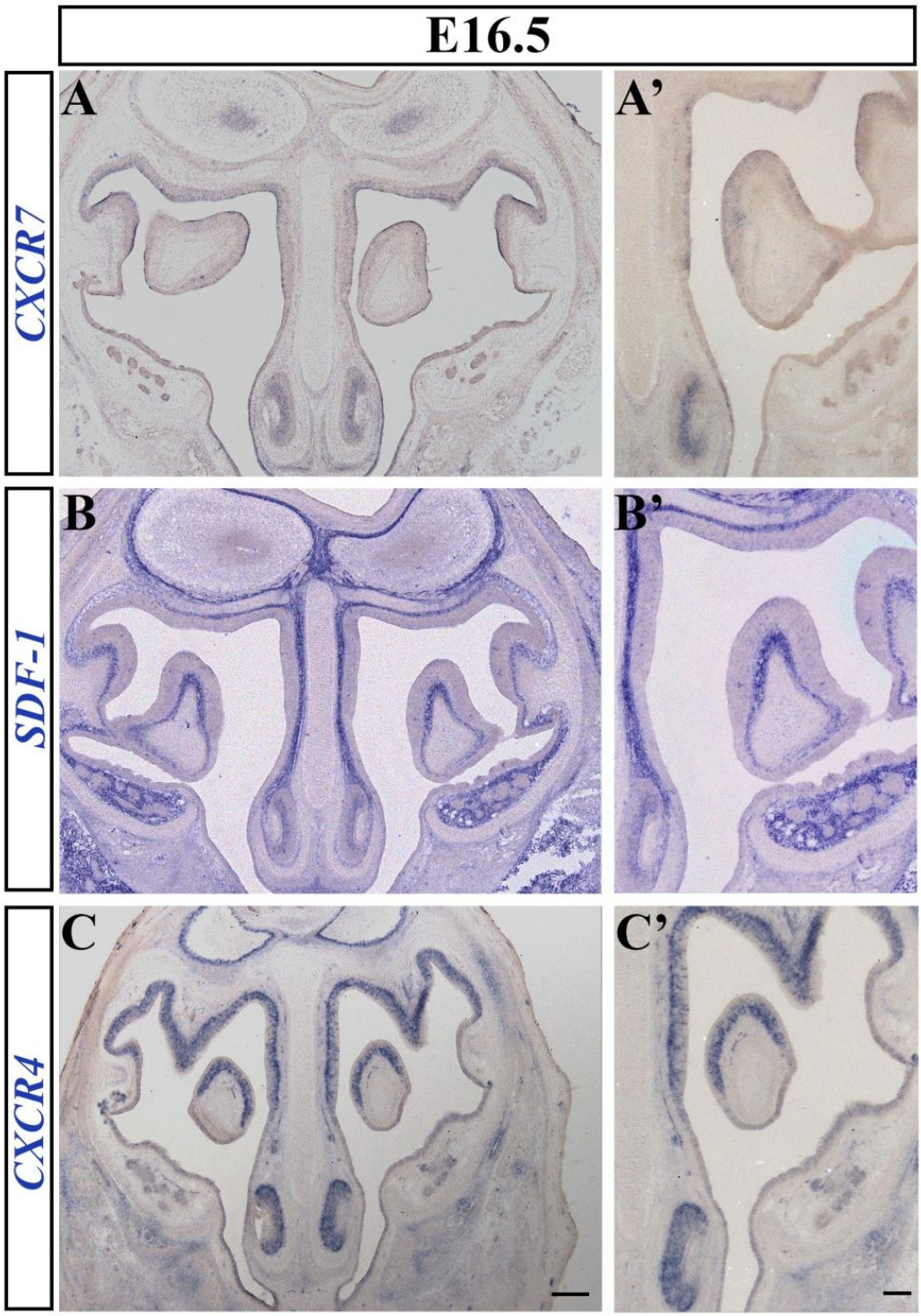

E14.5 nasal cavity
VNO
OE
CXCR7
SDF-1
CXCR4
SDF-1/GnRH
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
NM
OE
VNO
IZ
AZ
CB
CC
BZ

Figure 3.4 Comparison of expression of *SDF-1* and its receptors in the nasal area of E16.5 mice.

A,**A'**: Low and higher magnification of coronal sections processed for i*n situ* hybridization reveals expression of *CXCR7* in the AZ of the VNO and to a lesser extent in the AZ of the OE. **B**,**B'**: *SDF-1* expression in the NM and in the AZ of the VNO. **C**,**C'**: *CXCR4* was expressed in cells in the VNO and BZ and IZ of the OE. Abbreviations: OE, olfactory epithelium; VNO, vomeronasal organ; NM, nasal mesenchyme; AZ, apical zone; IZ, intermediate zone; BZ, basal zone. Scale bars: A-C: 600 µm; A-C': 300 µm.


E16.5
CXCR7
SDF-1
CXCR4
A
A'
B
B'
C
C'

3.3. CXCR7 is not expressed by migrating GnRH neurons nor their guiding axons

In order to determine the identity of CXCR7- and CXCR4-expressing cells in the developing nasal compartment, BAC transgenic mice carrying eGFP under the control of *CXCR7* and *CXCR4* promoters were used in double immunofluorescence for GFP and cell-specific markers. We found that the overall expression patterns of *CXCR7*- and *CXCR4*-eGFP matched the distribution of the respective mRNAs (Figure 3.5). In *CXCR7*-eGFP mice, double immunohistochemistry with GFP and GnRH antibodies showed no co-localization at the ages examined (E12.5 and E14.5; Figure3.6A-D'''), indicating that GnRH neurons do not express *CXCR7* during their migration. Since olfactory/vomeronasal axons serve as guides to migrating GnRH neurons, we assessed whether *CXCR7* was present in this axonal scaffold by staining sections through the nasal compartment of mice with peripherin. These experiments indicated that peripherin-labelled axons were also negative for *CXCR7*-eGFP (Figure 3.6E-F''). In similar experiments, using *CXCR4*-eGFP transgenic mice, we confirmed that, in contrast to *CXCR7*, the majority of GnRH neurons (Schwarting et al., 2006a) Figure 3.7A-C'') and their guiding axons (Toba et al., 2008b) Figure 3.7D-E'') express *CXCR4* at all ages examined.

Figure 3.5 Images of coronal head sections of E12.5 and E14.5 *CXCR7*- and *CXCR4*- eGFP transgenic mice reveal the relative expression patterns of *CXCR7* (A and B) and *CXCR4* (C and D) in the nasal area.

Abbreviations: NM, nasal mesenchyme; OB, olfactory bulb; OE, olfactory epithelium, Olf/VNN, olfactory/vomeronasal nerve; VNO, vomeronasal organ; CC, cartilaginous capsule. Scale bar: 100 µm.

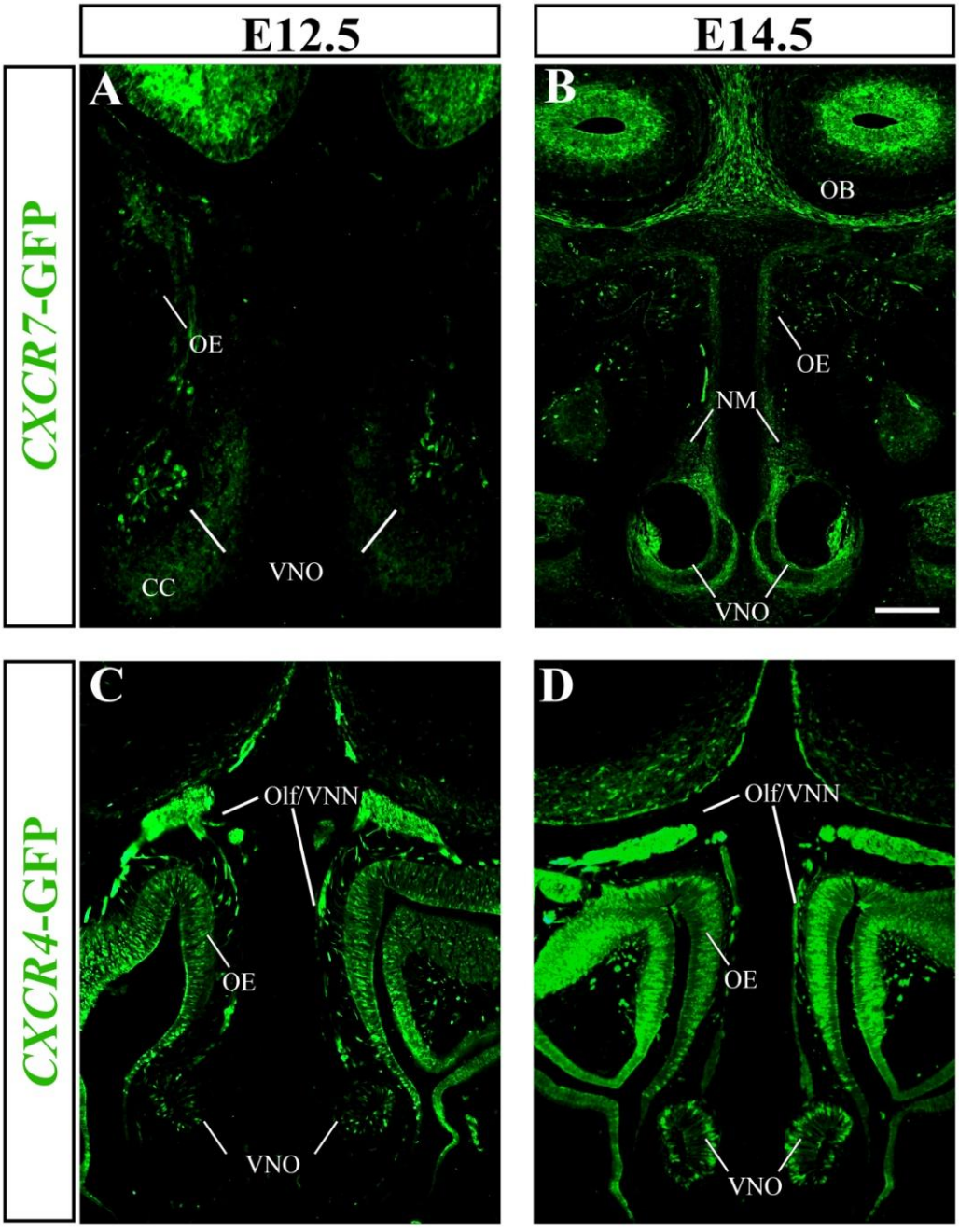

E12.5
E14.5
CXCR7-GFP
CXCR4-GFP
A
B
C
D
OE
CC
VNO
OB
NM
Olf/VNN

Figure 3.6. GnRH neurons and their axonal scaffold do not express CXCR7.

A: Coronal head section of E12.5 *CXCR7*-eGFP transgenic mice processed for immunohistochemistry, shows a number of GnRH cells (red) migrating through the nasal mesenchyme. These cells, shown at higher magnification in **B-B''**, were GFP-negative, suggesting lack of CXCR7. **C-D'''**: At 14.5, GnRH neurons still did not express CXCR7. **E-F''**: Similarly, the Olf/VNN axons used by migrating GnRH neurons and stained for peripherin (red) were CXCR7 negative. Scale bars: A,C,E: 100 µm; B-B'',D-D'',F-F'': 100 µm; D''': 50 µm.

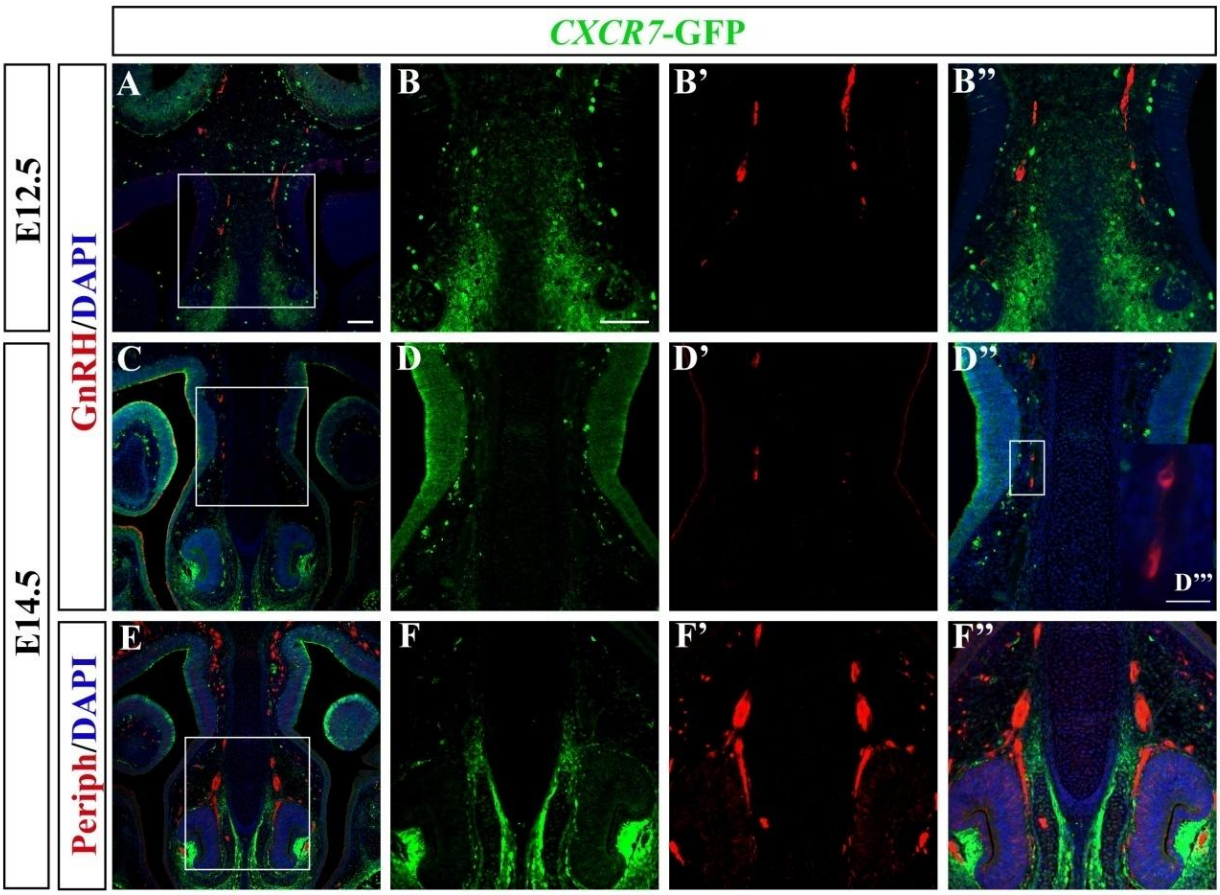
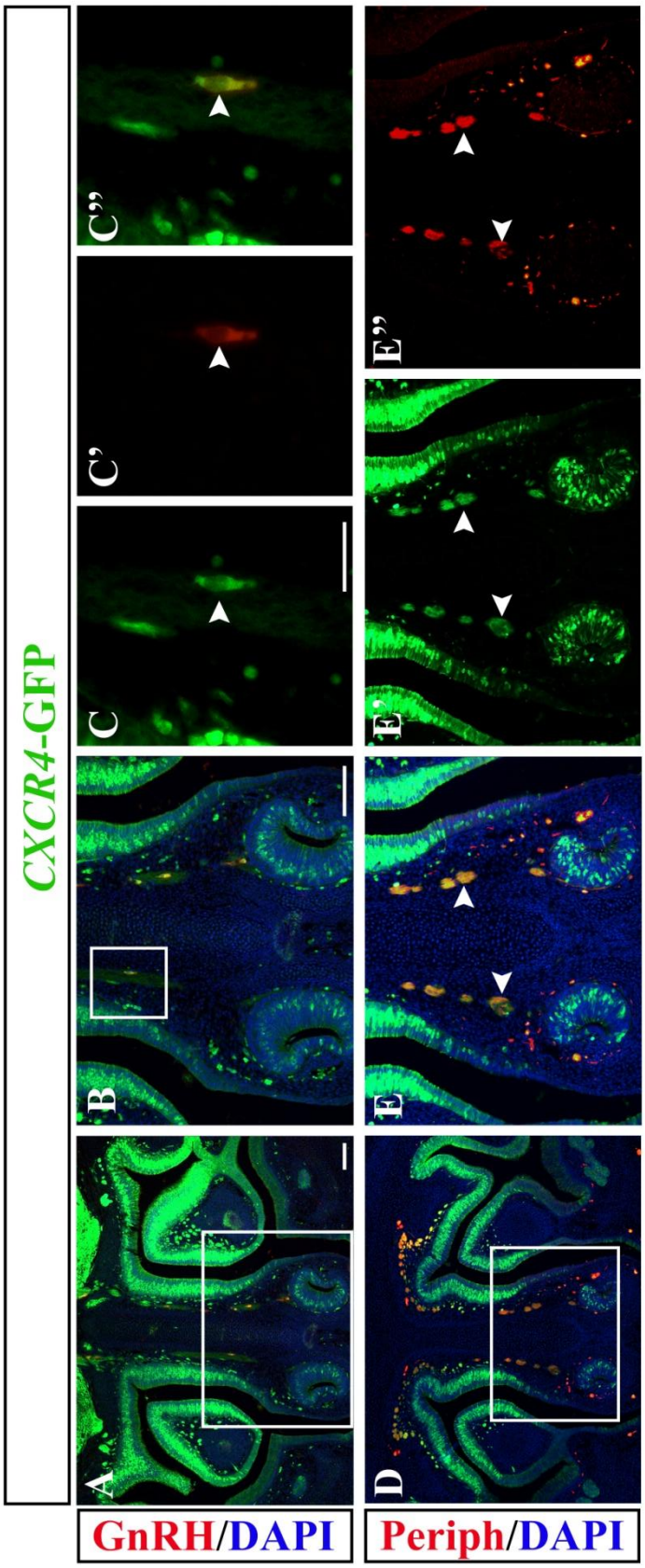

CXCR7-GFP
E12.5
E14.5
GnRH/DAPI
Periph/DAPI
A
B
B'
B''
C
D
D'
D''
D'''
E
F
F'
F''

Figure 3.7 GnRH neurons and their axonal scaffold express CXCR4.

A: Coronal head section of a *CXCR4*-eGFP mouse, shown in higher magnification in **B**, processed for GnRH immunohistochemistry, shows a number of GnRH cells migrating through the nasal mesenchyme. Cells, contained in the area outlined and shown at higher magnification in **C-C''**, were CXCR4-positive (yellow, arrowheads). Similarly, the Olf/VNN axons used by migrating GnRH neurons and stained for peripherin (red) were CXCR4-positive (yellow, **D-E''**). Scale bars: A,D: 100 µm; B,E-E'': 100 µm; C-C'': 30 µm.

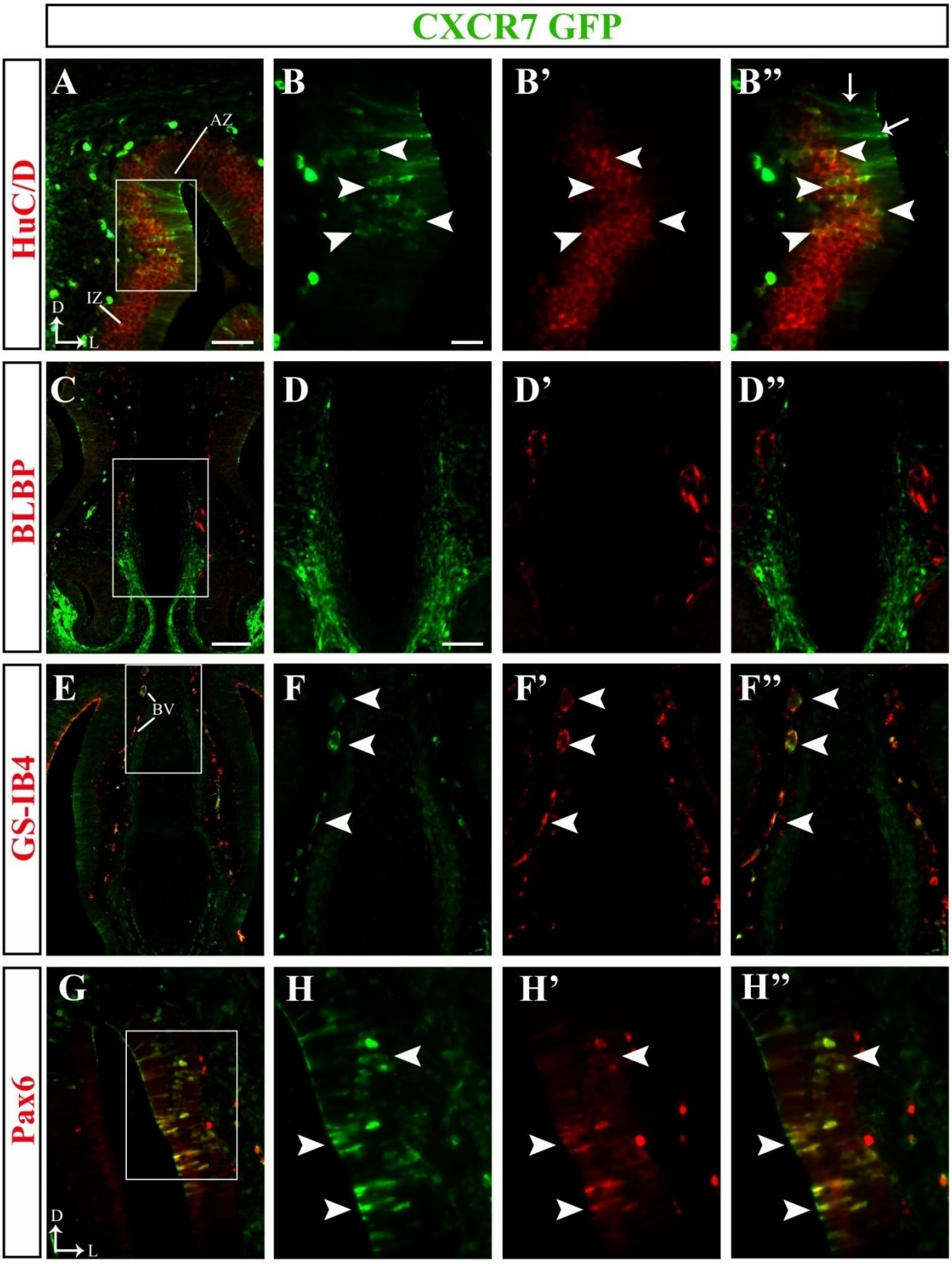

CXCR4-GFP
C''
C'
C
E''
E'
B
E
A
D
GnRH/DAPI
Periph/DAPI

In order to identify the molecular features of the *CXCR7*-expressing cells, a range of markers specific of cells present in the nasal area were used in double immunohistochemistry with GFP. Staining with HuC/D, an early neuronal marker, showed that, apart from neurons (Figure 3.8B'', arrowheads), bottle-shaped cells, resembling supporting cells, also expressed this receptor (Figure 3.8B'', arrows). This area also contained olfactory ensheathing glial cells, known to provide support to migrating GnRH neurons(Fink et al., 2012). Double immunohistochemistry with BLBP (a marker of glia cells) showed that CXCR7 is not expressed by these cells (Figure 3.8D''). However, CXCR7 was found in the endothelial wall of blood vessels as indicated by GS-Isolectin type B4 staining (Figure 3.8F''). Hence, of the cell types present in the nasal compartment that may influence GnRH neuron migration, only stromal/mesenchymal cells and blood vessels express CXCR7.

Since *CXCR7* mRNA was detected mostly in the proliferative apical layer of the OE, we assessed whether progenitor cells do express this receptor. Thus, double immunofluorescence with Pax6, a stem cell marker known to be expressed in both supporting cells and progenitor cells in the OE (Forni et al., 2011c, Guo et al., 2010, Purcell et al., 2005), showed co-expression with CXCR7 (Figure 3.8H'', arrowheads).

Figure 3.8 Characterization of cells in the OE of *CXCR7*-eGFP mice at E14.5.

A-**B´´**: Double staining for GFP and HuC/D, an early neuronal marker, showed expression of CXCR7 in both neuronal (IZ, arrowheads) and non-neuronal cells (AZ, arrows). **B-B''**: Rectangle in A shown at higher magnification. **C-D''**: Olfactory ensheathing cells stained for BLBP demonstrated no co-localization with CXCR7 (arrows). **E-F''**: Double staining for GFP and GS-IB4, a marker of blood vessels, indicated that CXCR7 was expressed by cells in the endothelial wall of blood vessels (BV). Blood vessels contained in the square in **E** are shown at higher magnification in **F-F''** (arrowheads). **G-H''**: Double labelling for the progenitor cell marker Pax6 and GFP showed double-positive cells throughout the dorso-lateral part of the olfactory epithelium (arrowheads). Scale bars: A,G: 50 µm; B,H: 30 µm**;** C,E: 200 µm; D,F: 100 µm.


CXCR7 GFP
HuC/D
BLBP
GS-IB4
Pax6
A
B
B'
B"
C
D
D'
D"
E
F
F'
F"
G
H
H'
H"
AZ
IZ
BV
D
L

3.4. Discussion

The expression pattern of CXCR7 in the nasal compartment during the time course of migration of GnRH neurons described above, suggests a role for this receptor in the migration of these neurons through the nose. Specifically, CXCR7 was found to be expressed within and around the VNO, where GnRH neurons emerge, and its expression in the nasal mesenchyme, where GnRH neurons migrate, diminished progressively in the rostro-caudal direction. Considering the previously proposed role of CXCR7 as SDF-1 scavenger (Boldajipour et al., 2008, Naumann et al., 2010), we hypothesized that CXCR7 is expressed in this particular pattern in the nasal area in order to shape the required SDF-1 gradient for directed GnRH neuron migration. The fact that GnRH neurons as well as their supporting axons of the olfactory/vomeronasal nerve do not express CXCR7, further supports the proposed scavenging role for this receptor in shaping SDF-1 gradients. The presence of CXCR7 in the OE also indicates a role for this molecule in this system. Specifically, CXCR7 was mainly localized in the apical zone of the OE, where supporting cells reside and proliferate, suggesting that it may be important for the maintenance of these cells.

It has been previously suggested (McIntyre et al., 2010) that CXCR4 in the OE may play a role in the transition from basal cell to mature ORN phenotype. Furthermore, it was proposed that the localization of SDF-1 in the underlying lamina propria may provide a signal to promote the extension of CXCR4-expressing ORN axons and their orientation out of the OE towards the olfactory bulb. The presence of SDF-1 in the apical zone of the OE, observed in our experiments, may suggest a similar role, guiding the ORN dendrites to the

olfactory cavity/mucus. Alternatively, as SDF-1 has been shown to play a role in cell proliferation and survival, it is possible that SDF-1, expressed in the apical zone of OE, plays a role in proliferation/survival of the cells residing in this area.

Chapter 4. The role of CXCR7 in GnRH neuron migration

4.1 Introduction

SDF-1 was previously shown to play an important role in the early development of the GnRH system (Schwarting et al., 2006, Toba et al., 2008) as CXCR4 deficient mice exhibit a decrease in GnRH neurons (45%) and impaired migration, as many of these cells fail to exit the VNO and subsequently die.

The identification of CXCR7 as a second receptor for SDF-1 prompted us to investigate whether it has a role in the development of the GnRH system. Our expression studies showed that CXCR7 is present in the area around the VNO, where GnRH neurons begin their migration, but not expressed by the neurons themselves. However, previous studies have shown that CXCR7 can affect the migration of cells that do not express it by regulating SDF-1 protein levels (scavenger function) available to CXCR4-expressing cells and, thus, controlling CXCR4 function (Boldajipour et al., 2008).

Our studies revealed that mice deficient for CXCR7, indeed, exhibit impaired migration and ectopic GnRH neurons in the OE when, at the same time, the total cell number was unaffected. This suggests that the receptor is unlikely to play a role in GnRH neurongenesis.

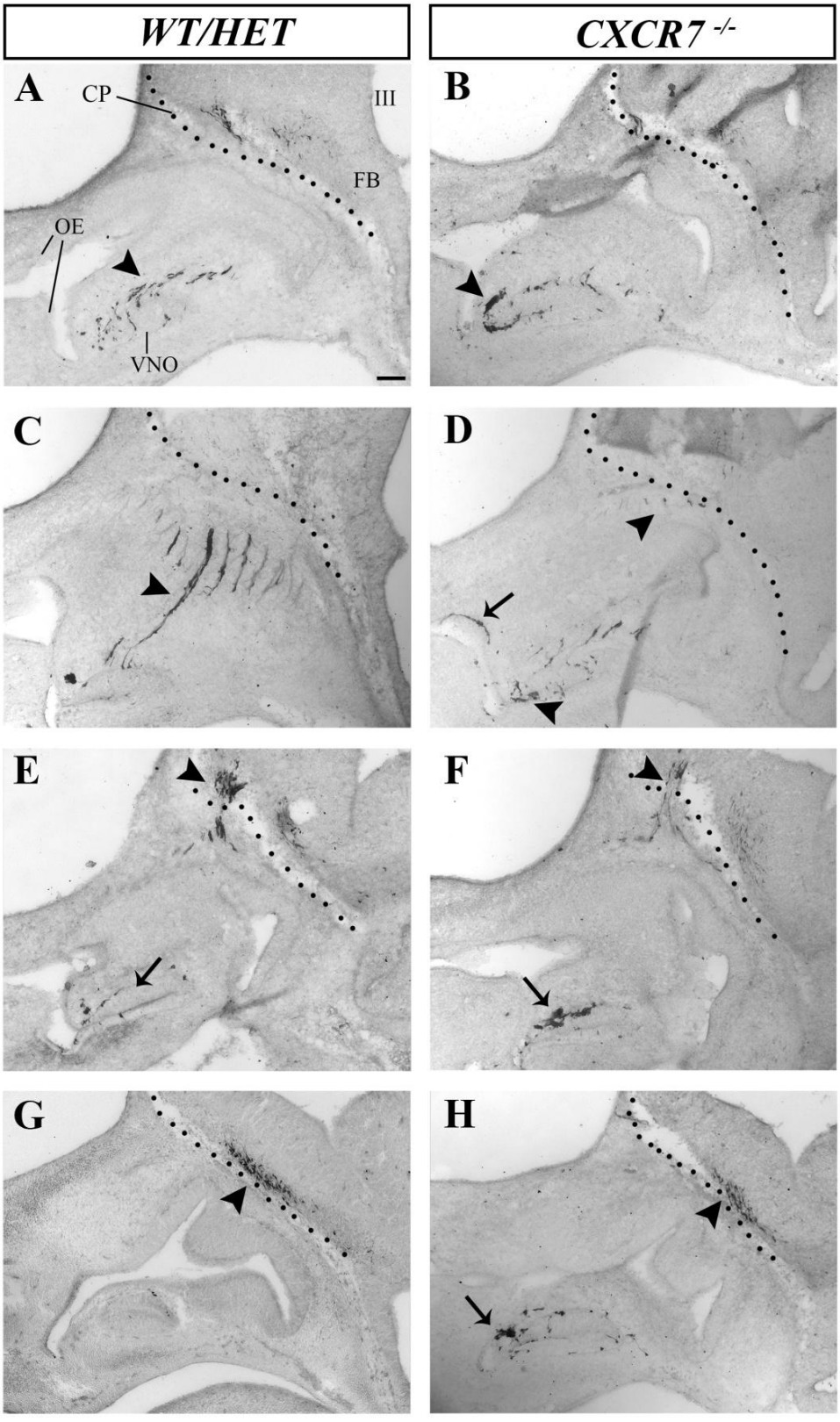
4.2. *CXCR7*-deficient mice exhibit impaired GnRH neuron migration

In order to assess whether CXCR7 is required for proper GnRH cell migration, the number and distribution of GnRH neurons in sagittal sections through the nose and forebrain of *CXCR7* deficient mice were compared to control littermates at three developmental stages (E12.5, E14.5 and E16.5).

At first glance, GnRH neurons in E12.5 *CXCR7* null mice seemed to concentrate around the VNO area, where they formed clusters (Figure 4.1 B, arrowhead), as opposed to control littermates in which GnRH cells migrated away from the VNO (Figure 4.1A,C, arrowheads), in a chain-like formation towards the brain (Figure 4.1 C, arrowhead). Comparison of sagittal sections (Figure 4.1E-H) revealed that the number of GnRH cells crossing the CP (Figure 4.1E, arrowhead) or present in the FB (Figure 4.1G, arrowhead) was significantly greater in control animal than in *CXCR7* knockout mice (Figure 4.1F,H arrowheads). In these animals, many cells appeared "trapped" in the VNO (Figure 4.1F,H arrows). Additionally, in *CXCR7* null mice, some GnRH neurons were found abnormally in the OE (Figure 4.1D, arrow).

Figure 4.1 Defective GnRH migration in CXCR7 deficient mice at E12.5.

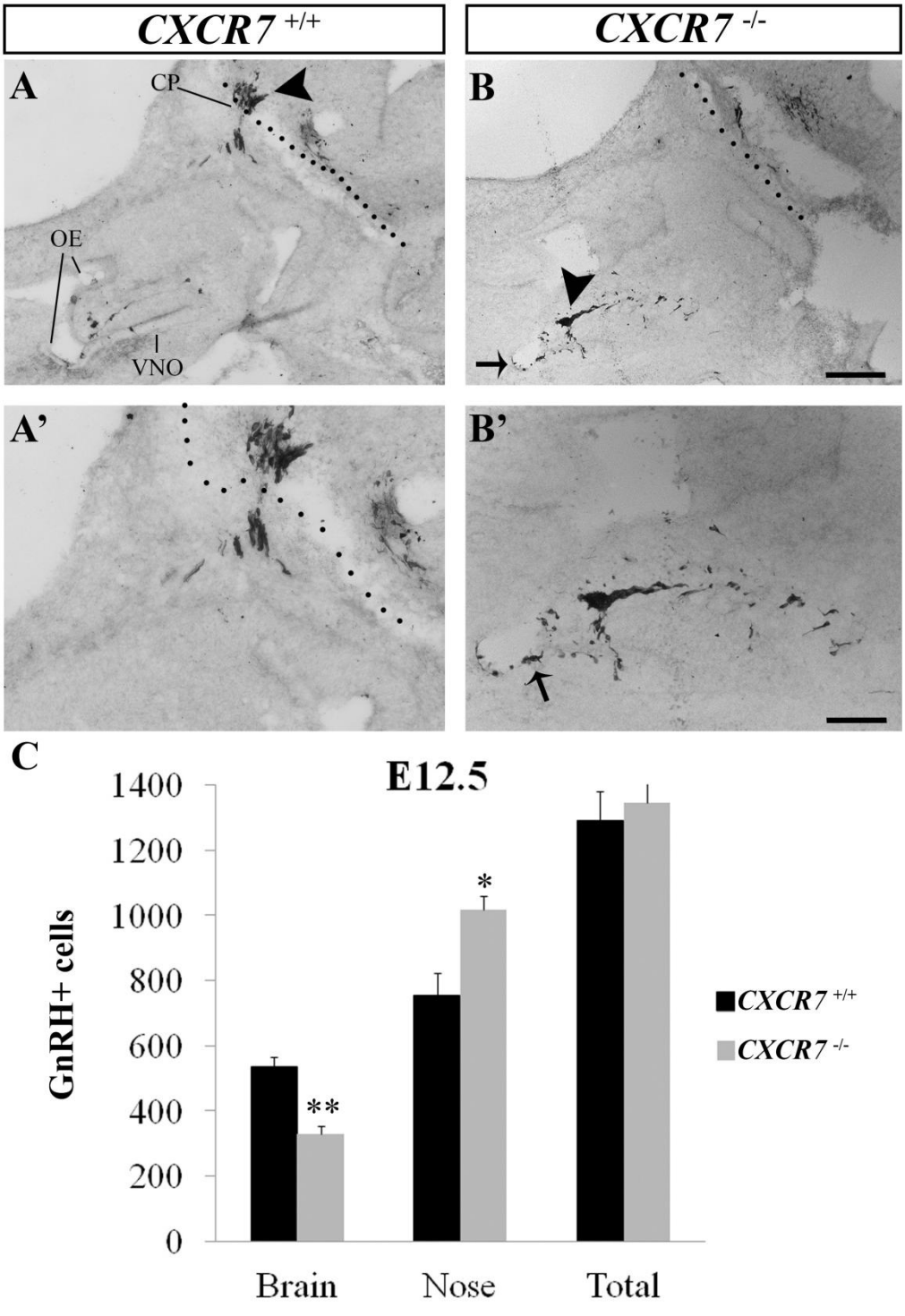
A-D: Images of sagittal sections through the nose at E12.5, stained for GnRH, show that cells tend to remain in clusters in the area of the VNO in *CXCR7* knockout mice (B,D, arrowheads) whereas, in the corresponding controls, they appear dispersed (A, arrowhead) or migrating in strings towards the brain (C, arrowhead). Black dots separate the nasal area from the brain. Moreover, some GnRH neurons in *CXCR7* knockout mice were abnormally present in the OE (D, arrow) and only a few close to the CP (D, top arrowhead). **E-H**: Most GnRH neurons in sections of control animals have crossed the CP (E,G, arrowheads) but, in similar sections of CXCR7 null mice, most cells were clustered in the VNO (F,H arrows) and only few have crossed the CP (F,H, arrowheads). Abbreviations: VNO, vomeronasal organ; OE, olfactory epithelium; CP, cribriform plate; FB, forebrain; III, third ventricle. Scale bar: 300 μm.


WT/HET
CXCR7 -/-
A
CP
III
FB
OE
VNO
B
C
D
E
F
G
H

As these findings suggest a defect in migration of GnRH neurons, a regional analysis/quantification was performed to assess whether this apparent defect can be interpreted in terms of statistical significant differences in the cell numbers in two different compartments, nose and brain, between *CXCR7* knockout and control littermates. Indeed, this analysis revealed an increase in the number of GnRH neurons in the nasal compartment and a concomitant decrease in the forebrain of the *CXCR7* deficient mice compared to controls at all ages examined. In detail, at E12.5, there was no significant difference between the total GnRH neuron population of *CXCR7* knockout mice and controls (KO: 1346±64 as compared to control: 1289±92, $p=0.57$; $n=4$; t-test; Figure 4.2C), implying that the production of these neurons is not affected by the absence of the receptor. Nonetheless, *CXCR7* knockout mice exhibited a significant increase in GnRH cell numbers in the nasal compartment (with cells accumulating around the VNO and found abnormally in the OE; Figure 4.2B, arrowhead and arrow, respectively) as compared to controls (Figure 4.2A, arrowhead) (KO: 1017±2, control: 754±68, $p^*=0.04<0.05$; Figure 4.2C) and a decrease in the FB (Figure 4.2B) (KO: 329±23, control: 535±29, $p^{**}=0.006068<0.01$; Figure 4.2C). This finding suggests a role for CXCR7 in the early steps of GnRH neuron migration, when these cells exit the VNO and start migrating towards the FB.

Figure 4.2 Migration of GnRH neurons is altered in *CXCR7* null mice.

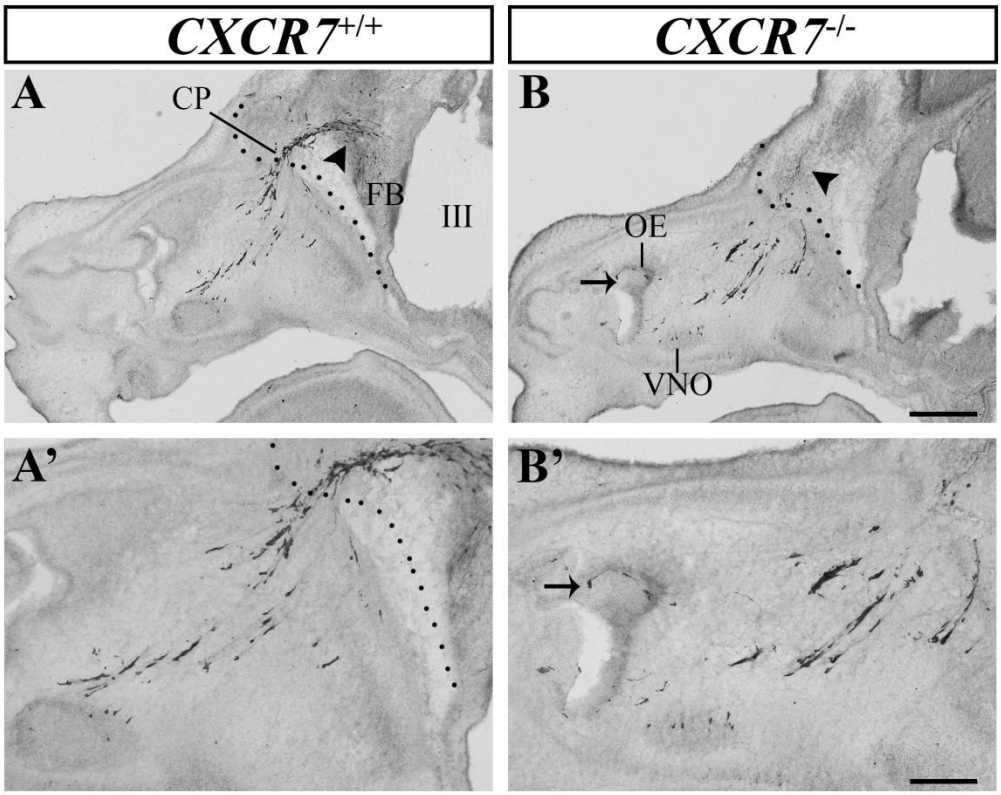
Sagittal sections (**A,B** and in higher magnification **A'** and **B'**) through the nose at E12.5, stained for GnRH, showed that cells tend to cluster in the area of the VNO in *CXCR7* null mice (B, arrowhead) whereas, in the corresponding controls, they appeared to cross the CP (A, arrowhead). Black dots separate the nasal area from the brain. **C**: Compartmental quantification of GnRH cells at E12.5 showed significant increase of neurons in the nose and a concomitant decrease in the brain of *CXCR7* null mice compared to control. However, the total number of cells was similar in the two groups. p*<0.05, p**<0.01; Abbreviations: VNO, vomeronasal organ; OE, olfactory epithelium; CP, cribriform plate; Scale bars: B: 200 µm; B':100 µm.


CXCR7 +/+
CXCR7 -/-
A
CP
OE
VNO
B
A'
B'
C
E12.5
GnRH+ cells
1400
1200
1000
800
600
400
200
0
**
*
Brain
Nose
Total
CXCR7 +/+
CXCR7 -/-

At E14.5, when more than 50% of GnRH neurons in control animals have already passed the CP and are directed towards the FB (Figure 4.3A,A'), the equivalent proportion of neurons in *CXCR7* deficient mice is 34% (KO: 367±37 as compared to control: 713±50, p***=0.001, n=6; Figure 4.3C; Figure 4.4A,B, arrowheads) with many GnRH cells remaining in the nasal compartment (Figure 4.3B,B') (KO: 793±23, control: 636±33, p**=0.009<0.01; Figure 4.3C). The phenotype was evident when coronal sections of the MPOA area- final destination of GnRH neurons- were compared between CXCR7 null mice and their control littermates. As depicted in Figure 4.4, the number of cells present in the MPOA of CXCR7 deficient mice was severely reduced when compared to control littermates. Additionally, GnRH neurons in *CXCR7* deficient mice often deviated from the chain-like formation observed in control animals (Figure 4.5) and migrated towards the OE (Figure 4.5B-E', arrowheads). Strikingly, large multicellular clusters, similar to those previously reported in GNR23 mice carrying a deletion in *Epha5* gene (Gamble et al., 2005), were observed in CXCR7 null mice (Figure 4.5F,F'). Interestingly, in many cases, GnRH cells were present in the lumen of the VNO (Figure 4.5D,D', arrows). Still at this stage (E14.5), the total GnRH cell number did not differ significantly between the two groups of animals, although there was a slight reduction in the mutants (KO: 1160±32, control: 1350±69, p=0.06>0.05; Figure 4.3E).

Figure 4.3 Impaired migration of GnRH neurons in *CXCR7* deficient mice.

Sagittal sections through the nose at E14.5, stained for GnRH, reveal that most cells are still migrating in the nasal area (**B**, and **B'** at higher magnification) in *CXCR7* knockout mice with only a few cells found in the brain (B, arrowhead) whereas, in the corresponding controls, they appear to cross the CP (arrowheads in **A**, and **A'** at higher magnification) and migrate towards the FB. Additionally, some cells are still present in the VNO and, abnormally, in the OE (B, B', arrows) in knockouts. Black dots separate the nasal area from the brain. **C**: Compartmental quantification of GnRH cells showed significant increase of neurons in the nose and a concomitant decrease in the brain of *CXCR7* knockout mice compared to control littermates, with the total number of cells similar in the two groups. p**<0.01, p***<0.001. Abbreviations: OE, olfactory epithelium; VNO, vomeronasal organ; CP, cribriform plate; FB, forebrain; III, third ventricle. Scale bars: A,B: 400 μm; A',B': 200 μm.


CXCR7+/+
CXCR7-/-
A
CP
FB
III
B
OE
VNO
A'
B'

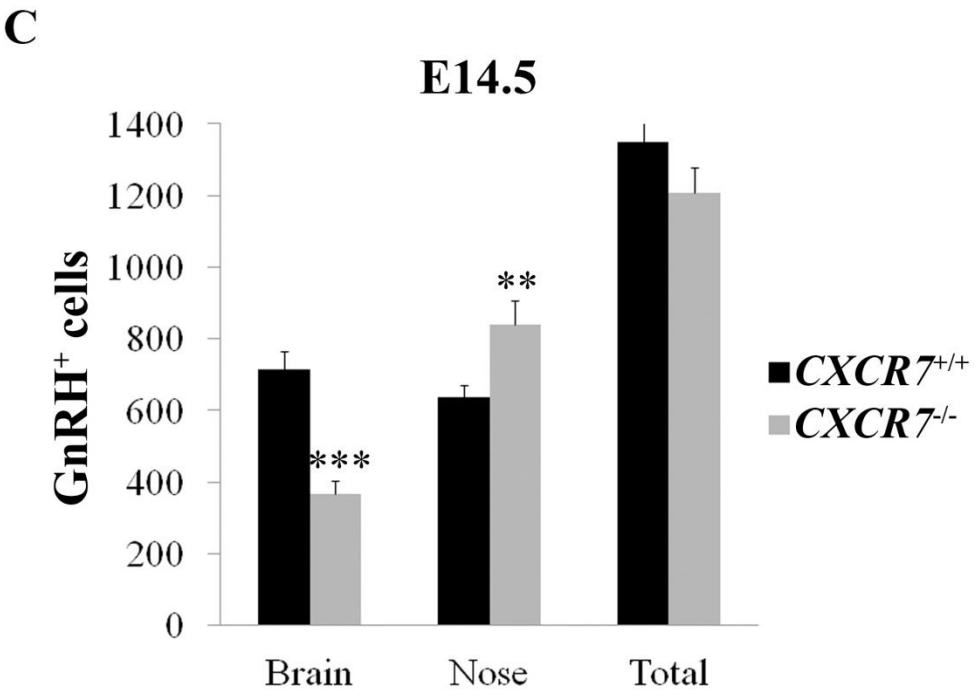

C
E14.5
GnRH+ cells
1400
1200
1000
800
600
400
200
0
***
**
Brain
Nose
Total
CXCR7+/+
CXCR7-/-

Figure 4.4 GnRH neurons in *CXCR7* deficient mice exhibit defective migration.

The number of GnRH neurons present in the MPOA of *CXCR7* deficient mice at E14.5 (**B,** and **B'** at higher magnification, arrowheads) is reduced as compared to control littermates (**A** and **A'**, arrowheads). Abbreviation: MPOA, medial preoptic area. Scale bars: A,B: 100 μm; A',B': 50 μm.

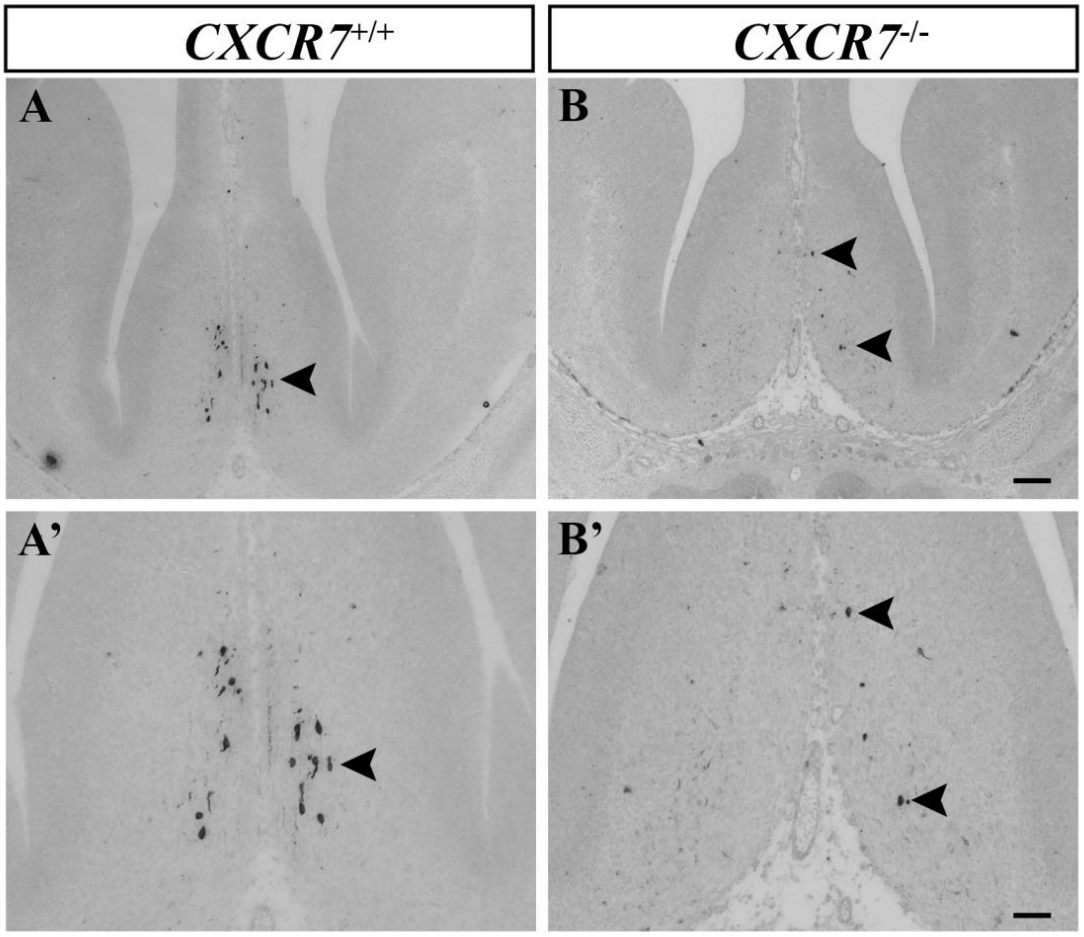
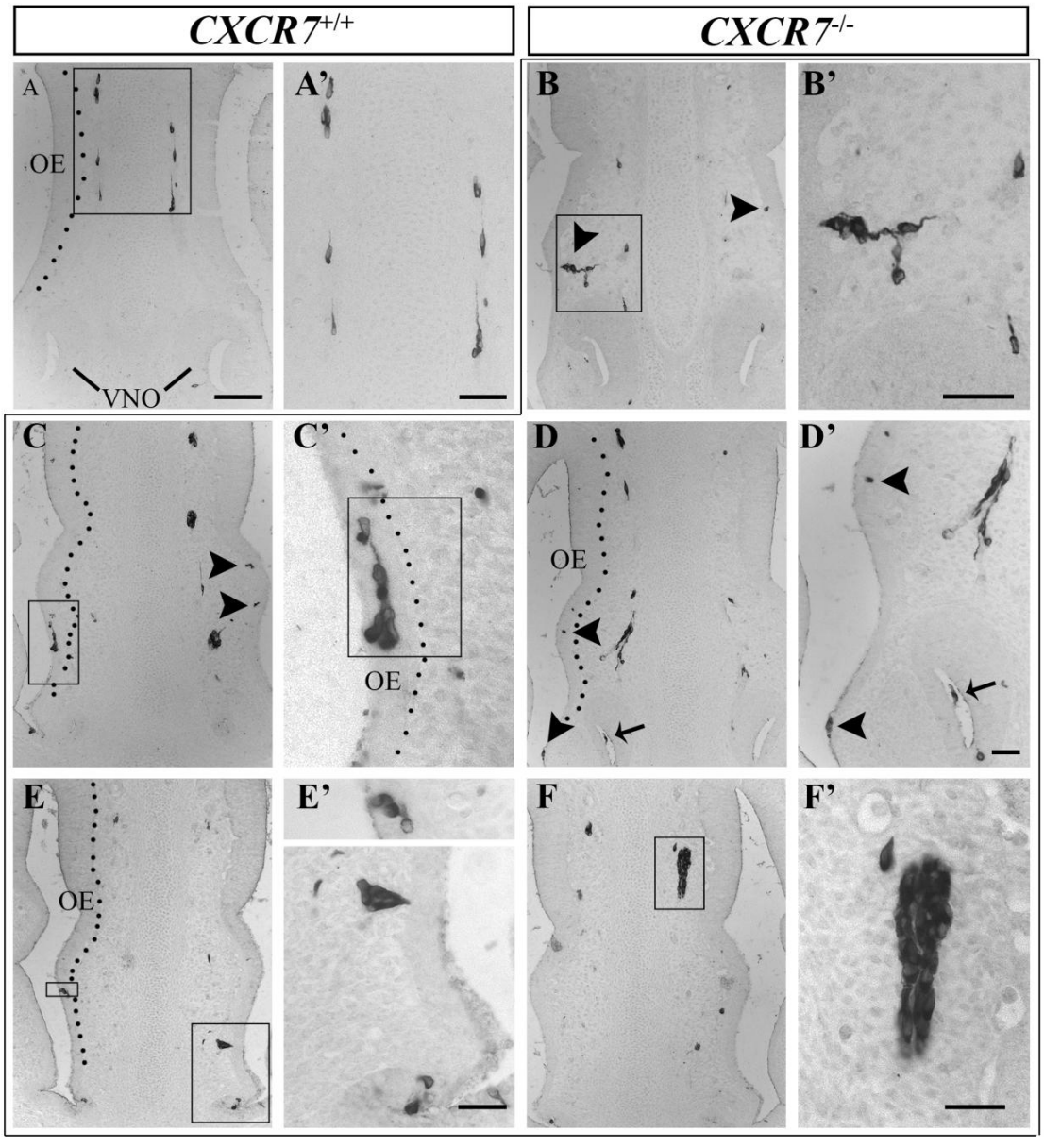

CXCR7+/+
CXCR7-/-
A
B
A'
B'

Figure 4.5 GnRH neurons in CXCR7 deficient mice are found abnormally in the OE and form multicellular clusters in the nasal mesenchyme.

Coronal sections stained for GnRH reveal that, in *CXCR7* knockout mice, migrating cells deviate from their normal chain-like formation (**A** and rectangle at higher magnification at **A'**) and, instead, appear in the OE (arrowheads in **B-E** and **B'-E'** at higher magnification) and, at times, in the lumen of the VNO (arrows in **D** and **D'**). Black dots separate the nasal area from the OE. Further, they form abnormal multicellular clusters in the nasal mesenchyme (rectangle in **F** and at higher magnification in **F'**). Abbreviations: OE, olfactory epithelium; VNO, vomeronasal organ. Scale bars: A,B,C,D,E,F: 100 µm; A',B',D': 50 µm; C',E',F',F'': 30 µm.


CXCR7+/+
CXCR7-/-
A
A'
B
B'
OE
VNO
C
C'
D
D'
OE
OE
E
E'
F
F'
OE

In order to determine the fate of the cells that accumulate in the nose, we analyzed *CXCR7* null mice of E16.5. By this age, the majority of GnRH neurons in control mice have migrated to the brain (Figure 4.6A), whereas in *CXCR7* null animals most GnRH neurons remained in the nasal area (KO: 500±34, control: 299±22, p***=0.000184<0.001; Figure 4.6E) and only a small percentage (~37%), similar to that observed at E14.5, had passed the CP (KO: 289±26 as compared to control: 837±27, p***=3.21E-09<0.001, t-test; Figure 4.6E). Interestingly, the mean total number of GnRH neurons in *CXCR7* knockout mice at this later age appeared significantly reduced, when compared to control animals (KO: 789±57, control: 1135±30, p***=0.00018<0.001; Figure 4.6E), implying that abnormally accumulating GnRH neurons in the nose could have been eliminated by programmed cell death.

We next examined the patterning of olfactory/vomeronasal axons stained for peripherin and confirmed that, in *CXCR7* null mice, the axonal substrates used by migrating GnRH neurons were normally fasciculated and properly positioned (Figure 4.7), suggesting that *CXCR7* is not involved in the patterning of these axons.

Figure 4.6 *CXCR7* knockout mice of E16.5 exhibit a defect in GnRH migration accompanied by a reduction in the total cell number when compared to controls.

A-D: Sagittal head sections of E16.5 mice stained for GnRH show that many GnRH neurons fail to reach the forebrain (B,D, arrowheads) as compared to control littermates (A,C, arrowheads). **E**: Compartmental quantification of GnRH cells showed significant increase of neurons in the nose and a concomitant decrease in the brain of *CXCR7* knockout mice compared to controls. Moreover, while the total number of cells was similar in the two groups at E12.5 and E14.5, it declined in the knockout mice at E16.5. p*<0.05, p**<0.01, p***<0.001. Scale bar: 200 μm.

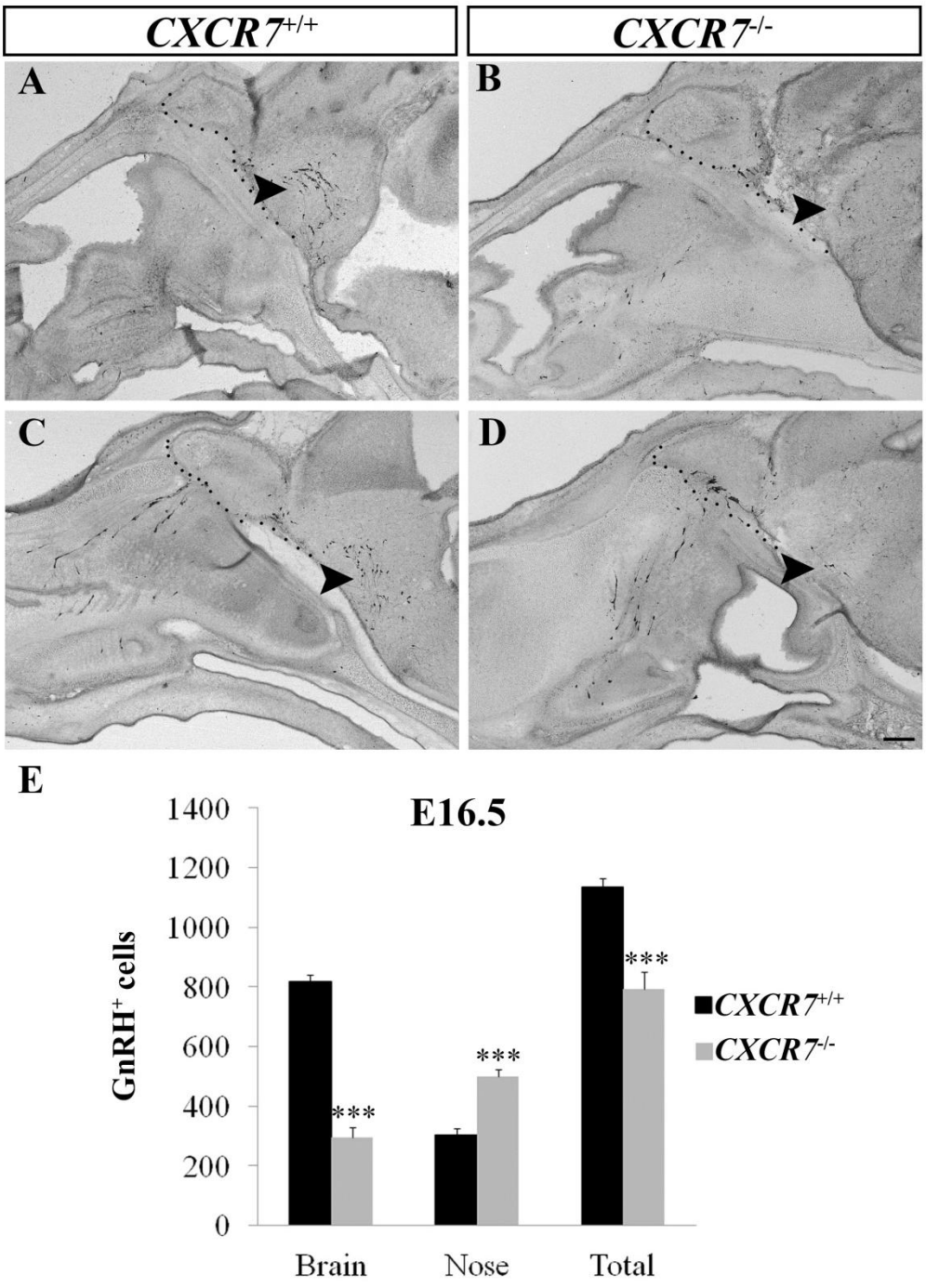
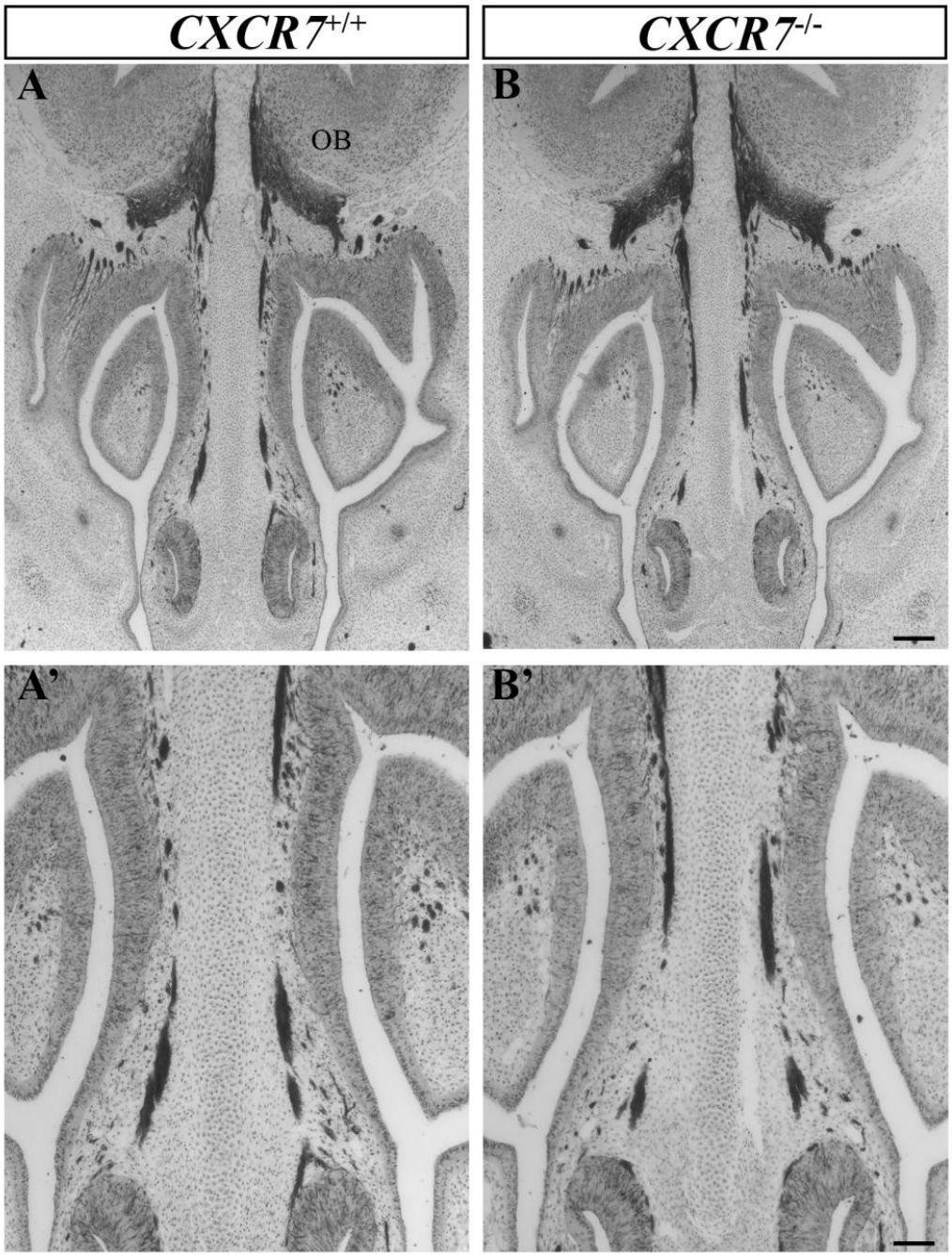

CXCR7+/+
CXCR7-/-
A
B
C
D
E
E16.5
GnRH+ cells
1400
1200
1000
800
600
400
200
0
***
***
***
Brain
Nose
Total
CXCR7+/+
CXCR7-/-

Figure 4.7 Olfactory/vomeronasal axons appear normal in *CXCR7* deficient mice.

A-B': Coronal sections through the nose of E14.5 mice, stained for peripherin, revealed that the axonal scaffold used by migrating GnRH neurons was unaffected in CXCR7 null mice (B, and B' at higher magnification) when compared to control littermates (A, and A' at higher magnification). No differences were observed in axonal fasciculation and targeting between the two groups. Abbreviation: OB: olfactory bulb. Scale bars: A-B: 200 μm; A'-B': 100 μm.


CXCR7+/+
CXCR7-/-
A
OB
B
A'
B'

4.3. Comparative analysis of *SDF-1*-, *CXCR4*- and *CXCR7* null mice

Previous studies have demonstrated that *CXCR4* deficient mice display decreased GnRH cell number and impaired migration (Schwarting et al., 2006, Toba et al., 2008). However, comparable analysis of mice lacking the ligand, SDF-1, has not been performed to date. After CXCR7 was identified as a second SDF-1 receptor (Balabanian et al., 2005b), we reasoned that examination of *SDF-1* null mice in comparison with animals lacking *CXCR4* or *CXCR7* would shed light on the role of each of these molecules in the development of the GnRH neuron system, and enhance our knowledge of the mechanisms that regulate this receptor system.

Thus, we analyzed *SDF-1* defective mice at E14.5 and found a gross decrease in the total number of GnRH neurons when compared to control littermates (KO: 480±25, control: 1187±16; p***=1.9826E-05<0.0001; n=3; Figure 4.8F), with the majority of the cells located in the nasal compartment and hardly any in the MPOA (Figure 4.8D,E). Moreover, GnRH cells were found in the lumen of the VNO (Figure 4.8B, arrowheads), and formed abnormal clusters in the lumen of the OE (at the level of the VNO, Figure 4.8 C,C', arrowheads) suggesting that many GnRH neurons do not manage to emerge from and die "trapped" in the lumen. It is worth noting that cell debris was always observed in the OE lumen of *SDF-1* knockout mice (Figure 4.8B,H, arrows), suggesting that this ligand is important for the survival of the nearby tissue. We then re-examined *CXCR4* knockout mice at the same age and, in agreement with previous studies (Schwarting et al., 2006), found reduced total GnRH cell number (KO: 628±12, control: 1227±25; p***=2.9633E-05; n=3; Figure 4.9E).

Figure 4.8 SDF-1 deficient mice exhibit impaired GnRH neuron migration and a gross decrease in their total number at E14.5.

A,B: In the nose, GnRH neurons migrated in a chain-like formation in control animals (A) but, in SDF-1 knockouts, they were found in the VNO (B, arrowheads) and in clusters in the lumen of the OE (**C,C'**, arrowheads). Moreover, cell debris in the OE lumen was observed only in knockout animals (arrows in B and H). **D,E:** GnRH staining of coronal sections at the level of the MPOA showed the distribution of these cells in control animals (D, arrowhead) and the relative reduction in numbers in the *SDF-1* knockout mice (E, arrowhead). **F**: Compartmental quantification of GnRH neurons in mice of E14.5 showed an overall decrease in *SDF-1* null mice, and a significant reduction in both nose and brain when compared to control animals. However, most GnRH cells were still localized in the nasal area as opposed to controls where more than half were found in the brain. **G,H**: No apparent differences were observed in the axonal scaffold between the SDF-1 knockout and control littermates. p*<0.05, p**<0.01, p***<0.001. Abbreviations: MPOA, medial preoptic area; OE, olfactory epithelium; VNO, vomeronasal organ. Scale bars: A,E,F,H: 100 μm; H': 50 μm.

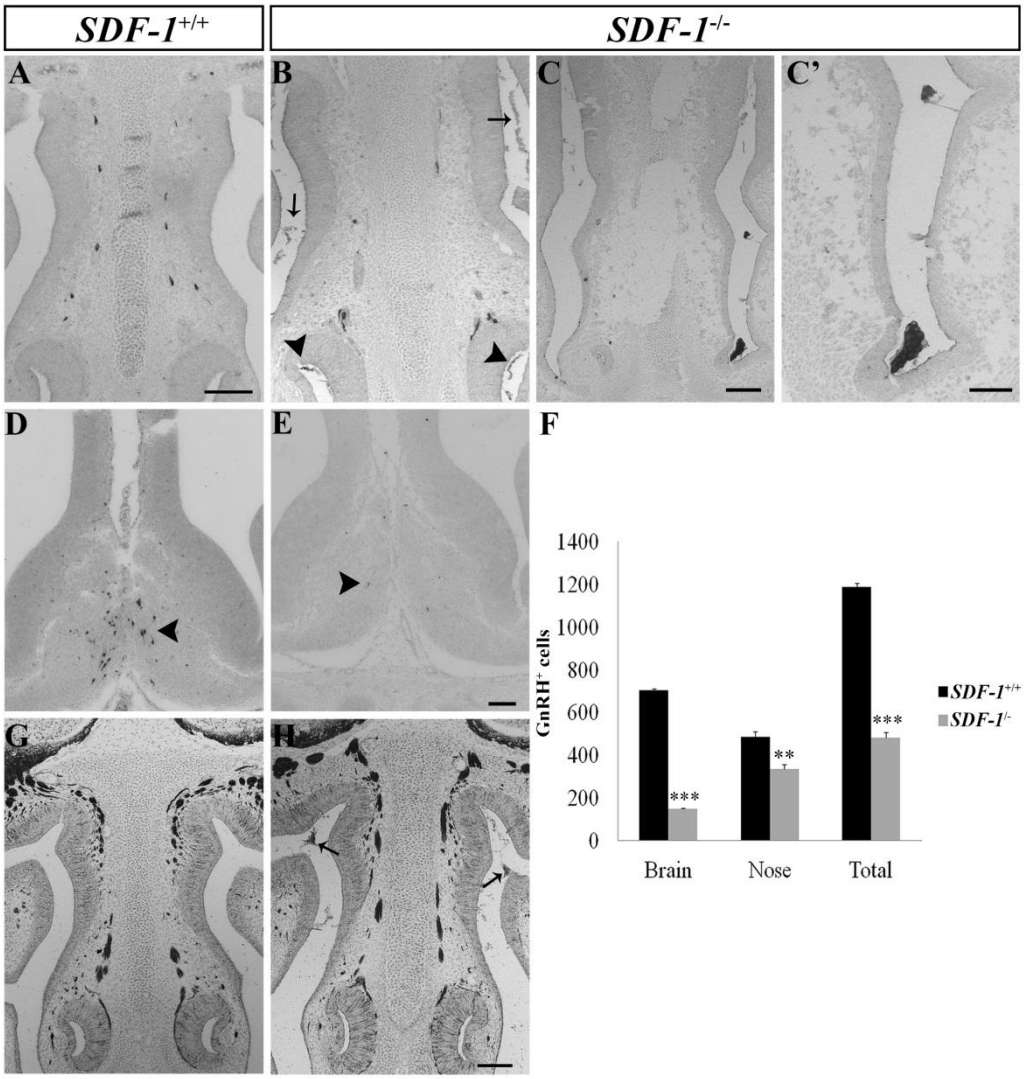
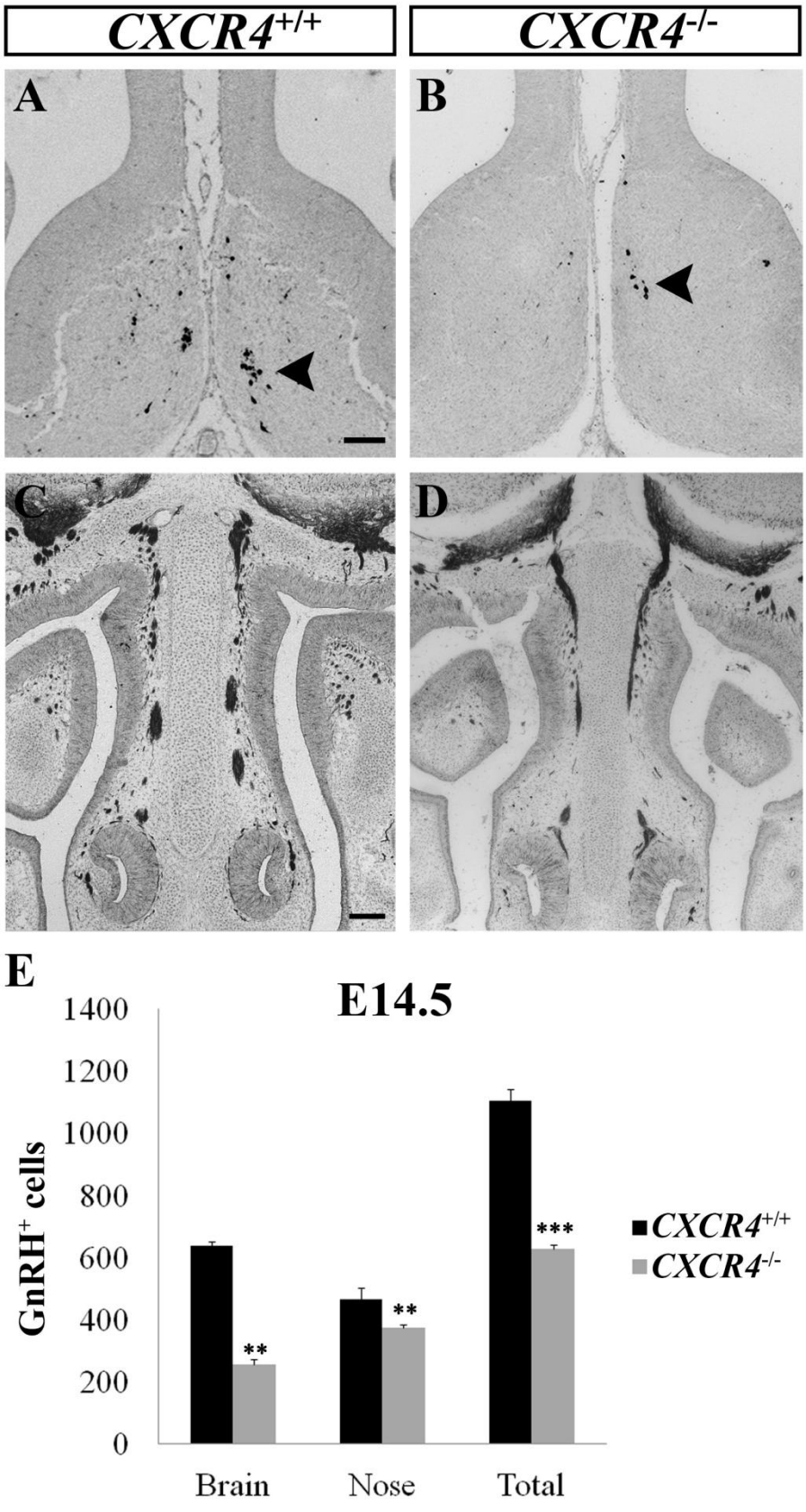

SDF-1+/+
SDF-1-/-
A
B
C
C'
D
E
F
G
H
1400
1200
1000
800
600
400
200
0
GnRH+ cells
***
**
***
Brain
Nose
Total
SDF-1+/+
SDF-1-/-

Figure 4.9 CXCR4 null mice displayed a decrease in the total GnRH cell number in the MPOA (A,B).

However, the axonal scaffold, revealed with peripherin staining, was similar between knockout and control mice (**C,D**). **E**: Compartmental quantification of GnRH neurons at E14.5 showed an overall decrease in *CXCR4* deficient mice and a significant reduction in both nose and brain when compared to control animals. p*<0.05, p**<0.01, p***<0.001. Abbreviation: MPOA, medial preoptic area. Scale bars: A-B: 100 µm; C-D: 100 µm


CXCR4+/+
CXCR4-/-
A
B
C
D
E
E14.5
GnRH+ cells
1400
1200
1000
800
600
400
200
0
**
**
***
Brain
Nose
Total
CXCR4+/+
CXCR4-/-

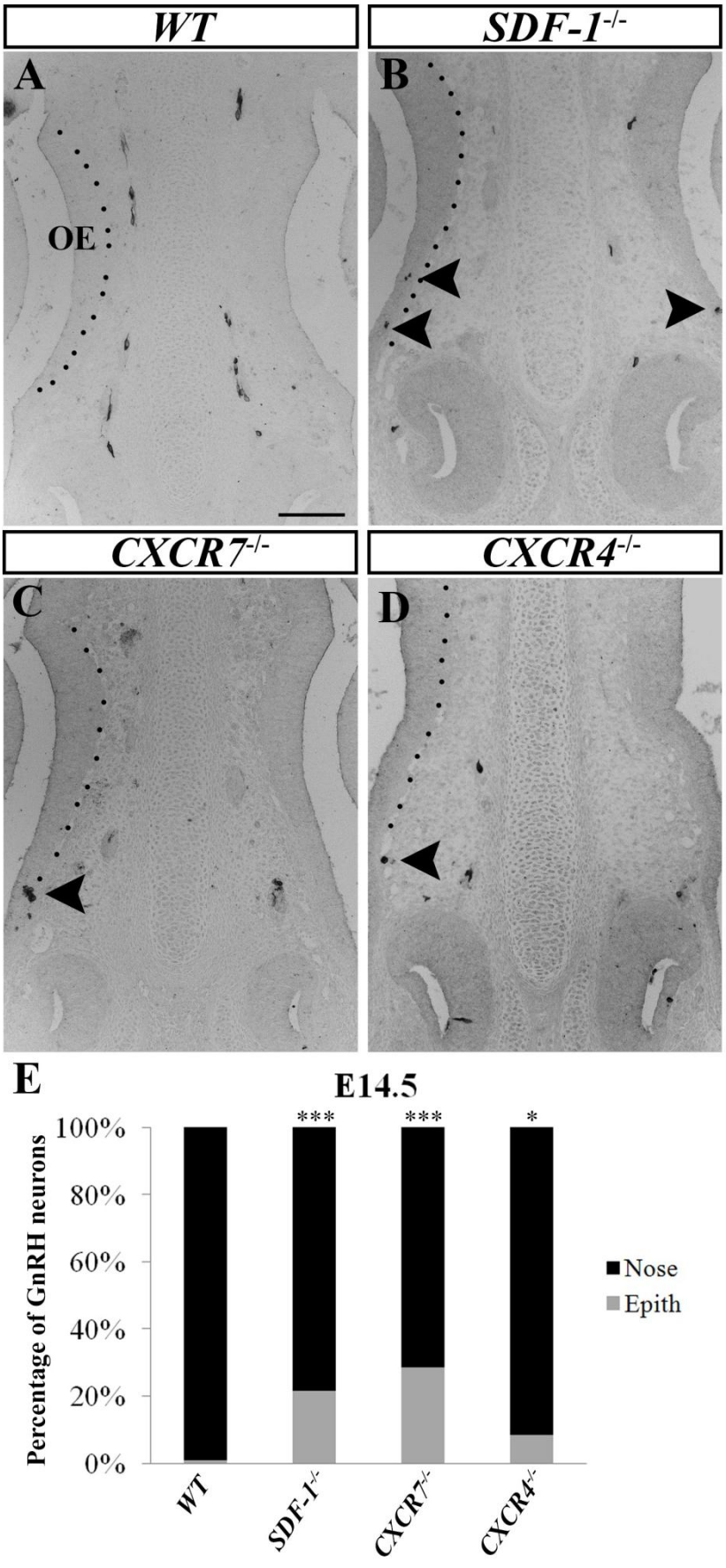
As CXCR7 was shown to be absent from GnRH neurons, at least within the timeframe of our analysis (E12.5-16.5; knockout mice die in late gestation), we considered CXCR4 to be the only receptor mediating SDF-1 signalling on GnRH neurons. The similarity between the two phenotypes (significant decrease in the total number of GnRH neurons in *CXCR4*- and *SDF-1* null mice) together with our finding that *CXCR7* null mice did not display such defect (Figure 4.3C) provide credence to this hypothesis.

In addition to the defect in total cell number in both *CXCR4*- and *SDF-1* null mice, the regional distribution of GnRH neurons differed from that of control animals. Specifically, similar to embryos lacking *CXCR7*, GnRH neurons in *SDF-1* deficient mice were found accumulated in the nasal compartment (area between the VNO and nasal/forebrain junction) and not migrating to the brain (Figure 4.8E,F). Thus, at E14.5, there were twice as many neurons in the nose compared to the brain when, in control mice, most of GnRH neurons had already crossed the nasal/forebrain junction and were directed towards the hypothalamus. Counts of cells residing in the forebrain revealed that control animals had five times as many GnRH neurons as *SDF-1* knockout mice (KO: 148± 5, control: 708± 11; p=0.00024; Figure 4.8F). Similarly, in *CXCR4*-lacking mice, the majority (60%) of the cells were still present in the nasal compartment as compared to 42% in control littermates (Figure 4.9E). Concomitantly, the number of GnRH neurons in the brain differed significantly from control mice (KO: 254± 17, control: 714± 36; p=0.0016; Figure 4.9B,E). It should be added that no apparent defect was found in the axonal scaffold of *SDF-1* and *CXCR4* knockout mice (Figure 4.8G,H; Figure 4.9C,D).

In order to gain greater insight into why GnRH neurons remain in the nasal compartment, we carefully examined their appearance and spatial distribution in this area. As depicted in Figure 4.10, considerably more cells in these animals were found abnormally in the OE (Figure 4.10B,C,D, arrowheads). Specifically, when we analyzed the positions of GnRH neurons in the three groups (*SDF-1*, *CXCR4*, *CXCR7*) of knockout mice, we observed that in *SDF-1* and *CXCR7* deficient mice, 20% and 28% of the total GnRH cell population present in the nose were aberrantly located in the OE; in the *CXCR4* knockouts, the percentage was 8.5%. In comparison, hardly any (0-1%) cells were identified in the OE in any of the control littermates. These observations strongly suggest that this chemokine/receptor system keeps GnRH neurons to their migratory path.

Figure 4.10 Spread analysis of GnRH neurons in the nasal area revealed disorganized cell distribution in the three knockouts.

A-D: Comparison of the position of GnRH neurons in coronal nose sections in E14.5 WT, *SDF-1*-, *CXCR7*- and *CXCR4* knockout mice, showed abnormal localization of GnRH cells in the OE only in the knockout animals (arrowheads in B-D). **E**: Quantification of GnRH neurons in the nose divided in two compartments, OE and nasal mesenchyme, revealed that 22% of cells were abnormally located in the OE of *SDF-1* knockout animals. The respective percentage was 28% in *CXCR7*- and 10% in *CXCR4* null mice. No GnRH neurons were found in the OE in control animals. p*<0.05, p**<0.01, p***<0.001. Abbreviations: OE, olfactory epithelium; WT, wild-type. Scale bar: 100 μm


WT
SDF-1-/-
A
B
OE
CXCR7-/-
CXCR4-/-
C
D
E
E14.5
***
***
*
100%
80%
60%
40%
20%
0%
Percentage of GnRH neurons
Nose
Epith
WT
SDF-1-/-
CXCR7-/-
CXCR4-/-

4.4 Discussion

Since CXCR7 has been shown to act as scavenger receptor to shape extracellular SDF-1 gradients (Luker et al., 2010, Boldajipour et al., 2008), the strong expression along the migratory path of GnRH neurons prompted us to investigate whether its loss might affect indirectly the migration of these neurons. Indeed, our analysis of *CXCR7* deficient mice revealed a defect in GnRH neuron migration when, at the same time, the total cell number was unaffected. This suggests that CXCR7 is not involved in the early generation of GnRH neurons. In these null mice, half of the GnRH neuron population failed to migrate to the hypothalamus and, instead, distributed randomly in the nasal area, formed clusters and positioned themselves ectopically in the OE, suggesting that CXCR7 is required for their directed migration towards the forebrain. Interestingly, although the absolute GnRH cell numbers in these mice did not differ at early developmental stages (E12.5-14.5) as compared to control littermates, the later (E16.5) GnRH neuron population was significantly reduced. It is likely that ectopic GnRH neurons that failed to migrate to the brain eventually die or cease to express GnRH gene (as GnRH is the only marker for these cells, this possibility cannot be ruled out). Unfortunately, examination of CXCR7 deficient mice or later developmental stages was hindered due to embryonic lethality.

As the GnRH system was never studied before in SDF-1 null mice, we reasoned that a comparative analysis of mice lacking SDF-1, CXCR4 and CXCR7 would provide valuable information on the role of each molecule in this system and their interactions. We found that the phenotype in *SDF-1* knockouts closely

resembled that of *CXCR4* null mice, both in terms of migration and overall decrease in cell number, strongly suggesting that SDF-1 signals through CXCR4 in this system to control GnRH neuron survival and migration. Moreover, the aberrant distribution of GnRH neurons in the nasal area of *SDF-1*, *CXCR4* and *CXCR7* null mice points to a direct role for SDF-1 in guiding the migration of these cells, especially since the supporting axonal scaffold was unaffected in the three knockout lines.

Chapter 5. CXCR7 indirectly regulates CXCR4 protein levels

5.1 Introduction

We have previously shown that *CXCR7* deficient mice exhibit impaired GnRH neuron migration. Since CXCR7 is not expressed by these cells, how can this receptor affect their migration? Earlier work showed that high levels of SDF-1 leads to downregulation of CXCR4 due to increased activation and desensitization (Marchese and Benovic, 2001). In accordance with this, CXCR7 has been shown to regulate CXCR4 function by controlling the extracellular levels of the chemokine due to its scavenging function. This can be done either in a cell-autonomous manner, when it is co-expressed with CXCR4 by the same cell, as demonstrated in other systems (Sanchez-Alcaniz et al., 2011) or even in a non-cell autonomous manner, when is expressed in the tissue surrounding CXCR4 positive cells (Boldajipour et al., 2008). Hence, we next assessed whether the cellular localization of CXCR4 differs in *CXCR7* null mice.

5.2 Altered subcellular distribution of CXCR4 in *CXCR7* null mice

In order to assess the subcellular distribution of CXCR4 in CXCR7 deficient mice, sections of the nasal area were stained with UMB-2 (anti-CXCR4 Ab) and compared to control littermates. As depicted in Figure 5.1, the levels of CXCR4 protein were reduced in the nasal compartment of CXCR7 deficient mice, when

compared to control. Careful analysis of these mice revealed that in both the OE and VNO, cells and axons expressing CXCR4 showed an altered subcellular distribution of the receptor. Specifically, CXCR4 was re-allocated in the cytoplasm of cells, where it appeared as dots (Figure 5.1A,B, arrowheads), suggesting increased internalisation. In SDF-1 knockout mice, on the other hand, CXCR4 appeared upregulated in the cell membrane (Figure 5.1C), possibly due to the absence of the ligand which leads to the presence of all protein produced in the membrane in its inactive form. Interestingly, in double CXCR7/SDF-1 null mice, the CXCR4 receptor phenotype was partially rescued, with CXCR4 present again in the membrane (Figure 5.1D), supporting the hypothesis that the role of CXCR7 in this system is to control SDF-1 protein levels acting as a scavenger.

Double immunohistochemistry with UMB-2 (for CXCR4) and βIII-tubulin that labels both soma and processes of neurons, illustrated better the different appearance of CXCR4 in somata and absence of the receptor from the olfactory/vomeronasal axons in *CXCR7* knockout mice (Figure 5.2D,E compared to A,B). This observation suggested that CXCR7 is important for proper CXCR4 function. In GnRH neurons, CXCR4 also appeared to be more internalized or even absent when compared to controls (Figure 5F'' compared to 5C''). In a similar experiment, SDF-1 knockout mice displayed elevated levels of the CXCR4 on the plasma membrane of cells in the VNO as well as axons, when compared to wild-type mice (Figure 5G,H,I''). The enhanced internalization of CXCR4 observed in *CXCR7* deficient mice, a typical response to high levels of

SDF-1, was reversed in an experiment that combined removal of SDF-1 and CXCR7 (Figure 5J,K,L'').

In order to confirm these findings and exclude the possibility that they are specific to this antibody (UMB-2), an alternative anti-CXCR4 antibody was used (2B11) and compared to UMB-2. As depicted in Figure 5.3, staining of CXCR4 with both antibodies was identical. Moreover, CXCR4 in *CXCR7* knockout mice appeared as dots when stained with both antibodies.

Figure 5.1 Altered subcellular distribution of CXCR4 protein in *CXCR7*- and *SDF-1* deficient mice.

A-**B**: Coronal nose sections of control and *CXCR7*-lacking mice stained with UMB-2 (anti-CXCR4 Ab), showed that CXCR4 exhibited reduced immunoreactivity in *CXCR7* null mice as compared to WT as well as increased internalization as evidenced by the appearance of dots in both the VNO and OE. On the other hand, in *SDF-1* knockout mice, the receptor seemed to be upregulated and localized on the cell membrane, possibly due to the absence of the ligand (**C**). Interestingly, combined deletion of *SDF-1* (and CXCR7) rescued the CXCR4 phenotype observed in *CXCR7* knockout mice, suggesting that CXCR7 acts as a SDF-1 scavenger (**D**). Abbreviations: OE, olfactory epithelium; VNO, vomeronasal organ; WT, wild-type. Scale bar: 100 μm.

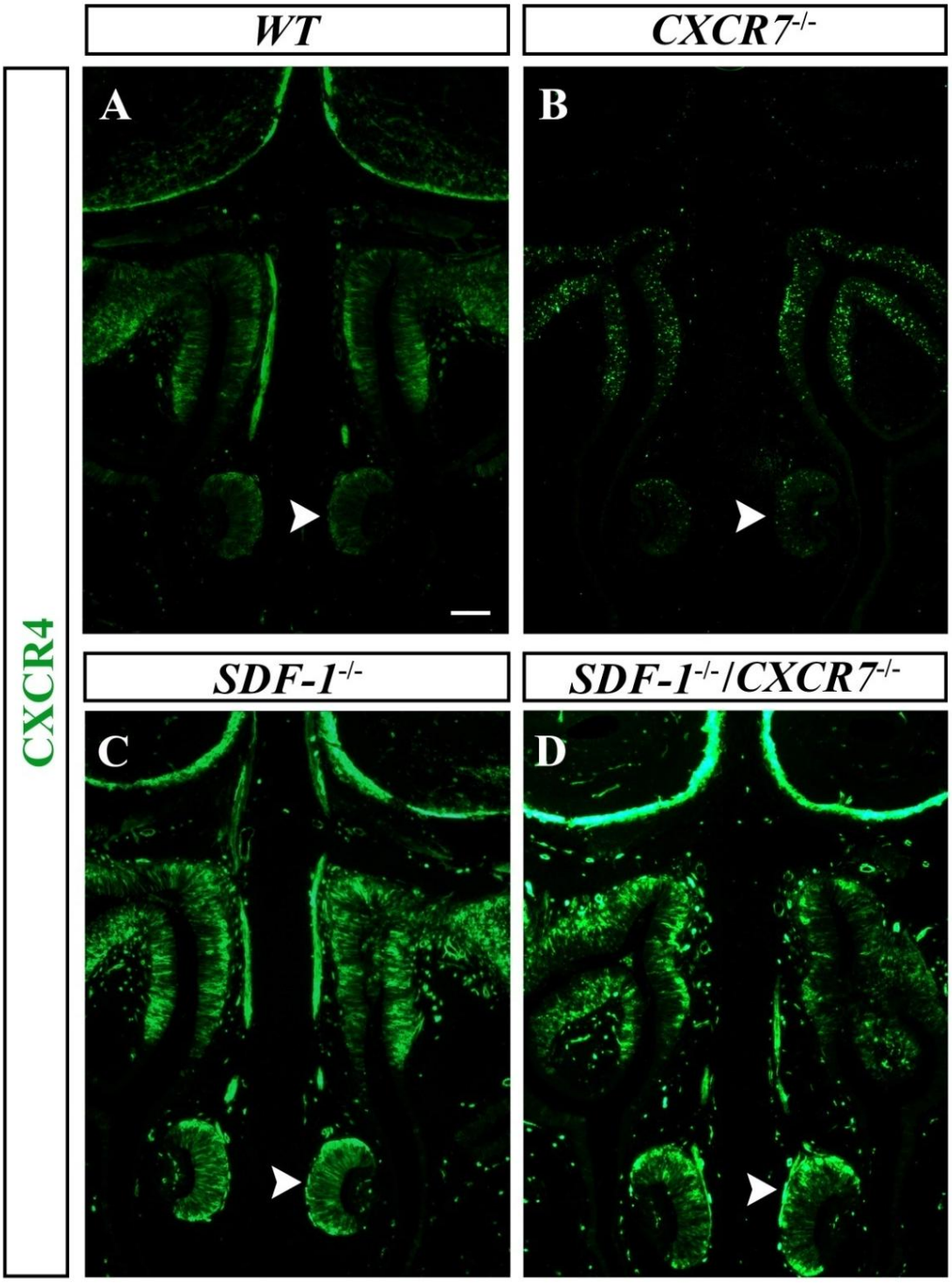

WT
CXCR7-/-
A
B
CXCR4
SDF-1-/-
SDF-1-/-/CXCR7-/-
C
D

Figure 5.2 Subcellular localization of CXCR4 is altered in *CXCR7*- and *SDF-1* deficient animals.

A,**B**: Double labelling for CXCR4 (green) and βIII tubulin (red) shows that in *WT* animals, CXCR4 is localized in axons (A, arrow) and in cells in the VNO (B, arrowheads). **D**,**E**: CXCR4 is not detectable in axons (D, arrow) and shows a punctate/internalized pattern in cells of the OE and VNO of *CXCR7* null animals (E, arrowheads). **G**,**H**, In contrast, CXCR4 is upregulated in axons (G, yellow, arrow) and in cells of the OE and VNO (H, arrowheads) in *SDF-1* null mice. **J,K**: The loss of CXCR4 observed in *CXCR7* KO animals (D,E) is reversed in double *CXCR7/SDF-1* knockout animals (J, arrow; K, arrowheads). **C**,**F**,**I**,**L:** Similar changes in CXCR4 localization were noted in GnRH neurons. For example, in one of these neurons of a control animal (C-C'', arrowheads), CXCR4 is clearly expressed on the cell membrane. However, in a GnRH neuron of *CXCR7* null, the receptor is localized in the cytoplasm and not detected on the plasma membrane (F-F'', arrowhead). In the absence of SDF-1, CXCR4 is upregulated on the cell membrane and CXCR4 internalization is not observed (I-L'', arrowhead). Scale bars: A,D,G,J, 200 μm; B,E,H,K, 50 μm; C,F,I,L, 20 μm.

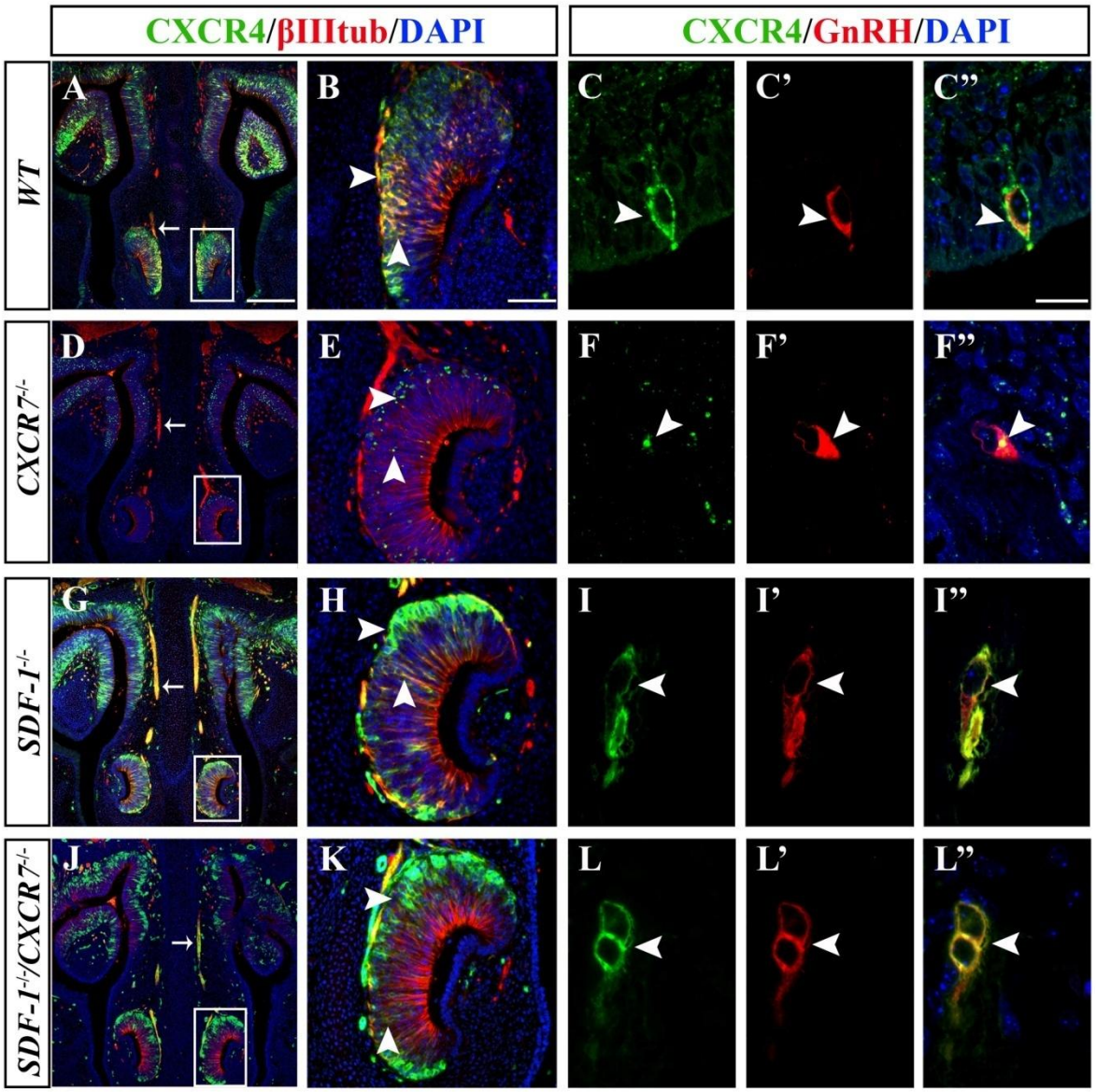
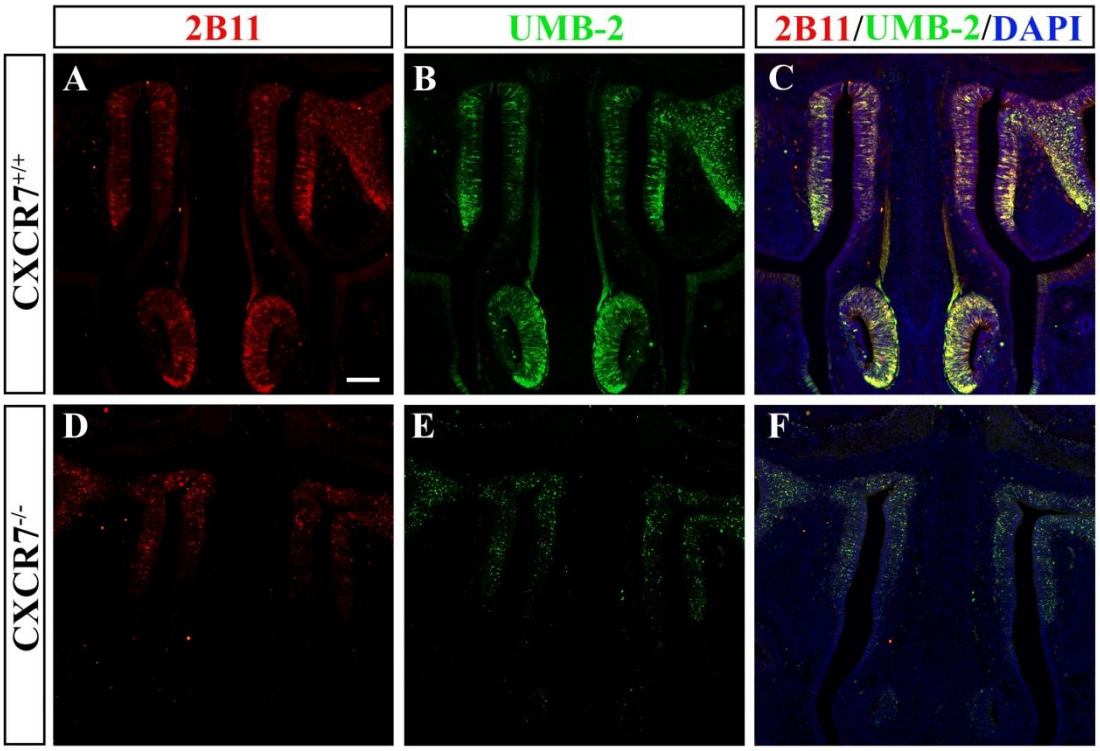

CXCR4/βIIItub/DAPI
CXCR4/GnRH/DAPI
WT
CXCR7-/-
SDF-1-/-
SDF-1-/-/CXCR7-/-
A
B
C
C'
C''
D
E
F
F'
F''
G
H
I
I'
I''
J
K
L
L'
L''

Figure 5.3 Staining with two different anti-CXCR4 antibodies (2B11 and UMB-2) produced identical results.

CXCR4 appeared internalized in CXCR7 knockout mice sections stained with two different antibodies (**D**, **E**) when compared to control littermates (**A**, **B**). Scale bar: A: 100 μm.


2B11
UMB-2
2B11/UMB-2/DAPI
A
B
C
D
E
F
CXCR7+/+
CXCR7-/-

5.3 Discussion

Previous studies have shown that the scavenger function of CXCR7 can exert strong influence on SDF-1/CXCR4 dependent guidance of migrating cells in two ways. First, CXCR7, expressed by non-migrating cells in zebrafish, contributes to the formation of a steep gradient of SDF-1 and prevents migration to non-desired locations (Boldajipour et al., 2008); second, CXCR7 expressed by migrating interneurons in mouse, helps to maintain cell responsiveness to SDF-1/CXCR4 signalling (Sanchez-Alcaniz et al., 2011). Here, we hypothesized that CXCR7 can affect levels of CXCR4 and thus, cell responsiveness to SDF-1, even when the receptors are not present on the same cell, by controlling the extracellular levels of the chemokine. In this concept, absence of SDF-1 would cause upregulation of CXCR4 with its localization on the plasma membrane (as most, if not all, protein produced would be inactive and present on the membrane).

Using the subcellular localization of CXCR4 as a measure for the amount of SDF-1 available in the extracellular matrix, we determined the distribution of the receptor when CXCR7 is absent from the environment. In such conditions (CXCR7 knockout mice), CXCR4 was found downregulated, as evidenced by the appearance of dots (puncta) intracellularly, suggesting that high levels of SDF-1 in the environment trigger the internalization of CXCR4 in cells of the VNO and OE, as well as in GnRH neurons and their guiding axons. Further evidence to support this hypothesis comes from the finding that removal of the ligand (SDF-1) rescues the CXCR4 receptor phenotype, which appears again, upregulated, at

the cell membrane, suggesting that the function of CXCR7 in this system is to control SDF-1 levels.

Chapter 6. General discussion

The migration of GnRH neurons represents a unique example of neuronal migration as these cells are born outside the brain, in the nasal placode, and during embryonic development they follow a long and tortuous route to their destinations in the forebrain. Failure of GnRH neurons to migrate results in delayed or absent pubertal maturation and infertility.

Chemokines expressed along migration corridors have been reported to serve as guidance cues for migrating neurons (Borrell and Marin, 2006, Stumm and Hollt, 2007). Accordingly, SDF-1 has been found to be expressed in an increasing gradient in the nasal mesenchyme and to guide migrating GnRH neurons towards the forebrain (Schwarting et al., 2006, Toba et al., 2008). SDF-1 exerts its functions by binding to CXCR4. In the developing brain, SDF-1 and its receptor CXCR4 are highly expressed in the neocortex, cerebellum and hippocampus, and regulate cell migration and axonal pathfinding (Ma et al., 1998, Chalasani et al., 2003). GnRH neurons express CXCR4 early in their development, as they leave the VNO to begin their migration to the brain. CXCR4 deficient mice exhibit impaired GnRH neuron migration and a significant decrease in their total number (Schwarting et al., 2006, Toba et al., 2008), pointing to the importance of SDF-1 signalling in this system. CXCR4 was for long considered the only receptor for SDF-1, until it was shown recently that it binds with higher affinity to an additional receptor, CXCR7 (Balabanian et al.,

2005). Although its signalling properties are presently controversial (Odemis et al., 2012, Rajagopal et al., 2010a, Thelen and Thelen, 2008), CXCR7 appears to act as SDF-1 scavenger controlling chemokine levels in the extracellular environment by ligand sequestration (Boldajipour et al., 2008, Naumann et al., 2010, Hoffmann et al., 2012). In this context, loss of CXCR7 leads to extracellular accumulation and perturbed gradients of the chemokine (Sanchez-Alcaniz et al., 2011, Boldajipour et al., 2008). Similar to SDF-1 and CXCR4, CXCR7 is expressed in the developing hematopoietic system, cardiovascular system and brain, and *CXCR7*-deficient mice die perinatally with cardiovascular defects (Sierro et al., 2007, Schonemeier et al., 2008). Here, evidence is provided that CXCR7 is important in the early steps of GnRH neuron migration, as lack of this receptor results in aberrant accumulation of cells in the nasal cavity. The defects are associated with severe reduction and perturbed subcellular distribution of CXCR4 protein in GnRH neurons and their guiding axonal scaffold.

6.1 *CXCR7* expression in the nasal area

The first indication that CXCR7 may be involved in GnRH neuron migration came from its expression at the start and along the well-defined migratory path of these cells in the nose. Specifically, we identified CXCR7 expression within and around the VNO, where GnRH neurons emerge, that progressively diminishes in the nasal mesenchyme where GnRH neurons migrate. It is tempting to suggest that the diminishing levels of CXCR7 contribute to the purported SDF-1 gradient in the nasal area (Schwarting et al., 2006). Our observation that GnRH neurons as well as the supporting axons of the

olfactory/vomeronasal nerve do not express CXCR7, is consistent with the previously proposed scavenging role for this receptor in shaping SDF-1 gradients. Interestingly, CXCR7 was also present in presumed supporting cells and immature neurons of the OE, a tissue normally avoided by migrating GnRH neurons.

6.2 Impaired migration and abnormal distribution of GnRH neurons in *CXCR7* null mice

If shaping the SDF-1 gradient by CXCR7 is important for GnRH neuron migration, then this process would be affected in mice lacking the receptor. Indeed, analysis of *CXCR7* deficient mice revealed that GnRH neurons remained in the nasal compartment, with fewer neurons detected in the forebrain when, at the same time, the total cell number was unaffected. This suggests that CXCR7 is not involved in the early generation of GnRH neurons. In these null mice, half of the GnRH neuron population failed to migrate to the hypothalamus and, instead, distributed randomly in the nasal area, formed disordered clusters along the pathway and positioned themselves ectopically in the OE, suggesting that CXCR7 is required for their directed migration towards the forebrain. The partial phenotype may be attributed to the reported heterogeneous makeup of the GnRH neuron population (Forni et al., 2011b, Jasoni et al., 2009). Interestingly, although the overall GnRH cell number did not differ from control littermates at early developmental stages (E12.5-E14.5), it did decline significantly at E16.5. It is likely that ectopic GnRH neurons that failed to migrate to the brain eventually die or cease to express the GnRH gene.

As the GnRH system has never been investigated in mice lacking *SDF-1*, we analyzed the number and distribution of GnRH neurons in these mice and compared their phenotype to that of *CXCR4*- and *CXCR7*-deficient animals. We found that the phenotype in *SDF-1* knockouts closely resembled that of *CXCR4* null mice, both in terms of migration and overall decrease in cell number, strongly suggesting that SDF-1 signals through CXCR4 in this system to control GnRH neuron survival and migration. Moreover, the aberrant distribution of GnRH neurons in the nasal area of *SDF-1*, *CXCR4* and *CXCR7* null mice points to a direct role for SDF-1 in guiding the migration of these cells, especially since the supporting axonal scaffold was unaffected in the three knockout lines.

6.3 CXCR7 controls CXCR4 protein levels

Previous studies have shown that sustained excess of SDF-1 leads to desensitization and degradation of CXCR4 (Marchese and Benovic, 2001). In addition, CXCR7 has been shown to regulate CXCR4 function by controlling the extracellular levels of the chemokine by rapid internalization and sequestration of the ligand. This can be done either in a cell autonomous manner, when it is co-expressed with CXCR4 in the same cell (Sanchez-Alcaniz et al., 2011), or in a non-cell autonomous manner, when it is expressed in the tissue surrounding CXCR4-positive cells (Boldajipour et al., 2008). Our present data support for the first time the latter hypothesis for migrating neurons and thus, are clearly distinct from earlier findings proposing a cell autonomous function for CXCR7 in neuronal migration (Tiveron et al., 2010, Wang et al., 2011). GnRH neurons express CXCR4, but not CXCR7 during their migration through the nasal

compartment, and are attracted by progressively higher levels of SDF-1 as they target the forebrain. In the case of compromised CXCR7 activity, we found increased internalization and, in some cases, loss of CXCR4 protein in GnRH neurons and in olfactory/vomeronasal cells and their axons. This finding together with the proposed function of CXCR7 as a scavenger of extracellular SDF-1 (Naumann et al., 2010), prompted us to suggest that CXCR4 re-allocation from the plasma membrane to the cytoplasm of neurons in the nasal compartment is an indicator for excessive SDF-1-promoted CXCR4 activation. Thus, if increased levels of SDF-1 in CXCR7 null mice result in CXCR4 internalization, then concomitant removal of the ligand (and CXCR7) would prevent this receptor phenotype. Indeed, the apparent internalization of CXCR4 in *CXCR7* deficient mice was reversed in double *CXCR7/SDF-1* knockout animals, strongly suggesting that the function of CXCR7 in this system is to control SDF-1 levels. It has been proposed that GnRH neurons switch off the CXCR4 pathway once they reach the forebrain (Schwarting et al., 2006, Toba et al., 2008). Here, we may suggest a rapid gene regulation-independent mechanism by which this may be achieved: GnRH neurons, while in the nasal compartment, migrate towards higher levels of SDF-1, retaining CXCR4 activity through CXCR7 function. When they reach the cribriform plate, which is rich in SDF-1, but almost devoid of CXCR7, CXCR4 starts to become highly internalized and degraded as a result of hyperactivation, rendering GnRH neurons no longer responsive to the effects of SDF-1. Once GnRH neurons enter the forebrain, they are subjected to the influences of other molecules as they negotiate their migration towards the hypothalamus. In summary, our studies have shown that CXCR7 is required for GnRH neuron migration. Our data support a mechanism,

whereby CXCR7 controls CXCR4 activity in migrating GnRH neurons by regulating the available levels of SDF-1. It is pertinent to note that recent studies in zebrafish have implicated β-arrestin in this mechanism (Mahabaleshwar et al., 2012). It would be interesting to assess whether this process is conserved in mice, and whether mice with defects in β-arrestin/CXCR7 interaction exhibit a similar phenotype to *CXCR7* null animals.

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