Universidade do Minho
Escola de Ciências da Saúde

Ashley da Cruz Novais

Unraveling the biological role of neudesin, a novel neurotrophic factor

setembro de 2013

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Tese de Doutoramento
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Trabalho realizado sob a orientação do
Professor Doutor João Carlos Sousa

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“Si no nos dejais soñar, no os dejaremos dormir!”

To S...

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And because those who mind do not matter and those who matter do not mind, let's move on!

Abstract

Neudesin, also known as neuron derived neurotrophic factor (NENF), is a non-canonical neurotrophic factor. Since it presents a heme/steroid binding domain in its structure, NENF is also a member of the membrane associated progesterone receptor (MAPR) family. *In vitro* studies have shown that recombinant NENF *per se* is able to induce proliferation and differentiation of embryonic cortical neurons and respective precursor cells. Of note, the addition of hemin to the media significantly increased the neurotrophic capacity of NENF *in vitro*, showing that heme successfully binds to NENF primary structure and indicating that heme is a prosthetic group needed for NENF activity, at least *in vitro*. Nevertheless, still much is unknown on the mechanism of action of NENF. Furthermore *in vivo* studies on NENF function are yet scarce.

In this thesis, we have evaluated the importance of NENF for central nervous system (CNS) postnatal development and in adulthood, specifically at the behavioral, neurochemical and structural levels. Furthermore we characterized the adult neurogenic niches in the absence of NENF. For that, we have taken advantage of a unique mouse model with a target deletion of the entire neudesin gene, from now on designated as neudesin-null mice. We observed that in the absence of NENF, adult males have an anxious-like behavior, as it was assessed in the elevated plus maze (EPM), light/dark box (LDB) and novelty suppressed feeding (NSF) tests; surprisingly we have not seen the same anxious phenotype in the acoustic startle (AS) test. These results reveal a role for NENF in the regulation of state anxiety but not in generalized anxiety, at least in males. On the other hand, neudesin-null males do not show any motor, exploratory, depressive or cognitive alterations. This anxious phenotype was accompanied by impoverished dendritic arborization of ventral hippocampal granular cells as well as impaired dopaminergic activity, and shorter dendrites at the anterior medial division of the bed nucleus of the stria terminals (amBNST). These observations reveal a critical role for NENF in the maintenance of the anxiety circuitry, especially at the ventral hippocampus. Since neurodevelopment is highly regulated by neurotrophic factors, and might impact on adult behavior we have studied the postnatal acquisition of developmental milestones of neudesin-null animals during the first 21 days of postnatal life. We observed sex-differences regarding specific neurological reflexes dependent on the vestibular area maturation, with a mild delay on neurodevelopment observed only in neudesin-null males. This was followed by a neurochemical imbalance, evaluated in postnatal day 21, in body righting and position regulating brain regions. Specifically we observed that the brain stem of neudesin-null males showed increased serotonin and norepinephrine, a decreased

serotonergic and dopaminergic activity in the cerebellum, diminished norepinephrine in the motor cortex and the vestibular area revealed less dopaminergic activity.

Lastly we have characterized the proliferative capacity of neudesin-null mice neurogenic niches, *in vivo* and *in vitro*. We observed a sex difference in proliferation occurring in the sub granular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. Neudesin-null male mice showed less labeling of BrdU cells than littermate controls, which was not observed in females. Assessing neurogenesis ratio by doublecortin (DCX) and BrdU positive cells, we have observed that neudesin-null males showed less neurogenesis only in the ventral division of the hippocampus. Furthermore, *in vitro* we observed that neurospheres formed by neural stem cells derived from the hippocampus of neudesin-null mice had an inferior clonal expansion capacity. All these evidences prompt us to propose that NENF might also have neurotrophic capacity *in vivo*, specifically at the ventral division of the hippocampus, but interestingly its importance is primordial in males.

With this work we contributed to further unravel the biological role of NENF in the CNS.

Resumo

A neudesina, também designada por factor neurotrófico derivado de neurónios (NENF), é uma proteína com atividade neurotrófica. Esta tem na sua estrutura primária um domínio de ligação a grupos heme e esteroides que a classifica como um membro da família de proteínas dos receptores de progesterona associados à membrana (MAPR). Estudos *in vitro* demonstraram que a NENF *per se* é capaz de induzir proliferação e diferenciação de neurónios derivados do córtex embrionário e também das respetivas células precursoras desses neurónios. De salientar que a adição de hemina ao meio de cultura aumentou significativamente a capacidade neurotrófica da NENF, demonstrando que *in vitro,* a hemina se liga ao domínio da NENF e que atua como um grupo protético necessário para a atividade da NENF. No entanto, ainda muito pouco se sabe sobre o mecanismo de ação da NENF *in vivo*.

Neste estudo, avaliamos a importância da NENF no desenvolvimento do sistema nervoso central (SNC) adulto e pós-natal, especificamente ao nível do comportamento, neuroquímica e estrutura. Adicionalmente caracterizamos os nichos neurogénicos adultos na ausência de NENF. Para tal, utilizamos um modelo animal de murganho sem o gene da neudesina, que daqui em diante designaremos de $NENF^{-/-}$. Observamos que na ausência de NENF, os machos adultos têm um comportamento ansioso, observado em 3 diferentes testes que aferem os níveis de ansiedade contextualizada mas não num outro teste que afere a ansiedade generalizada. Isto sugere um papel para a NENF como modelador do comportamento ansioso contextualizado, o que envolve circuitos cerebrais específicos. Por outro lado, animais sem NENF não mostraram qualquer problema motor/exploratório, depressivo ou cognitivo. O fenótipo ansioso foi acompanhado por uma atrofia da arborização dos neurónios granulares do girus denteado do hipocampo ventral e também por uma desregulação da atividade dopaminérgica nesta região e assim como por dendrites mais curtas dos neurónios da divisão anterior medial do núcleo da estria terminal. Estas alterações revelam um papel crítico para a NENF na manutenção do circuito da ansiedade, especialmente no hipocampo ventral.

Como o neurodesenvolvimento é regulado por fatores neurotróficos, estudamos a aquisição de marcos específicos do desenvolvimento pós-natal dos murganhos $NENF^{-/-}$ durante os primeiros 21 dias de vida. Observamos uma diferença dependente do género em alguns reflexos neurológicos dependentes da maturação da área vestibular. Apenas os machos apresentam um atraso na aquisição destes reflexos. Este atraso foi acompanhado por uma desregulação

neuroquímica avaliada no dia pós-natal 21, em áreas do cérebro envolvidas em mecanismos de equilíbrio e posição do corpo. Especificamente, observamos um aumento de serotonina e norepinefrina no tronco cerebral de animais NENF$^{-/-}$, assim como um aumento da atividade serotonérgica e dopaminérgica no cerebelo, diminuição de norepinefrina no córtex motor e uma diminuição da atividade dopaminérgica na área vestibular.

Por fim, caracterizamos a capacidade proliferativa dos nichos neurogénicos de animais NENF$^{-/-}$, *in vivo* e *in vitro*. Observamos uma diferença dependente do género na proliferação na zona sub granular do girus denteado do hipocampo. Os machos NENF$^{-/-}$ apresentam menos células BrdU positivas do que os controlos, o que não se observa nas fêmeas. Na avaliação da neurogénese pela contagem de neuroblastos em proliferação, observamos que os machos NENF$^{-/-}$ apresentam menos neurogenese a ocorrer apenas na divisão ventral do hipocampo. Adicionalmente, *in vitro* observamos que neuroesferas formadas a partir de células estaminais do hipocampo de animais NENF$^{-/-}$ apresentam uma capacidade clonal inferior. Este conjunto de evidências, leva-nos a propor que a NENF possui também capacidade neurotrofica *in vivo*, especificamente na divisão ventral do hipocampo apenas em machos.

Com este trabalho contribuímos para clarificar um pouco mais o papel da neudesina no sistema nervoso central.

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List of abbreviations

5HT - serotonin

amBNST – anterior medial bed nuclea of the stria terminalis

amy – amygdala

aSVZ - adult subventricular zone

BAT - brown adipose tissue

BDNF - brain derivated neurotrophic factor

BrdU - bromodeoxyuridin

CeA - central amygdala

CORT – corticosteroid

CNS - central nervous system

CNTF - ciliary neurotrophic factor

CSF - cerebrospinal fluid

DA - dopamine

DCX - doublecortin

DG - dentate gyrus

EPM - elevated plus maze

FBS - fetal bovine serum

FGF-2 - fibroblast growth factor-2

$GABA_AR$ - GABA A receptors

GDNF - Glial derivired neurotrophic factor

GFAP - glial fibrilary acidic protein

Glu - glutamate

HO1- heme oxigenase 1

HPZ - high proliferative zone

LC - locus coeruleus

LV - lateral ventricles

MAP - mitogen-activated protein kinases

MAPR - membrane associated progesterone receptor family

MAP-2 - microtubule-associated protein 2

ME - median eminence

NE - norepinephrine

Nenf - neuron derived neurotrophic factor

Neuro2a - mouse neuroblastoma cell line

NMDA - N-methyl-d-aspartate

NGF - nerve growth factor

NSC - neuron stem cells

OF - open field arena test

PAQR - progestin and adipoQ receptor

PGRMC1 - progesterone receptor membrane component 1

PI3-K - phosphatidylinositide 3-kinase

PKA - protein kinase A

PSA-NCAM - polysialylated form of the neural cell adhesion molecule

PTX - pertussis toxin

RMS - rostral migratory stream

RN - raphe nuclei

ROS - reactive oxygen species

SCIRP10 - spinal cord injury related protein 10

SGZ - subgranular zone

SNP - single nucleotide polymorphism

SSRI - Selective serotonin reuptake inhibitor

TAP's - transient amplifying progenitors

TBI - traumatic brain injury

vHPC – ventral hippocampus

VTA - ventral tegmental area

WAT - white adipose tissue

1. Introduction

1. Neudesin

Neudesin, also known as neuron derived neurotrophic factor (NENF), spinal cord injury related protein 10 (SCIRP-10), cell immortalization-related protein 2 or secreted protein of unknown function, was firstly described in 2005 as a novel neuron secreted protein (Kimura et al., 2005). The multi designations for this gene are in part due to the fact that the *neudesin* gene was independently detected in a few genomic/transcriptome studies (Magdaleno et al., 2006;Neubauer et al., 2006;Han et al., 2012;Byerly et al., 2013). It is of notice that, possibly given the relatively short time since the first description of the protein, scarce literature is available on the function and/or characterization of this protein.

1.1 NENF gene and protein structure

The *neudesin* mouse gene is a 11.41 kilobases (kb) sequence located in chromosome 1 with 4 exons and 3 introns and a coding DNA sequence that ranges from nucleotides 27 to 542, which are 515 nucleotide long (figure 1). This sequence encodes for the mouse NENF protein that contains 171 aminoacids long. This amino acidic sequence is highly homologous between species but its N-terminal part differs between mammals and other classes of vertebrates. From the initial predictive analysis of the primary structure of the protein no similarities to any other secreted protein known at the time was found, and thus the protein was named neudesin (or neuron derived neurotrophic factor) and proposed as a novel secreted protein with neurotrophic activity over neurons but not astrocytes (Kimura et al., 2005), as will be described later in more detail. Further bioinformatic analysis by multiple sequence alignments on the protein structure revealed the existence in the mouse NENF protein of 3 amino acids residues that act as ligands for the iron-binding heme group (Kimura et al., 2008). In fact this work revealed the existence of a cytochrome-b5 like heme/steroid binding domain in the NENF protein (Kimura et al., 2008) which was the key element to classify NENF as a member of the membrane associated progesterone receptor family (MAPR). More recently, another secreted protein of the MAPR family was also identified, and named neuferricin (Kimura et al., 2010); this protein also has a cytochrome-b5 like heme/steroid binding domain, and similar to NENF, displays neurogenenic inducing activity. A putative role for MAPR family members in the development and function of the central nervous system (CNS) (Kimura et al., 2012) will be later discussed in this chapter.

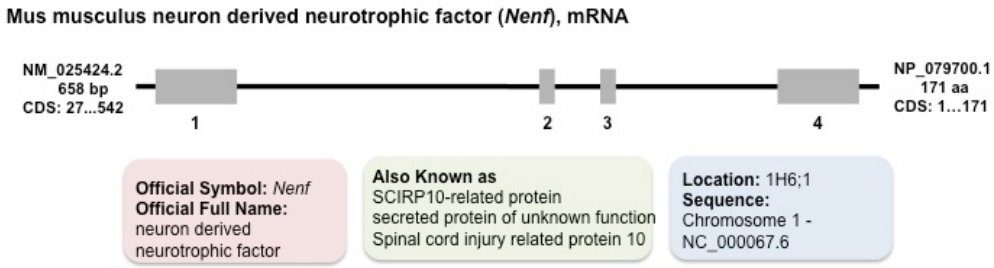


Figure 1 – Neudesin gene schematization. Neudesin gene has 4 exons and 3 introns and there is no alternative splicing form known.

Neudesin contains a heme/steroid-binding domain similar to the one of the cytochrome b5 family, with clearly demonstrated heme-binding properties (Kimura et al., 2008). As indicated above this resulted in the classification of neudesin into the MAPR family, a subfamily of cytochrome b5 family, along with progesterone receptor membrane component 1 and 2 (PGRMC1 and 2) and neuferricin. All these proteins share a homologous primary structure, especially at the cytochrome-b5 like domain and all depend on the binding of heme, as a prosthetic group, to its cytochrome b5 domain to exert their function. Although PGRMC1 and NENF share 40% of homology, at the heme-binding domain, they present different patterns of expression and cellular locations, being NENF a secreted protein and PGRMC1 is transmembranar (Rohe et al., 2009).

1.2 NENF expression

A complete characterization of the NENF ontogenic expression profile is not yet available. Embryonically, *Nenf* major expression sites are the brain, spinal cord, heart and lungs (Kimura et al., 2005). It is described that *Nenf*'s expression in the developing brain starts from E12.5 and was shown to be present up to E18.5. Specifically, *Nenf* was weakly detected at E12.5, clearly present at E13.5 and its expression levels increased until E18.5. At E13.5 it was observed that the pre-plate, where newly post-mitotic neurons are present, is the region where *Nenf* expression is higher, interestingly this region is constituted by neurons that migrated from the subventricular zone, where *Nenf* expression is lower (Kimura et al., 2006). Apparently, *Nenf* expression pattern in the developing mouse brain is coincident with the late maturation of neural precursors (E15.5) but starts prior to the appearance of mature neuronal marker the microtubule-associated protein 2 (MAP-2) (Kimura et al., 2006). Similarly, its expression on early phases of cortical development seems restricted to post-mitotic neurons. Surprisingly, *in vitro*, neural precursor cells isolated at

E13.5 from the cerebral cortex, cultured for 48h in differentiating conditions, indicated that *Nenf* expression diminishes, inversely to the MAP-2 expression pattern (Kimura et al., 2006) (figure 2). This inconsistent pattern of expression between *in vivo* and *in vitro* conditions might suggest a paracrine control of *Nenf* expression by surrounding cells, that are not present *in vitro*; these cells are most probably post-mitotic and/or committed cells.

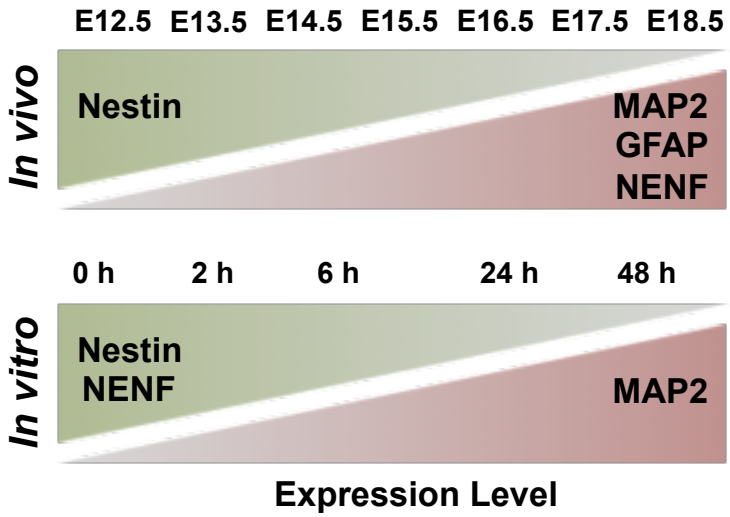


Figure 2 – Schematic view of neudesin and different cellular markers expression in mouse embryonic cortex throughout development (*in vivo*) and in 48 h cultures (*in vitro*).

It is of relevance that in the adult brain (P49) *Nenf* expression, evaluated by *in situ* hybridization, was found to be ubiquitous but restricted to neurons (Kimura et al., 2005). Nevertheless, *Nenf* expression pattern was not homogeneous; the hippocampus, cortex, thalamus and hypothalamus were the regions where *Nenf* expression was detected to be the highest. It should be noted that in adulthood (P49), detectable levels of *Nenf* mRNA were also present in other organs/systems other than the nervous system, such as the heart, lung, kidney, and in the white adipose tissue (WAT) (table 1) (Kimura et al., 2005). In case of pathology, particularly breast carcinoma, there is a report that showed NENF to be up regulated in estrogen receptor$^+$/progesterone receptor$^+$ tumors but not in estrogen receptor$^+$/progesterone receptor$^-$ tumors (Neubauer et al., 2006). This observation suggests that NENF might play a role in other organ/systems besides the nervous system, which is similar to what is described for classical neurotrophins.

Table1 – Expression profile of NENF in adult and embryonic mice.

time point	brain	heart	lung	WAT	smooth muscle	testis	stomach
PND49	++	+++	++	++	+	+	+
E18.5	+++	++	-	?	+	?	-

1.3 NENF function

When NENF was initially described a body of evidence for its function as a "putative neurotrophic factor" was found (Kimura et al., 2005). As indicated previously, posterior analysis of its primary structure revealed the presence of a heme/steroid binding domain, which resulted in the classification of NENF as a member of the MAPR family, and prompted for other potential functions of neudesin. Given this, we will next independently approach each function of NENF.

1.3.1 Neudesin role as a neurotrophic factor

Evidence for a potential role of neudesin as a neurotrophic factor was born from *in vitro* studies that showed that recombinant NENF protein enhances proliferation and survival of E18.5 cerebral cortex neuronal cultures in a dose dependent manner. This was partially similar to what was described for the effect of fibroblast growth factor-2 (FGF-2), since neudesin only displayed neurotrophic activity in neuronal cultures but not in astrocytic cell cultures (Kimura et al., 2005). Proliferation and survival mechanisms *in vitro* are associated to the activation of the Mitogen-activated protein kinases (MAP) and phosphatidylinositide 3-kinases (PI3-K) pathways. FGF-2 and neurotrophins in general activate these pathways similarly to what was observed for NENF. The effect of NENF in these signaling pathways was further demonstrated since its activity was abolished when specifically ERK1/2 and Akt inhibitors were used in the same conditions (Kimura et al., 2005). In order to explore a possible receptor upstream of the phosphorylation of ERK1/2 and Akt, inhibition of tyrosine kinase receptors for FGF, EGF and IGF-I was tested, however when stimulated with recombinant NENF phosphorylation of ERK1/2 and Akt still occurred. This suggests that NENF's activity does not cross with the activation of other neurotrophic factors receptors. Interestingly, Gi/Go-protein inhibitor pertussis toxin (PTX) was able to disrupt ERK1/2

and Akt phosphorylation as well as recombinant NENF's neurotrophic effect (Kimura et al., 2005). This indicated that, at least in neurons, the most probable receptor candidate for NENF is a Gi/Go-protein-coupled receptor (Kimura et al., 2005). In addition to these neurotrophic function, recombinant NENF was also able to decrease terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells in primary cultures of cortical neurons, showing also a neuroprotective effect (Kimura et al., 2005), but the mechanism involved in this process is still not understood.

On the other hand, NENF might modulate early stages of neural development, namely by stimulating neurogenesis and promoting the differentiation of neurons in E13.5 precursors cell cultures (Kimura et al., 2006). MAPK, PI3-K and protein kinase A (PKA) pathways mediate these actions but, contrary to what was demonstrated in E18.5 isolated cells, this activation was not promoted by the Gi/Go-protein-coupled receptor. Indeed, even in the presence of FGF-2, recombinant NENF is able to potentiate the differentiation of neural precursors into MAP-2 positive neurons, specifically by PI3-K and PKA pathways (Kimura et al., 2006). On the other hand, the proliferation of E13.5 isolated cortical neural precursor cells *in vitro* was not observed in the presence of recombinant NENF for longer than 48h, but a similar proliferative effect to the one induced by FGF-2 was observed in the first 24 h in culture (Kimura et al., 2006). Of notice, MAPK and PKA pathways, but not the PI3-K pathway, mediated this transient induction of cell proliferation by the action of recombinant NENF (figure 3).

Another interesting finding related to NENF action was the fact that recombinant NENF, in the presence of fetal bovine serum (FBS), inhibits the differentiation of astrocytes (Kimura et al., 2006). This suggests that NENF's role in neurogenesis is dual: it maintains the stemness capacity of uncommitted neural stem cells and also, once they enter a differentiated state, NENF is able to potentiate differentiation into a neuronal but not astrocytic phenotype, at least *in vitro*.

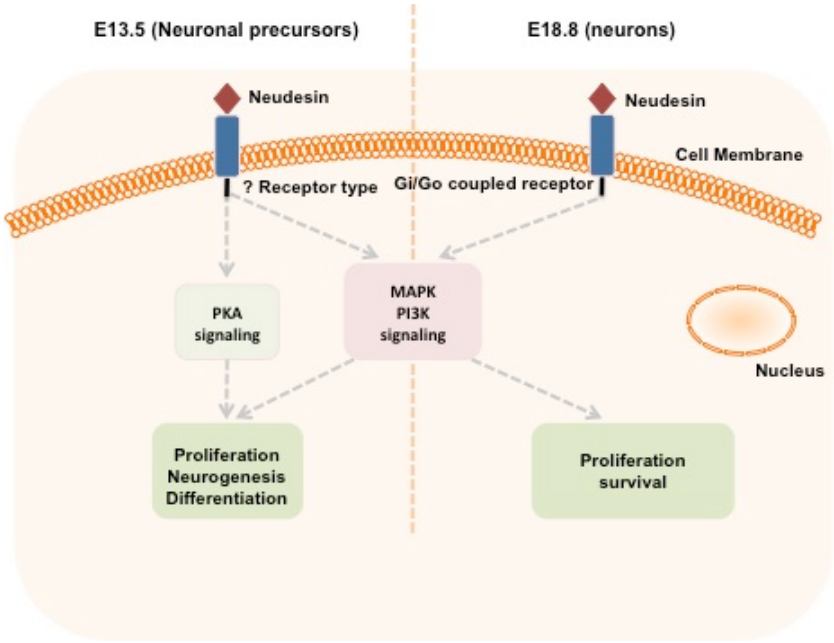


Figure 3 - Schematic representation of neudesin activation pathways *in vitro.*

Evidence exists with cell types other than neural cells demonstrating the proliferative inducing capacity of NENF. For instance, the same properties were observed with experiments in a mouse neuroblastoma cell line (Neuro2a). Specifically, *Nenf* siRNA knock down was able to disrupt the phosphorylation of ERK1/2 and Akt of this cell line (Kimura et al., 2008). Another study showed a evidence contributing for the signaling activation pattern of NENF, the cDNA transcript of *Nenf*, at the time denominated as CIR2, was described to be over expressed in immortalized cells (Ma et al., 1998), specifically in the human SV-40 transformed human embryonic kidney (HEK) cell line. But further functional analysis on the relevance of CIR2 (or NENF) in the immortalization and proliferation inducing process was not pursued. NENF has also been described to participate in the maintenance of the proliferative status of adipose precursor cells. Namely, recombinant NENF, *in vitro*, inhibits adipogenesis of an adipocyte-like cell line derived from 3T3 classic fibroblast cell line, the 3T3-L1 cell line. This inhibition occurs by preventing adipocytes precursors to differentiate into mature adipocytes. The signaling cascade by which this continuous proliferative state occurs is via phosphorylation of ERK1/2 or MAPK activation, but not Akt phosphorylation. This maintains the stemness properties of adipocyte precursors (Kimura et al., 2009).

As described above the activation pattern involved in the mechanism of action of NENF occurs, although not simultaneously, through MAP and PI3 kinase and AKT pathways. This is similar to the activation patterns of neurotrophins and other neurotrophic factors. Ultimately this activation results in the survival, proliferation and/or differentiation of both mature and precursors cells. These evidences favored the classification of NENF as a neurotrophic factor. Nevertheless, it is not known through what receptor, or any other partner proteins, these properties are elicited by NENF. On the other hand and given that these actions of NENF were demonstrated *in vitro*, it is not clear if NENF induces the same effects *in vivo*.

1.3.2 Neudesin role as a heme-binding protein

Heme acts as a prosthetic group of several heme-binding proteins such as cytochromes, hemoglobin, myoglobin and others. Importantly, NENF was the first extracellular protein, with heme-binding activity, to be described to activate intrinsic signal transduction. As described previously (section 1.1) it was shown that mouse NENF presents 3 residues that act as ligands for the iron-binding heme group (Kimura et al., 2008). Moreover *in vitro* studies using the Neuro2a cell line have shown that, in fact, recombinant NENF requires hemin binding to its primary structure to exert survival and anti-apoptotic actions. Contrarily from what was seen in primary brain cultures, proliferation of Neuro2a was enhanced in the presence of recombinant NENF but this effect was significantly boosted when free hemin was added to the culture medium. Of notice, hemin *per se* was not able to promote any of these actions (Kimura et al., 2008).

Interestingly not only recombinant NENF potentiated proliferation and survival of Neuro2a cells but also, when endogenous *Nenf* was knocked-down by siRNA, proliferation and survival of cells was halted, thus eliminating the possible artifact of external addition of NENF to cell media. Moreover it was shown that this cell line secretes NENF into the media. Endogenous NENF was then quantified for its binding capacity to heme using a heme-affinity chromatography, which revealed that all endogenous NENF was bound to hemin (Kimura et al., 2008).

1.3.3 Neudesin role as a steroid-binding protein

While NENF displays a steroid-binding domain, NENF binding to steroids was not detected (Kimura et al., 2008). It should be noted that the later experiments were performed by dot blot analysis of recombinant NENF incubation with different steroids, and this methodology might not be the most appropriate if NENF binding to any steroid occurs with low affinity. Nevertheless the *in vivo* interaction of NENF with steroids is still a matter of dispute, especially since NENF primary structure holds 30% of homology to membrane associated progesterone receptor component 1 (PMGRC1) (Cahill, 2007;Kimura et al., 2012), which activates P450 proteins, that are involved in drug, hormone and lipid metabolism (Losel et al., 2008). Similarly to NENF, in the case of PGRMC1 the binding of progesterone still suffers from conflicting evidence, with some authors demonstrating direct binding (Meyer et al., 1996;Peluso et al., 2008;Peluso et al., 2009) and others arguing in favor of PGRMC1 not bind directly to progesterone but instead forming a complex with proteins that together bind progesterone (Rohe et al., 2009). The same holds true for other steroids such as, corticosterone, testosterone and cortisol and drugs (Meyer et al., 1996;Min et al., 2005;Rohe et al., 2009). Progesterone binding to PGRMC1 complex was shown to promote proliferation and cell cycle gene expression of neural precursor cells through PGRMC1 and 2 by activation of MAP kinase pathways, similarly to the activation induced by NENF (Liu et al., 2009). The possibility of NENF forming a complex with other proteins and then binding progesterone has not been demonstrated yet. But indeed it has been shown that NENF signiling pathway activation is blocked by inhibition of Gi/Go-protein-coupled receptor. And in fact, another group of membrane proteins known to bind to progesterone (mPR) that belong to the progestin and adipoQ receptor (PAQR) family, are thought to be unique G protein-coupled receptors that act through cAMP (Thomas and Pang, 2012).

The general mechanism of action of progesterone occurs via genomic regulation; for this progesterone enters the cells and binds nuclear receptors that in turn modulate gene transcription. On the other hand progesterone also induces non-genomic actions. Given the lipophilic nature of progesterone, Kimura and colleagues (2013) have hypothesized that NENF might be important to retain progesterone from entering the cell. In this way and by forming a complex (NENF-progesterone), NENF would be able to induce the non-genomic actions of progesterone via activation of membrane receptors (figure 4). The need of NENF as a stabilizer of progesterone at the cellular membrane or a complex formation in order for non-genomic

signaling activation of progesterone to occur is nevertheless still an elegant hypothesis (Kimura et al., 2013). Despite the speculative nature of this mechanism of NENF action, it is of note that recently a non-genomic signaling activation induced by progesterone binding to membrane receptors (PGRMC1 and mPR) has been demonstrated (Singh et al., 2013).

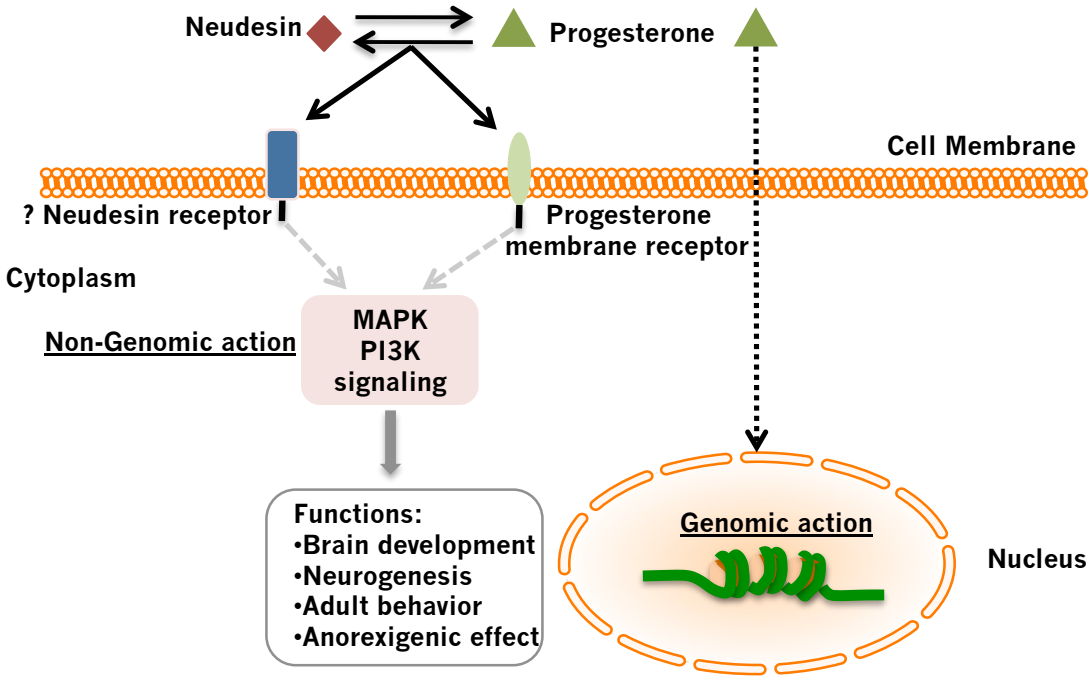


Figure 4 - Schematic representation of the hypothesis of neudesin and progesterone interaction.

1.3.4 Other roles of neudesin

Despite the *in vitro* evidence, NENF physiological role is still largely unknown. Very recently the first report showing an *in vivo* function for NENF was published. NENF was observed to be highly expressed in some nucleus of the hypothalamus involved in feeding behavior regulation, as the arcuate nucleus (Arc) and the paraventricular nucleus (PVN). Furthermore, it was shown that interacerebroventricular delivery of recombinant NENF to the brain had and anorexigenic like effect, diminishing animals appetite and body weight by the activation of melanocortin signaling (Byerly et al., 2013). The NENF function shown in this work has a neurotrophic support but in a more selective form, namely by modulating specific neuronal populations (proopiomelanocortin^{+} (POMC) neurons), as well as, the BDNF receptor expression, tyrosine kinase B (trkB). The modulatory actions of NENF shown in this work were very similar to the ones observed by BDNF

in the same conditions (Byerly et al., 2013), which adds in favor of the potential of NENF as a neurotrophic factor *in vivo*. This study was the first to bring insight for NENF neurotrophic modulation of physiologic states such as anorexia and orexigenia (Byerly et al., 2013).

Given the singular nature of NENF, the rather minute reference to it in the literature and the many gaps still existing in what concerns NENF action, we will next give further background and insight into the general functions of other molecular players that display properties similar to the ones of NENF.

2. Neurotrophic factors

Classically, neurotrophic factors can be defined as molecules (peptides or proteins) essential for the differentiation, maturation or survival of specific populations of neurons from embryonic to adult life (Henderson, 1996). There are 3 major families of classical neurotrophic factors. The neurotrophins family which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and 4/5 (NT-4/5); the neurokines, including ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF); and, the transforming and fibroblast growth factor families, which includes TGF-β1, TGF-β2, TGF-β3, FGF family members and their distant relative glial cell line derived neurotrophic factor (GDNF) (Segal and Greenberg, 1996).

2.1 Role of neurotrophic factors in brain development

The "neurothrophic hypothesis" postulates that a neurotrophic factor might be essential for the correct development of a certain population of neurons and that it exists in less quantity than the necessary for the maintenance of the entire neuronal population (Davies, 1996). In this sense, knockout (KO) models for neurotrophic factors and their receptors have been an exceptional tool to study neurotrophic action mainly during nervous system development (Snider, 1994).

The levels of neurotrophins and its receptors are generally high throughout the mammalian CNS development. Being expressed in different stages in various structures of the nervous system, since the neuroepithilium and neural tube stage until adult brain structures, as hippocampus, striatum and basal ganglia (Bartkowska et al., 2010). During embryonic development,

sympathetic and sensory ganglia neurons are the most vulnerable structures to neurotrophic fluctuations, with the absence of neurotrophic support resulting in severe cell loss (Levi-Montalcini, 1987;Purves et al., 1988). In the CNS, the absence of neurotrophins results in a milder phenotype, with a bland neuronal loss, suggesting that neurotrophins, per se, do not play a crucial role in the survival and differentiation of CNS neurons at least during embryonic development. Instead the absence of certain or combined neurotrophins and/or their receptors result in abnormal neuronal phenotypes. For example, BDNF KO mice present increased death of cerebellar granule cells, resulting in abnormal cerebellar patterning and foliation (Schwartz et al., 1997). In the same line, absence of trkB or trkC neurotrophin receptors induces cell loss, specially in the dentate gyrus, during the first postnatal weeks and this phenotype is worse in double KO trkB /trkC (Minichiello and Klein, 1996). NGF or trkA KO mice do not show cell loss in the CNS throughout the fourth postnatal week, instead they shown an abnormal cholinergic system, resulting in specifically loss of cholinergic neurons in the striatum and septal region of young adults trkA KO mice (Fagan et al., 1997).

Knockout mouse models for single neurotrophins, combination of 2 or knockout of the combination of a neurotrophin and their receptor, show a poor viability with developmental abnormalities (Agerman et al., 2003) where the vestibular and the cochlear nucleus, along with the nodose-petrosal and dorsal root glangia are main structures suffering cell lose (reviewed at (Huang and Reichardt, 2001).

It should be noted that most neurotrophins and their respective receptors, with the exceptions of NT3/4 and p75, are essential for postnatal life, since homozygous mutant mice die prenatally or within the initial postnatal weeks (Bartkowska et al., 2010). The severity of the phenotypes observed suggests that a variety of organ systems fail due to the lack of neurotrophin signaling, and not all entirely related to neuronal abnormalities (Bartkowska et al., 2010). Another potent neurotrophic factor, FGF2, is known to be highly expressed in embryonic and postnatal ventricular zones as well as in neural stem cells. Indeed KO for FGF2 show an abnormal cortical and hippocampal development (Raballo et al., 2000) and importantly this has consequences in adulthood.

All of these cellular modulatory actions are essential for the correct neuro development and ultimately have consequences in adulthood. For instance, vestibular neurons require BDNF and NT3 for survival while nodose-petrosal ganglion complex sensory neurons require BDNF and NT4/5 (Ernfors et al., 1995). A conditional depletion of BDNF solely in the embryonic brain

results in a pronounced effect in adulthood, namely depressive-like behavior and deficits in serotonin A2 receptor, which underlines the importance of this neurotrophin during development (Chan et al., 2006).

2.2 Role of neurotrophic factors in brain function and behavior

Neurotrophins are important modulators of adult CNS. Several lines of evidence have shown that neurotrophins play a major role in the modulation of synaptic transmission, long-term potentiation and depression, neuronal excitability, structural synaptic plasticity, axonal and dendritic branching, synapse number, size, and maturity (reviewed in (McAllister et al., 1999;Hagg, 2009).

Neurotrophins expression throughout the adult CNS supports the understanding of their crucial role in the adult brain. Although BDNF, NT3 and NT4/5 and the receptors TrkB and C are widely expressed throughout the adult brain, NGF is restricted to striatal and basal forebrain cholinergic neurons (Bartkowska et al., 2010). On the other hand BDNF, NT3, NT4/5, TrkB and C expression increases in abundance postnatally, peaking at P14 (in the rat) (Ringstedt et al., 1993;Knusel et al., 1994), which, in fact, correlates with maximal neuronal growth, differentiation, and synaptogenesis processes in the brain.

Of all the neurotrophic factors referred above, BDNF is the most studied in both short-term and long-term neurotrophic cellular modulation in the adult brain. In adulthood, BDNF is mostly expressed throughout the entire hippocampus, amygdala, pre-frontal cortex (PFC) and hypothalamus (Broad et al., 2002). The strong expression of BDNF in the hippocampus and its increased presence at the synapse upon glutamatergic stimulus, points to a particular role for BDNF in hippocampal glutamate projections modulation (Hartmann et al., 2001). Indeed, during chronic stress exposure there is a reduction of BDNF expression [as well as NGF and NT3 (Ueyama et al., 1997)] at important stress response regions that recovers to normal levels upon effective treatment. (Mallei et al., 2002;Khawaja et al., 2004). Furthermore, these alterations are associated with the pathological hallmarks of stress such as neuronal death, dendrite atrophy and synaptic loss (Hill et al., 2012). While a link between BDNF, glutamatergic neurotransmission and depression has been established, a similar relationship between BDNF, GABAergic neurons and other psychiatric disorders, such as anxiety (GABAergic alterations) has not been demonstrated. In fact, GABAergic neurons express BDNF at very low levels (Brunig et

al., 2001). Whether other neurotrophic factors might play a more predominant role in this case is unknown. It would certainty be interesting to explore a role for NENF action in the modulation of these circuits/behavior.

BDNF was also shown to be involved in depression and anxiety modulation since epidemiologic studies have shown that humans that carry the BDNF Val66Met polymorphism have reduced serum levels of BDNF, as well as 11% hippocampal atrophy (Egan et al., 2003;Bueller et al., 2006). This was also seen in knock in BDNF Val66Met homozygous mice, which showed to have reduced levels of BDNF, hippocampal atrophy and a depressive and anxious like behaviors (Chen et al., 2006).

Other neurotrophic factors, besides BDNF, also play a relevant role in affective and anxiety related disorders. Indeed FGF family proteins have been shown to modulate behaviors of anxiety, depression, or substance abuse (Turner et al., 2012). For instance, FGF2 was associated to the modulation of affective disorders, such as anxiety; specifically ablation of FGF2 in the hippocampus (using lentivirus) display an anxious-like behavior (Salmaso and Vaccarino, 2011). NGF is another neurotrophin whose expression is altered in particular brain regions under certain conditions. For instance, in bipolar disorder, lithium treatment increases NGF in hippocampus and other brain regions (Hellweg et al., 2002). Indeed, in a rodent model of chronic i.c.v. delivery of NGF results in enhanced recognition memory and neurogenesis (Birch and Kelly, 2013). In fact, neurotrophic factors such as CNTF participate in the cellular mechanisms involved in anxiety and depression, as CNTK KO adult mice show to have loss of GABA neurons and impaired serotonergic activity, which results in an anxious and depressive like behaviors (Peruga et al., 2012).

Undeniably, neurotrophic factors are well known regulators of the adult neurogenic niches as one of their important functions is the maintenance of the stemness capacity and correct differentiation of the niches. Adult neurogenesis at the hippocampus takes place at the sub granular zone (SGZ) through out all dorsal/ventral axis of the dentate gyrus (DG) and the adult subventricular zone (aSVZ) lining the lateral ventricles of the adult brain.

Neurogenesis occurring, specifically at the DG has a functional output in the adult brain, specifically in psychiatric disorders such as mood disorders (Duman and Monteggia, 2006). It is well described that the successful treatment of mood disorders with anti-depressants, specially fluoxetine has its major out comes after 3 to 4 weeks, which can be correlated with the time

needed for new neurons to rise on the DG, accompanied by a significant increase of BDNF expression. Interestingly, chronic treatment (28 days) with anti-depressant can increase neurogenesis in the SGZ (Santarelli et al., 2003). On the other hand, it is observed a correlatable trend of decrease in hippocampal neurogenesis with stress related episodes associated with depressive like behavior and with a decrease expression of BDNF and NT-3 in stress regulatory related regions (Duman and Monteggia, 2006). Other neurotrophic factors have been shown to increase adult neurogenesis, as IGF-I (Aberg et al., 2000), associated with effective anti depressant treatment (Hoshaw et al., 2005). Vascular endothelial growth factor (VEGF) also fluctuates with anti-depressive treatment and regulates the rate of neurogenesis (Fabel K et al., 2003). It is also known that absence of specific neurotrophic factors during development, such as FGF2 lead to deficits in hippocampal neurogenesis that cannot be reverted (Werner et al., 2011). But there is also reports showing that neurotrophic factors negatively control neurogenesis; for instance, TGF1 is found to be up regulated in aging and that its over expressing in the brain blocks adult neurogenesis (Buckwalter et al., 2006) and that chronic delivery of GDNF induces the formation of new neural cells even when delivered to non neurogenic niches, such as the substancia nigra (Chen et al., 2005). Importantly, physical exercise and enriched environment are known to be potent inducers of hippocampal neurogenesis and this modulation is known to also over express BDNF or NGF (Jha et al., 2011;Birch et al., 2013). Considering all these findings, it is known that the adult neurogenic niches are under high regulation of neurotrophic factors and that their expression fluctuation has functional implication, namely in the hippocampal DG.

3. Heme/steroid binding proteins

There is a family of proteins that share common structural evidence: the presence of a heme and steroid binding domain in the primary protein structure. These proteins generally depend on the binding of heme to their cytochrome-b5 domain as a prosthetic group for full activity and function (Lederer, 1994). Hemoglobin, cytochromes, and guanylate cyclases, all belong to this family, although they do not share much functional properties between them. The major heme-binding proteins in mammals are hemoglobin and myoglobin, which need heme for oxygen transportation (Reedy et al., 2008). Heme-binding proteins participate in several different functions, such as

oxygen transportation, catalytic activity, electron transfer, and signaling functions as well as microRNA processing.

Regarding steroids, they are a class of organic compounds that contain 4 alkanes with methyl groups. Classical types of steroids are cholesterol, sex hormones (estrogen, progesterone, testosterone) and the stress hormone cortisol (corticosterone in rodents). They are classically involved in neuroendocrine regulation but also in non-classical functions, especially in the brain. Here we will focus on the role of sex steroids, particularly progesterone, in the brain.

3.1 Steroids in brain development

Physiologically the rise of different sex steroid/hormonal systems appears during postnatal development (Gore, 2008). This requires the development and maturation of a steroid-sensitive network between the reproductive organs and the brain hypothalamus during critical developmental windows. In fact, the mammalian brain is quite sensitive to sex hormone during late embryonic and early postnatal periods. Importantly, the mother's estrogens do not intervene in the masculinization of the brain due to alpha-fetoprotein (De Mees et al., 2006).

The developing brain exposure to sex hormones has a crucial influence in the sexual dimorphism of brain development and this process is closely regulated resulting in dimorphic development of brain regions that later in adulthood will result in female and male behaviors (Simerly, 1989). As an example, the anteroventral periventricular nucleus is bigger in females than males, and it is known that postnatal stress results in an increase of this nucleus in males, rising in a feminization of the brain (Sumida et al., 1993). Nuclear sex hormones receptors as estrogen receptors (ERα, ERβ), androgen receptors (AR) and progesterone receptors (PR) are expressed in the brain throughout development and participate in the epigenetical changes inherent to the dimorphic development of males and females (Tsai and O'Malley, 1994). In fact neuroendocrinology studies were among the first to reveal that embryonic and postnatal environment play a key role in determining adult physiology and behavior in mammals (Gore, 2008). Although the gonads only begin to produce large amounts of sex hormones in puberty, the brain dimorphic differentiation occurring early in life is fundamental for current reproductive capacity (Gore, 2008). Meanwhile, puberty is another time window of development in which the brain is sensitive to the organizational effects of steroid hormones. This period is characterized by neurite and dendritic growth and synaptogenesis of hormonal sensitive brain regions such as the

hypothalamus (Matsumoto and Arai, 1977;Arai and Matsumoto, 1978). As ascribed, steroids have a particular important role in the development of the brain. The classic PR is shown to be transiently expressed in the embryonic and postnatal periods, especially at the hippocampus and caudate putamen, suggesting an exclusive developmental function for progesterone (Wagner, 2008).

Unquestionably, all mechanisms involving the steroid system regulation during theses critical developmental periods will have somber influence in the correct orchestration of the entire brain gonadal network.

3.2 Steroids in adult brain

Finger imprinting, epigenetical changes and neuro-endocrine regulation by sex hormones in the brain are of extreme importance for the correct reproduction and survival of the species. But recently additional modulatory functions have arised, other than reproductive related, provided by sex hormones, in particular estrogen and progesterone, which involve non-genomic regulatory actions such as, neurogenesis, neuroprotection, mood alteration, oxidative metabolism and cancer progression (McEwen, 1991).

Here we will focus on the modulatory function of progesterone in these non-reproductive functions, specifically in the brain. In adulthood, the ovaries in females and the testis and adrenal cortex in males produce the majority of progesterone (Strauss and Barbieri, 2009). Surprisingly, the levels of progesterone in males and females are identical, but surprisingly males seem to have much higher expression of PR than females in the brain (Wagner, 2008).

Although the involvement of progesterone in these function is in a non-classical way, involving other membrane proteins, such as, membrane progesterone receptor-alpha (mPRα, mPRβ, mPRγ), progesterone receptor membrane component-1 (PGRMC1), PGRMC2 and maybe NENF that are known to activate downstream cytoplasmic kinase signaling thus resulting in both transcription-independent and transcription dependent effects. One of the potential functions of the MAPR family members (PGRMC1 and 2) is the regulation of non-genomic actions of progesterone. MAPR members are known to activate cell viability mechanisms involving cellular cascades, but the upstream mechanism by which these cascades are activated is still not totally clear with progesterone being a strong candidate. Among the several non-genomic cellular cascades that progesterone activates are the MAPK, cAMP/protein kinase A (PKA) and PI3 K/Akt

pathways (Simoncini and Genazzani, 2003).

In fact, the classical progesterone receptors activation also has been shown to be neuroprotective, namely by activating the transcription of BDNF. Surprisingly knockout mice for the classical nuclear PR also showed progesterone activity dependence (Krebs et al., 2000) that is membrane PR dependent but not nuclear PR dependent. This study revealed that although it is not yet known how progesterone exerts non-genomic function, they are as much important as the classic ones. One strong possible way by which the neuroprotective effects might be modulated is the activation of neurotrophic factors expression. Progesterone can induce BDNF expression not only by the nuclear PR but also through PGRMC1 (Su et al., 2012).

Two membrane receptors able to interact with progesterone have been identified, membrane progesterone receptors (mPRs) and the progesterone membrane receptor component (PGMRC). The mPR has a high affinity for progesterone binding and although nuclear PR and mPR are expressed in the same brain regions, such has hippocampus, cortex, hypothalamus, and cerebellum, they have different cellular expression patterns; mPR are preferentially expressed by neurons and not glia in basal conditions. But wide cellular expression of mPR is known to occur in traumatic brain injury, showing an anti-inflammatory role in the brain (Meffre et al., 2013). On the other hand, PGRMC (1 and 2) binding to progesterone is still a matter of debate; most probable PGRMC1 participate in the formation of a membrane complex, with affinity to bind progesterone and other steroids and same drugs as well (Thomas, 2008). Both of these membrane receptors are expressed in the brain and studies revealed that the two independently have been associated to progesterone non-genomic function, such as in progesterone-increased neural progenitor proliferation or decreased starvation-induced apoptosis in hippocampal neuronal cells (Liu et al., 2009;Pang et al., 2013). Indeed progesterone's non-genomic actions have been implicated in the regulation of the adult hippocampal neurogenesis since progesterone increases neural progenitor cells cell cycle *in vitro* (Liu et al., 2009) and *in vivo* it was seen that progesterone intra peritoneal delivery promoted survival of new born neurons in the SGZ, while the inhibition of 5alpha-reductase by finasteride did not affect this progesterone action, indicating that the progesterone effect is exerted by itself and not its metabolites (Zhang et al., 2010).

The first observation that a faster and non-genomic mechanism of action for progesterone existed was seen by its rapid anesthetic effects (Selye and Masson, 1942) and in fact progesterone has modulatory effects in neurotransmitter systems, specially the GABAergic. *In vivo* progesterone actions can also occur by its metabolization into neuro active neurosteorids. In fact, the most

studied neurosteroid is allopregnalone or 3α, 5α tetrahydroprogesterone, synthesized from progesterone by the 5α-recuctase enzyme. Nevertheless, neurosteroids are well-known modulators of anxiety by allosteric modulation of $GABA_AR$, mediating chloride influx, and indeed there has been observed a significant increase of neurosteroids in patients with generalized anxiety disorder (Semeniuk et al., 2001). It is believed that this increase might be a coping mechanism for a negative feedback (Strohle et al., 2003). Although neurosteroids were observed to interact with different receptors besides $GABA_AR$, like 5-HT3 receptors, oxytocin receptors, N-methyl-d-aspartate (NMDA) or sigma1 receptors, the exact output of these interaction is not understood (Meyer et al., 2002). It is also known that stress elevates the levels of specific neurosteroids and this way a possible modulation of the depressive behavior has been hypothesized, saying that the long lasting effect shown by selective serotonin reuptake inhibiters (SSRI) treatment would not be through elevation of serotonin but instead by stimulation of the allopreganalone catalytic enzymes 5α-reductase and 3 α-hydroxysteroid oxidoreductase (Serra et al., 2001). All these evidences make the role of neurosteroids an extremely exciting opportunity for understanding the modulation of brain physiology. But specifically, allopregnalone has also been found to participate in the protecting effect mediated of progesterone by participating in the inhibition of apoptosis during brain injury (Sayeed et al., 2009), as well as, to have potential effects on neurogenesis (Wang et al., 2008) and participate in the regulation of BDNF (Nin et al., 2011).

These evidences show the potential role for steroids, particularly progesterone, in adult brain physiology and function.

References

Aberg, M.A., Aberg, N.D., Hedbacker, H., Oscarsson, J., and Eriksson, P.S. (2000). Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. *J Neurosci* 20**,** 2896-2903.

Agerman, K., Hjerling-Leffler, J., Blanchard, M.P., Scarfone, E., Canlon, B., Nosrat, C., and Ernfors, P. (2003). BDNF gene replacement reveals multiple mechanisms for establishing neurotrophin specificity during sensory nervous system development. *Development* 130**,** 1479-1491.

Arai, Y., and Matsumoto, A. (1978). Synapse formation of the hypothalamic arcuate nucleus during post-natal development in the female rat and its modification by neonatal estrogen treatment. *Psychoneuroendocrinology* 3**,** 31-45.

Bartkowska, K., Turlejski, K., and Djavadian, R.L. (2010). Neurotrophins and their receptors in early development of the mammalian nervous system. *Acta Neurobiol Exp (Wars)* 70**,** 454-467.

Birch, A.M., and Kelly, A.M. (2013). Chronic intracerebroventricular infusion of nerve growth factor improves recognition memory in the rat. *Neuropharmacology* 75C**,** 255-261.

Birch, A.M., Mcgarry, N.B., and Kelly, A.M. (2013). Short-term environmental enrichment, in the absence of exercise, improves memory, and increases NGF concentration, early neuronal survival, and synaptogenesis in the dentate gyrus in a time-dependent manner. *Hippocampus* 23**,** 437-450.

Broad, K.D., Mimmack, M.L., Keverne, E.B., and Kendrick, K.M. (2002). Increased BDNF and trk-B mRNA expression in cortical and limbic regions following formation of a social recognition memory. *Eur J Neurosci* 16**,** 2166-2174.

Brunig, I., Penschuck, S., Berninger, B., Benson, J., and Fritschy, J.M. (2001). BDNF reduces miniature inhibitory postsynaptic currents by rapid downregulation of GABA(A) receptor surface expression. *Eur J Neurosci* 13**,** 1320-1328.

Buckwalter, M.S., Yamane, M., Coleman, B.S., Ormerod, B.K., Chin, J.T., Palmer, T., and Wyss-Coray, T. (2006). Chronically increased transforming growth factor-beta1 strongly inhibits hippocampal neurogenesis in aged mice. *Am J Pathol* 169**,** 154-164.

Bueller, J.A., Aftab, M., Sen, S., Gomez-Hassan, D., Burmeister, M., and Zubieta, J.K. (2006). BDNF Val66Met allele is associated with reduced hippocampal volume in healthy subjects. *Biol Psychiatry* 59**,** 812-815.

Byerly, M.S., Swanson, R.D., Semsarzadeh, N.N., Mcculloh, P.S., Kwon, K., Aja, S., Moran, T.H., Wong, G.W., and Blackshaw, S. (2013). Identification of hypothalamic neuron-derived neurotrophic factor as a novel factor modulating appetite. *Am J Physiol Regul Integr Comp Physiol* 304**,** R1085-1095.

Cahill, M.A. (2007). Progesterone receptor membrane component 1: an integrative review. *J Steroid Biochem Mol Biol* 105**,** 16-36.

Chan, J.P., Unger, T.J., Byrnes, J., and Rios, M. (2006). Examination of behavioral deficits triggered by targeting Bdnf in fetal or postnatal brains of mice. *Neuroscience* 142**,** 49-58.

Chen, Y., Ai, Y., Slevin, J.R., Maley, B.E., and Gash, D.M. (2005). Progenitor proliferation in the adult hippocampus and substantia nigra induced by glial cell line-derived neurotrophic factor. *Exp Neurol* 196**,** 87-95.

Chen, Z.Y., Jing, D., Bath, K.G., Ieraci, A., Khan, T., Siao, C.J., Herrera, D.G., Toth, M., Yang, C., Mcewen, B.S., Hempstead, B.L., and Lee, F.S. (2006). Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* 314**,** 140-143.

Davies, A.M. (1996). The neurotrophic hypothesis: where does it stand? *Philos Trans R Soc Lond B Biol Sci.* 351**,** 389-394.

De Mees, C., Laes, J.F., Bakker, J., Smitz, J., Hennuy, B., Van Vooren, P., Gabant, P., Szpirer, J., and Szpirer, C. (2006). Alpha-fetoprotein controls female fertility and prenatal development of the gonadotropin-releasing hormone pathway through an antiestrogenic action. *Mol Cell Biol* 26**,** 2012-2018.

Duman, R.S., and Monteggia, L.M. (2006). A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* 59**,** 1116-1127.

Egan, M.F., Kojima, M., Callicott, J.H., Goldberg, T.E., Kolachana, B.S., Bertolino, A., Zaitsev, E., Gold, B., Goldman, D., Dean, M., Lu, B., and Weinberger, D.R. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112**,** 257-269.

Ernfors, P., Van De Water, T., Loring, J., and Jaenisch, R. (1995). Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron* 14**,** 1153-1164.

Fabel K, Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo Cj, and Td, P. (2003). VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci* 18**,** 2803-2812.

Fagan, A.M., Garber, M., Barbacid, M., Silos-Santiago, I., and Holtzman, D.M. (1997). A role for TrkA during maturation of striatal and basal forebrain cholinergic neurons in vivo. *J Neurosci* 17**,** 7644-7654.

Gore, A.C. (2008). Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Front Neuroendocrinol* 29**,** 358-374.

Hagg, T. (2009). From neurotransmitters to neurotrophic factors to neurogenesis. *Neuroscientist* 15**,** 20-27.

Han, K.H., Lee, S.H., Ha, S.A., Kim, H.K., Lee, C., Kim, D.H., Gong, K.H., Yoo, J., Kim, S., and Kim, J.W. (2012). The functional and structural characterization of a novel oncogene GIG47 involved in the breast tumorigenesis. *BMC Cancer* 12**,** 274.

Hartmann, M., Heumann, R., and Lessmann, V. (2001). Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO J* 20**,** 5887-5897.

Hellweg, R., Lang, U.E., Nagel, M., and Baumgartner, A. (2002). Subchronic treatment with lithium increases nerve growth factor content in distinct brain regions of adult rats. *Mol Psychiatry* 7**,** 604-608.

Henderson, C.E. (1996). Role of neurotrophic factors in neuronal development. *Curr Opin Neurobiol* 6**,** 64-70.

Hill, M.N., Hellemans, K.G., Verma, P., Gorzalka, B.B., and Weinberg, J. (2012). Neurobiology of chronic mild stress: parallels to major depression. *Neurosci Biobehav Rev* 36**,** 2085-2117.

Hoshaw, B.A., Malberg, J.E., and Lucki, I. (2005). Central administration of IGF-I and BDNF leads to long-lasting antidepressant-like effects. *Brain Res* 1037**,** 204-208.

Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24**,** 677-736.

Jha, S., Dong, B., and Sakata, K. (2011). Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-derived neurotrophic factor in mice lacking its expression through promoter IV. *Transl Psychiatry* 1**,** e40.

Khawaja, X., Xu, J., Liang, J.J., and Barrett, J.E. (2004). Proteomic analysis of protein changes developing in rat hippocampus after chronic antidepressant treatment: Implications for depressive disorders and future therapies. *J Neurosci Res* 75**,** 451-460.

Kimura, I., Konishi, M., Asaki, T., Furukawa, N., Ukai, K., Mori, M., Hirasawa, A., Tsujimoto, G., Ohta, M., Itoh, N., and Fujimoto, M. (2009). Neudesin, an extracellular heme-binding protein, suppresses adipogenesis in 3T3-L1 cells via the MAPK cascade. *Biochem Biophys Res Commun* 381**,** 75-80.

Kimura, I., Konishi, M., Miyake, A., Fujimoto, M., and Itoh, N. (2006). Neudesin, a secreted factor, promotes neural cell proliferation and neuronal differentiation in mouse neural precursor cells. *J Neurosci Res* 83**,** 1415-1424.

Kimura, I., Nakayama, Y., Konishi, M., Kobayashi, T., Mori, M., Ito, M., Hirasawa, A., Tsujimoto, G., Ohta, M., Itoh, N., and Fujimoto, M. (2010). Neuferricin, a novel extracellular heme-binding protein, promotes neurogenesis. *J Neurochem* 112**,** 1156-1167.

Kimura, I., Nakayama, Y., Konishi, M., Terasawa, K., Ohta, M., Itoh, N., and Fujimoto, M. (2012). Functions of MAPR (membrane-associated progesterone receptor) family members as heme/steroid-binding proteins. *Curr Protein Pept Sci* 13**,** 687-696.

Kimura, I., Nakayama, Y., Yamauchi, H., Konishi, M., Miyake, A., Mori, M., Ohta, M., Itoh, N., and Fujimoto, M. (2008). Neurotrophic activity of neudesin, a novel extracellular heme-binding protein, is dependent on the binding of heme to its cytochrome b5-like heme/steroid-binding domain. *J Biol Chem* 283**,** 4323-4331.

Kimura, I., Nakayama, Y., Zhao, Y., Konishi, M., and Itoh, N. (2013). Neurotrophic effects of neudesin in the central nervous system. *Front Neurosci* 7**,** 111.

Kimura, I., Yoshioka, M., Konishi, M., Miyake, A., and Itoh, N. (2005). Neudesin, a novel secreted protein with a unique primary structure and neurotrophic activity. *J Neurosci Res* 79**,** 287-294.

Knusel, B., Rabin, S.J., Hefti, F., and Kaplan, D.R. (1994). Regulated neurotrophin receptor responsiveness during neuronal migrationand early differentiation. *J Neurosci* 14**,** 1542-1554.

Krebs, C.J., Jarvis, E.D., Chan, J., Lydon, J.P., Ogawa, S., and Pfaff, D.W. (2000). A membrane-associated progesterone-binding protein, 25-Dx, is regulated by progesterone in brain regions involved in female reproductive behaviors. *Proc Natl Acad Sci U S A* 97**,** 12816-12821.

Lederer, F. (1994). The cytochrome b5-fold: an adaptable module. *Biochimie* 76**,** 674-692.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science* 237**,** 1154-1162.

Liu, L., Wang, J., Zhao, L., Nilsen, J., Mcclure, K., Wong, K., and Brinton, R.D. (2009). Progesterone increases rat neural progenitor cell cycle gene expression and proliferation via extracellularly regulated kinase and progesterone receptor membrane components 1 and 2. *Endocrinology* 150**,** 3186-3196.

Losel, R.M., Besong, D., Peluso, J.J., and Wehling, M. (2008). Progesterone receptor membrane component 1–many tasks for a versatile protein. *Steroids* 73**,** 929-934.

Ma, L., Broomfield, S., Lavery, C., Lin, S.L., Xiao, W., and Bacchetti, S. (1998). Up-regulation of CIR1/CROC1 expression upon cell immortalization and in tumor-derived human cell lines. *Oncogene* 17**,** 1321-1326.

Magdaleno, S., Jensen, P., Brumwell, C.L., Seal, A., Lehman, K., Asbury, A., Cheung, T., Cornelius, T., Batten, D.M., Eden, C., Norland, S.M., Rice, D.S., Dosooye, N., Shakya, S., Mehta, P., and Curran, T. (2006). BGEM: an in situ hybridization database of gene expression in the embryonic and adult mouse nervous system. *PLoS Biol* 4**,** e86.

Mallei, A., Shi, B., and Mocchetti, I. (2002). Antidepressant treatments induce the expression of basic fibroblast growth factor in cortical and hippocampal neurons. *Mol Pharmacol* 61**,** 1017-1024.

Matsumoto, A., and Arai, Y. (1977). Precocious puberty and synaptogenesis in the hypothalamic arcuate nucleus in pregnant mare serum gonadotropin (PMSG) treated immature female rats. *Brain Res* 129**,** 375-378.

Mcallister, A.K., Katz, L.C., and Lo, D.C. (1999). Neurotrophins and synaptic plasticity. *Annu Rev Neurosci* 22**,** 295-318.

Mcewen, B.S. (1991). Non-genomic and genomic effects of steroids on neural activity. *Trends Pharmacol Sci* 12**,** 141-147.

Meffre, D., Labombarda, F., Delespierre, B., Chastre, A., De Nicola, A.F., Stein, D.G., Schumacher, M., and Guennoun, R. (2013). Distribution of membrane progesterone receptor alpha in the male mouse and rat brain and its regulation after traumatic brain injury. *Neuroscience* 231**,** 111-124.

Meyer, C., Schmid, R., Scriba, P.C., and Wehling, M. (1996). Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes. *Eur J Biochem* 239**,** 726-731.

Meyer, D.A., Carta, M., Partridge, L.D., Covey, D.F., and Valenzuela, C.F. (2002). Neurosteroids enhance spontaneous glutamate release in hippocampal neurons. Possible role of metabotropic sigma1-like receptors. *J Biol Chem* 277**,** 28725-28732.

Min, L., Strushkevich, N.V., Harnastai, I.N., Iwamoto, H., Gilep, A.A., Takemori, H., Usanov, S.A., Nonaka, Y., Hori, H., Vinson, G.P., and Okamoto, M. (2005). Molecular identification of adrenal inner zone antigen as a heme-binding protein. *FEBS J* 272**,** 5832-5843.

Minichiello, L., and Klein, R. (1996). TrkB and TrkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. *Genes Dev* 10**,** 2849-2858.

Neubauer, H., Clare, S.E., Kurek, R., Fehm, T., Wallwiener, D., Sotlar, K., Nordheim, A., Wozny, W., Schwall, G.P., Poznanovic, S., Sastri, C., Hunzinger, C., Stegmann, W., Schrattenholz, A., and Cahill, M.A. (2006). Breast cancer proteomics by laser capture microdissection, sample pooling, 54-cm IPG IEF, and differential iodine radioisotope detection. *Electrophoresis* 27**,** 1840-1852.

Nin, M.S., Martinez, L.A., Pibiri, F., Nelson, M., and Pinna, G. (2011). Neurosteroids reduce social isolation-induced behavioral deficits: a proposed link with neurosteroid-mediated upregulation of BDNF expression. *Front Endocrinol (Lausanne)* 2**,** 73.

Pang, Y., Dong, J., and Thomas, P. (2013). Characterization, neurosteroid binding and brain distribution of human membrane progesterone receptors delta and {epsilon} (mPRdelta and mPR{epsilon}) and mPRdelta involvement in neurosteroid inhibition of apoptosis. *Endocrinology* 154**,** 283-295.

Peluso, J.J., Liu, X., Gawkowska, A., and Johnston-Macananny, E. (2009). Progesterone activates a progesterone receptor membrane component 1-dependent mechanism that promotes human granulosa/luteal cell survival but not progesterone secretion. *J Clin Endocrinol Metab* 94**,** 2644-2649.

Peluso, J.J., Romak, J., and Liu, X. (2008). Progesterone receptor membrane component-1 (PGRMC1) is the mediator of progesterone's antiapoptotic action in spontaneously immortalized granulosa cells as revealed by PGRMC1 small interfering ribonucleic acid treatment and functional analysis of PGRMC1 mutations. *Endocrinology* 149**,** 534-543.

Peruga, I., Hartwig, S., Merkler, D., Thone, J., Hovemann, B., Juckel, G., Gold, R., and Linker, R.A. (2012). Endogenous ciliary neurotrophic factor modulates anxiety and depressive-like behavior. *Behav Brain Res* 229**,** 325-332.

Purves, D., Snider, W.D., and Voyvodic, J.T. (1988). Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature* 336**,** 123-128.

Raballo, R., Rhee, J., Lyn-Cook, R., Leckman, J., Schwartz, M., and Vaccarino, F. (2000). Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *journal of neuroscience* 20**,** 5012-5023.

Reedy, C.J., Elvekrog, M.M., and Gibney, B.R. (2008). Development of a heme protein structure-electrochemical function database. *Nucleic Acids Res* 36**,** D307-313.

Ringstedt, T., Lagercrantz, H., and Persson, H. (1993). Expression of members of the trk family in the developing postnatal rat brain. *Brain Res Dev Brain Res* 72**,** 119-131.

Rohe, H.J., Ahmed, I.S., Twist, K.E., and Craven, R.J. (2009). PGRMC1 (progesterone receptor membrane component 1): a targetable protein with multiple functions in steroid signaling, P450 activation and drug binding. *Pharmacol Ther* 121**,** 14-19.

Salmaso, N., and Vaccarino, F.M. (2011). Toward a novel endogenous anxiolytic factor, fibroblast growth factor 2. *Biol Psychiatry* 69**,** 508-509.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., and Hen, R. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301**,** 805-809.

Sayeed, I., Parvez, S., Wali, B., Siemen, D., and Stein, D.G. (2009). Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism for better neuroprotective effects of allopregnanolone over progesterone. *Brain Res* 1263**,** 165-173.

Schwartz, P.M., Borghesani, P.R., Levy, R.L., Pomeroy, S.L., and Segal, R.A. (1997). Abnormal cerebellar development and foliation in BDNF-/- mice reveals a role for neurotrophins in CNS patterning. *Neuron* 19**,** 269-281.

Segal, R.A., and Greenberg, M.E. (1996). Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci* 19**,** 463-489.

Selye, H., and Masson, G. (1942). Additional Steroids with Luteoid Activity. *Science* 96**,** 2494-2358.

Semeniuk, T., Jhangri, G.S., and Le Melledo, J.M. (2001). Neuroactive steroid levels in patients with generalized anxiety disorder. *J Neuropsychiatry Clin Neurosci* 13**,** 396-398.

Serra, M., Pisu, M.G., Muggironi, M., Parodo, V., Papi, G., Sari, R., Dazzi, L., Spiga, F., Purdy, R.H., and Biggio, G. (2001). Opposite effects of short- versus long-term administration of fluoxetine on the concentrations of neuroactive steroids in rat plasma and brain. *Psychopharmacology (Berl)* 158**,** 48-54.

Simerly, R.B. (1989). Hormonal control of the development and regulation of tyrosine hydroxylase expression within a sexually dimorphic population of dopaminergic cells in the hypothalamus. *Brain Res Mol Brain Res* 6**,** 297-310.

Simoncini, T., and Genazzani, A.R. (2003). Non-genomic actions of sex steroid hormones. *Eur J Endocrinol* 148**,** 281-292.

Singh, M., Su, C., and Ng, S. (2013). Non-genomic mechanisms of progesterone action in the brain. *Frontiers in Neuroscience* 7.

Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* 77**,** 627-638.

Strauss, J.F., and Barbieri, R.L. (2009). *Yen and Jaffe's reproductive endocrinology: physiology, pathophysiology and clinical managment* Elsevier.

Strohle, A., Romeo, E., Di Michele, F., Pasini, A., Hermann, B., Gajewsky, G., Holsboer, F., and Rupprecht, R. (2003). Induced panic attacks shift gamma-aminobutyric acid type A receptor modulatory neuroactive steroid composition in patients with panic disorder: preliminary results. *Arch Gen Psychiatry* 60**,** 161-168.

Su, C., Cunningham, R.L., Rybalchenko, N., and Singh, M. (2012). Progesterone increases the release of brain-derived neurotrophic factor from glia via progesterone receptor membrane component 1 (Pgrmc1)-dependent ERK5 signaling. *Endocrinology* 153**,** 4389-4400.

Sumida, H., Nishizuka, M., Kano, Y., and Arai, Y. (1993). Sex differences in the anteroventral periventricular nucleus of the preoptic area and in the related effects of androgen in prenatal rats. *Neurosci Lett* 151**,** 41-44.

Thomas, P. (2008). Characteristics of membrane progestin receptor alpha (mPRalpha) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Front Neuroendocrinol* 29**,** 292-312.

Thomas, P., and Pang, Y. (2012). Membrane progesterone receptors: evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells. *Neuroendocrinology* 96**,** 162-171.

Tsai, M.J., and O'malley, B.W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63**,** 451-486.

Turner, C.A., Watson, S.J., and Akil, H. (2012). The fibroblast growth factor family: neuromodulation of affective behavior. *Neuron* 76**,** 160-174.

Ueyama, T., Kawai, Y., Nemoto, K., Sekimoto, M., Tone, S., and Senba, E. (1997). Immobilization stress reduced the expression of neurotrophins and their receptors in the rat brain. *Neurosci Res* 28**,** 103-110.

Wagner, C.K. (2008). Progesterone receptors and neural development: a gap between bench and bedside? *Endocrinology* 149**,** 2743-2749.

Wang, J.M., Liu, L., Irwin, R.W., Chen, S., and Brinton, R.D. (2008). Regenerative potential of allopregnanolone. *Brain Res Rev* 57**,** 398-409.

Werner, S., Unsicker, K., and Von Bohlen Und Halbach, O. (2011). Fibroblast growth factor-2 deficiency causes defects in adult hippocampal neurogenesis, which are not rescued by exogenous fibroblast growth factor-2. *J Neurosci Res* 89**,** 1605-1617.

Zhang, Z., Yang, R., Zhou, R., Li, L., Sokabe, M., and Chen, L. (2010). Progesterone promotes the survival of newborn neurons in the dentate gyrus of adult male mice. *Hippocampus* 20**,** 402-412.

2. Aims and goals

2. Aims and goals

The goal of this work is to understand the role of neudesin (NENF) in the central nervous system (CNS). For that we have taken advantage of a unique mouse model with a deletion of the neudesin gene, neudesin-null mice, to study the impact of NENF *in vivo*, particularly in the brain.

Specifically we aim at:

I) Understand the impact of NENF ablation in CNS functioning, namely in adult behavior, neurotransmission and brain morphology.

II) Characterize the postnatal neurological developmental of neudesin-null mice.

III) Unravel whether NENF plays a role as neurotrophic factor *in vivo*, namely in the adult neurogenic niches.

3. Neudesin is involved in anxiety behavior: structural and neurochemical correlates

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Neudesin is involved in anxiety behavior: structural and neurochemical correlates

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Neudesin (also known as neuron derived neurotrophic factor, Nenf) is a scarcely studied putative non-canonical neurotrophic factor. In order to understand its function in the brain, we performed an extensive behavioral characterization (motor, emotional, and cognitive dimensions) of neudesin-null mice. The absence of neudesin leads to an anxious-like behavior as assessed in the elevated plus maze (EPM), light/dark box (LDB) and novelty suppressed feeding (NSF) tests, but not in the acoustic startle (AS) test. This anxious phenotype is associated with reduced dopaminergic input and impoverished dendritic arborizations in the dentate gyrus granule neurons of the ventral hippocampus. Interestingly, shorter dendrites are also observed in the bed nucleus of the stria terminalis (BNST) of neudesin-null mice. These findings lead us to suggest that neudesin is a novel relevant player in the maintenance of the anxiety circuitry.

Keywords: neudesin, anxiety, dopamine, ventral hippocampus, bed nucleus of the stria terminalis

INTRODUCTION

Neudesin (also known as neuron derived neurotrophic factor, Nenf) is a 21 kiloDalton secreted protein with 171 aminoacids (Kimura et al., 2005). Neudesin was classified as a member of the membrane-associated progesterone receptor family since its primary structure contains a cytochrome b5-heme/steroid binding domain (Kimura et al., 2012). Studies in mice showed that neudesin is most abundantly expressed in the brain and spinal cord (Kimura et al., 2005). While in the developing mouse brain neudesin is predominantly expressed in neurons with scattered presence in other cell types, in the adult brain its expression seems to be restricted to neurons (Kimura et al., 2005). Neudesin expression starts at approximately embryonic day 12.5 (E12.5), as evaluated by real time PCR (RT-PCR) in neural precursor cells; its expression increases during the rest of the embryonic period in inverse correlation with the expression of markers of dividing neural precursor cells (nestin) and in direct correlation with that of microtubule-associated protein 2 (MAP-2) (a marker for mature neurons) (Kimura et al., 2005). Of notice, neudesin expression is higher in the cortical preplate, an area that participates in the formation of the cerebral cortex. During embryonic and postnatal development, neurotrophic factors main functions are to provide survival and differentiation of nervous cells through activation of the p75 and tyrosine kinase (Trk) receptors and by downstream pathways such as MAP and PI-3 kinases (Russell and Duman, 2002). In adulthood, these downstream cascades activate different functional responses such as axon growth, dendrite pruning, cell fate decisions (Gray et al., 2013), as well as the modulation of neurotransmitters, thus regulating synaptic plasticity (McAllister, 2001). Importantly, studies in primary cultures of neurons revealed that neudesin display a neurotrophic activity. Specifically, neudesin was shown to induce the proliferation of cortical neural precursor cells early in development, and their subsequent differentiation into neurons (Kimura et al., 2005, 2006). Nevertheless, the identification of a receptor for neudesin is still elusive. Even less information is available on the exact function of neudesin in the adult brain, but *in vitro* experiments indicate that it may promote the maintenance of neurons in an autocrine/paracrine mode (Kimura et al., 2005). This neurotrophic activity has been shown to depend on the attachment of hemin to its cytochrome b5-heme/steroid domain, while a similar relevant action on the binding of steroids failed to be demonstrated (Kimura et al., 2008).

The importance of neurotrophic factors in brain maturation and function was extensively demonstrated (Snider, 1994). Furthermore, it is well-known that neurotrophic factors such as brain derived neurotrophic factor (BDNF) and fibroblast growth factor (FGF) play an important role in the etiology of mood disorders, such as depression, and in modulating emotional responses, including anxiety (Masi and Brovedani, 2011; Turner et al., 2012). In accordance, the limbic brain regions known to be involved in the modulation of emotional responses, that include the ventral hippocampus, amygdala and the bed nucleus of the stria terminalis (BNST), were shown to present an altered neurotrophins levels after an exposure to harmful stimuli, such as chronic stress, thus culminating in neurotransmission imbalance and in synaptic plasticity impairment (Taylor et al., 2011; Jung et al., 2012). Noteworthy, altered monoaminergic neurotransmission was found in BDNF-null mice (Ren-Patterson et al., 2005).

Given the *in vitro* available evidence on the potential neurotrophic properties of neudesin, in this study we addressed the role of Nenf in modulating behavior (emotional and cognitive) brain cytoarchitecture and neurotransmission (namely monoaminergic), for which we used neudesin-null mice.

MATERIALS AND METHODS

ANIMALS

A mouse strain with targeted deletion of the Nenf gene, provided by Merck-Serono under a material transfer agreement, was used. The neudesin-null ($Nenf^{-/-}$) mouse strain was generated by using a 129/SvEv genomic library from a BAC clone and the target construct was made by deletion of the entire coding sequence of the neudesin gene (exons 1–4, ∼12 KB) and replacing it by a *LacZ-neomicin* cassette. The BAC targeting vector was then inserted into embryonic stem cells (FiH4 ES cells), where the homologous pieces of DNA were recombined. The cells identified as homologous recombinant clones were microinjected into C57BL/6F1 blastocysts to generate chimeric mouse. Initial genotyping was performed using a loss-of-native-allele assay. Animals used in this study were backcrossed in a C57BL/6F background.

$Nenf^{-/-}$ and $Nenf^{+/+}$ were obtained by *crossing heterozygous animals*. Mice genotype was confirmed by PCR using two independent sets of primers: one for the *LacZ* cassette, specific for the genotype: LacZ-foward 5′-GGTAAACTGGCTCGGATTAGGG-3′ and LacZ-reverse 5′-TTGACTGTAGCGGCTGATGTTG-3′; and another for the Nenf gene, specific for $Nenf^{+/+}$ animals: Nenf-intron3 5′- CTTGGAGTTTGGGGCTGATA-3′, Nenf-exon4 5′-TGGCTTTGTACACCTTGCTG-3′. The amplified fragments were of 210 and 176 bp, respectively, and distinguishable by electrophoresis through a 1.5% agarose gel. Confirmation of loss of neudesin synthesis in $Nenf^{-/-}$ was also obtained by performing immunohistochemistry with a neudesin-specific antibody (Sigma, St. Louis, USA) in brain samples of both control and neudesin-null mice; neuronal expression of neudesin was not detected in neudesin-null mice.

Neudesin-null homozygous mice are viable, fertile, grow normally and do not display apparent morphological alterations. Adult animals, at the beginning of behavioral analysis no differences in body weight between littermate controls and neudesin-null mice were found ($Nenf^{+/+} = 21.13 \pm 1.98$ vs. $Nenf^{-/-} = 21.28 \pm 2.08$ g).

Animals were maintained under 12 h light/dark cycles at 22 ± 1°C, 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. This study was approved by the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (permission ID: DGV9457). All experiments were performed in accordance with the guidelines for the care and handling of laboratory animals, as described in the Directive 2010/63/EU of the European Parliament and of the Council.

ADULT BEHAVIOR

Adult behavior was assessed in 3 months-old male mice, in 3 independent sets of animals, of which one is presented here. Eight neudesin-null and 10 littermates control animals were analyzed in the open field (OF), elevated plus maze (EPM), forced swim test (FST) and Morris water maze (MWM) tests, performed by this sequential order, as an initial behavioral characterization. A 24 h time interval was used between OF, EPM, and FST tests; a 96 h time interval was used between FST and MWM. After the first behavioral characterization, the acoustic startle (AS), light/dark box (LDB) and novelty suppress feeding (NSF) tests were performed, by this sequence, in order to further evaluate the anxious-like behavior (see results section below); for this additional evaluation, another 3 independent sets of animals were analyzed, and one representative set of 10 neudesin-null males and 10 control littermate mice is presented here.

Adult behavior tests were performed in all animals during the light phase of the light/dark cycle at the same period of the day to avoid physiological differences related to the circadian cycle. The tests were performed as described next:

Open field

Animals were placed in a room adjacent to the experimental room 1 h before the test. Locomotor activity was assessed in a brightly illuminated square arena with 43.2×43.2 cm size surrounded by walls to prevent escape. Animals were placed in the center of the arena and allowed to explore it for 5 min. Data collected through the infrared system (MedAssociates Inc., St Albans, VT) contained total distance travelled, and distance and time spent in the center vs. the periphery of the arena.

Forced swim test

Animal learned helplessness behavior was analyzed using the FST in 2 consecutive days. Mice were placed for 5 min in a glass cylinder filled with water (24°C) at a depth of 30 cm. Twenty-four hours later mice repeated the test in the same conditions (Porsolt et al., 1977). Trials were video recorded and manually analyzed using the Etholog V2.2 software (Ottoni, 2000). The 5 min of the second day were analyzed. Data collected consisted in the duration of swimming and of immobility time.

Morris water maze

MWM was used to evaluate mice spatial reference memory. In this test, animals were placed in a circular white pool (170 cm in diameter and 50 cm in height) filled with tap water (24 ± 1°C) placed in a poorly lit room. The pool was divided in 4 imaginary quadrants and a transparent plexiglas platform (14 cm in diameter) was hidden 0.5 cm bellow surface in the center of one of the quadrants. For each quadrant, external clues were placed in the walls of the room. The test consisted of 4 trials per day for 4 consecutive days. In each trial, animals were randomly placed in each one of the quadrants and were allowed to swim for 120 s. Mice that failed to reach the platform within this time-period were gently guided to the platform. The distance and time animals took to find the platform were recorded using a video camera connected to a video-tracking system (Videotrack, Viewpoint, Champagne au Mont d'Or, France).

In the fifth day animals performed the probe and reversal tests. The probe test consisted of a unique trial without the platform where the animals were allowed to swim for 120 s. Time and distance swum in each quadrant were collected and analyzed for the first 60 s. To test memory flexibility we performed the reversal test, which consisted in changing the initial position of the platform to

the opposite quadrant of the pool. Animals were given 3 trials of 120 s each to learn the new position. The percentage of distance swum in each quadrant is represented.

Elevated plus maze

Anxious behavior was assessed using an apparatus composed of two opposite brightly illuminated open arms (51 × 10 cm) and two opposite dark closed arms (51 × 10 × 40 cm) and a central platform, 74 cm above the floor (NIR plus maze, MedAssociates Inc.). Animals were placed in the center of the maze and allowed to explore it for 5 min. Data collected consisted of the number of entries (four paws) in each arm as well as the time spent in each arm (MedPCIV, MedAssociates software).

Acoustic startle

Startle reflex was measured in a startle response apparatus (SR-LAB, San Diego Instruments, San Diego, CA, USA), consisting of a non-restrictive plexiglas cylinder (inner diameter 2.8 cm, length 8.9 cm), mounted on a plexiglas platform and placed in a ventilated sound-attenuated chamber. Animals were habituated to the apparatus (5 min) 1 day before actual testing. Cylinder movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system (San Diego Instruments, San Diego, CA, USA) was used to ensure comparable startle magnitudes. Startle stimuli were presented through a high frequency speaker located 33 cm above the startle chamber. Startle magnitudes were sampled every ms over a period of 200 ms, beginning with the onset of the startle stimulus. A startle response is defined as the peak response during 200 ms recording period. A higher startle reflex reflects an increased anxious state of the animal.

Light/dark box

For this test the OF arena was divided in half. One part was open and the other consisted of a black plexiglas with an entrance at the center of the arena facing the bright side. Each animal was placed alone at the center of the arena facing the lateral wall and allowed to explore it for 10 min. An infrared system (MedAssociates Inc) registered the time spent in each compartment.

Novelty suppressed feeding

Animals were food deprived for 24 h before being placed in the corner of the OF arena (MedAssociates Inc) and left to explore it for 10 min. A single pellet of food was placed in the center of the novel environment and the latency the animal took to leave the corner and feed was recorded. Upon reaching and start eating the pellet the animal was placed back in the home cage, where it was allowed to eat pre-weighted food. Food intake was recorded after 5, 15, and 30 min, as a measure of appetite drive.

GOLGI-COX STAINING

One week after completion of the behavioral tests mice (5 $Nenf^{+/+}$ and 5 $Nenf^{-/-}$) were transcardialy perfused with 0.9% saline under deep anesthesia and brains were removed for Golgi-Cox staining (Gibbs et al., 1998). Briefly, brains were immersed in Golgi-Cox solution (a 1:1 solution of 5% potassium dichromate (Merck, Darmstadt, Germany) and 5% mercuric chloride (Merck) diluted 4:10 with 5% potassium chromate (Merck) for 14 d; then transferred to 30% sucrose isolution in 0.5% sodium azide where they were kept until processing. Coronal vibrotome 200 μm thick sections were collected in 6% sucrose and blotted dry onto clean, gelatin-coated microscope slides, alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed, dehydrated through a graded series of ethanol, cleared in xylene, mounted with entellan and coverslipped.

DENDRITIC TREE ANALYSIS

Basolateral amygdala (BLa) pyramidal neurons, anterior medial bed nucleus of the stria terminalis (amBNST), lateral dorsal bed nucleus of the stria terminalis (ldBNST) bipolar neurons, ventral and dorsal dentate gyrus (DG) granular and ventral CA1 pyramidal-neurons of the hippocampus were chosen randomly, 3 per section; regional boundaries were defined as previously outlined (Dong et al., 2001; Paxinos and Franklin, 2001). The criteria to choose perfect Golgi impregnated neurons were the same as described previously (Uylings et al., 1986): (1) dendritic branches were not incomplete, broken or non-impregnated; (2) dendrites did not show overlap with other branches; (3) neurons were visually well-isolated. Twenty five to 30 neurons per experimental group were studied, i.e., 5–6 neurons per animal were analyzed for each of the 5 animals in the experimental groups. For each selected neuron, all branches of the dendritic tree were reconstructed at 600× magnification using a motorized microscope (BX51, Olympus), with oil objectives attached to a camera (Microbrigthfield Bioscience, Madgedurg, Germany) and using the Neurolucida software (Microbrightfield). Dendritical parameters analyzed were the total dendritic length and 3D version of the Sholl analysis (Sholl, 1956); where, the number of dendritical intersections with concentric spheres positioned at 20 μm intervals from the neurons soma was counted using the NeuroExplorer software (MicroBrightField).

NEUROCHEMICAL ANALYSIS

Monoamines' levels were measured using high performance liquid chromatography with electrochemical detection (HPLC-ED). Naive 3 months old male mice were killed by decapitation (10 $Nenf^{+/+}$ and 8 $Nenf^{-/-}$). Skulls were snap frozen in liquid nitrogen to avoid degradation during macrodissection. Brains were carefully dissected for ventral hippocampus, amygdala, BNST. Dissection was performed on ice with the help of a stereomicroscope.

Dissected tissues were weighed and then homogenized and deproteinized in 100 μL of 0.2 N perchloric acid solution (Applichem, Darmstadt, Germany) containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA (Riedel-de Haën AG, Seelze, Germany), centrifuged at 20000 g for 45 min at 4°C and the supernatant was stored at −80°C until analysis.

The analysis was performed using a GBC LC1150 HPLC pump (GBC Scientific Equipment, Braeside, Victoria, Australia) coupled with a BAS-LC4C (Bioanalytical Systems Inc., USA) electrochemical detector, as previously described (Kokras et al., 2009). The working electrode of the electrochemical detector was set at +800 mV. In all samples reverse phase ion pairing chromatography was used to assay dopamine (DA) and its metabolites 3,4 dihydroxyphenylacetate (DOPAC) and

homovanillic acid (HVA), serotonin (5HT) and its metabolite 5-hydroxyindoleatic (5HIAA) and norepinephrine (NE). The mobile phase consisted of a 50 mM phosphate buffer regulated at pH 3.0, containing 5-octylsulfate sodium salt at a concentration of 300 mg/L as the ion pairing reagent and Na_2EDTA at a concentration of 20 mg/L (Riedel-de Haën AG); acetonitrile (Merck, Darmstadt, Germany) was added at a 9% concentration. The reference standards were prepared in 0.2 N perchloric acid solution containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA. The column used was an Aquasil C18 HPLC Column, 100 × 1 mm, 5μm Particle Size (Thermo Electron, UK). Samples were quantified by comparison of the area under the curve against known external reference standards using a PC compatible HPLC software package (Chromatography Station for Windows ver.17 Data Apex Ltd). The limit of detection was 1 pg/20 μL (of injection volume). In addition to the assay of 5-HT and 5-HIAA tissue levels, the 5-HT turnover rate was also calculated, separately for each chromatograph, as the ratio of 5-HIAA/5-HT. Similarly, the ratios of DOPAC/DA and HVA/DA were calculated as an index of DA turnover rates. Turnover rates estimate the serotonergic and dopaminergic activities better than individual neurotransmitter and metabolite tissue levels as they reflect 5-HT and DA release and/or metabolic activity as described elsewhere (Dalla et al., 2008; Kokras et al., 2009; Mikail et al., 2012).

STATISTICAL ANALYSIS

All values presented are expressed as the mean ± SEM and significance was verified by using the Mann–Whitney test for independent samples for all behavior, dendritic length, sholl analysis and neurochemical data. Differences were considered significant when $p < 0.05$.

RESULTS

ABLATION OF NEUDESIN INDUCES AN ANXIOUS-LIKE PHENOTYPE IN ADULT MICE

Despite the described neuronal expression profile of neudesin in the adult brain, no information is available on the relevance of this protein for the central nervous system (CNS) functioning. To tackle this gap we performed a wide behavioral characterization of neudesin-null mice.

From the behavioral analyses performed we observed that the ablation of neudesin induces a striking anxious-like phenotype as revealed by a series of tests that specifically assess anxiety-like behavior. We first used the EPM to analyze anxiety-like behavior and found that the percentage of time neudesin-null mice spent in the open arms (22%) was significantly lower than that of controls (37%) (**Figure 1A**) and, conversely, more time was spent in the closed arms (67%) when compared to controls (50%) ($p < 0.05$). Of relevance, the number of entries in the closed arms ($Nenf^{+/+} = 10.8 \pm 1.5$ and $Nenf^{-/-} = 8.5 \pm 1.8$ s) did not significantly differ between the groups, indicating that the exploratory activity is preserved in neudesin-null mice. This anxious phenotype was further confirmed in other contextual behavioral paradigms, namely the LDB and the NSF. In the LDB test neudesin-null male mice spent more time in the dark zone (396.6 ± 27.5 s) when compared to controls (296.1 ± 25.0 s) ($p < 0.05$) and significantly less time in the light zone ($Nenf^{+/+} = 303.4 \pm 24.9$ vs. $Nenf^{-/-} = 202.8 \pm 27.5$ s) ($p < 0.05$) (**Figure 1B**); importantly there were no differences regarding the total distance travelled (data not shown). In the NSF test animals have an extra motivational cue—they are fastened for 24 h—so when they are placed in a novel environment, the latency to feed is a measurement of anxiety. In this test we observed that neudesin-null mice displayed increased latency time to eat in the OF arena (416.8 ± 63.6 s) when compared to controls (253.8 ± 35.9 s) ($p < 0.05$) (**Figure 1C**). The previously described tests are based on conflicts between the animal's innate

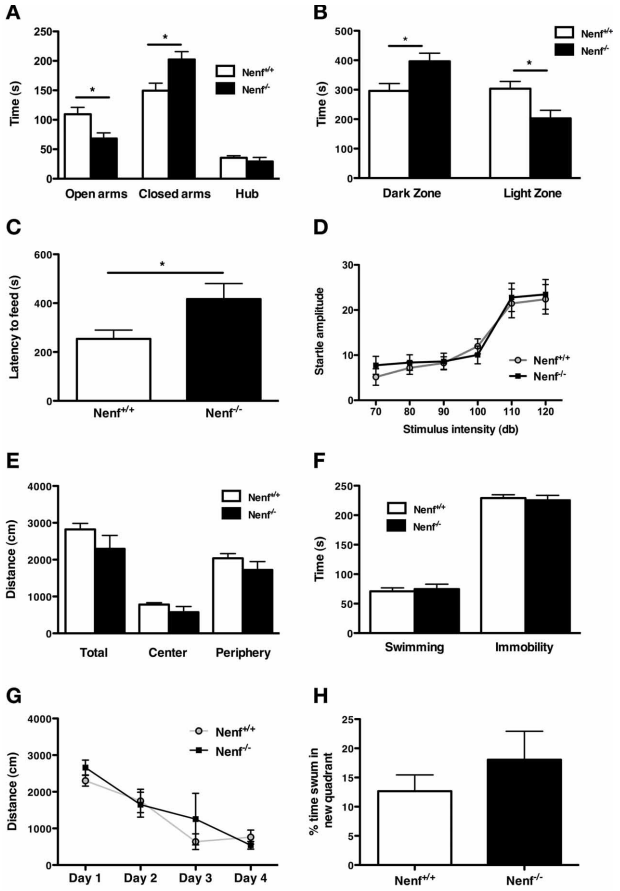


FIGURE 1 | Neudesin-null mice ($Nenf^{-/-}$) display an anxious-like behavior in contextual paradigms. (A) $Nenf^{-/-}$ mice spend significantly less time exploring the open arms of the EPM than control mice ($Nenf^{+/+}$). **(B)** The LDB test shows that $Nenf^{-/-}$ mice spend more time in the dark zone than in the light zone. **(C)** Also, in the NSF $Nenf^{-/-}$ mice take longer to feed in the novel environment when compared with $Nenf^{+/+}$. **(D)** The AS test shows that $Nenf^{-/-}$ mice have a similar startle response to higher intensities of sound not reflecting an anxious like behavior in this anxiety dimension. **(E)** In the OF no differences were observed between $Nenf^{-/-}$ and $Nenf^{+/+}$ in the total distance traveled in the arena. **(F)** Performance in the FST, which assessed learned helplessness behavior does not reveal differences between the two groups. **(G)** Cognition, as assessed using the MWM test, is identical between groups, as revealed by the time taken to learn the platform position. **(H)** The % of distance swum in the new quadrant in the reversal trials of the MWM test did not reveal any differences between groups. Data are presented as mean ± SEM. *$p < 0.05$. OA, open arms; CA, closed arms; H, hub; DZ, dark zone; LZ, light zone?

exploratory/feeding behavior and its aversion for open brightly lit spaces. These are often considered tests for state anxiety and are dependent on cortical processing. On the other hand, the AS test is based on a reflex response to aversive stimuli that tests for a different dimension of anxiety, a more generalized and innate response—trait anxiety. Therefore, a more anxious animal will respond with a bigger startle at higher intensities of sound. Interestingly, the AS test failed to show an anxious like behavior in neudesin-null mice (**Figure 1D**), even at the higher intensities of sound (120 dB) ($Nenf^{+/+} = 22.37 \pm 3.26$ vs. $Nenf^{-/-} = 23.45 \pm 3.29$ ms) (**Figure 1D**).

Regarding other behavioral traits, neudesin-null mice did not reveal any phenotype. The OF arena was used to assess locomotor activity. The analysis revealed that neudesin-null mice did not display motor impairments, when compared to control animals (**Figure 1E**). We used the FST to measure helplessness behavior, a behavioral dimension relevant for depression. In the FST (**Figure 1F**) both controls and neudesin-null animals spent a similar amount of time immobile ($Nenf^{+/+} = 229 \pm 5$ vs. $Nenf^{-/-} = 225 \pm 8$ s), thus indicating that neudesin-null mice do not display a depressive-like behavioral phenotype. Cognition, specifically spatial reference memory, was analyzed in the MWM. Both neudesin-null and control mice learned the position of the hidden platform as they similarly decreased the latency of time required to perform the task, indicating an absence of cognitive impairment (**Figure 1G**); this was further confirmed in the probe test (data not shown). Regarding performance in the reverse learning task of the MWM, the percentage of distance swum in the new quadrant by neudesin-null mice was similar to that of control mice (**Figure 1H**).

NEUDESIN-NULL ADULT MALE MICE HAVE ALTERED NEURONAL MORPHOLOGY

Neuronal morphology, dendrite formation and the establishment of synaptic contacts are dependent of trophic support. Due to the potential role of neudesin as a neurotrophic factor we studied the 3D-morphology of neurons in neudesin-null mice. In light of the anxiety-like phenotype described before, we focused the analysis in brain regions implicated in the modulation of this behavioral trait, namely the ventral hippocampus, the amygdala (BLa nuclei) and the BNST. When compared to control mice, neudesin-null mice presented shorter dendritic length of ventral hippocampal DG granular neurons ($Nenf^{+/+} = 741.8 \pm 32.8$ vs. $Nenf^{-/-} = 463.9 \pm 83.3\,\mu m$) ($p < 0.05$) (**Figure 2A** left panel). Sholl analysis also revealed fewer intersections of the dendritic tree (with the imaginary spheres) at distances between 60 and 120 μm from the soma in neudesin-null mice ($p < 0.001$) (**Figure 2A** right panel). Nevertheless, in ventral hippocampal CA1 pyramidal-like neurons no difference was found between control and neudesin-null mice in dendritic length or dendritic arborization, both for the basal and for the apical dendrites (**Figure 2B**). Noteworthy the differences found for ventral hippocampal DG neurons were not observed in the dorsal DG granular neurons ($Nenf^{+/+} = 644.7 \pm 29.3$ vs. $Nenf^{-/-} = 606.3 \pm 39.9\,\mu m$). Regarding the BNST, two different divisions were analyzed, the anterior medial BNST (amBNST) and the lateral dorsal BNST (ldBNST); neudesin-null mice had a statistically significant reduction in dendritic length in the anterior medial ($Nenf^{+/+} = 584.8 \pm 36.7$ vs. $Nenf^{-/-} = 412.6 \pm 14.7\,\mu m$) (**Figure 2C** left panel), but no differences in the lateral dorsal division ($Nenf^{+/+} = 628.7 \pm 64.5$ vs. $Nenf^{-/-} = 485.2 \pm 34.1\,\mu m$), as well as no differences regarding dendritic arborization of neurons in both BNST divisions. Finally, the morphology of pyramidal-like neurons of the BLa was analyzed and no differences were observed between control and neudesin-null mice (**Figure 2D**).

NEUROTRANSMITTER ACTIVITY PROFILE IN ANXIETY RELATED REGIONS

Given the behavioral and structural alterations found in neudesin-null mice we next characterized the monoaminergic profile (major findings presented **Table 1** for complete data) of several anxiety-related brain regions (amygdala, BNST, ventral hippocampus). We observed a reduction of DA levels in the ventral hippocampus of neudesin-null animals ($p < 0.05$) (**Table 1**), which was accompanied by an elevated dopaminergic turnover of HVA/DA ($p < 0.05$) (**Table 1**). Regarding the amygdala, there was a trend for a reduction in DA, DOPAC, and NE levels (**Table 1**); in the BNST no alterations were observed in monoamines levels or turnover (**Table 1**).

Regarding serotonin, a significant reduction of the serotonin metabolite 5HIAA ($p < 0.05$) (**Table 1**) was seen in the ventral hippocampus, but this difference did not translate into any alterations regarding serotonin turnover. An ∼40–50% reduction in the levels of both 5HT and 5HIAA in neudesin-null mice was also observed in the amygdala but these differences did not reach statistical significance for both metabolites. In the BNST no differences were observed for 5HT metabolites and respective turnover rates.

DISCUSSION

This work provides the first *in vivo* demonstration of the relevance of the neurotrophic factor neudesin for CNS normal function. In the absence of neudesin, mice display a contextual anxious like phenotype that is accompanied by impairment in the dopaminergic activity of the ventral hippocampus, where the dendritic arborization in granular neurons branching is impoverished; in addition, they also present a dendritic atrophy in the amBNST nucleus.

We first addressed the role of neudesin by exploring the behavioral consequences of its ablation. Of the several behavioral dimensions assessed, only anxiety-related phenotypes were clearly affected by the absence of neudesin, as demonstrated by the shorter time spent in the open arms of the EPM and in the light zone of the LDB and the higher latency to eat in the NSF test. Interestingly, no deficits were found in the AS, which reveals that the increased anxiety occurs specifically in contextual conflict paradigms, described to involve modulation by different neuronal circuits (Koch and Schnitzler, 1997). Anxiety in the presence of contextual anxiogenic environments, such as those observed here, is shown to be mediated by the BNST (Ventura-Silva et al., 2012) under the modulation of different cortical regions namely the hippocampus and the prefrontal cortex (Ventura-Silva et al., 2013). The BNST is closely involved in stress and in the HPA axis-dependent modulation of emotional behaviors (Herman and

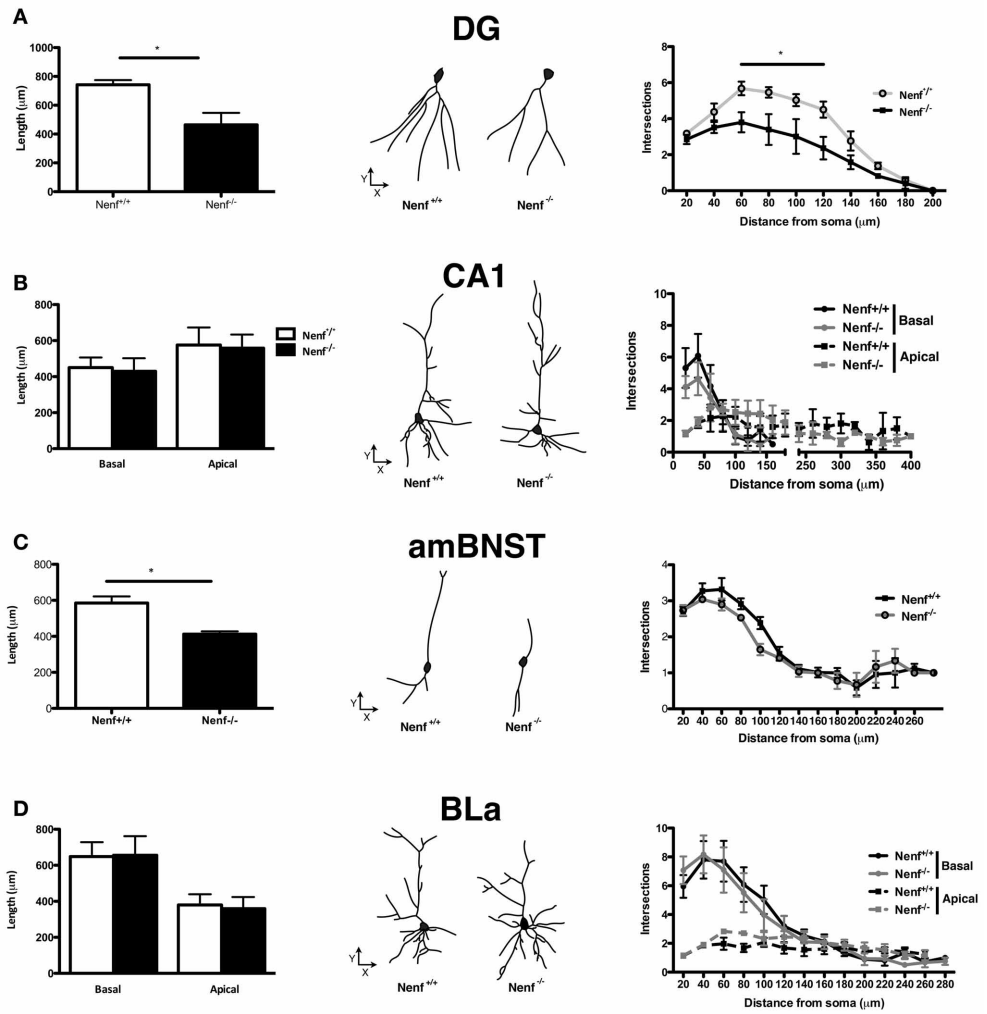


FIGURE 2 | Three-dimensional morphometric analysis of Golgi-Cox impregnated neurons of the ventral hippocampus, BNST and amygdala. Nenf$^{-/-}$ mice have shorter dendrites than controls in the dentate gyrus (DG) of the ventral hippocampus (**A**, left panel) as well as less intersections between dendrites (**A,** right panel). These differences in dendrite morphology and neuron arborization are not observed for the hippocampal CA1 region (**B**, left and right panel, respectively). Neudesin-null mice have shorter dendritic length in the anterior medial division of the BNST (amBNST) and no differences in the number of intersections in the sholl analysis (**C**, left and right panel, respectively). Analysis of the basolateral nucleus of the amygdala (BLa) revealed no differences concerning dendritic length or arborization (**D,** left and right panels, respectively). The middle panels correspond to a representative 3D reconstructed neuron from each region analyzed from both Nenf$^{+/+}$ (left) and Nenf$^{-/-}$ (right). Data are presented as mean ± SEM. $^{*}p < 0.05$.

Cullinan, 1997). It has been reported that anxiety triggered by different stress-inducing paradigms is related to hypertrophy of the amBNST neurons (Pego et al., 2008; Oliveira et al., 2012). Surprisingly, however, neudesin-null mice display shorter dendrites in the amBNST, which suggests this might be a stress-specific effect and that other factors beyond the pure structure of these neurons might underlie the behavioral changes displayed by neudesin-null mice.

Interestingly, the same holds true for the ventral hippocampus, where we also found dendritic atrophy, specifically in granule cells. The role of the ventral hippocampus in anxiety has been a matter of debate, with some studies showing that an increased activity in this brain region is related with increased emotionality (McHugh et al., 2004, 2011), while others studies do not confirm this association (Marrocco et al., 2012). Recently, however, the activity of ventral granule cells has been specifically implicated in anxiety behavior. Using optogenetics tools to activate and to inhibit DG granular cells in the ventral hippocampus, Fournier et al saw that elevating activity in this area suppresses anxiety in the EPM (Fournier and Duman, 2013). The present results seem to support the later study, in as much as we show that neudesin-null mice present an impoverished arborization in granule cells of the ventral hippocampus. Noteworthy, this later effect is specific to the ventral hippocampus since we found no differences between control and neudesin-null mice in the arborization of dorsal DG granular cells. Given the recently shown role of dorsal

Table 1 | Levels of monoamines in brain areas of littermate control (Nenf$^{+/+}$) and neudesin-null (Nenf$^{-/-}$) mice.

	Nenf$^{+/+}$	Nenf$^{-/-}$
VENTRAL HIPPOCAMPUS		
NE	1.49 ± 0.22	1.06 ± 0.31
DA	0.49 ± 0.10	0.27 ± 0.09*
DOPAC	0.43 ± 0.15	0.35 ± 0.15
HVA	0.32 ± 0.06	0.27 ± 0.10
DOPAC/DA	0.78 ± 0.13	1.17 ± 0.22
HVA/DA	0.61 ± 0.07	1.02 ± 0.08**
5HT	8.69 ± 1.51	5.21 ± 1.31
5HIAA	10.59 ± 2.40	6.28 ± 1.92*
5HIAA/5HT	0.94 ± 0.09	1.08 ± 0.09
BED NUCLEUS OF THE STRIA TERMINALIS		
NE	9.44 ± 1.49	13.58 ± 4.33
DA	6.77 ± 1.51	9.54 ± 3.85
DOPAC	8.32 ± 2.57	9.89 ± 3.95
HVA	5.38 ± 1.15	6.52 ± 1.85
DOPAC/DA	0.96 ± 0.10	1.33 ± 0.20
HVA/DA	0.72 ± 0.10	1.16 ± 0.21
5HT	13.12 ± 3.69	16.12 ± 4.88
5HIAA	6.72 ± 1.41	9.80 ± 2.35
5HIAA/5HT	0.60 ± 0.06	0.72 ± 0.07
AMYGDALA		
NE	2.50 ± 0.69	1.57 ± 0.41
DA	6.76 ± 1.56	3.00 ± 1.09
DOPAC	7.54 ± 2.40	3.16 ± 0.86
HVA	3.33 ± 1.04	1.38 ± 0.35
DOPAC/DA	0.99 ± 0.14	1.38 ± 0.45
HVA/DA	0.50 ± 0.07	0.56 ± 0.12
5HT	15.08 ± 3.31	6.50 ± 0.98
5HIAA	10.15 ± 2.81	5.97 ± 1.58
5HIAA/5HT	0.64 ± 0.07	0.91 ± 0.21

*Levels of catecholamines and monoamines are in μg/g tissue and expressed as means ± S.E.M. *$p < 0.05$ and **$p < 0.01$.*

DG cells in the modulation of learning (Kheirbek et al., 2013), this result is in accordance with the absence of a cognitive phenotype in neudesin-null mice. This specific alteration in the atrophy of DG granule cells along the dorsal-ventral axis in the absence of neudesin is of relevance and deserves further investigation.

Going further into the molecular levels, we next studied the monoaminergic profile of anxiety-related brain regions. Interestingly, the absence of neudesin is associated with low levels of DA and increased HVA/DA turnover ratio in the ventral hippocampus. Importantly, DA levels are described to be higher in the ventral than in the dorsal hippocampus (Eisenhofer et al., 2004) and DA is known to play a crucial role in modulating plasticity in the ventral portion of the hippocampus (Belujon and Grace, 2011). In addition, dopamine 1 receptor (D1) is highly expressed in the dendrites of the granular cells of the ventral DG (Mansour et al., 1992), and dopamine release from projecting ventral tegmental area neurons impacts on the modulation of synaptic plasticity and synapse strenght in this brain region (Hamilton et al., 2010). Whether decreased DA release in the ventral hippocampus, as observed in neudesin-null mice, contributes to the dendritic atrophy in ventral DG granular neurons is unknown. Nevertheless, the role of dopamine in anxiety is also endowed in controversy: while pioneer studies with systemic apomorphine (a D1/D2 agonist) administration were shown to decrease anxiety (Hjorth et al., 1986), more recent studies using specific administration of this drug in the ventral hippocampus increased anxiety in the EPM (Zarrindast et al., 2010). Regarding the levels of 5HT, it is of notice that the ventral hippocampus of neudesin-null mice displays a decrease (although not significant) of 40% in the levels of 5HT and a sharp reduction in the levels of its derived metabolite 5HIAA. This is of relevance since selective knock-down of auto 5HT-1A receptors in the raphe nuclei of mice results in a direct increase of anxiety levels in contextual paradigms, and in a concomitant decrease in the extracellular levels of 5HT in the ventral hippocampus (Richardson-Jones et al., 2011). Thus, the alteration in serotonergic metabolites observed in the ventral hippocampus may contribute to the anxiety state observed in neudesin-null mice, in accordance to previous studies in stressed animals (Dalla et al., 2008). Also interesting, although not significant, is the reduction in the 5HT levels in the amygdala of neudesin-null mice. This may be of relevance when considering the fundamental role of 5HT as a player in the crosstalk between amygdala and the ventral hippocampus in the modulation of anxiety behavior (Asan et al., 2013). Overall, these results further highlight a possible role for neudesin in the establishment and/or maintenance of hippocampal circuitry and in the modulation of contextual anxiety-behavior.

Both during embryonic and postnatal brain development several external signals including neurotrophic factors, neurotransmitters and hormones are involved in the genesis and maturation of new neurons and in their integration into functional circuitries (Abrous et al., 2005). One of the unique roles previously ascribed to neudesin, as shown *in vitro*, is its neurotrophic activity (Kimura et al., 2005, 2006). Thus, it is plausible that the herein described functional alterations in neuronal arborization result from the absence of neurotrophic support conveyed by neudesin. On the other hand, neudesin structure displays a heme and/or steroid-binding site (Kimura et al., 2005), which seems necessary for its function (Kimura et al., 2008). Free heme is a powerful oxidative stressor (Jeney et al., 2002; Craven et al., 2007; Abraham and Kappas, 2008) and under physiological conditions it is bound to extracellular heme binding proteins such as Nenf. Thus, we cannot discard the possibility that the deleterious effects of free heme due to the absence of neudesin might have implications in the maintenance of neurons as previously suggested (Burmester and Hankeln, 2004).

The data presented in this study suggest that neudesin modulates anxiety behavior mainly through the DG ventral hippocampus and altered dopaminergic activity. Thus, the neurotrophic action described previously for neudesin per ser, or in association with its ligands, might be of therapeutical and/or pharmacological potential in anxiety-related disorders. The modulatory role of neudesin in anxiety might be associated with its putative neurotrophic role but why this effect is specific for the anxiety circuits is still to be determined. Nevertheless, since the mouse model used in this study is a constitutive knock-out of the Nenf gene, we

cannot exclude potential developmental determinants (Stevens et al., 2010) resulting from the absence of neudesin in adult anxiety circuits, which should next be investigated.

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REFERENCES

Abraham, N. G., and Kappas, A. (2008). Pharmacological and clinical aspects of heme oxygenase. *Pharmacol. Rev.* 60, 79–127. doi: 10.1124/pr.107.07104

Abrous, D. N., Koehl, M., and Le Moal, M. (2005). Adult neurogenesis: from precursors to network and physiology. *Physiol. Rev.* 85, 523–569. doi: 10.1152/physrev.00055.2003

Asan, E., Steinke, M., and Lesch, K. P. (2013). Serotonergic innervation of the amygdala: targets, receptors, and implications for stress and anxiety. *Histochem. Cell Biol.* 139, 785–813. doi: 10.1007/s00418-013-1081-1

Belujon, P., and Grace, A. A. (2011). Hippocampus, amygdala, and stress: interacting systems that affect susceptibility to addiction. *Ann. N.Y. Acad. Sci.* 1216, 114–121. doi: 10.1111/j.1749-6632.2010.05896.x

Burmester, T., and Hankeln, T. (2004). Neuroglobin: a respiratory protein of the nervous system. *News Physiol. Sci.* 19, 110–113.

Craven, R. J., Mallory, J. C., and Hand, R. A. (2007). Regulation of iron homeostasis mediated by the heme-binding protein Dap1 (damage resistance protein 1) via the P450 protein Erg11/Cyp51. *J. Biol. Chem.* 282, 36543–36551. doi: 10.1074/jbc.M706770200

Dalla, C., Antoniou, K., Kokras, N., Drossopoulou, G., Papathanasiou, G., Bekris, S., et al. (2008). Sex differences in the effects of two stress paradigms on dopaminergic neurotransmission. *Physiol. Behav.* 93, 595–605. doi: 10.1016/j.physbeh.2007.10.020

Dong, H. W., Petrovich, G. D., and Swanson, L. W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res. Rev.* 38, 192–246. doi: 10.1016/S0165-0173(01)00079-0

Eisenhofer, G., Kopin, I. J., and Goldstein, D. S. (2004). Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol. Rev.* 56, 331–349. doi: 10.1124/pr.56.3.1

Fournier, N. M., and Duman, R. S. (2013). Illuminating hippocampal control of fear memory and anxiety. *Neuron* 77, 803–806. doi: 10.1016/j.neuron.2013.02.017

Gibbs, S. J., Bird, A. C., Owens, S., and Plowman, P. N. (1998). Radiotherapy for age-related macular degeneration. *Clin. Oncol. (R. Coll. Radiol.)* 10, 356–360. doi: 10.1016/S0936-6555(98)80029-0

Gray, J. D., Milner, T. A., and McEwen, B. S. (2013). Dynamic plasticity: the role of glucocorticoids, brain-derived neurotrophic factor and other trophic factors. *Neuroscience* 239, 214–227. doi: 10.1016/j.neuroscience.2012.08.034

Hamilton, T. J., Wheatley, B. M., Sinclair, D. B., Bachmann, M., Larkum, M. E., and Colmers, W. F. (2010). Dopamine modulates synaptic plasticity in dendrites of rat and human dentate granule cells. *Proc. Natl. Acad. Sci. U.S.A.*107, 18185–18190. doi: 10.1073/pnas.1011558107

Herman, J. P., and Cullinan, W. E. (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci.* 20, 78–84. doi: 10.1016/S0166-2236(96)10069-2

Hjorth, S., Engel, J. A., and Carlsson, A. (1986). Anticonflict effects of low doses of the dopamine agonist apomorphine in the rat. *Pharmacol. Biochem. Behav.* 24, 237–240. doi: 10.1016/0091-3057(86)90344-8

Jeney, V., Balla, J., Yachie, A., Varga, Z., Vercellotti, G. M., Eaton, J. W., et al. (2002). Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 100, 879–887. doi: 10.1182/blood.V100.3.879

Jung, S., Lee, Y., Kim, G., Son, H., Lee, D. H., Roh, G. S., et al. (2012). Decreased expression of extracellular matrix proteins and trophic factors in the amygdala complex of depressed mice after chronic immobilization stress. *BMC Neurosci.* 13:58. doi: 10.1186/1471-2202-13-58

Kheirbek, M. A., Drew, L. J., Burghardt, N. S., Costantini, D. O., Tannenholz, L., Ahmari, S. E., et al. (2013). Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. *Neuron* 77, 955–968. doi: 10.1016/j.neuron.2012.12.038

Kimura, I., Konishi, M., Miyake, A., Fujimoto, M., and Itoh, N. (2006). Neudesin, a secreted factor, promotes neural cell proliferation and neuronal differentiation in mouse neural precursor cells. *J. Neurosci. Res.* 83, 1415–1424. doi: 10.1002/jnr.20849

Kimura, I., Nakayama, Y., Konishi, M., Terasawa, K., Ohta, M., Itoh, N., et al. (2012). Functions of MAPR (membrane-associated progesterone receptor) family members as heme/steroid-binding proteins. *Curr. Protein Pept. Sci.* 13, 687–696. doi: 10.2174/138920371120907068 7

Kimura, I., Nakayama, Y., Yamauchi, H., Konishi, M., Miyake, A., Mori, M., et al. (2008). Neurotrophic activity of neudesin, a novel extracellular heme-binding protein, is dependent on the binding of heme to its cytochrome b5-like heme/steroid-binding domain. *J. Biol. Chem.* 283, 4323–4331. doi: 10.1074/jbc.M706679200

Kimura, I., Yoshioka, M., Konishi, M., Miyake, A., and Itoh, N. (2005). Neudesin, a novel secreted protein with a unique primary structure and neurotrophic activity. *J. Neurosci. Res.* 79, 287–294. doi: 10.1002/jnr.20356

Koch, M., and Schnitzler, H. U. (1997). The acoustic startle response in rats–circuits mediating evocation, inhibition and potentiation. *Behav. Brain Res.* 89, 35–49. doi: 10.1016/S0166-4328(97)02296-1

Kokras, N., Antoniou, K., Dalla, C., Bekris, S., Xagoraris, M., Ovestreet, D. H., et al. (2009). Sex-related differential response to clomipramine treatment in a rat model of depression. *J. Psychopharmacol. (Oxford)* 23, 945–956. doi: 10.1177/0269881108095914

Mansour, A., Meador-Woodruff, J. H., Zhou, Q., Civelli, O., Akil, H., and Watson, S. J. (1992). A comparison of D1 receptor binding and mRNA in rat brain using receptor autoradiographic and *in situ* hybridization techniques. *Neuroscience* 46, 959–971. doi: 10.1016/0306-4522(92)90197-A

Marrocco, J., Mairesse, J., Ngomba, R. T., Silletti, V., Van Camp, G., Bouwalerh, H., et al. (2012). Anxiety-like behavior of prenatally stressed rats is associated with a selective reduction of glutamate release in the ventral hippocampus. *J. Neurosci.* 32, 17143–17154. doi: 10.1523/JNEUROSCI.1040-12.2012

Masi, G., and Brovedani, P. (2011). The hippocampus, neurotrophic factors and depression: possible implications for the pharmacotherapy of depression. *CNS Drugs* 25, 913–931. doi: 10.2165/11595900-000000000-00000

McAllister, A. K. (2001). Neurotrophins and neuronal differentiation in the central nervous system. *Cell. Mol. Life Sci.* 58, 1054–1060. doi: 10.1007/PL00000920

McHugh, S. B., Deacon, R. M., Rawlins, J. N., and Bannerman, D. M. (2004). Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav. Neurosci.* 118, 63–78. doi: 10.1037/0735-7044.118.1.63

McHugh, S. B., Fillenz, M., Lowry, J. P., Rawlins, J. N., and Bannerman, D. M. (2011). Brain tissue oxygen amperometry in behaving rats demonstrates functional dissociation of dorsal and ventral hippocampus during spatial processing and anxiety. *Eur. J. Neurosci.* 33, 322–337. doi: 10.1111/j.1460-9568.2010.07497.x

Mikail, H. G., Dalla, C., Kokras, N., Kafetzopoulos, V., and Papadopoulou-Daifoti, Z. (2012). Sertraline behavioral response associates closer and dose-dependently with cortical rather than hippocampal serotonergic activity in the rat forced swim stress. *Physiol. Behav.* 107, 201–206. doi: 10.1016/j.physbeh.2012.06.016

Oliveira, M., Rodrigues, A. J., Leao, P., Cardona, D., Pego, J. M., and Sousa, N. (2012). The bed nucleus of stria terminalis and the amygdala as targets of antenatal glucocorticoids: implications for fear and anxiety responses. *Psychopharmacology (Berl)* 220, 443–453. doi: 10.1007/s00213-011-2494-y

Ottoni, E. B. (2000). EthoLog 2.2: a tool for the transcription and timing of behavior observation sessions. *Behav. Res. Methods. Instrum. Comput.* 32, 446–449. doi: 10.3758/BF03200814

Paxinos, G. and Franklin, K. B. (2001). *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.

Pego, J. M., Morgado, P., Pinto, L. G., Cerqueira, J. J., Almeida, O. F. X., and Sousa, N. (2008). Dissociation of the morphological correlates of stress-induced anxiety and fear. *Eur. J. Neurosci.* 27, 1503–1516. doi: 10.1111/j.1460-9568.2008.06112.x

Porsolt, R. D., Bertin, A., and Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther.* 229, 327–336.

Ren-Patterson, R. F., Cochran, L. W., Holmes, A., Sherrill, S., Huang, S. J., Tolliver, T., et al. (2005). Loss of brain-derived neurotrophic factor gene allele exacerbates brain monoamine deficiencies and increases stress abnormalities of serotonin transporter knockout mice. *J. Neurosci. Res.* 79, 756–771. doi: 10.1002/jnr.20410

Richardson-Jones, J. W., Craige, C. P., Nguyen, T. H., Kung, H. F., Gardier, A. M., Dranovsky, A., et al. (2011). Serotonin-1A autoreceptors are necessary and sufficient for the normal formation of circuits underlying innate anxiety. *J. Neurosci.* 31, 6008–6018. doi: 10.1523/JNEUROSCI.5836-10.2011

Russell, D. S., and Duman, R. S. (2002). "Neurotrophic factors and intracellurar signal transduction pathways," in *Neuropsychopharmacology: The Fifth Generation of Progress.* eds K. L. Davis, D. Charney, J. T. Coyle, and C. Nemeroff (Philadelphia, PA: American College of Neuropsychopharmacology), 207–215.

Sholl, D. A. (1956). The measurable parameters of the cerebral cortex and their significance in its organization. *Prog. Neurobiol.* 324–333.

Snider, W. D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* 77, 627–638. doi: 10.1016/0092-8674(94)90048-5

Stevens, H. E., Smith, K. M., Rash, B. G., and Vaccarino, F. M. (2010). Neural stem cell regulation, fibroblast growth factors, and the developmental origins of neuropsychiatric disorders. *Front. Neurosci.* 4, 1–14. doi: 10.3389/fnins.2010.00059

Taylor, S. L., Stanek, L. M., Ressler, K. J., and Huhman, K. L. (2011). Differential brain-derived neurotrophic factor expression in limbic brain regions following social defeat or territorial aggression. *Behav. Neurosci.* 125, 911–920. doi: 10.1037/a0026172

Turner, C. A., Watson, S. J., and Akil, H. (2012). The fibroblast growth factor family: neuromodulation of affective behavior. *Neuron* 76, 160–174. doi: 10.1016/j.neuron.2012.08.037

Uylings, H. B., Ruiz-Marcos, A., and Van Pelt, J. (1986). The metric analysis of three-dimensional dendritic tree patterns: a methodological review. *J. Neurosci. Methods* 18, 127–151. doi: 10.1016/0165-0270(86)90116-0

Ventura-Silva, A. P., Melo, A., Ferreira, A. C., Carvalho, M. M., Campos, F. L., Sousa, N., et al. (2013). Excitotoxic lesions in the central nucleus of the amygdala attenuate stress-induced anxiety behavior. *Front. Behav. Neurosci.* 7:32. doi: 10.3389/fnbeh.2013.00032

Ventura-Silva, A. P., Pego, J. M., Sousa, J. C., Marques, A. R., Rodrigues, A. J., Marques, F., et al. (2012). Stress shifts the response of the bed nucleus of the stria terminalis to an anxiogenic mode. *Eur. J. Neurosci.* 36, 3396–3406. doi: 10.1111/j.1460-9568.2012.08262.x

Zarrindast, M. R., Naghdi-Sedeh, N., Nasehi, M., Sahraei, H., Bahrami, F., and Asadi, F. (2010). The effects of dopaminergic drugs in the ventral hippocampus of rats in the nicotine-induced anxiogenic-like response. *Neurosci. Lett.* 475, 156–160. doi: 10.1016/j.neulet.2010.03.069

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4. Neurodevelopmental impairment as a consequence of neudesin ablation

(Manuscript to be submitted)

Neurodevelopmental impairment as a consequence of neudesin ablation.

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Abstract

Neudesin (NENF), a neuron-secreted protein, with a distinctive cytochrome b5-like heme/steroid-binding domain, that belongs to the membrane-associated progesterone receptor family, displays neurotrophic proprieties. Nevertheless, information regarding neudesin function is scarce. *In vitro* evidences show that nenf modulates proliferation and differentiation of mice cortical neurons in specific time windows. *Nenf* expression and neurotrophic activity appears to be restricted to neurons. In fact, Nenf mRNA is described to be more abundant in embryo brain and spinal cord, but also in postnatal organs, including brain. The presence of NENF on mouse embryonic brain suggests a role for nenf during development. In this study we aimed at exploring if the absence of Nenf impairs brain physiology and function, namely in behavior. We performed developmental milestones of neudesin-null mice to evaluate the correct acquisition of age specific neurological functions. The early postnatal neurological reflexes have specific time windows of maturation. A delay in the correct acquisition of these developmental hallmarks, although transient, can reveal impairments in more mature functions. This broad characterization was performed during the first 21 postnatal days.

We observed sex differences between neudesin-null and control animals, namely a delay in the acquisition of 3 of the 5 vestibular dependent tests. These alterations indicate altered vestibular system function, the area responsible for providing the correct information about spatial and movement position of the body and one of the brain regions that mature in first 3 weeks of postnatal life. Furthermore, we tackled these behavior differences with a monoaminergic profile of related brain regions, which revealed that the brain stem of neudesin-null males present increased serotonin and decreased serotonergic activity this was also observed in the cerebellum. It was also observed an increase in the levels of norepinephrine in the brain stem while it was decreased in the motor cortex. Regarding dopaminergic activity, it is decreased in the vestibular area and the cerebellum. These delays and the neurochemical alterations observed can have implications in adult behavior dimensions, contributing to unravel the function of neudesin in the nervous system.

Introduction

Neurotrophic factors are important for nervous cells function and homeostasis from embryonic stages through adulthood (Park and Poo, 2013). In fact, several *in vivo* studies using knockout and transgenic mice for specific neurotrophic factors and/or the respective receptors have contributed to demonstrate the importance of this protein family during diverse brain developmental stages. For instance, the absence of particular neurotrophic factors, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), yields mild to severe impairment in the development of the CNS (Snider, 1994;Tessarollo, 1998;Binder and Scharfman, 2004;Binder, 2007;Iwata and Hevner, 2009).

Similarly to several neurotrophic factors, neudesin (also known as neuron derived neurotrophic factor, NENF) was shown to be a secreted protein that displays trophic actions in neuronal cells (Kimura et al., 2005). Namely, using different *in vitro* approaches, it was shown that NENF is able to potentiate the proliferation of primary cell cultures of mouse embryonic cortex in a dose dependent manner, and differentiate them only into neurons and not astrocytes, throughout the involvement of MAP kinase, PI-3 kinase and PKA pathways (Kimura et al., 2006). Furthermore, NENF promoted mitogenic activity in primary cultures of neurons but not in astrocytes; this activity was shown to occur via the activation of MAP kinase and PI-3 Kinase pathways and inhibited by the inhibition of Gi/Go-protein-coupled receptor. Of notice, NENF displays a cytochrome b5-like heme/steroid-binding domain in its primary structure and it was shown *in vitro* that heme-binding was relevant for neudesin neurotrophic actions (Kimura et al., 2008). While in adult mice (PND49), *Nenf* is expressed in other tissues such as the heart, lungs, kidney and brain, during embryonic development (E18.5) it is highly expressed specifically in the brain and spinal cord (Kimura et al., 2005). During embryonic development, *Nenf* expression increases from E12.5 until E18.5 in the mouse cerebral cortex, and the main region expressing mouse *Nenf* at E13.5 stage is the preplate, where most post mitotic neurons exist (Kimura et al., 2006). In adulthood we have shown that *Nenf* is involved in the modulation of anxiety circuitries. Specifically, in the absence of neudesin, mice display an anxious-like phenotype exclusively in novel contextual paradigms of anxiety. This behavioral impairment was correlated with an imbalance of the monoaminergic system in brain regions involved in the control of emotional responses. Furthermore, NENF was shown to be relevant for the proper arborization of ventral hippocampus and bed nuclei of the stria terminalis (BNST) neurons (Novais et al., 2013).

Although several lines of evidence support the potential relevance of neudesin for the nervous system development this was still not demonstrated. This is of relevance since abnormal development of neural circuitries makes individuals prone to neuropsychiatric disorders (Cicchetti and Walker, 2003). In order to evaluate the postnatal development of neudesin-null mice, we used a set of specific behavioral paradigms to analyze the neurological early postnatal development of mice (Fox, 1965;Santos et al., 2007;Lim et al., 2008;Castelhano-Carlos et al., 2010). Furthermore, we have analyzed the neurochemical profile of the monoaminergic system in brain regions involved in the maturation of neurological postnatal milestones.

Methods

Animals

A mouse strain with targeted deletion of the neudesin gene, provided by Merck Serono under a material transfer agreement, was used in all experiments. Briefly, the neudesin-null mouse strain was generated by using a 129/SvEv genomic library from a BAC clone and the targeting construct was made by deleting the entire coding sequence (exons 1 to 4, approximately 12KB) and replacing it with a LacZ-neomicin cassette. The target DNA was then inserted into embryonic stem cells (FiH4 ES cells), where the homologous pieces of DNA recombined. The cells identified as homologous recombinant clones were microinjected into C57BL/6F1 blastocysts to generate chimeric mouse and then backcrossed to a C57BL6 background.

Animals were maintained under 12h light/dark cycle at 22±1ºC, 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. The Portuguese national authority for animal experimentation - Direcção Geral de Veterinária - approved the study (permission ID: DGV9457). All experiments were performed in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council. Neudesin null mice ($Nenf^{-/-}$) and their respective littermate controls ($Nenf^{+/+}$) were obtained by mating $Nenf^{+/-}$ females with $Nenf^{+/-}$ males. Mice genotype was confirmed by PCR using two sets of primers. One for the lacZ cassette: lacZ-Forward 5'- GGTAAACTGGCTCGGATTAGGG -3', lacZ-Reverse 5'- TTGACTGTAGCGGCTGATGTTG -3' and another for the nenf gene, nenf_intron 3 5'-CTTGGAGTTTGGGGCTGATA -3', nenf-Exon 4 5'- TGGCTTTGTACACCTTGCTG -3'. The amplified fragments were of 210bp and 176bp, respectively, and identified by electrophoresis through a 1.5% agarose gel.

Developmental milestones

Developmental milestones were performed in 26 males and 26 females, 16 $Nenf^{-/-}$ (8 females and 8 males) and 36 $Nenf^{+/+}$ (18 females and 18 males) from nine different litters. Neonatal neurodevelopmental behavior was evaluated by using an adapted protocol that was previously validated in mice (Fox, 1965;Santos et al., 2007;Lim et al., 2008;Castelhano-Carlos et al., 2010). Briefly, after birth animals were daily observed and scored for the acquisition of maturity in somatic parameters, reflexes, strength, coordination and spatial reference tests during the first 21 postnatal days. The researcher performing the assay was blind for animal's genotype during the protocol. Specifically, the following parameters were evaluated.

Somatic parameters

Newborn animals were examined daily for correct maternal care and well being, skin appearance, activity and presence of milk in the stomach (milk spot) to confirm feeding. Animals were weighted and anogenital distance (ANGD) was measured daily. From PND1 onwards animals were observed for eye opening, defined as the initial break in the membrane sealing the lids of both eyes, fur appearance and incisor eruption.

Neurological reflexes

The evaluation of neurologic reflexes was performed daily from PND 1 to PND 21 at the same circadian rhythm hour (2-4 pm). The cages were taken from the housing room and the pups were left in the same room as the mother during the tests and returned to the mother's cage immediately after the tests ended. A list and brief description of the tests performed follows.

Cliff aversion: From PND1 to 14. Tests for spatial reflex and coordination. Animals were placed on the edge of a cliff (box) with forepaws and face over the edge. Mature response was observed when the animals turn and crawl away from the cliff.

Negative geotaxis: From PND1 to 14. Tests for spatial, body righting and labyrinthine mechanisms. Animals were placed face down on a 45^{o} grid. Mature response was observed when animals turn 180^{o} up and walk to the top of the grid.

Surface righting: From PND1 to 13. Tests for spatial, body righting and labyrinthine mechanisms. Animals were placed in their back in the table. Mature response was observed when the animals turns to normal position in 4 paws.

Wire suspension: From PND5 to 14. Tests for strength. Animals were placed vertically to hold a 2 mm metal bar with forelimbs. Mature response was observed when animals could hold body weight alone without falling.

Walking: From PND5 to 21. Tests for locomotion and coordination. Progression of animals pivoting to crawling and finally walking was analyzed. Mature response was observed when animals walk straight forward with 4 paws.

Grasping: From PND4 to 18. Tests for strength. Animals forelimb was stroked with a blunt instrument. Mature response was observed when animals flex paws and grasp instrument.

Postural reflex: From PND5 to 21. Tests for spatial and body righting mechanisms. The animal s were placed inside a box and gently shaken up and down, left and right. Mature response was observed when animals maintain straight posture by opening the base between limbs.

Auditory startle: From PND7 to 18. Tests for auditory reflex. Startle response observed after a handclap.

Ear twitch: From PND7 to 15. Tests for tactile and auditory reflex. With the end of a cotton tip, animals ear was scrubbed. Mature response was observed when animals flat the ear against the side of the head.

Air righting: From PND8 to 21. Tests for spatial and body righting mechanisms. Animals was held upside down at a 13cm distance from the bedding and dropped. Mature response was observed when animals lands on four paws.

Developmental milestones were evaluated according to a score for each test. Tests that evaluated presence/no presence were scored as 1/0 (rooting, wire suspension, postural reflex, eye opening, ear twitch, auditory startle, air righting). The tests where a gradual evolution was observable were scored from 0 to 3 (cliff aversion, negative geotaxis, surface righting, walking, grasping), with 0 being not present and 3 being mature response.

Neurochemical analysis

Monoamines' levels were measured using high performance liquid chromatography with electrochemical detection (HPLC-ED). Male mice after developmental milestones protocol at PND21 were killed by decapitation ($Nenf^{-/-}$=8 and $Nenf^{+/+}$=10). Skulls were snap frozen in liquid nitrogen to avoid degradation during macrodissection. Brains were carefully dissected for cerebellum, brainstem, vestibular area and motor cortex on ice and with the help of a stereomicroscope (SZX7; Olympus).

Dissected tissues were weighed and then homogenized and deproteinized in 100 µL of 0.2 N perchloric acid solution (Applichem, Darmstadt, Germany) containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA (Riedel-de Haën AG, Seelze, Germany), centrifuged at 20000 g for 45 min at 4°C

and the supernatant was stored at - 80°C until analysis.

The analysis was performed using a GBC LC1150 HPLC pump (GBC Scientific Equipment, Braeside, Victoria, Australia) coupled with a BAS-LC4C (Bioanalytical Systems Inc., USA) electrochemical detector, as previously described (Kokras et al., 2009). The working electrode of the electrochemical detector was set at +800 mV. In all samples reverse phase ion pairing chromatography was used to assay dopamine (DA) and its metabolites 3,4 dihydroxyphenylacetate (DOPAC) and homovanillic acid (HVA), serotonin (5HT) and its metabolite 5-hydroxyindoleatic (5HIAA), and norepinephrine (NE). The mobile phase consisted of a 50 mM phosphate buffer regulated at pH 3.0, containing 5-octylsulfate sodium salt at a concentration of 300 mg/L as the ion pairing reagent and Na_2EDTA at a concentration of 20 mg/L (Riedel-de Haën AG); acetonitrile (Merck, Darmstadt, Germany) was added at a 9% concentration. The reference standards were prepared in 0.2 N perchloric acid solution containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na_2EDTA. The column used was an Aquasil C18 HPLC Column, 100 mm X 1 mm, 5 μm Particle Size (Thermo Electron, UK). Samples were quantified by comparison of the area under the curve (AUC) against known external reference standards using a PC compatible HPLC software package (Chromatography Station for Windows ver.17 Data Apex Ltd). The limit of detection was 1 pg/20 μL (of injection volume). In addition to the assay of 5-HT and 5-HIAA tissue levels, the 5-HT turnover rate was also calculated, separately for each chromatograph as the ratio of 5-HIAA/5-HT. Similarly, the ratios of DOPAC/DA and HVA/DA were calculated as an index of DA turnover rates. Turnover rates estimate the serotonergic and dopaminergic activities better than individual neurotransmitter and metabolite tissue levels as they reflect 5-HT and DA release and/or metabolic activity as described elsewhere (Dalla et al., 2008;Kokras et al., 2009;Mikail et al., 2012).

Statistical analysis

Values presented in table 1 are expressed as mean±standard deviation and significance was verified by using the Mann-Whitney test for independent samples. Differences were considered significant when $p<0.05$. Monoamines quantification are expressed as mean±standart error of the mean (SEM) and significance was verified by the Mann-Whitney test for independent samples. The day in which each animal achieves maturity for each test has been normalized to z-scores; z-normalization is a methodology that standardizes observations obtained at different times. It indicates how many standard deviations and animal performance is above or below the

mean. We used this normalization factor since we have used animals from 9 different litters (always with the same observer). By calculating z-scores for each individual vestibular and strength test, we then calculated the average of each z-score values across tests, which lessens potential biases induced by a single test. Vestibular score was calculated from the average scores of the cliff aversion, surface righting, air righting, postural reflex and negative geotaxis tests; the strength score was calculated from the scores on the walking, grasping and wire suspension tests.

Results

Neudesin-null animals somatic development

All newborn mice of both genders and genotypes were observed daily, for the first 21 days of early life. Somatic parameters, such as body temperature, milk spot and anogenital distance, were observed and no differences were found between mouse genotypes, suggesting a correct maternal care and well being of all litters analyzed.

Neudesin-null males and females gain body weight in a healthy manner (figure 1A and B) but a significant difference between neudesin-null males and their respective control littermates was found from PND19 to PND21 ($p<0,05$) (figure 1A). Nonetheless, this weight difference is transitory since in adulthood it is not present (data not shown). ANGD was measured as a parameter of animal growth. ANGD did not differ between sex and genotype demonstrating that no abnormalities concerning general growth were observed (figure 1C and 1D). Regarding eye opening, besides previously described inherent differences between males and females (Cancedda et al., 2004), no significant differences were observed between genotypes, although neudesin-null females when compared to littermate controls show a slighter but non-significant delay (figures 1E and F).

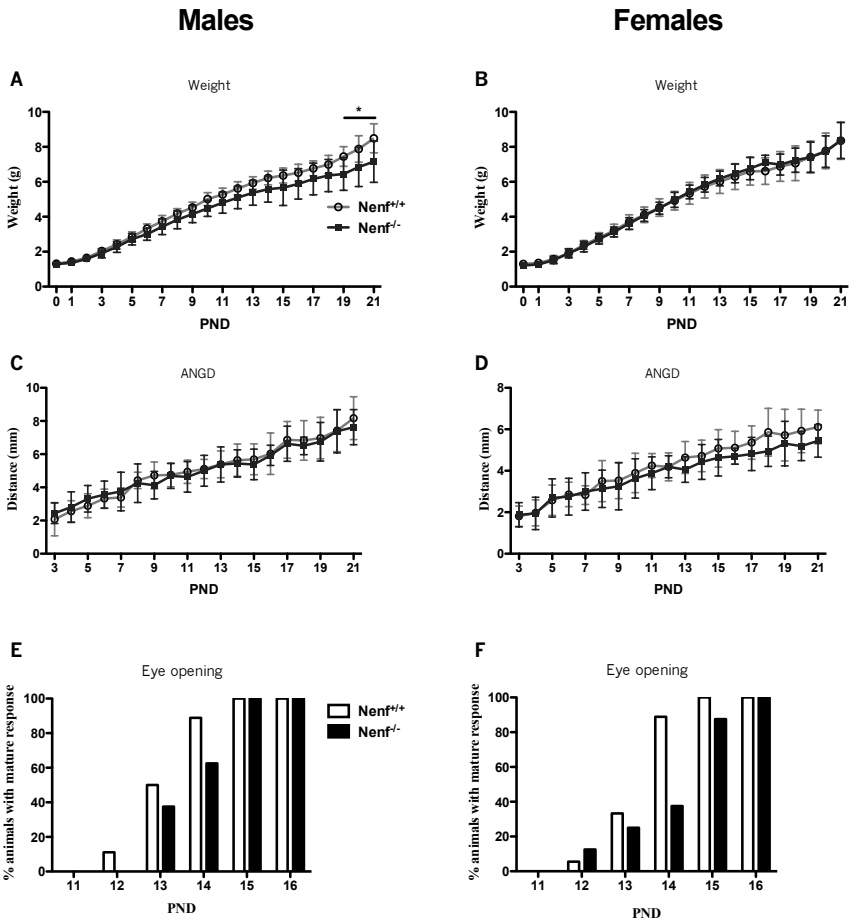


Figure 1 - Evaluation of somatic parameters during the developmental milestones protocol in neudesin-null mice (Nenf$^{-/-}$) and littermate controls (Nenf$^{+/+}$). A diminished body weight in the days previous to weaning was observed in neudesin-null male mice (**A**) but not in females (**B**). The anogenital distance (ANGD) was also measured during the 21 days of protocol in males (**C**) and females (**D**) with no differences being observed between genotypes. The percentage of animals that open both eyes per postnatal day in males (**E**) and females (**F**) does not significantly differ between Nenf$^{-/-}$ and Nenf$^{+/+}$ mice. Values are means±SEM except for **E** and **F**. * $p<0.05$.

Neudesin-null males have a mild developmental neurologic delay

Sensory-motor reflexes, locomotion, coordination and strength milestones were analyzed and compared between neudesin-null mice and control littermates. The neurologic developmental milestones tests performed fit 3 categories: vestibular area dependent (cliff aversion, surface righting, air righting, postural reflex and negative geotaxis); strength and motor dependent (walking, grasping and wire suspension) and auditory dependent (auditory startle and ear twitch). Mean days of mature response for all performed milestones are summarized in table 1. All animals reached mature scores for all tests performed during the period analyzed (0-3 weeks).

Regarding vestibular area dependent tests, we observed that neudesin-null males displayed an overall delay in the acquisition of maturity when compared to control littermates, but the same was not observed in females (figure 2). Nevertheless, in some tests, namely the cliff aversion test (figure 2A and 2B) and the surface righting test (figures 2C and 2D), the percentage of neudesin-null mice that reach maturity earlier than their control littermates was slightly higher. Nevertheless, these differences between genotypes did not reflect a significant delay in the acquisition of these reflexes when the average day of maturity is calculated for both genders (table1). On the other hand, the remaining vestibular area dependent tests, showed that neudesin-null mice have a marked delay in the acquisition of these reflexes as evaluated by both the percentage of animals reaching maturity in a given day of test and the average day of maturity, but, interestingly only in males (figure 2E, 2G, 2I and table 1). In fact, while in the air righting test, only males showed a significant delay ($Nenf^{+/+}$=PND11 vs $Nenf^{-/-}$= PND13) ($p<0.05$) (table1, figure 2E), the same was not observed in neudesin-null females in the acquisition of this reflex (table1, figure 2F). Similarly, in the postural reflex, neudesin-null males achieved maturity on average only at PND9 while control littermates reached maturity earlier at PND6 ($p<0,001$) (table1 and figure 2G); in the case of the postural reflex, a comparable delay ($Nenf^{+/+}$=PND7 vs $Nenf^{-/-}$= PND9) was observed in females (figure 2H) but it did not reach significance (table 1). In the negative geotaxis test, neudesin-null males showed a significant delay when compared to controls ($Nenf^{+/+}$=PND7 vs $Nenf^{-/-}$=PND9) ($p<0.05$), but this difference was not observed in females (table1 and figures 2I and 2J). Overall neudesin-null male mice achieved maturity with a significant delay in 3 of the 5 developmental milestones evaluated, while neudesin-null females did not show any significant delay in the same tests. Regarding motor tests, namely the walking test, no difference between genotypes was observed in the first time animals walked with four paws without dragging the belly. In the grasping test, both genotypes and genders reached maturity approximately at PND13 (table1). In the wire suspension test, that tests forelimb paw strength, both neudesin-null males and females achieved maturity, but not significantly, earlier than littermate controls (table1). Besides tests that evaluate vestibular and motor function, we tested other dimensions of postnatal neurodevelopment. In the auditory startle response, that reflects the opening of the ear canal, only neudesin-null male mice showed a mild delay achieving maturity, with at PND15 100% of controls having the ear canal open comparing to only 75% of neudesin-null mice; maturity in all (100%) neudesin-null male mice was only achieved at PND18; however this difference is not significant (table1). In females this difference was not

present and neudesin-null females even achieve maturity slightly earlier than controls (table1). In the ear twitch, neudesin-null mice revealed a minor delay in the acquisition of this tactile reflex (not significant) in both males and females (table1). Overall, the ablation of NENF had an impact in the timely acquisition of development milestones in males, but not in females.

Table 1 – Summary of developmental milestones average maturation day and body weight (at PND21) for males and females neudesin-null ($Nenf^{-/-}$) and littermate control ($Nenf^{+/+}$) mice.

	Males		**Females**	
Parameters	**$Nenf^{+/+}$**	**$Nenf^{-/-}$**	**$Nenf^{+/+}$**	**$Nenf^{-/-}$**
Weigth (g)	8,4±0,8	**7,2±1,1***	8,3±1,0	8,3±1,0
Eye Opening	13,5±0,8	13,9±1,1	13,7±0,7	14,1±1,1
Cliff Aversion	9,3±1,8	8,4±1,5	9,4±3,2	7,1±2,6
Surface righting	7,3±1,8	7,0±1,6	7,0±2,1	5,4±0,8
Air righting	11,3±2,0	**13,5±2,0***	12,7±2,0	12,1±2,1
Postural Reflex	5,8±1,1	**9,5±1,9****	7,2±1,5	8,6±1,3
Negative Geotaxis	6,9±1,2	**9,0±0,8***	8,4±1,6	8,4±2,0
Auditory Startle	13,8±1,2	15±2,1	14,4±1,3	13,2±2,0
Ear twitch	10,9±1,1	12,1±0,6	11,4±0,8	12,3±1,7
Grasping	13,9±2,2	12,8±2,3	12,5±1,7	13,2±1,1
Wire suspension	9,4±1,3	9,0±2,2	8,9±1,2	7,0±1,2
Walking	9,7±1,4	9,6±1,5	9,5±1,9	10,2±1,9

Mean ± SD; *p<0.05; **p<0.001

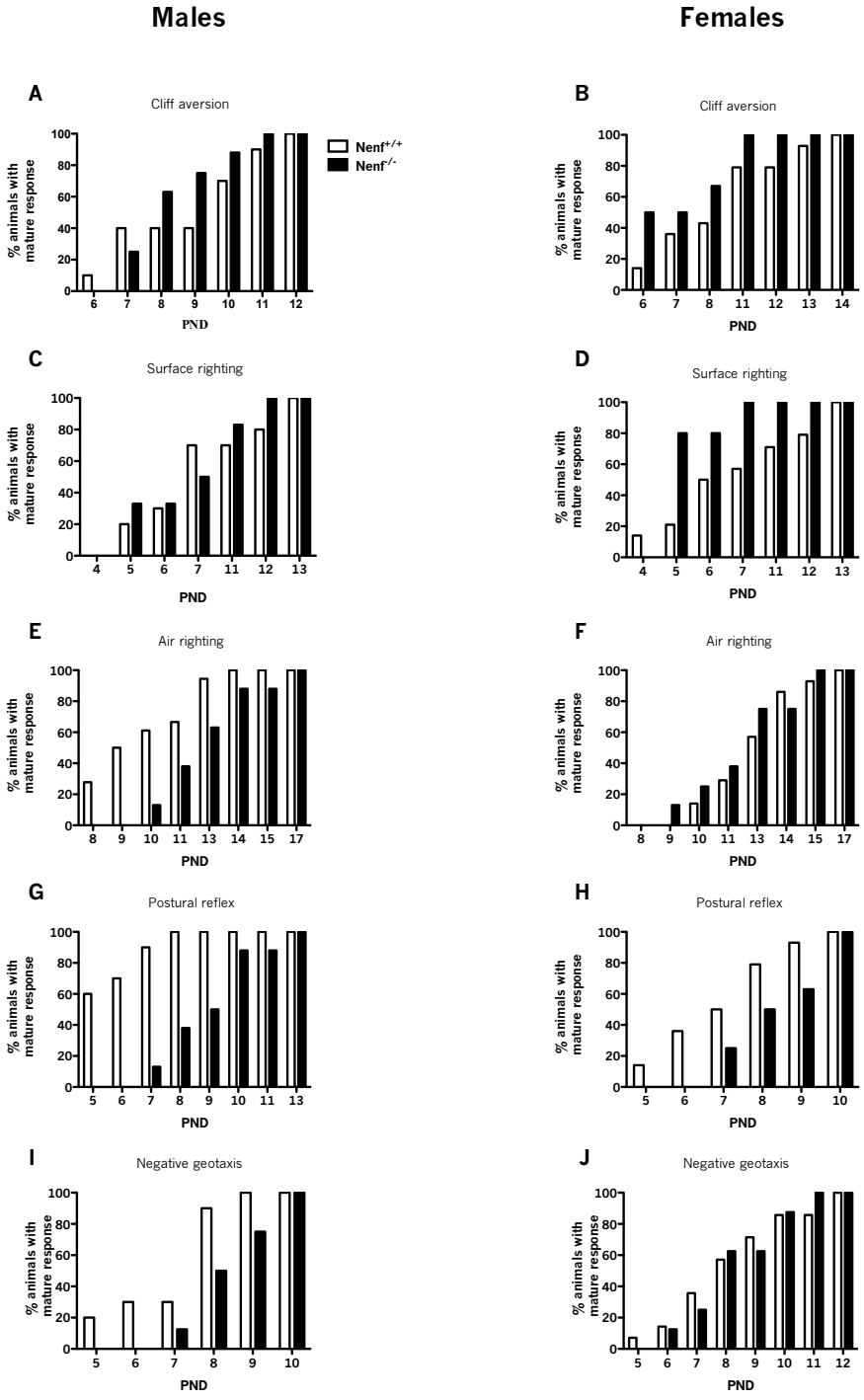


Figure 2 - Neudesin ablation resulted in a delay in the acquisition of neurologic reflexes during postnatal days only in males. No major differences in the acquisition of maturity were observed between littermate controls and neudesin-null mice in the cliff aversion (**A, B**) and the surface righting tests (**C, D**). On the other hand, neudesin-null male mice reach maturity later than littermate controls in the air righting, postural reflex and negative geotaxis tests (**E, G, I**); the same was not observed in females (**F, H, J)**.

Mouse behavior is multimodal, quantifying it is only possible when the same animal is exposed to multiple behavioral tests covering a wide range of behaviors over several days (Crawley and Paylor, 1997). We performed a z-normalization (Guilloux et al., 2011) that allowed us to group tests of the same behavioral dimension using z-scores. Grouping all vestibular dependent reflexes tested in a single vestibular score showed a significant difference between neudesin-null male mice and respective controls ($p<0.05$); neudesin-null mice had higher vestibular z-score ($Nenf^{-/-}$= 0,39±0,13 vs $Nenf^{+/+}$=-0,15±0,09) (fig.3A) which reflects that in the absence of NENF animals reach vestibular maturity later. This difference was not observed between genotype when strength related tests were evaluated together using the z-score (figure 3B).

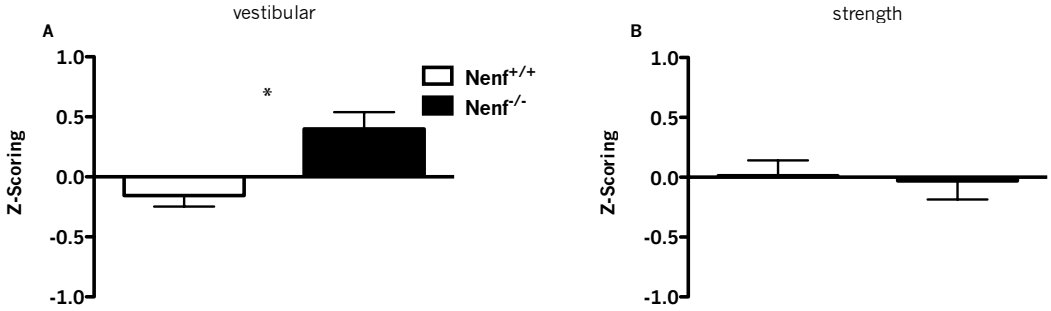


Figure 3 - Influence of neudesin ablation ($Nenf^{-/-}$) in the acquisition of neurodevelopmental milestones in male mice. Z-score translates the power of the sum of 5 tests for vestibular maturation (A) and the power of the sum of 2 tests for strength maturation (B). *p<0.05

Nenf ablation disrupts monoaminergic neurotransmission

Taking into account the sex differences above described, with an increased sensitivity in male mice in the absence of neudesin in vestibular area dependent tests, together with the anxious-like phenotype observed only in adult male neudesin-null mice (Novais et al., 2013), we next searched at the neurochemical level for alterations that may support the mild delay described in vestibular dependent tasks during post natal development in males. We performed HPLC to quantify monoamines in brain regions (vestibular area, brain stem, cerebellum and motor cortex) involved in acquisition of neurological milestones of neudesin-null males at PND21; relevant findings are represented in figure 4. The brain stem was the brain region analyzed that displayed the major alterations in monoaminergic neurotransmission. In this region, neudesin-null animals showed significantly more 5HT (figure 4A) when compared to littermate controls ($Nenf^{+/+}$=1,36±0,31 vs $Nenf^{-/-}$= 2,63±0,25) ($p<0,001$); this was translated in a significant 50% decrease of the 5HIAA/5HT ratio in neudesin-null mice ($p<0,05$) (figure 4C). A similar increase in

the levels of NE ($Nenf^{+/+}$=0,85±0,13 vs $Nenf^{-/-}$= 1,25±0,10) (p<0,05) was also observed in the brainstem. NE was also decreased in 21% in the motor cortex of neudesin-null mice (p<0,05) (figure 2B). In the cerebellum neudesin-null mice displayed a 42% decrease in serotonergic activity, reflected by the ratio of 5HIAA/5HT, as well as a 60% decrease, when compared to control littermates, in dopaminergic activity reflected by the ratio HVA/DA (p<0,05) (figure 2D). Another finding was the 60% decrease in dopaminergic activity in the vestibular area of neudesin-null mice revealed by the ratio HVA/DA in 60% (p<0,05) (figure 2E). No significant differences were found for the other neurotransmission parameters analyzed in the remaining brain regions.

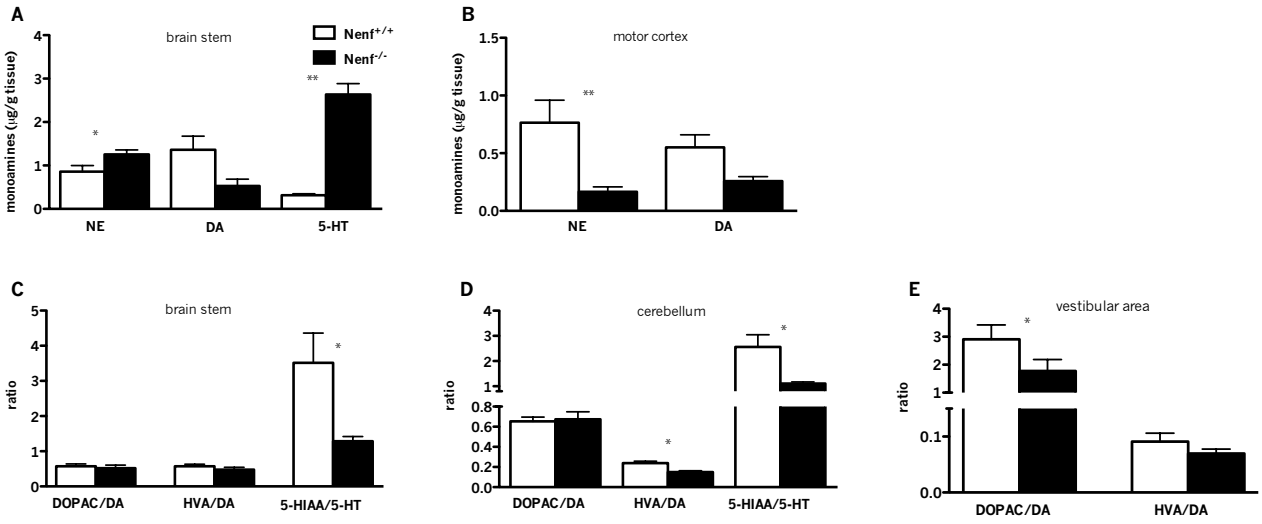


Figure 4 - Monoaminergic neurotransmission in brain region of neudesin-null ($Nenf^{-/-}$) and respective control littermate ($Nenf^{+/+}$) mice at 21 days of age. Quantification of monoamines levels (µg/g of tissue) in the brain stem (**A**) and motor cortex (**B**) brain regions. Monoaminergic activity is presented as the neurotratransmitter/respective metabolite ratio in the brain stem (**C**) cerebellum (**D**) and vestibular area (**E**). NE- noradrenaline, DA- dopamine, 5HT- serotonin, DOPAC- 3,4 dihydroxyphenylacetate, HVA- homovanillic acid and 5HIAA- 5-Hydroxy-indole-acetic acid. Values are mean±SEM. *p<0,05, **p<0.001.

Discussion

Given the altricial nature of rodents, the early postnatal period is an informative time window to study the maturation process of neural systems. In fact, the three first postnatal weeks are a critical time window for correct neurodevelopment in rodents. For instance, it has been reported that both mild and severe stressors during this time period have irreversible consequences in the adult brain function (Mesquita et al., 2006;Mesquita et al., 2007). *Nenf* is expressed in the

developing brain (Kimura et al., 2006), but no studies addressed its importance in the brain maturation process from birth until adulthood. Here we studied, for the first time, the postnatal development of a mouse strain with the constitutive ablation of the *Nenf* gene. We performed a developmental milestone analysis that evaluates the acquisition of postnatal neurological reflexes and found it is mildly delayed in neudesin-null mice. Most interestingly, this delay in postnatal development was sex-specific, since it was only observed in males. To discard possible confounding effects, we used neudesin-null and littermate controls reared by heterozygous dams. Neudesin heterozygous female mice do not present reduced fertility and raise normal litters; mother-litter contact was observed everyday during the milestones examination.

In the somatic development, neudesin-null mice showed a sex-specific difference in weight; males weight being significantly less than females weight from PND19 to PND21. Nevertheless this difference did not interfere in the delay observed in vestibular maturation in neudesin-null mice, since the time window in which animals achieve maturity in these tests ranges from PND9 to PND14. Most importantly this weight difference is transient since adult neudesin-null males do not show weight differences (Novais et al., 2013). Contradictorily it has been reported that intracerebroventricular delivery of recombinant NENF has an anorexic effect in adult mice, reducing food intake and body weight and this was observed to be regulated by BDNF signaling (Byerly et al., 2013). On the other hand, NENF was shown to modulate adipogenesis *in vitro* (Kimura et al., 2009), thus it is possible that NENF is transiently relevant in adipose tissue maturation during development, at least in males.

Postnatal changes are associated to progressive maturation of sensory and motor skills, which are closely associated with the vestibular system (Khan et al., 2004). Regarding vestibular dependent tests, we could observe gender-specific retardation in reaching maturity in the air righting and negative geotaxis tests, with only males displaying the delay. In the postural reflex test both genders (only significant in males) showed a delay in maturation, while no delay was observed in motor development (walking test); this evidence might suggest a compensatory mechanism by brain regions involved in motor functions for the vestibular impairment. The adequate postural control is essential for the acquisition of motor and strength of fine movements, which occurs later in development (Geisler and Gramsbergen, 1998). Thus, this delay in the postural and body awareness dependent of the vestibular system, with no difference between genotypes in motor skills acquisition, suggests a region and/or time window specificity of NENF influence, but only in males.

Neurotrophic support is important for the correct development of the CNS; namely it participates in the correct formation of functional afferent connections between brain regions. For instance, neurotrophic factors are known to modulate monoaminergic activity in the brain (Mamounas et al., 1995;Juric et al., 2006) and neurotrophins delivery to specific brain regions was correlated with altered monoaminenergic activity (Altar et al., 1994). To further dissect the molecular alterations responsible for the developmental delay observed in postural and body righting mechanisms, we have evaluated the monoaminergic profile of neudesin-null mice. Regions chosen for this analysis were the ones closely involved in movement output: brain stem, cerebellum, motor cortex and vestibular area in which monoamines have been described to participate in the maturation of spinal reflexes (Anden et al., 1964;Herregodts et al., 1990). We observed a very significant difference in 5HT in the brain stem and consequent defective serotonergic turnover, as well as a higher turnover in the cerebellum. Serotonin is produced in the raphe nuclei from where it projects to form the serotoninergic neural system. Descending serotonergic projections to spinal cord achieve maturation around PND10 concomitant with motor activity maturation (Ballion et al., 2002). Neudesin-null mice did not show impairment in the ontogeny of motor activity, suggesting preserved serotonergic descending projections. Importantly, neurotrophic factors, such as BDNF, are known to be upregulated in response to several types of neuronal disturbance during the postanatal period (Scheepens et al., 2003) and this upregulation was correlated with serotonergic terminal loss and neurochemical content alteration in regions such as the brain stem (Koprich et al., 2003). Norepinephrine is mainly produced at the locus coeruleus of the brain stem and establishes adult innervation in the first 2 postnatal weeks of age. It is known that spinal descendent projections play an important role in reflex control (Coradazzi et al., 2010). In this study we observed a significant increase in NE in the brain stem in the absence of NENF. On the other hand, NE was found to be decreased at the motor cortex, which suggests that both descending and ascending NE projections (Waterhouse et al., 1983) can be compromised in neudesin-null mice. The increased quantity of NE and 5HT in the cellular bodies of the brain stem might suggest that these neurotransmitters are not being effectively synthetized and/or trafficked to the synaptic cleft. Of notice, it was shown that BDNF could increase 5HT synthesis in raphe neurons by increasing expression of tryptophan hydroxylase (Eaton et al., 1995). Interestingly NENF can upregulate BDNF levels, at least in the hypothalamus (Byerly et al., 2013). It is possible that a compensatory mechanism (BDNF) might arise in the absence of an important player, in this case neudesin, during development.

Nevertheless, this compensation might still not correct the absence of neudesin and thus become an incorrect adaptation. It is tempting to hypothesize that the mechanism by which NENF might influence 5HT levels in the brain stem at PND21 involves, directly or indirectly, BDNF action.

The dopaminergic system matures later in juvenile life, after the third week (Herlenius and Lagercrantz, 2004) and thus the low levels of dopamine we detected are related to immature dopaminergic inputs. Nevertheless in the absence of NENF, dopaminergic activity, especially at the vestibular area and the cerebellum, is decreased. Whether the genotype differences in the levels of dopamine are related to the behavioral phenotype observed is uncertain but possible since treatment with dopamine agents ameliorates vestibular compensation (Petrosini and Dell'Anna, 1993).

The most striking finding in the neurodevelopment of neudesin-null mice is the gender-specific difference observed in vestibular dependent tests. Different sex steroids participate in brain postnatal development processes, particularly in the cerebellum (Dean and McCarthy, 2008). For instance, it is known that during early neonatal development, estrogen and progesterone, as well as their neurosteroid precursors play a role in cerebellar dendritic sprouting, spinogenesis and synaptogenesis (Tsutsui, 2012). Also, it was shown that estrogen modulates tryptophan hydroxylase expression in raphe neurons (Clark et al., 2012). As described previously, NENF is a heme/steroid binding protein (Kimura et al., 2008). Although binding to steroids, both *in vitro* and *in vivo,* is still to be proven, physiologically the interaction between NENF and sex steroids can be of relevance for this gender difference observed. In particular, it was recently proposed that NENF has a role in the non genomic actions of progesterone in the CNS (Singh et al., 2013). The mechanism by which NENF absence results in the postnatal developmental and neurochemical differences observed is yet to be determined and should next be addressed. Furthermore, we should next explore how this delay and differences in neurochemical content observed in neudesin-null male pups impacts adult life and is related with the behavioral phenotype observed in adult neudesin-null (Novais et al., 2013).

Conflict of interest: The authors declare no competing financial interests.

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References

Altar, C.A., Boylan, C.B., Fritsche, M., Jackson, C., Hyman, C., and Lindsay, R.M. (1994). The neurotrophins NT-4/5 and BDNF augment serotonin, dopamine, and GABAergic systems during behaviorally effective infusions to the substantia nigra. *Exp Neurol* 130**,** 31-40.

Anden, N.E., Jukes, M.G., and Lundberg, A. (1964). Spinal Reflexes and Monoamine Liberation. *Nature* 202**,** 1222-1223.

Ballion, B., Branchereau, P., Chapron, J., and Viala, D. (2002). Ontogeny of descending serotonergic innervation and evidence for intraspinal 5-HT neurons in the mouse spinal cord. *Brain Res Dev Brain Res* 137**,** 81-88.

Binder, D.K. (2007). Neurotrophins in the dentate gyrus. *Prog Brain Res* 163**,** 371-397.

Binder, D.K., and Scharfman, H.E. (2004). Brain-derived neurotrophic factor. *Growth Factors* 22**,** 123-131.

Byerly, M.S., Swanson, R.D., Semsarzadeh, N.N., Mcculloh, P.S., Kwon, K., Aja, S., Moran, T.H., Wong, G.W., and Blackshaw, S. (2013). Identification of hypothalamic neuron-derived neurotrophic factor as a novel factor modulating appetite. *Am J Physiol Regul Integr Comp Physiol* 304**,** R1085-1095.

Cancedda, L., Putignano, E., Sale, A., Viegi, A., Berardi, N., and Maffei, L. (2004). Acceleration of visual system development by environmental enrichment. *J Neurosci* 24**,** 4840-4848.

Castelhano-Carlos, M.J., Sousa, N., Ohl, F., and Baumans, V. (2010). Identification methods in newborn C57BL/6 mice: a developmental and behavioural evaluation. *Lab Anim* 44**,** 88-103.

Cicchetti, D., and Walker, E. (2003). *Neurodevelopmental Mechanisms in Psychopathology.* Cambridge University Press.

Clark, J.A., Alves, S., Gundlah, C., Rocha, B., Birzin, E.T., Cai, S.J., Flick, R., Hayes, E., Ho, K., Warrier, S., Pai, L., Yudkovitz, J., Fleischer, R., Colwell, L., Li, S., Wilkinson, H., Schaeffer, J., Wilkening, R., Mattingly, E., Hammond, M., and Rohrer, S.P. (2012). Selective estrogen receptor-beta (SERM-beta) compounds modulate raphe nuclei tryptophan hydroxylase-1 (TPH-1) mRNA expression and cause antidepressant-like effects in the forced swim test. *Neuropharmacology* 63**,** 1051-1063.

Coradazzi, M., Gulino, R., Garozzo, S., and Leanza, G. (2010). Selective lesion of the developing central noradrenergic system: short- and long-term effects and reinnervation by noradrenergic-rich tissue grafts. *J Neurochem* 114**,** 761-771.

Crawley, J.N., and Paylor, R. (1997). A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. *Horm Behav* 31**,** 197-211.

Dalla, C., Antoniou, K., Kokras, N., Drossopoulou, G., Papathanasiou, G., Bekris, S., Daskas, S., and Papadopoulou-Daifoti, Z. (2008). Sex differences in the effects of two stress paradigms on dopaminergic neurotransmission. *Physiol Behav* 93**,** 595-605.

Dean, S.L., and Mccarthy, M.M. (2008). Steroids, sex and the cerebellar cortex: implications for human disease. *Cerebellum* 7**,** 38-47.

Eaton, M.J., Staley, J.K., Globus, M.Y., and Whittemore, S.R. (1995). Developmental regulation of early serotonergic neuronal differentiation: the role of brain-derived neurotrophic factor and membrane depolarization. *Dev Biol* 170**,** 169-182.

Fox, W.M. (1965). Reflex-ontogeny and behavioural development of the mouse. *Anim Behav* 13**,** 234-241.

Geisler, H.C., and Gramsbergen, A. (1998). Motor development after vestibular deprivation in rats. *Neurosci Biobehav Rev* 22**,** 565-569.

Guilloux, J.P., Seney, M., Edgar, N., and Sibille, E. (2011). Integrated behavioral z-scoring increases the sensitivity and reliability of behavioral phenotyping in mice: relevance to emotionality and sex. *J Neurosci Methods* 197**,** 21-31.

Herlenius, E., and Lagercrantz, H. (2004). Development of neurotransmitter systems during critical periods. *Exp Neurol* 190 Suppl 1**,** S8-21.

Herregodts, P., Velkeniers, B., Ebinger, G., Michotte, Y., Vanhaelst, L., and Hooghe-Peters, E. (1990). Development of monoaminergic neurotransmitters in fetal and postnatal rat brain: analysis by HPLC with electrochemical detection. *J Neurochem* 55**,** 774-779.

Iwata, T., and Hevner, R.F. (2009). Fibroblast growth factor signaling in development of the cerebral cortex. *Dev Growth Differ* 51**,** 299-323.

Juric, D.M., Miklic, S., and Carman-Krzan, M. (2006). Monoaminergic neuronal activity up-regulates BDNF synthesis in cultured neonatal rat astrocytes. *Brain Res* 1108**,** 54-62.

Khan, Z., Carey, J., Park, H.J., Lehar, M., Lasker, D., and Jinnah, H.A. (2004). Abnormal motor behavior and vestibular dysfunction in the stargazer mouse mutant. *Neuroscience* 127**,** 785-796.

Kimura, I., Konishi, M., Asaki, T., Furukawa, N., Ukai, K., Mori, M., Hirasawa, A., Tsujimoto, G., Ohta, M., Itoh, N., and Fujimoto, M. (2009). Neudesin, an extracellular heme-binding protein, suppresses adipogenesis in 3T3-L1 cells via the MAPK cascade. *Biochem Biophys Res Commun* 381**,** 75-80.

Kimura, I., Konishi, M., Miyake, A., Fujimoto, M., and Itoh, N. (2006). Neudesin, a secreted factor, promotes neural cell proliferation and neuronal differentiation in mouse neural precursor cells. *J Neurosci Res* 83**,** 1415-1424.

Kimura, I., Nakayama, Y., Yamauchi, H., Konishi, M., Miyake, A., Mori, M., Ohta, M., Itoh, N., and Fujimoto, M. (2008). Neurotrophic activity of neudesin, a novel extracellular heme-binding protein, is dependent on the binding of heme to its cytochrome b5-like heme/steroid-binding domain. *J Biol Chem* 283**,** 4323-4331.

Kimura, I., Yoshioka, M., Konishi, M., Miyake, A., and Itoh, N. (2005). Neudesin, a novel secreted protein with a unique primary structure and neurotrophic activity. *J Neurosci Res* 79**,** 287-294.

Kokras, N., Antoniou, K., Dalla, C., Bekris, S., Xagoraris, M., Ovestreet, D.H., and Papadopoulou-Daifoti, Z. (2009). Sex-related differential response to clomipramine treatment in a rat model of depression. *J Psychopharmacol* 23**,** 945-956.

Koprich, J.B., Campbell, N.G., and Lipton, J.W. (2003). Neonatal 3,4-methylenedioxymethamphetamine (ecstasy) alters dopamine and serotonin neurochemistry and increases brain-derived neurotrophic factor in the forebrain and brainstem of the rat. *Brain Res Dev Brain Res* 147**,** 177-182.

Lim, M.A., Stack, C.M., Cuasay, K., Stone, M.M., Mcfarlane, H.G., Waschek, J.A., and Hill, J.M. (2008). Regardless of genotype, offspring of VIP-deficient female mice exhibit developmental delays and deficits in social behavior. *Int J Dev Neurosci* 26**,** 423-434.

Mamounas, L.A., Blue, M.E., Siuciak, J.A., and Altar, C.A. (1995). Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *J Neurosci* 15**,** 7929-7939.

Mesquita, A.R., Pego, J.M., Summavielle, T., Maciel, P., Almeida, O.F., and Sousa, N. (2007). Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience* 147**,** 1022-1033.

Mesquita, A.R., Tavares, H.B., Silva, R., and Sousa, N. (2006). Febrile convulsions in developing rats induce a hyperanxious phenotype later in life. *Epilepsy Behav* 9**,** 401-406.

Mikail, H.G., Dalla, C., Kokras, N., Kafetzopoulos, V., and Papadopoulou-Daifoti, Z. (2012). Sertraline behavioral response associates closer and dose-dependently with cortical rather than hippocampal serotonergic activity in the rat forced swim stress. *Physiol Behav* 107**,** 201-206.

Novais, A., Ferreira, A.C., Marques, F., Pego, J.M., Cerqueira, J.J., David-Pereira, A., Campos, F.L., Dalla, C., Kokras, N., Sousa, N., Palha, J.A., and Sousa, J.C. (2013). Neudesin is involved in anxiety behavior: structural and neurochemical correlates. *Front Behav Neurosci* 7**,** 119.

Park, H., and Poo, M.M. (2013). Neurotrophin regulation of neural circuit development and function. *Nat Rev Neurosci* 14**,** 7-23.

Petrosini, L., and Dell'anna, M.E. (1993). Vestibular compensation is affected by treatment with dopamine active agents. *Arch Ital Biol* 131**,** 159-171.

Santos, M., Silva-Fernandes, A., Oliveira, P., Sousa, N., and Maciel, P. (2007). Evidence for abnormal early development in a mouse model of Rett syndrome. *Genes Brain Behav* 6**,** 277-286.

Scheepens, A., Wassink, G., and Blanco, C.E. (2003). The effect of a global birth asphyxia on the ontogeny of BDNF and NGF protein expression in the juvenile brain. *Brain Res Dev Brain Res* 140**,** 215-221.

Singh, M., Su, C., and Ng, S. (2013). Non-genomic mechanisms of progesterone action in the brain. *Front Neurosci* 7.

Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* 77**,** 627-638.

Tessarollo, L. (1998). Pleiotropic functions of neurotrophins in development. *Cytokine Growth Factor Rev* 9**,** 125-137.

Tsutsui, K. (2012). Neurosteroid biosynthesis and action during cerebellar development. *Cerebellum* 11**,** 414-415.

Waterhouse, B.D., Lin, C.S., Burne, R.A., and Woodward, D.J. (1983). The distribution of neocortical projection neurons in the locus coeruleus. *J Comp Neurol* 217**,** 418-431.

5. Neudesin is involved in the regulation of the adult hippocampal neurogenesis

(Manuscript in preparation)

Neudesin is involved in the regulation of the adult hippocampal neurogenesis

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Abstract

Neudesin, also known as neuron derived neurotrophic factor (NENF), is a heme/steroid binding protein. *In vitro* it has been shown to be a potent stimulator of embryonic neuronal precursors proliferation and differentiation, as well as a survival factor for neurons. Surprisingly, *in vivo* neudesin's putative neurotrophic action is unknown. In this study, we have used neudesin-null mice and performed a global characterization of the proliferation state of the adult neurogenic niches of male and female neudesin-null animals. We have found a decrease in the hippocampal subgranular zone (SGZ) of the dentate gyrus proliferation in neudesin-null mice but only in males. We further assessed neurogenesis taking into consideration the dorsal-ventral axis of the hippocampus and found that neudesin-null males have less proliferating neuroblasts in the ventral division but not in the dorsal division of the hippocampus. Nevertheless, this difference in the number of proliferating neuroblasts at the ventral division of the hippocampus did not translate into a functional difference evaluated by the amount of glutamate released. Additionally, we have performed neurospheres cultures of adult subventricular zone (aSVZ) and SGZ of neudesin-null mice and observed that the clonal expansion capacity of neurospheres isolated from the SGZ, but not from the aSVZ, of neudesin-null mice is lower when compared to controls. All together, this study represents the first *in vivo* confirmation for the neurotrophic capacity of neudesin at the neurogenic niches, particularly the SGZ of the hippocampus.

Introduction

Neuron derived neurotrophic factor (NENF), commonly known as neudesin, is a secreted neuronal protein with 21 KDa and 171 aminoacids (Kimura et al., 2005;Bath and Lee, 2010). Since neudesin presents a cytochrome b5-like heme/steroid binding domain in it's primary structure, it is classified as a member of the membrane associated progesterone receptor family (MAPR) (Cahill, 2007;Kimura et al., 2008). While neudesin is highly expressed in neurons at embryonic stages in the brain and spinal cord, in adult mice neudesin is also expressed in other organs besides the nervous system, namely in the heart, lung and kidneys, among others (Kimura et al., 2005). In the central nervous system (CNS), neurons differentiate between E13.5 and E16.5, while glia appears later in development; interestingly, neudesin is expressed in all of these embryonic stages (Kimura et al., 2006). Of notice, in the embryonic brain, during the cortical formation neudesin is highly expressed but this expression is restricted to the pre-plate region, where post-mitotic neurons are located; hence, it was not found in regions of precursors proliferation and migration (Kimura et al., 2006). The specific pattern of neudesin expression and secretion by neurons suggests that it plays a role exclusively over neurons and not other neural cell types. In fact, *in vitro*, neudesin was shown to promote proliferation and differentiation of neural precursors into a neuronal lineage as well as to promote the survival of mature neurons (Kimura et al., 2005;Kimura et al., 2006). Given these properties elicited by recombinant neudesin *in vitro* and the pathways activated by neudesin, this protein was proposed to be a neurotrophic factor (Kimura et al., 2005).

While extensive *in vitro* evidence exists for a potential neurotrophic action of neudesin in the proliferation and differentiation of embryonic neural precursor cells (NPCs), no studies have addressed neudesin action on adult NPCs. In the adult brain there are two main neurogenic niches, the subventricular zone (aSVZ) of the lateral ventricles of the brain and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Seaberg and van der Kooy, 2002). Although both have different patterns of differentiation and regulation, they share similar embryonic cellular ancestors and stages of differentiation (Zhao et al., 2008). aSVZ neurogenesis mainly feeds the olfactory bulbs, since neuroblats migrate from the aSVZ through the rostral migratory stream until they reach the olfactory bulb, where some differentiate and integrate the olfactory neurocircuitry (Tramontin et al., 2003). In the SGZ, neuroblats migrate to the granular zone of the DG, where they differentiate into young granular neurons that project to the CA3 region and integrate the hippocampal circuitry (Piatti et al., 2011). For both neurogenic niches,

several neurotrophic factors play different roles in all of the above-described stages (Lee and Son, 2009;Bath and Lee, 2010).

As described, neudesin ability to promote proliferation, differentiation and survival were demonstrated *in vitro* in embryonic neural precursors and early post-natal neurons. Whether neudesin neurotrophic potential also participates in the modulation of adult neurogenesis and *in vivo* is unknown. In this study we assess the role of neudesin in the regulation of the two adult neurogenic niches, the aSVZ and the SGZ, taking advantage of mice with a target deletion of the neudesin gene.

Methods

Animals

A mouse strain with targeted deletion of the neudesin gene, provided by Merck-Serono under a material transfer agreement, was used. The neudesin-null ($Nenf^{-/-}$) mouse strain was generated by using a 129/SvEv genomic library from a BAC clone and the target construct was made by deletion of the entire coding sequence of the neudesin gene (exons 1 to 4, approximately 12KB) and replacing it by a *LacZ-neomicin* cassette. The target DNA was then inserted into embryonic stem cells (FiH4 ES cells), where the homologous pieces of DNA were recombined. The cells identified as homologous recombinant clones were microinjected into C57BL/6F1 blastocysts to generate chimeric mouse. Initial genotyping was performed using a loss-of-native-allele assay. Animals used in this study were backcrossed to a C57BL/6F background.

Littermate controls ($Nenf^{+/+}$) and neudesin-null ($Nenf^{-/-}$) animals were obtained by crossing heterozygous animals. Mice genotype was confirmed by PCR using two independent sets of primers: one for the *LacZ* cassette, specific for the $Nenf^{-/-}$ genotype: LacZ-foward 5'-GGTAAACTGGCTCGGATTAGGG-3' and LacZ-reverse 5'-TTGACTGTAGCGGCTGATGTTG-3'; and another for the Nenf gene, specific for $Nenf^{+/+}$ animals: Nenf-intron3 5'-CTTGGAGTTTGGGGCTGATA-3', Nenf-exon4 5'-TGGCTTTGTACACCTTGCTG-3'. The amplified fragments were of 210bp and 176bp, respectively, and distinguishable by electrophoresis through a 1,5% agarose gel. Confirmation of loss of neudesin synthesis in $Nenf^{-/-}$ was also obtained by performing immunohistochemistry with a neudesin-specific antibody (Sigma, Aldrich, St. Louis, USA) in brain samples of both control and neudesin-null mice; neuronal expression of neudesin was not detected in neudesin-null mice.

Animals were maintained under 12 h light/dark cycles at 22±1°C, 55% humidity and fed with

regular rodent's chow and tap water *ad libitum*. This study was approved by the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (permission ID: DGV9457). All experiments were performed in accordance with the guidelines for the care and handling of laboratory animals, as described in the Directive 2010/63/EU of the European Parliament and of the Council.

Administration of 5-bromo-2'-deoxyuridine (BrdU) for proliferation assessment

BrdU is a thymidine analogue that is incorporated in DNA during the S phase of the mitotic cycle. BrdU is often used to label proliferative cells in the brain. To detect proliferating cells, BrdU (Sigma Aldrich) (50mg/Kg) was delivered intraperitoneally (i.p.) 24h prior to sacrifice.

Immunohistochemistry

Animals were transcardially perfused, under anesthesia [ketamine hydrochloride (150 mg/Kg) with medetomidine (0.3 mg/Kg)], with 4% of paraformaldehyde (PFA) in phosphate-buffered saline (PBS). After perfusion, brains were removed from the skull, left 1h in 4% PFA solution, 12h in 30% sucrose solution and then embedded in O.C.T. compound and snap-frozen in liquid nitrogen. Immunohistochemistry was performed on coronal sections (20 μm) with an anti-BrdU primary antibody at a dilution of 1:50 (Mouse Anti-Bromodeoxyurine, Clone Bu20a, DAKO, Spain). This was then detected using Ultravision Detection System (Lab Vision, Freemont, CA, USA), and the reaction developed with 3,3'-diaminobenzidine substrate (Sigma Aldrich). Sections were subsequently counterstained with 25% hematoxylin diluted solution. For this analysis 8-week old littermate controls and neudesin-null mice (n=7 in each group) of each gender were used.

Immunofluorescence analysis was performed in the same conditions as the immunohistochemistry, the primary antibodies used were: doublecortin (DCX) (rabbit polyclonal, Abcam, UK) at a dilution of 1:500 and BrdU (rat anti-BrdU, BU1/75 clone, Abcam) at a dilution of 1:100. Fluorescent secondary antibodies (Invitrogen, Carlsbad, CA, USA), anti-rabbit and anti-rat, respectively, were used to detect the primary antibodies at a dilution of 1:1000. To label the nucleus, incubation with 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) at a dilution of 1:1000 was performed. Antibodies were diluted in PBS-0.5% Tween/10% FBS and incubated overnight at 4°C, for the primary antibodies, and 2 hours at room temperature, for the secondary antibodies. For the immunofluorescence analysis, we used 6 neudesin-null and 6 littermate control male animals with 8 weeks of age.

Stereology

The number of BrdU positive cells was assessed in the neurogenic niches (aSVZ and SGZ) using an Olympus BX51 microscope (Olympus, Hamburg, Germany) associated to the Visiopharm Integrator system (VIS) software (version 2.12.3.0). Coronal sections comprised aSVZ between bregma 0.86 mm and 0.14 mm and SGZ -1.22 mm to -2.30 mm coordinates (Paxinos G., 2001). The number of BrdU positive cells was normalized for the area (mm^2).

Confocal imaging and quantitative analysis

To assess the number of proliferating neuroblasts ($DCX^+/BrdU^+$) at the SGZ, the hippocampus was separated in 2 divisions: dorsal and ventral hippocampus; the dorsal hippocampus ranged from coordinates -1.22 mm to -2.30 mm and the ventral from -3.40 mm to -3.80 coordinates (Paxinos G., 2001). Six animals per genotype ($Nenf^{+/+}$ and $Nenf^{-/-}$) were assessed and 3 sections from each animal were analyzed for both hemispheres. BrdU positive cells were identified using a confocal microscope (FV1000; Olympus) and the total number of double $DCX^+/BrdU^+$ positive cells was counted. The number of $DCX^+/BrdU^+$ was normalized for each SGZ area analyzed using a BX51 Olympus microscope attached to a camera with the StereoInvestigator software (MBF Bioscience, Vermont, USA).

Glutamate quantification

The levels of glutamate were measured using high performance liquid chromatography, combined with electrochemical detection (HPLC/EC).

Neudesin-null male mice were sacrificed by decapitation ($Nenf^{+/+}$=10 and $Nenf^{-/-}$=8). Skulls were rapidly snap frozen in liquid nitrogen to avoid degradation during macro dissection. Brains were carefully dissected for dorsal and ventral hippocampus on ice with the help of a 2X magnifying lens (SZX7, Olympus), following a stereotaxic brain atlas (Paxinos G., 2001). Dissected tissues were weighed and then homogenized and deproteinized in 100 µL of 0.2 N perchloric acid solution (Applichem, Darmstadt, Germany) containing 7.9 mM $Na_2S_2O_5$ and 1.3 mM Na2EDTA (Riedel-de Haën AG, Seelze, Germany), centrifuged at 15,000 r.p.m. for 45 min at 4 °C and the supernatant was stored at −80 °C until analysis. Glutamate levels were measure in a reverse phase ion-pair chromatography with precolumn derivatization on a Pharmacia-LKB 2248 high-performance liquid chromatography (HPLC) pump coupled with a BAS LC4B electrochemical detector, as previously described (Kokras et al., 2009)

Neurospheres assay

Hippocampi and aSVZ from 3-6 days old Nenf$^{+/+}$ and Nenfmice were dissected carefully and meninges and choroid plexus were removed. Tissue was triturated in DMEM F12 (Gibco, Rockville, MD, USA) with 10% Fetal Bovine Serum (FBS) with the help of a P1000 pipette; upon completion of tissue dissociation, cell suspension was centrifuged at 900 r.p.m. and supernatant carefully discarded. Single cells were plated in DMEM F12 media supplemented with GlutaMAX (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 1% B27 (Gibco), 10 ng/ml epidermal growth factor (Gibco), and 10 ng/ml basic fibroblast growth factor (Gibco). Neurospheres were allowed to form and grow for 5 days. Splitting of neurospheres was performed using 0.05% of trypsin in DMEM F12 and mechanical dissociation with a P1000 pipette followed by incubation for 3 minutes at 37°C; trypsin was then inactivated with 10% FBS, cells centrifuged at 1000 r.p.m. and supernatant discarded. Single cells were counted using a Neubauer chamber and 1 cell/μL was plated in the same media with growth factors in 48 well plates, 6 wells per each condition for the clonal density assay. 10 cells/μl were plated in DMEM F12 with 10% FBS for the differentiation assay in 24 well poly-D-lysine coverslips for 5 days, 3 wells per each condition.

Immunocytochemistry

To assess in which cell type the neurospheres differentiate, we analysed the number of astrocytes using immunocytochemistry technique. For that, cells were fixated for 20 minutes in 4% PFA and blocked in 0.4% FBS diluted in PBS at room temperature for 1 hour. A glial fibrillary acidic protein (GFAP) antibody (polyclonal rabbit anti-GFAP, DAKO) at a dilution of 1:200 was incubated at room temperture for 1 hour. Fluorescent secondary antibody (Invitrogen), anti-rabbit at a dilution of 1:1000 was used to detect primary antibody. To label the nucleus, incubation with DAPI (Sigma Aldrich) at a dilution of 1:1000 was performed. Primary and secondary antibodies were diluted in PBS and all washes were performed with PBS as well.

Statistical analysis

All values presented are expressed as the mean ± SEM and significance was verified by using the Mann-Whitney test for independent samples. Differences were considered significant when $p<0.05$.

Results

The absence of neudesin impairs proliferation in the SGZ of males but not females

In this work, we aimed at evaluating, *in vivo* and *in vitro*, the effect of neudesin ablation in the adult neurogenic niches. We initially injected the DNA thymidine-analog BrdU 24h before sacrifice, which mainly labels fast dividing cells.

We found that proliferation occurring in the SGZ in the absence of neudesin ($Nenf^{-/-}$ mice) was significantly reduced by approximately 50% when compared to littermate controls ($Nenf^{+/+}$) in both brain hemispheres (figure 1A). Most interestingly, this difference is gender specific since no differences were observed between mouse strains in the SGZ proliferation of females (figure 1B).

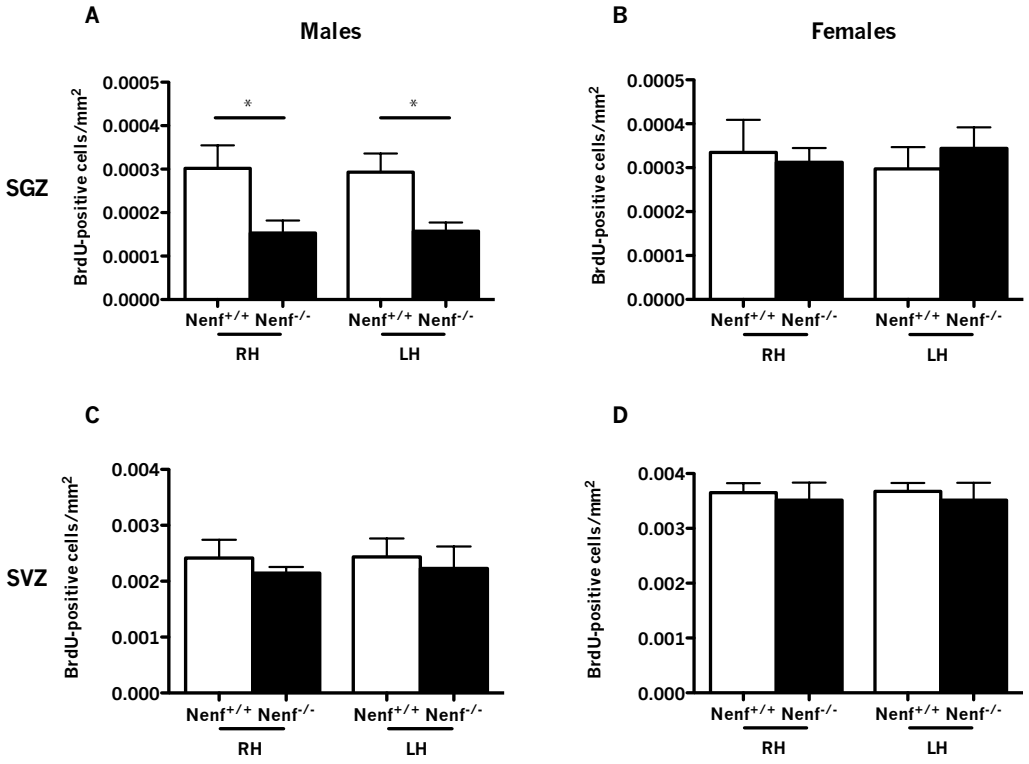


Figure 1 - Characterization of the proliferation profile of the SGZ and aSVZ neurogenic niches of neudesin-null mice. BrdU labeling shows significantly less BrdU positive cells in the sub-granular zone (SGZ) of the dentate gyrus of male neudesin-null ($Nenf^{-/-}$) animals when compared to control littermates ($Nenf^{+/+}$) (**A**). On the other hand similar numbers of mitotic cells incorporating BrdU are observed in the adult subventricular zone (aSVZ) of male $Nenf^{+/+}$ and $Nenf^{-/-}$ mice (**C**). In female mice proliferation at both the SGZ (**B**) and the aSVZ (**D**) does not differ between genotypes. Data is presented as mean ± SEM. *p<0.05. LH= left hemisphere and RH= right emisphere.

When total cell proliferation was analyzed in the other adult neurogenic niche, the aSVZ, no differences were observed between neudesin-null and littermate control mice, in both males and females (Figures 1C and 1D).

Abnormal proliferation in the SGZ of neudesin-null male mice is restricted to the ventral dentate gyrus

The diminished proliferation observed in the SGZ of neudesin-null male mice prompted us to further characterize neurogenesis in this neurogenic niche. We labeled proliferative neuroblasts by using both DCX and BrdU immunostaining ($BrdU^+DCX^+$) throughout the dorsal/ventral axis of the DG. While no differences were observed between mouse strains in dorsal SGZ proliferating neuroblasts (Figure 2A), neudesin-null mice ventral SGZ displayed significantly less $BrdU^+DCX^+$ cells in both right (60% decrease) and left (70% decrease) hemispheres when compared to control littermates, ($p<0.05$) (Figure 2B).

Although no differences were observed in the proliferation in the aSVZ, we analyzed the number of proliferating neuroblasts in this niche taking in consideration the topographic specificities of this region (Falcao et al., 2012). Nevertheless, we failed to observe any differences between control and neudesin-null mice in the density of $BrdU^+DCX^+$ cells in any of the divisions (anterior, intermediate, posterior) or regions (rostral migratory stream, dorsal, dorsolateral, ventral) of the aSVZ (data not shown).

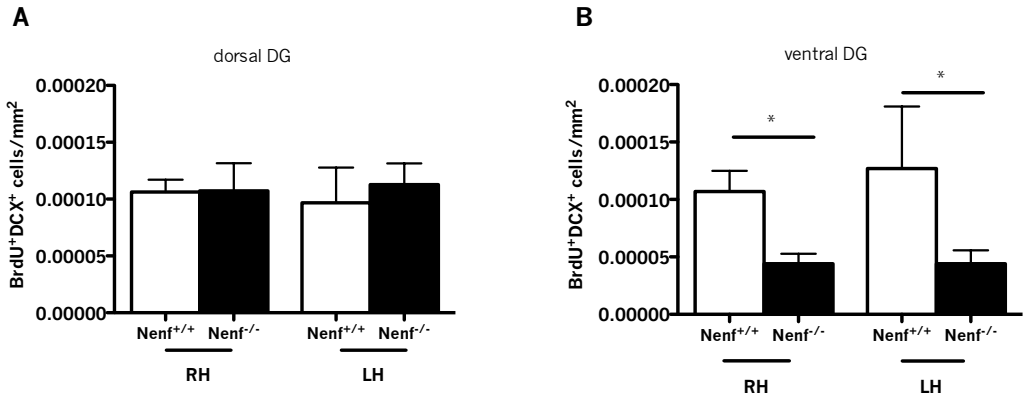


Figure 2 - Proliferating neuroblasts at the dorso-ventral axis of the subgranular zone (SGZ) of the dentate gyrus (DG) of neudesin-null mice. The density of proliferating neuroblasts ($BrdU^+DCX^+$) in the dorsal SGZ (**A**) in neudesin-null ($Nenf^{-/-}$) males is similar to littermate controls ($Nenf^{+/+}$), but a marked decrease is observed in the ventral SGZ (**B**) of neudesin-null mice in both right and left hemispheres (RH and LH, respectively). Data is presented as mean ± SEM. *$p<0.05$.

Altered pattern of proliferation in the dorsal-ventral axis of the DG does not reflect in the glutamatergic quantity

The ventral axis specific sharp decrease in proliferative neuroblasts observed in the adult hippocampal DG of neudesin-null mice prompted us to evaluate if this would be translated functionally. Since proliferating neuroblasts in the SGZ give rise to glutamatergic granule neurons that integrate into the granule cell layer we quantified glutamate in both the dorsal and the ventral hippocampus of neudesin-null adult males and respective littermate controls. No differences between genotypes were observed in the dorsal division of the hippocampus ($Nenf^{+/+}$ =6801 ± 1264 vs $Nenf^{-/-}$=5247 ± 1103) (Figure 3A). Despite a marked decrease in the levels of glutamate in the ventral hippocampus ($Nenf^{+/+}$=2489 ± 561 vs $Nenf^{-/-}$=1203 ± 255) (Figure 3B), it did not reach statistical significance.

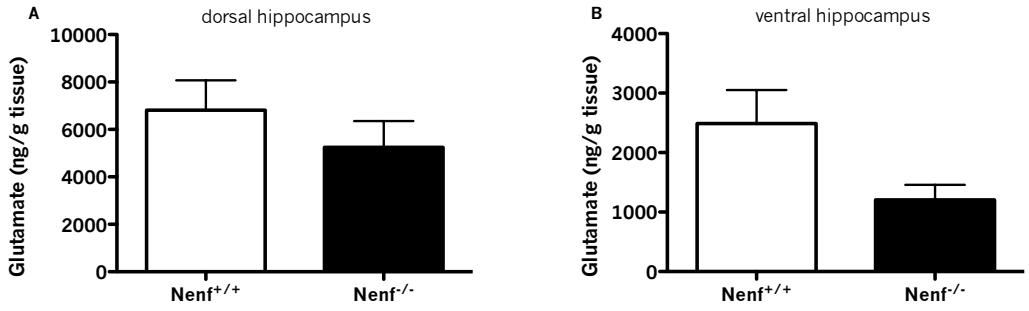


Figure 3 – Quantification of glutamate in the dorsal and ventral division of the hippocampus of neudesin-null mice. Control ($Nenf^{+/+}$) and neudesin-null ($Nenf^{-/-}$) mice show similar levels of glutamate at the dorsal (**A**) and ventral (**B**) hippocampus. Data is presented as mean ± SEM.

Neurospheres forming capacity of DG neural stem cells is diminished in neudesin-null mice

Dentate gyrus derived and aSVZ neural stem cells from pups were cultured *in vitro* and formed neurospheres, and clonal expansion capacity was assessed. Neural stem cells isolated from neudesin-null mice hippocampi formed significantly less neurospheres when compared to controls ($p<0.05$) (Figure 4A) while no difference was observed in the clonal expansion of neurospheres isolated from the aSVZ (Figure 4B). After 5 days in culture we have split the hippocampal neurospheres, plated single cells and allowed it to grow in culture for 5 days in astrocytic differentiation conditions. Then, after performing GFAP immunocytochemistry, we evaluated differentiation into astrocytes from both neudesin-null and control littermate mice. We

observed that neural stem cells from controls under these culture conditions form 75%, while neudesin-null mice derived neural stem cells form 50% of astrocytes when compared to control(Figure 4C).

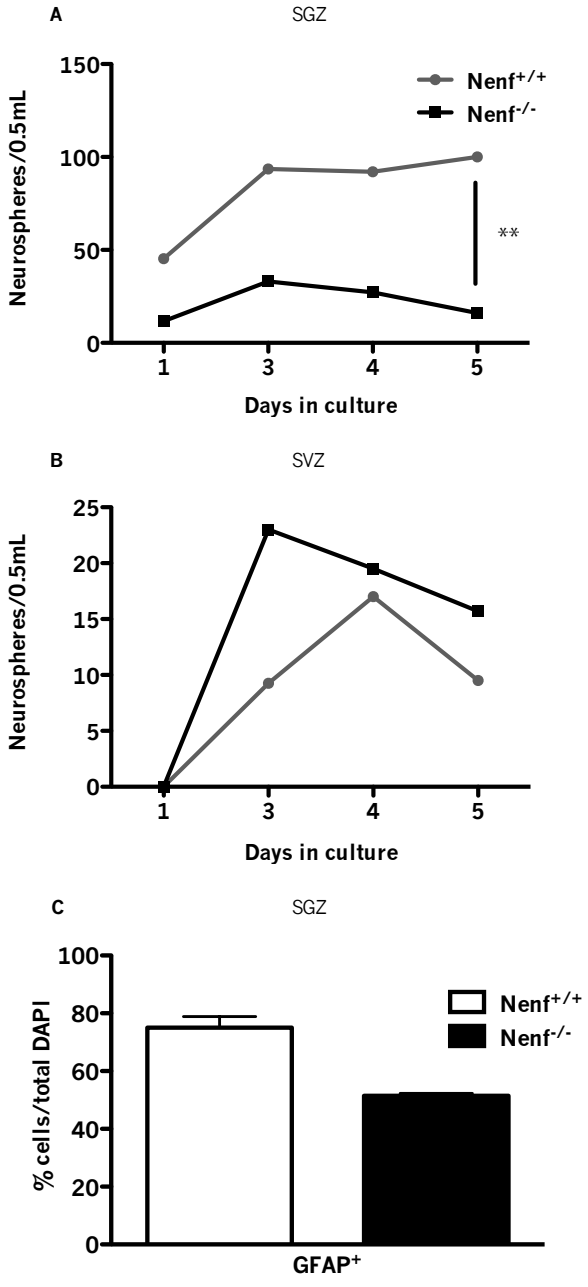


Figure 4 - Neurosphere forming and differentiation assays. Neural stem cells isolated from postnatal hippocampi of neudesin-null ($Nenf^{-/-}$) form significantly less neurospheres than those isolated from control ($Nenf^{+/+}$) mice (**A**). No differences were observed when analyzing neural stem cells isolated from postnatal aSVZ (**B**). $Nenf^{-/-}$ neural stem cells differentiate less into astrocytes ($GFAP^{+}$) than $Nenf^{+/+}$ (**C**). Data is presented as mean ± SEM. *$p<0.05$.

Discussion

This study is the first to show *in vivo* the initially described *in vitro* neurotrophic properties of neudesin. We have demonstrated this by analyzing the effects of NENF ablation in the adult mice neurogenic niches, the SGZ and the aSVZ, of mice. We show that neudesin-null male mice do not display differences in the proliferation in the aSVZ when compared to control littermates, but in the SGZ the absence of neudesin leads to decreased cell proliferation. Surprisingly, this difference is sex specific, since neudesin-null females do not show abnormal proliferation in both neurogenic niches, when compared to control animals. Since only males revealed a decrease in SGZ proliferation, we have further characterized neurogenesis throughout the dorsal-ventral axis of the SGZ in neudesin-null mice, and revealed a significant decrease in neurogenesis exclusively in the ventral division of the SGZ. Furthermore, *in vitro* we show that neural stem cells isolated from the hippocampus of neudesin-null mice form less neurospheres, and that these adult neural stem cells display an altered pattern of differentiation, at least in the astrocytic lineage. These findings support an additional role for neudesin as a neurotrophic player in the adult brain.

It was previously demonstrated that recombinant neudesin has potent neurotrophic capacity, as demonstrated *in vitro* in cultures of embryonic neural precursor cells and neurons (Kimura et al., 2005;Kimura et al., 2006). Noteworthy not much was known about such neudesin properties *in vivo* and particularly in adulthood. Both aSVZ and SGZ neural stem cells embryonically have origin in radial glia from germinative layers of the telencephalon (Tramontin et al., 2003) and are comprised of three similar main cell types: stem cells, progenitor cells and neuroblasts (Ming and Song, 2011). Specifically, in the aSVZ the quiescent cells are slow dividing radial glia stem cells (type B), which give rise to type C cells, also known as transient amplifying progenitors (TAP's), that in turn differentiate into type A cells (also designated neuroblasts). In turn, in the SGZ, proliferating radial glia stem cells and nonradial precursors (type I and II) give rise to intermediate progenitor cells that will differentiate into neuroblasts (Zhao et al., 2006). TAP's, intermediate progenitors and neuroblasts are fast dividing cells, and are easily labeled by short pulses of BrdU, the thymidine analogue. Although the cell population of these niches, aSVZ and SGZ, is very similar, they are regulated in distinctive ways. For instance, it is known that sex hormones (Tanapat et al., 1999) and chronic anti-depressant treatment (Malberg et al., 2000) have modulatory effects in hippocampal neurogenesis but not in aSVZ neurogenesis. Here we show that neudesin plays a different role in this two neurogenic niches: at the aSVZ it does not seem to be essential for the maintenance of neural precursor cells whereas in the SGZ the absence of

neudesin yields a significant decrease in cell proliferation.

Considering the differentiation process of neural stem cells, the transition from transient amplifying progenitors into neuroblasts is a critical period in the niche maintenance and in the neurogenesis process; it is a selective period closely regulated by intrinsic and extrinsic factors (Sierra et al., 2010). Importantly, we observed that neudesin might play a crucial role in this stage of neurogenesis, given the observed decrease in proliferating neuroblasts, at least in the ventral part of the SGZ. Of notice, we demonstrated previously that adult neudesin-null male mice have an anxious like behavior as well as impoverished dendritic branching of granular neurons of the ventral DG (Novais et al., 2013), but whether the decreased population of proliferative neuroblasts is related to the anxious-like phenotype is yet to be determined.

Newly formed neurons migrate to the granular zone of the DG and project an axon to the CA3 layer of the hippocampus, thus integrating into the hippocampal circuitry (Zhao et al., 2008;Piatti et al., 2011), which mainly is composed of glutamatergic neurons. This newly born granular cell integration in the circuitry is highly modulated by neurotrophins and particularly BDNF (Lessmann, 1998;Lu, 2003)). BDNF not only regulates glutamatergic synaptic transmission (Lu, 2003) but also regulates proliferation in this region (Sairanen et al., 2005). Of note, functionally, while the absence of neudesin decreased neurogenesis in the ventral DG, it does not seem to interfere in glutamatergic neurotransmission in the hippocampus, in both dorsal and ventral division. This indicates that neudesin, contrarily to what is known for BDNF, seems to participate only in the regulation of the neurogenic process rather than influencing the integration of newborn neurons into hippocampus circuits. Complementary studies with newly born neurons survival and integration into the hippocampal circuitry of neudesin-null mice should be performed to verify this hypothesis.

Neural stem cells have been extensively characterized for their *in vitro* phenotype and behavior (Richards et al., 1992;Barkho et al., 2006;Pastrana et al., 2011) and provide a powerful tool to better understand proliferation and differentiation profiles. We observed that neural stem cells isolated from neudesin-null mice hippocampi form less neurospheres, when compared to controls, in the clonal expansion assay. Interestingly this difference was only observed in the clonal expansion assay with hippocampal precursors and not with aSVZ neurospheres. This reinforces the selective role for neudesin as a putative modulator of the hippocampal neurogenic niche. When SGZ neural precursors were cultured in astrocytic differentiation conditions, a decrease in astrocyte differentiation was observed in the absence of neudesin. Interestingly,

recombinant neudesin *in vitro* was shown to differentiate precursor cells exclusively into neurons, as well as having selective modulation over neurons and not astrocytes (Kimura et al., 2005). In contrast, we show that, at least in astrocytic differentiation conditions, adult neudesin-null mice precursor cells differentiate into astrocytes, which suggests that physiological levels of neudesin are also important in the astrocytic lineage differentiation.

Steroid hormones and altered physiological states such as stress, pregnancy and lactation, have important effects on hippocampal neurogenesis (Tanapat et al., 1999;Galea et al., 2013). In fact, during the estrous cycle, the estrogen peak correlates with high proliferative rates in the hippocampus (Pawluski et al., 2009;Velazquez-Zamora et al., 2012). Moreover it was shown that the sex steroid progesterone regulates proliferation of neural stem cells (Liu et al., 2009). Despite being a member of the MAPR family, binding of steroids to the steroid-binding domain of neudesin was not shown to be necessary for its neurotrophic activity, at least *in vitro* (Kimura et al., 2008). Importantly, although still a matter of debate across mammalian species, the SGZ is distinctively regulated in males and females (Lagace et al., 2007). Noteworthy, neudesin-null male mice show less hippocampal proliferation when compared to littermate controls but the same genotype effect was not observed in females; this observation might point for a role for neudesin in sex steroids action at least in the SGZ niche, which should next be explored.

Herein, we provide the first characterization of the adult neurogenic niches (aSVZ and SGZ) of neudesin-null mice. We show that neudesin's regulation of embryonic neurons and its precursors reported previously (Kimura et al., 2006) is dissimilar from the observed in the modulation of the adult niches. Furthermore we observe a selective effect in the hippocampus in the absence of neudesin, specifically an impairment in neural progenitor cells proliferation and differentiation is only observed in the ventral DG; interestingly these observations correlate with the atrophy of granular cells previously described in the ventral hippocampus of adult neudesin-null mice (Novais et al., 2013). We suggest that neudesin plays a role in the maintenance of ventral hippocampus neurogenesis and this might be related to granular cells plasticity and the increased anxiety state of male neudesin-null mice.

Conflict of interest: The authors declare no competing financial interests.

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References

Barkho, B.Z., Song, H., Aimone, J.B., Smrt, R.D., Kuwabara, T., Nakashima, K., Gage, F.H., and Zhao, X. (2006). Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. *Stem Cells Dev* 15**,** 407-421.

Bath, K.G., and Lee, F.S. (2010). Neurotrophic factor control of adult SVZ neurogenesis. *Dev Neurobiol* 70**,** 339-349.

Cahill, M.A. (2007). Progesterone receptor membrane component 1: an integrative review. *J Steroid Biochem Mol Biol* 105**,** 16-36.

Falcao, A.M., Palha, J.A., Ferreira, A.C., Marques, F., Sousa, N., and Sousa, J.C. (2012). Topographical analysis of the subependymal zone neurogenic niche. *PLoS One* 7**,** e38647.

Galea, L.A., Wainwright, S.R., Roes, M.M., Duarte-Guterman, P., Chow, C., and Hamson, D.K. (2013). Sex, hormones, and neurogenesis in the hippocampus: Hormonal modulation of neurogenesis and potential functional implications. *J Neuroendocrinol*.

Kimura, I., Konishi, M., Miyake, A., Fujimoto, M., and Itoh, N. (2006). Neudesin, a secreted factor, promotes neural cell proliferation and neuronal differentiation in mouse neural precursor cells. *J Neurosci Res* 83**,** 1415-1424.

Kimura, I., Nakayama, Y., Yamauchi, H., Konishi, M., Miyake, A., Mori, M., Ohta, M., Itoh, N., and Fujimoto, M. (2008). Neurotrophic activity of neudesin, a novel extracellular heme-binding protein, is dependent on the binding of heme to its cytochrome b5-like heme/steroid-binding domain. *J Biol Chem* 283**,** 4323-4331.

Kimura, I., Yoshioka, M., Konishi, M., Miyake, A., and Itoh, N. (2005). Neudesin, a novel secreted protein with a unique primary structure and neurotrophic activity. *J Neurosci Res* 79**,** 287-294.

Kokras, N., Antoniou, K., Polissidis, A., and Papadopoulou-Daifoti, Z. (2009). Antidepressants induce regionally discrete, sex-dependent changes in brain's glutamate content. *Neurosci Lett* 464**,** 98-102.

Lagace, D.C., Fischer, S.J., and Eisch, A.J. (2007). Gender and endogenous levels of estradiol do not influence adult hippocampal neurogenesis in mice. *Hippocampus* 17**,** 175-180.

Lee, E., and Son, H. (2009). Adult hippocampal neurogenesis and related neurotrophic factors. *BMB Rep* 42**,** 239-244.

Lessmann, V. (1998). Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. *Gen Pharmacol* 31**,** 667-674.

Liu, L., Wang, J., Zhao, L., Nilsen, J., Mcclure, K., Wong, K., and Brinton, R.D. (2009). Progesterone increases rat neural progenitor cell cycle gene expression and proliferation via extracellularly regulated kinase and progesterone receptor membrane components 1 and 2. *Endocrinology* 150**,** 3186-3196.

Lu, B. (2003). BDNF and activity-dependent synaptic modulation. *Learn Mem* 10**,** 86-98.

Malberg, J.E., Eisch, A.J., Nestler, E.J., and Duman, R.S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 20**,** 9104-9110.

Ming, G.L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 70**,** 687-702.

Novais, A., Ferreira, A.C., Marques, F., Pego, J.M., Cerqueira, J.J., David-Pereira, A., Campos, F.L., Dalla, C., Kokras, N., Sousa, N., Palha, J.A., and Sousa, J.C. (2013). Neudesin is involved in anxiety behavior: structural and neurochemical correlates. *Front Behav Neurosci* 7**,** 119.

Pastrana, E., Silva-Vargas, V., and Doetsch, F. (2011). Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell* 8**,** 486-498.

Pawluski, J.L., Brummelte, S., Barha, C.K., Crozier, T.M., and Galea, L.A. (2009). Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. *Front Neuroendocrinol* 30**,** 343-357.

Paxinos G., F., K.B. (2001). *The Mouse Brain in Stereotaxic Coordinates.* San Diego: Academic Press.

Piatti, V.C., Davies-Sala, M.G., Esposito, M.S., Mongiat, L.A., Trinchero, M.F., and Schinder, A.F. (2011). The timing for neuronal maturation in the adult hippocampus is modulated by local network activity. *J Neurosci* 31**,** 7715-7728.

Richards, L.J., Kilpatrick, T.J., and Bartlett, P.F. (1992). De novo generation of neuronal cells from the adult mouse brain. *Proc Natl Acad Sci U S A* 89**,** 8591-8595.

Sairanen, M., Lucas, G., Ernfors, P., Castrén, M., and Castrén, E. (2005). Brain-Derived Neurotrophic Factor and Antidepressant Drugs Have Different But Coordinated Effects on Neuronal Turnover, Proliferation, and Survival in the Adult Dentate Gyrus. *J Neurosci* 25.

Seaberg, R.M., and Van Der Kooy, D. (2002). Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J Neurosci* 22**,** 1784-1793.

Sierra, A., Encinas, J.M., Deudero, J.J., Chancey, J.H., Enikolopov, G., Overstreet-Wadiche, L.S., Tsirka, S.E., and Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell* 7**,** 483-495.

Tanapat, P., Hastings, N.B., Reeves, A.J., and Gould, E. (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci* 19**,** 5792-5801.

Tramontin, A.D., Garcia-Verdugo, J.M., Lim, D.A., and Alvarez-Buylla, A. (2003). Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. *Cereb Cortex* 13**,** 580-587.

Velazquez-Zamora, D.A., Gonzalez-Tapia, D., Gonzalez-Ramirez, M.M., Flores-Soto, M.E., Vazquez-Valls, E., Cervantes, M., and Gonzalez-Burgos, I. (2012). Plastic changes in dendritic spines of hippocampal CA1 pyramidal neurons from ovariectomized rats after estradiol treatment. *Brain Res* 1470**,** 1-10.

Zhao, C., Deng, W., and Gage, F.H. (2008). Mechanisms and functional implications of adult neurogenesis. *Cell* 132**,** 645-660.

Zhao, C., Teng, E.M., Summers, R.G., Jr., Ming, G.L., and Gage, F.H. (2006). Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J Neurosci* 26**,** 3-11.

6. General Discussion and future perspectives

Discussion and future perspectives

Since neudesin (NENF) was first described, just 8 years ago (Kimura et al., 2005), as being a putative novel neurotrophic factor, it has not received much attention. In this period, studies were confined to two research groups that performed different approaches studying the function of NENF by using recombinant protein delivery strategies *in vitro* and *in vivo* (Kimura et al., 2005;Kimura et al., 2008;Byerly et al., 2013). In total 10 manuscripts were published (based in a PubMed search for the terms "neudesin" or any of the other designations attributed to this protein as indicated previously in the introduction section) in which NENF is referred. In this sense the lack of a neudesin-null mouse strain precluded further advances in the understanding of NENF physiological relevance. In this work we performed an extensive characterization of the impact of NENF ablation in the CNS. Specifically we evaluated the:

I) Impact of NENF ablation on adult behavior;

II) Postnatal neurodevelopment of neudesin-null mice;

III) Modulation of adult neurogenic niches by NENF.

Each of these topics and its interconnectivity will next be discussed in an attempt to provide further insight into the nature of NENF and its relevance in CNS function.

Many constitutive knockout mouse models for neurotrophins and/or their receptors die prematurely at the first stages of postnatal development (Snider, 1994). Of note, constitutive NENF ablation in rodents, the model used in this study, did not result in premature death and neudesin-null mice seem as healthy as littermate controls. This observation indicates that the potential role of NENF as a neurotrophic factor is certainly not as determinant for global body physiology as many of the classical neurotrophic factors.

It is known that NENF is broadly expressed in the adult brain (Kimura et al., 2005). The extensive expression profile of the neurotrophins family in the brain, is in accordance with its relevance in the correct maintenance of the CNS (Huang and Reichardt, 2001). In this sense, and although it has not yet been performed, a broad ontogenic neudesin expression characterization is of interest to better understand if spatial and temporal variations in the NENF expression pattern are determinant for region specific brain function. This is even more relevant given the behavioral phenotypes of neudesin-null mice described previously (Chapters 3 and 4). Despite the lack of this knowledge it is certain that NENF is only secreted by neurons (Kimura et al., 2005) and, although a possible membrane receptor was not found, it is known that its neurotrophic ability is

neuron specific (Kimura et al., 2005). Furthermore while NENF is expressed in neurons throughout the whole brain, apparently there are specific brain regions where neurons are more vulnerable to NENF's absence than others. Particularly, in this study we have observed that the amBNST and the ventral DG neurons have impoverished dendritic arborization in the absence of NENF (chapter 3). These alterations in cytoarchitecture were concomitant with the adult anxiety behavior phenotype observed in neudesin-null mice. The main brain regions involved in the anxiety process modulation are the ventral hippocampus, the amygdala and the BNST. The anxiety like behavior observed in neudesin-null males was in novel contextual paradigms such as the EPM, the LDB and the NSF. These are considered tests for state anxiety that rely on cortical processing, involving the hippocampus and the prefrontal cortex (Adhikari et al., 2010). Surprisingly neudesin-null mice did not present an anxious profile in the AS test. This test is based on a reflex response to an aversive stimulus that tests for a different dimension of anxiety, a more generalized and innate response (trait anxiety) highly dependent on the amygdala-BNST communication (Walker and Davis, 2008). In fact the ventral hippocampus might be the key structure in the behavioral phenotype observed in neudesin-null mice. Indeed, the ventral hippocampus appears to be involved in state anxiety processing that involves alterations in the HPA axis control providing negative feedback (Herman et al., 1995). The anteromedial division of the BNST (amBNST) receives projections mainly from the central and medial amygdala (CeA, MeA) and in turn projects to the PVN; the amBNST is the principal division of the BNST involved in the neuroendocrine regulation during stress response (Dong et al., 2001). This could explain that the decrease dendritic length observed in the amBNST of neudesin-null male animals does not translate in an impaired PVN activation, as it was not observed any other stress related alteration, like mood or cognition on neudesin-null animals. Given the alterations observed in the ventral hippocampus DG granular neurons and the amBNST neurons it would be interesting to evaluate if the stress response in neudesin-null mice is impaired upon stress exposure (such as chronic unpredictable stress).

Besides neurotrophic factors, there are many other molecular players that take place in the modulation of anxiety behavior. Examples of those players are neurosteroids, which are synthetized from cholesterol and/or steroids in the periphery and the CNS. Allopregnanolone, a progesterone metabolite, is one of the most common active steroids in the brain. These compounds act as allosteric modulators of neurotransmitter receptors, such as $GABA_AR$, NMDA and sigma receptors, acting as potent sedatives, anesthetics, anxiolytic or even anti-depressant

molecules (Reddy, 2010). Progesterone alone and its metabolites (allopregnanolone) also functions as a neurosteroid with anesthetics effect through the non-genomic activation of membrane PR (Korneyev and Costa, 1996;Reddy and Apanites, 2005). Regarding anxiety modulation, neurosteroids have revealed to be potent players. The GABAergic system is an important neurotransmitter system in the regulation of anxiety and actually anxiolytics, as benzodiazepines, are GABA receptors agonists. NENF is a member of the membrane associated progesterone receptor (MAPR) family, and although NENF binding to progesterone was not yet demonstrated, it was proposed to be a player in progesterone non-genomic actions (Kimura et al., 2013). Given the anxious-like phenotype of neudesin-null mice and since progesterone and its metabolites play a role in anxiety, it would be interesting to analyze the levels of progesterone and its precursors, such as allopregnanolone as well as the activity of the respective 5α reductase metabolizing enzyme, in brain regions involved in anxiety processing in neudesin-null mice. Most importantly, the fact that in neudesin-null mice only males appear to be susceptible to the hyper-anxious state further prompts for the analysis of these steroid metabolites.

As described previously, NENF's main cellular effect is the ability to induce embryonic neuron differentiation and survival in culture (Kimura et al., 2006). Here we studied the adult neurogenic niches of neudesin-null mice to evaluate if the absence of NENF resulted in impairment in proliferation and differentiation in the neurogenic niches. In this work we show that neudesin-null males display less proliferating neuroblasts at the ventral division of the hippocampus (chapter 5), which might translate into decreased integration of new neurons in the hippocampal circuitry. Furthermore we observed that, in the adult brain NENF modulates differently the adult SVZ and the SGZ neurogenic niches. Even further interesting, the absence of NENF results in a differential modulation of the neural stem cell niche at the dorsal and ventral DG axis. Also in this case, the ventral hippocampus revealed to be the most vulnerable structure to the absence of NENF. Importantly in disorders such as depression and anxiety (Evans et al., 2012;Sah et al., 2012) neurogenesis was also observed to be decreased, although the contribution of neurogenesis occurring at the dorsal or ventral division of the SGZ to these disorders is still a matter of debate (Banasr et al., 2006;Eadie et al., 2009;Lacefield et al., 2012;O'Leary et al., 2012). Nonetheless since dorso-ventral divisions of the hippocampus are functionally different neurogenesis occurring in each of these divisions has been implicated differentially: dorsal hippocampal neurogenesis in

cognition and ventral hippocampal neurogenesis in mood disorders (Kheirbek and Hen, 2011). Whether the decrease in ventral hippocampal neurogenesis is involved in the anxious-like phenotype observed in neudesin-null mice should next be studied in further detail. Namely how neudesin modulates neurogenesis and by rescuing neudesin's expression it reverts the observed effects in the DG and in the anxiety phenotype observed in neudesin-null mice.

The mismatch between neudesin-null males and females in the neural cell proliferation pattern at the hippocampal SGZ is another striking finding. Neudesin-null males have less cell proliferation in this neurogenic niche than their respective littermate controls, while in females this difference is not observed. Several intrinsic and extrinsic factors that modulate the neurogenic niche might explain gender differences, including life experiences, sexual hormones, pregnancy and lactation (Galea et al., 2013). As indicated above in the absence of NENF, males display less neural cell proliferation at the ventral division of the hippocampus and they present an anxious like behavior. Both findings were not observed in female neudesin-null mice. These observations were made in adult neudesin-null females nulliparous not synchronized for the estrous cycle. Given the fact that NENF was suggested to play a role in progesterone non-genomic actions (Kimura et al., 2013) it would be interesting to study if NENF absence affects neurogenesis and neuron morphology of the hippocampus, during different phases of the female adult life such as adolescence, pregnancy and post partum. It would then be possible to correlate NENF's function with different levels of sex hormones in particular progesterone. On the other hand, it would be interesting to study if chronic treatment with progesterone, reverts the differences observed in neudesin-null males

Neurotransmitters are key players in the modulation of complex behaviors and disruption in any neurotransmitter system balance can culminate in mild to severe behavior impairments. Anxiety behavior is also characterized by an imbalance in neurotransmission. In agreement, treatment with selective serotonin reuptake inhibiters (SSRIs) and norepinephrine reuptake inhibitors (SNRIs) were shown to have positive results in treating patients with generalized anxiety disorder (De Salas-Cansado et al., 2012) reinforcing a role for monoamines in this condition. The monoamine dopamine (DA) is a relevant modulator of the mesolimbic and mesocortical systems, which have been implicated in the regulation of mood (Diehl and Gershon, 1992). Interestingly parkinsonian patients exhibit anxiety states as social phobia (Stein et al., 2002). In this work we

show that neudesin-null mice have less DA reaching the ventral hippocampus, which was translated into a higher DA turnover ratio of HVA/DA. Previous studies have shown the differential contribution of dopamine D1 and D2 receptors for anxiety, suggesting a closer involvement of D1 activation in an anxiolytic effect (Rodgers et al., 1994). Importantly the ventral part of the hippocampus shows higher D1 expression, specifically in the DG granular neurons dendrites (Mansour et al., 1992). Neudesin-null mice present an atrophy of DG granular neurons. It is possible that the lack of neurotrophic support at the ventral hippocampal DG leads to a downregulation of D1 and hence impaired dopaminergic activity. On the other hand, it is known that DA modulates synaptic plasticity in this brain region (Hamilton et al., 2010), and thus diminished DA input might indirectly influence DG neurons morphology in neudesin-null mice.

The anxiety response mechanism is highly modulated by several different and complementary systems, making it hard to decipher the crucial elements for its regulation because of its heterogeneous nature. Levels of circulating neurotrophins, for instance BDNF, were shown to be altered in patients with impaired anxious response (Suliman et al., 2013). Furthermore a polymorphism in the human BDNF gene was correlated with a susceptibility to develop anxiety and depression (Montag et al., 2010;Li et al., 2013). The neurotrophic support provided to specific populations of neurons influences correct synapse formation and neurotransmission (Cohen-Cory, 2002). NENF is expressed in tyrosine hydroxylase (TH) neurons, particularly at the substantia nigra and the ventral tegmental area (VTA) (or own observation). It is possible that NENF is providing trophic support and thus is modulating DA afferent projections. On the other, hand the absence of NENF during development might result in subtle and specific alterations in dopaminergic circuitry formation. Another relevant fact, is that a GABAergic system dysregulation has been implicated in impaired DA release, pointing in a crosslink between anxiety and DA (Diehl and Gershon, 1992). Moreover, neurosteroids are known to modulate $GABA_AR$, and expression levels of the rate-limiting enzyme 5α reductase depends on dopaminergic system during stress paradigms (Bortolato et al., 2011). Some authors have shown a strong crosstalk between progesterone signaling and the dopaminergic system, with DA and some DA analogues being able to activate the progesterone nuclear receptor (PR) and hence influence gene expression (Foreman and Moss, 1979;Mani et al., 1994;Mani et al., 2009). It is not know if a similar cross-talk exists in what concerns the non-genomic actions of progesterone to which NENF is thought to be involved (Kimura et al., 2013).

As indicated above, many factors contribute to the development of an anxious-like state. The spatial (circuits involved) and the temporal (time window imprinting) interplay of the factors that elicit an anxiety state is also relevant. Here we observed that the absence of NENF manifests in an anxious like phenotype only when animals are exposed to novel environment paradigms. Although this phenotype is observed in adult males, the developmental contribution cannot be forsaken. The mouse model used in this work is a constitutive knockout model, which implies that from gestation homozygous knockout animals do not synthetize neudesin. Thus the CNS has to rearrange in order to deal with the absence of the protein during the apparent normal development of animals. Whether the impairments observed are due to i) the system response to deal with the absence of a relevant protein, or ii) the direct absence of the protein in the time point of analysis, are two questions that cannot be answered using this mouse model. We will next focus on the first aspect, in order to comprehend if neurodevelopment could have a possible contribution for the adult anxious phenotype observed.

Undeniably, in psychiatric disorders, the effect of genetic and environmental factors may converge on molecular pathways and brain circuits related to neurotrophic factors functioning. For instance it is known that the neurotrophic factor FGF2 has crucial role in brain development and that in the absence of it animals develop hyperactivity, as well as increased anxiety behavior (Terwisscha van Scheltinga et al., 2013). On the other hand it is known that early postnatal stress, such as maternal separation, leads to an adult anxious phenotype (Mesquita et al., 2007) and decreased BDNF expression in the hippocampal formation (Roceri et al., 2002). We studied if the absence of NENF influences behavior by affecting postnatal neurodevelopment. For that we have evaluated the postnatal acquisition of neurodevelopment reflexes in neudesin-null animals. We have observed that only neudesin-null males show a mild impairment in the acquisition of vestibular dependent neurological reflexes when compared to littermate controls. This was accompanied by altered monoaminergic activity in brain regions involved in the vestibular reflex maturation. These differences are transitory since all animals achieve maturity, which points for an adaptive time window of maturation that involves the adjustment of the system to the absence of NENF. Again, only neudesin-null males presented this delay in maturation, a further evidence reinforcing a possible sex-specific role for neudesin. The gender variance in neudesin-null mice calls our attention to the potential NENF action in progesterone activity (Kimura et al., 2013).

Nuclear PRs are more expressed in the developing (from E18 until PND10) male than female brain (Wagner, 2008), but after this period sex differences in the PR expression were not studied. PR is also transiently expressed in the posnatal dentate gyrus of the hippocampus, motor, sensory and reticular core nuclei (from P1 until P14) and in specific dopaminergic neuronal populations (Quadros et al., 2008). Although the perinatal ovary does not produce progesterone until the second week of life, high levels of progesterone are detectable in breast milk, and *de novo* synthesis of progesterone occurs in the male and female brain (Ingram et al., 1999;Micevych and Sinchak, 2008); this prompts for an important involvement of progesterone in brain development. NENF has 30% homology to the membrane progesterone receptor PGRMC1 (Kimura et al., 2012) and is thought to participate in progesterone non-genomic signaling (Kimura et al., 2013) but no evidence exists for its participation in progesterone genomic (PR dependent) actions. PGRMC1 is highly expressed and needed in the cerebellar cortex postnatal development (Sakamoto et al., 2008). We found that, the cerebellum of neudesin-null males shows a decrease in the activity of the serotonergic and dopaminergic systems. If the mild latency in neurologic reflexes acquisition presented by neudesin-null males depends more in the trophic action or in the progesterone signaling of NENF is yet to be deciphered. Furthermore, with the present evidence it is not possible to discriminate if this impairment contributes to the anxious like phenotype observed in adult neudesin-null males.

With the work developed in this thesis we demonstrate that neudesin is relevant for normal CNS functioning. Furthermore by pinpointing several novel roles for neudesin in the brain, we have elicited novel challenges that deserve further clarification. For instance, finding a link between the sex-specific modulatory actions of neudesin in the adult hippocampal neurogenic process and the anxiety-like behavior is certainly relevant and should next be studied.

References

Adhikari, A., Topiwala, M.A., and Gordon, J.A. (2010). Synchronized activity between the ventral hippocampus and the medial prefrontal cortex during anxiety. *Neuron* 65**,** 257-269.

Banasr, M., Soumier, A., Hery, M., Mocaer, E., and Daszuta, A. (2006). Agomelatine, a new antidepressant, induces regional changes in hippocampal neurogenesis. *Biol Psychiatry* 59**,** 1087-1096.

Bortolato, M., Devoto, P., Roncada, P., Frau, R., Flore, G., Saba, P., Pistritto, G., Soggiu, A., Pisanu, S., Zappala, A., Ristaldi, M.S., Tattoli, M., Cuomo, V., Marrosu, F., and Barbaccia, M.L. (2011). Isolation rearing-induced reduction of brain 5alpha-reductase expression: relevance to dopaminergic impairments. *Neuropharmacology* 60**,** 1301-1308.

Byerly, M.S., Swanson, R.D., Semsarzadeh, N.N., Mcculloh, P.S., Kwon, K., Aja, S., Moran, T.H., Wong, G.W., and Blackshaw, S. (2013). Identification of hypothalamic neuron-derived neurotrophic factor as a novel factor modulating appetite. *Am J Physiol Regul Integr Comp Physiol* 304**,** R1085-1095.

Cohen-Cory, S. (2002). The developing synapse: construction and modulation of synaptic structures and circuits. *Science* 298**,** 770-776.

De Salas-Cansado, M., Olivares, J.M., Alvarez, E., Carrasco, J.L., Barrueta, A., and Rejas, J. (2012). Pregabalin versus SSRIs and SNRIs in benzodiazepine-refractory outpatients with generalized anxiety disorder: a post hoc cost-effectiveness analysis in usual medical practice in Spain. *Clinicoecon Outcomes Res* 4**,** 157-168.

Diehl, D.J., and Gershon, S. (1992). The role of dopamine in mood disorders. *Compr Psychiatry* 33**,** 115-120.

Dong, H.W., Petrovich, G.D., and Swanson, L.W. (2001). Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res Brain Res Rev* 38**,** 192-246.

Eadie, B.D., Zhang, W.N., Boehme, F., Gil-Mohapel, J., Kainer, L., Simpson, J.M., and Christie, B.R. (2009). Fmr1 knockout mice show reduced anxiety and alterations in neurogenesis that are specific to the ventral dentate gyrus. *Neurobiol Dis* 36**,** 361-373.

Evans, J., Sun, Y., Mcgregor, A., and Connor, B. (2012). Allopregnanolone regulates neurogenesis and depressive/anxiety-like behaviour in a social isolation rodent model of chronic stress. *Neuropharmacology* 63**,** 1315-1326.

Foreman, M.M., and Moss, R.L. (1979). Role of hypothalamic dopaminergic receptors in the control of lordosis behavior in the female rat. *Physiol Behav* 22**,** 283-289.

Galea, L.A., Wainwright, S.R., Roes, M.M., Duarte-Guterman, P., Chow, C., and Hamson, D.K. (2013). Sex, hormones, and neurogenesis in the hippocampus: Hormonal modulation of neurogenesis and potential functional implications. *J Neuroendocrinol*.

Hamilton, T.J., Wheatley, B.M., Sinclair, D.B., Bachmann, M., Larkum, M.E., and Colmers, W.F. (2010). Dopamine modulates synaptic plasticity in dendrites of rat and human dentate granule cells. *Proc Natl Acad Sci U S A* 107**,** 18185-18190.

Herman, J.P., Adams, D., and Prewitt, C. (1995). Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. *Neuroendocrinology* 61**,** 180-190.

Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24**,** 677-736.

Ingram, J.C., Woolridge, M.W., Greenwood, R.J., and Mcgrath, L. (1999). Maternal predictors of early breast milk output. *Acta Paediatr* 88**,** 493-499.

Kheirbek, M.A., and Hen, R. (2011). Dorsal vs ventral hippocampal neurogenesis: implications for cognition and mood. *Neuropsychopharmacology* 36**,** 373-374.

Kimura, I., Konishi, M., Miyake, A., Fujimoto, M., and Itoh, N. (2006). Neudesin, a secreted factor, promotes neural cell proliferation and neuronal differentiation in mouse neural precursor cells. *J Neurosci Res* 83**,** 1415-1424.

Kimura, I., Nakayama, Y., Konishi, M., Terasawa, K., Ohta, M., Itoh, N., and Fujimoto, M. (2012). Functions of MAPR (membrane-associated progesterone receptor) family members as heme/steroid-binding proteins. *Curr Protein Pept Sci* 13**,** 687-696.

Kimura, I., Nakayama, Y., Yamauchi, H., Konishi, M., Miyake, A., Mori, M., Ohta, M., Itoh, N., and Fujimoto, M. (2008). Neurotrophic activity of neudesin, a novel extracellular heme-binding protein, is dependent on the binding of heme to its cytochrome b5-like heme/steroid-binding domain. *J Biol Chem* 283**,** 4323-4331.

Kimura, I., Nakayama, Y., Zhao, Y., Konishi, M., and Itoh, N. (2013). Neurotrophic effects of neudesin in the central nervous system. *Front Neurosci* 7**,** 1-11.

Kimura, I., Yoshioka, M., Konishi, M., Miyake, A., and Itoh, N. (2005). Neudesin, a novel secreted protein with a unique primary structure and neurotrophic activity. *J Neurosci Res* 79**,** 287-294.

Korneyev, A., and Costa, E. (1996). Allopregnanolone (THP) mediates anesthetic effects of progesterone in rat brain. *Horm Behav* 30**,** 37-43.

Lacefield, C.O., Itskov, V., Reardon, T., Hen, R., and Gordon, J.A. (2012). Effects of adult-generated granule cells on coordinated network activity in the dentate gyrus. *Hippocampus* 22**,** 106-116.

Li, Y.J., Xu, M., Gao, Z.H., Wang, Y.Q., Yue, Z., Zhang, Y.X., Li, X.X., Zhang, C., Xie, S.Y., and Wang, P.Y. (2013). Alterations of serum levels of BDNF-related miRNAs in patients with depression. *PLoS One* 8**,** 63648.

Mani, S.K., Allen, J.M., Clark, J.H., Blaustein, J.D., and O'malley, B.W. (1994). Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. *Science* 265**,** 1246-1249.

Mani, S.K., Portillo, W., and Reyna, A. (2009). Steroid hormone action in the brain: cross-talk between signalling pathways. *J Neuroendocrinol* 21**,** 243-247.

Mansour, A., Meador-Woodruff, J.H., Zhou, Q., Civelli, O., Akil, H., and Watson, S.J. (1992). A comparison of D1 receptor binding and mRNA in rat brain using receptor autoradiographic and in situ hybridization techniques. *Neuroscience* 46**,** 959-971.

Mesquita, A.R., Pego, J.M., Summavielle, T., Maciel, P., Almeida, O.F., and Sousa, N. (2007). Neurodevelopment milestone abnormalities in rats exposed to stress in early life. *Neuroscience* 147**,** 1022-1033.

Micevych, P., and Sinchak, K. (2008). Synthesis and function of hypothalamic neuroprogesterone in reproduction. *Endocrinology* 149**,** 2739-2742.

Montag, C., Basten, U., Stelzel, C., Fiebach, C.J., and Reuter, M. (2010). The BDNF Val66Met polymorphism and anxiety: support for animal knock-in studies from a genetic association study in humans. *Psychiatry Res* 179**,** 86-90.

O'leary, O.F., O'connor, R.M., and Cryan, J.F. (2012). Lithium-induced effects on adult hippocampal neurogenesis are topographically segregated along the dorso-ventral axis of stressed mice. *Neuropharmacology* 62**,** 247-255.

Quadros, P.S., Schlueter, L.J., and Wagner, C.K. (2008). Distribution of progesterone receptor immunoreactivity in the midbrain and hindbrain of postnatal rats. *Dev Neurobiol* 68**,** 1378-1390.

Reddy, D.S. (2010). Neurosteroids: endogenous role in the human brain and therapeutic potentials. *Prog Brain Res* 186**,** 113-137.

Reddy, D.S., and Apanites, L.A. (2005). Anesthetic effects of progesterone are undiminished in progesterone receptor knockout mice. *Brain Res* 1033**,** 96-101.

Roceri, M., Hendriks, W., Racagni, G., Ellenbroek, B.A., and Riva, M.A. (2002). Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Mol Psychiatry* 7**,** 609-616.

Rodgers, R.J., Nikulina, E.M., and Cole, J.C. (1994). Dopamine D1 and D2 receptor ligands modulate the behaviour of mice in the elevated plus-maze. *Pharmacol Biochem Behav* 49**,** 985-995.

Sah, A., Schmuckermair, C., Sartori, S.B., Gaburro, S., Kandasamy, M., Irschick, R., Klimaschewski, L., Landgraf, R., Aigner, L., and Singewald, N. (2012). Anxiety- rather than depression-like behavior is associated with adult neurogenesis in a female mouse model of higher trait anxiety- and comorbid depression-like behavior. *Transl Psychiatry* 2**,** e171.

Sakamoto, H., Ukena, K., Kawata, M., and Tsutsui, K. (2008). Expression, localization and possible actions of 25-Dx, a membraneassociated putative progesterone-binding protein, in the developing Purkinje cell of the cerebellum: a new insight into the biosynthesis, metabolism and multiple actions of progesterone as a neurosteroid. *Cerebellum* 7**,** 18-25.

Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* 77**,** 627-638.

Stein, D.J., Westenberg, H.G., and Liebowitz, M.R. (2002). Social anxiety disorder and generalized anxiety disorder: serotonergic and dopaminergic neurocircuitry. *J Clin Psychiatry* 63 Suppl 6**,** 12-19.

Suliman, S., Hemmings, S.M., and Seedat, S. (2013). Brain-Derived Neurotrophic Factor (BDNF) protein levels in anxiety disorders: systematic review and meta-regression analysis. *Front Integr Neurosci* 7**,** 1-11.

Terwisscha Van Scheltinga, A.F., Bakker, S.C., Kahn, R.S., and Kas, M.J. (2013). Fibroblast Growth Factors in Neurodevelopment and Psychopathology. *Neuroscientist* 5**,** 479-494.

Wagner, C.K. (2008). Progesterone receptors and neural development: a gap between bench and bedside? *Endocrinology* 149**,** 2743-2749.

Walker, D.L., and Davis, M. (2008). Role of the extended amygdala in short-duration versus sustained fear: a tribute to Dr. Lennart Heimer. *Brain Struct Funct* 213**,** 29-42.