# Effects of neural nitric oxide synthase gene inactivation on the neuroendocrine stress response in mice

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*To my family*

# CONTENTS

# 1 Introduction

## 1.1 The concept of stress

The ability of mammals to adequately respond to environmental challenges by physiological and behavioural alterations is a prerequisite for their survival in a permanently changing environment. All these responses are aimed at controlling the disturbing stimuli and, thus, reducing their potential harmfulness (for review, see Koolhaas *et al.*, 1999). Relevant stimuli are emotionally evaluated by the limbic system that controls the endocrine and the autonomic nervous system simultaneously with the behavioural response. The outcome of the emotional evaluation depends on the genetic background, earlier conditioning and individual past life experiences. Thus, the interpretation of stimuli as "challenging" or "disturbing" is a highly subjective event. If, because of innate or acquired capabilities, the individual is able to efficiently respond to a defined stimulus, no significant physiological or behavioural efforts will be required. However, if a stimulus was never encountered before or a known stimulus is perceived in an unexpected strength or duration, the individual must either refine an available strategy or develop a new one. The immediate responses that are set in motion in the latter case are defined coping strategies (Chrousos & Gold, 1992). Active coping mechanisms help animals to successfully control sudden aversive encounters: physiological (tachycardia, increased blood pressure) and behavioural (startle, fight or flight) adaptive responses are linked to the activation of the sympatho-adrenal system (SAS), and are characterised by the release of catecholamine into the blood. If the potentially threatening stimulus can not be controlled by "fight or flight" behaviour, animals adopt passive coping strategies. These are linked to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which triggers the release of glucocorticoids into the bloodstream, leading to significant physiological changes, such as lipolysis, gluconeogenesis, inhibition of growth, immune and reproductive systems, suppression of feeding, increased state of vigilance, arousal and cognition.

Hans Selye was the first who introduced the concept of stress to characterise the responses of the organism to challenging stimuli. He named it more specifically "general adaptive syndrome", since he observed that, although different stimuli evoke distinct patterns of adaptive responses, their specificity is gradually reduced as the gravity of the stressor augments (Selye, 1936; 1950; 1955). Although Selye's approach has been widely accepted, his definition of "stress" and "stressor" have been criticised as circular by other researches

(Lazarus & Folkman, 1984) and alternative definitions were proposed. Since there is still no general agreement among the scientific community concerning the definition of "stress" and "stressor", the present work will be referring to Chrousos (1998) who defined stress as "an animal threatened homeostasis", and stressor as "the disturbing forces or threats to homeostasis". Since in animals, unlike in humans, it is not possible to evaluate directly the emotional interpretation of an external stimulus, a physiological parameter must be used as an indicator to determine whether a defined stimulus has threatened the animal homeostasis and, thus, elicited stress. In the present study we defined Cort blood values higher than 100 ng/ml to be a sufficient sign of an ongoing stress reaction, since the occurrence of a significant rise of blood corticosterone (Cort) levels is regarded to as the principal endocrine marker of the stress response (Maier *et al.*, 1986; Mormede *et al.*, 1988),.

As an acute and time-limited phenomenon, the physiological consequences of a stress response have beneficial effects to gain control over a stressor. However, if the stress is protracted for a long time, or if its magnitude exceeds a certain threshold, a severe pathological state can arise predominantly due to a sustained action of the HPA-axis. For instance, a prolonged state of stress-related arousal can lead to anxiety, and increased vigilance can become insomnia, which are characteristic traits of melancholic depression (Gold *et al.*, 1988b; a). Similarly, decreased attention to feeding, which is advantageous for the organism in the context of the stress response, becomes exaggerated in illnesses like anorexia nervosa (Gold *et al.*, 1986; Kaye *et al.*, 1987). Also panic disorder (Roy-Byrne *et al.*, 1986; Gold *et al.*, 1988c) and obsessive-compulsive disorders (Insel *et al.*, 1982) have been associated with a pathological activation of the systems involved in the stress. Thus, understanding the mechanisms that modulate the stress response may help develop new tools to successfully treat a number of psychiatric diseases.

Under laboratory conditions, several experimental stressor paradigms have been established to examine the activity of the SAS, the HPA-axis and also the hypothalamic-neurohypophysial system (HNS), which has been postulated to be involved in coordinating the neuroendocrine response (Engelmann & Ludwig, 2004) to acute or chronic exposure to stressors. Stressors are generally categorised as inducing two different types of stress, "systemic" (also referred to as physical) and "neurogenic" (also referred to as predictive, emotional or psychological). Systemic stress is the response to real, physical threats that cannot be consciously appreciated, since they are perceived through distinct central or peripheral receptors and relayed via hard-wired viscero-somatic pathways: Inflammatory reactions, pain and osmotic alterations are examples of this type of stressors. By contrast,

neurogenic stress, for instance induced by immobilisation or electrical footshock, are predominantly emotional perturbations that involve cognitive and limbic processing. Although the two types of stress may result in selective activation of defined brain areas, both culminate in increasing the level of circulating glucocorticoids (Pacak & Palkovits, 2001). If these adaptive responses are sufficient to re-establish control over the stressor, the state of stress has no deleterious effects on the organism. The overall response remains physiological and is, therefore, called "eustress". Conversely, states of chronic hyper- or hypoactivation of the HPA-axis are termed "distress", as they are harmful to the body, thus leading to a pathological state (Chrousos, 1998).

As indicated above, a thorough understanding of the mechanisms underlying the regulation of a normal stress response under physiological conditions might help to delineate new therapeutic approaches for the treatment of stress-related psychiatric disorders. Therefore, we decided to employ an experimental approach, namely forced swimming, that leads to an "eustress" state. By exposure to this stressor, the lab animal is forced to cope with a new, acute challenge from which it can not escape, but with which it can cope. Moreover, forced swimming, inducing both systemic and neurogenic stress, resembles most naturally occurring stressors, and allows to measure simultaneously different variables of the stress response, including behaviour of the animals and their neuroendocrine activity. Thus, a more complete answer to the questions posed can be provided. Previous studies have shown that, in rodents, forced swimming activates brain regions deputed to control the stress response (Engelmann *et al.*, 1998; Wotjak *et al.*, 1998; Wotjak *et al.*, 2001; Salchner *et al.*, 2004; Drugan *et al.*, 2005). Therefore, this stressor seems to be an appropriate stimulus to investigate the regulatory mechanisms that modulate the endocrine stress response.

## 1.2 The sympatho-adrenal system

The neuroendocrine stress response follows a determined time course, which begins with the activation of the SAS within a few seconds from stressor onset and proceeds with the glucocorticoid peak around 30 min later. The activation of the SAS is characterised by the increased release of catecholamines into the bloodstream to allow for and modulate the "fight or flight" reaction. In laboratory rodents, a subpopulation of oxytocin (OXT)-containing parvocellular neurones of the hypothalamic paraventricular nucleus (PVN) regulates the SAS activity (Swanson & Sawchenko, 1980). These neurones provide the main input to the medulla, where they make synaptic contacts to neurones that descend to the sympathetic

system in the spinal cord (Swanson, 1987). Via this pathway, the hypothalamus gains direct control over catecholamine release since the adrenal medulla receives preganglionic sympathetic innervation from the greater (major supply) and lesser thoracic splanchnic nerve (Fig. 1). The synaptic nature of this transmission renders the “fight or flight” response an immediate reaction. The release of norepinephrine (NE) and epinephrine (E) into the bloodstream in response to stressor exposure is aimed at promoting alertness and elevated perfusion of skeletal muscle, brain, and liver, increased heartbeat and blood pressure, rise in blood sugar. Catecholamines are produced by sequential enzymes located in chromaffin cells: tyrosine hydroxylase (TH), which is the rate-limiting enzyme in catecholamine biosynthesis, aromatic L-amino acid decarboxylase and dopamine beta-hydroxylase. The final biosynthetic step occurs inside catecholamine storage vesicles, while the other biosynthetic enzymes are cytosolic. In chromaffin cells of the adrenal medulla (as well as epinephrinergic neurons of the central nervous system), another biosynthetic step occurs in the cytosol: phenylethanolamine N-methyltransferase (PNMT) N-methylates NE to form E. Forced swimming and chronic isolation are among the stressors that have been reported to enhance catecholamine plasma levels (Itoh *et al.*, 2006; for review, see Nankova & Sabban, 1999).

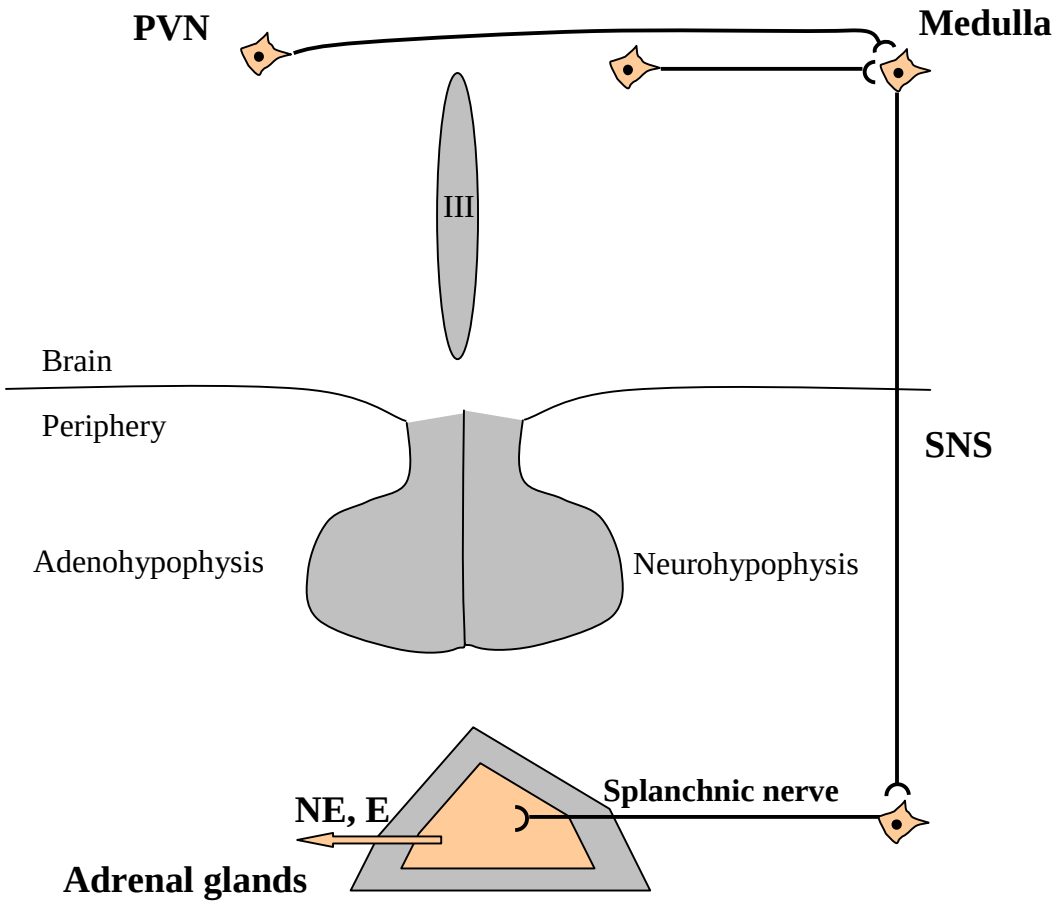


Fig 1. Schematic representation of the SAS. The synaptic pathways originating in the PVN lead to catecholamine (NE, E) exocytosis from the adrenal medulla into the blood (arrow). PVN neurones synaptically contact a population of neurones in the medulla, which in turn project to sympathetic pre-ganglionic neurones of the spinal cord that, through the splanchnic nerve, directly relay the information to chromaffin cells. PVN: paraventricular nucleus; III: third ventricle; SNS: sympathetic nervous system; NE: norepinephrine; E: epinephrine.

## 1.3 The hypothalamic-pituitary–adrenal axis

In addition to the cells that constitute the central origin of the SAS, the PVN harbours also another population of parvocellular neurosecretory neurones to which stress-related information is relayed. These neurones comprise the central nervous structure of the HPA-

axis, and initiate the neuroendocrine stress cascade by secreting corticotropin-releasing hormone into the portal blood (CRH; Vale *et al.*, 1981). CRH consists of 41-amino acid residues and is produced by cleavage of the 196-amino acid C-terminus of prepro-CRH in the parvocellular neurosecretory neurones of the PVN. Corticotrope cells of the anterior pituitary express high levels of the G-protein-coupled receptor subtype designated CRH-$R_1$ (Sanchez *et al.*, 1999b), which mediates CRH-induced adrenocorticotropic hormone (ACTH) release into the peripheral blood that in turn promotes the release of glucocorticoids (Cort in rodents and cortisol in humans) from the adrenal cortex (Fig. 2; for review, see Angelucci, 2000; Korte, 2001; Makara & Haller, 2001).

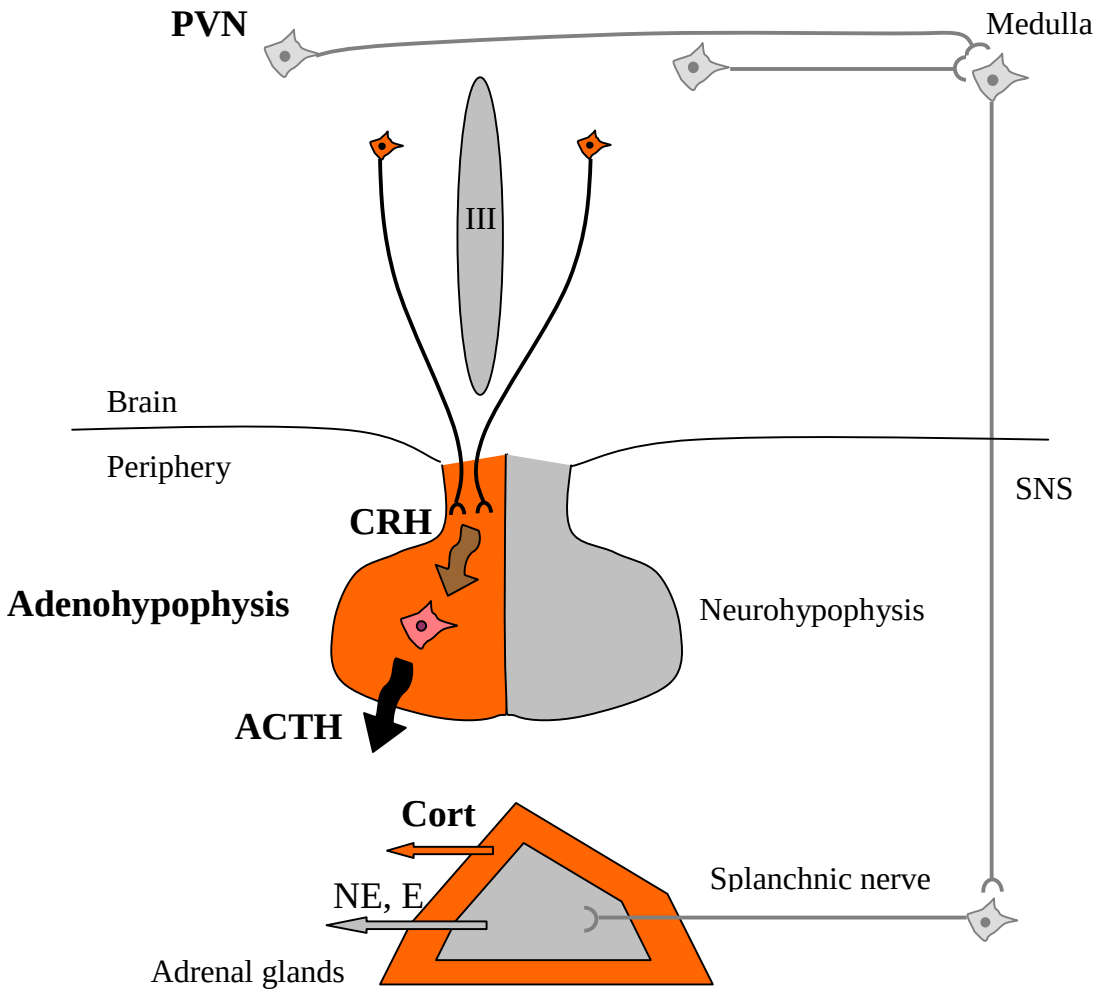


Fig. 2. Schematic representation of the SAS and the HPA-axis. CRH-containing neurones (red somata) of the PVN project to the adenohypophysis, where they release CRH into the portal blood (brown arrow). Upon CRH stimulation, corticotropes secrete ACTH into the general circulation (black arrow), which in turn elicits Cort release from the adrenal cortex (red arrow): CRH: corticotrophin-releasing hormone; ACTH:

adrenocorticotropin; Cort: corticosterone.; arrows represent neurohormones released into the portal blood and into the peripheral blood. See Fig. 1 for more details.

The levels of CRH in the hypophyseal blood peak within 1 minute from stressor onset (Plotsky *et al.*, 1987). CRH mRNA levels in the parvocellular cells of the PVN increase between 2 to 4 h in response to acute stressor exposure to replenish cellular stores (Ma *et al.*, 1997). Chronic stress conditions, however, produce a more pronounced increase of plasma ACTH compared to that under acute stress (Antoni, 1986). This is due to the action of vasopressin (AVP), which is co-secreted by parvocellular neurones into the portal blood (for review, see Antoni, 1993; Aguilera & Rabadan-Diehl, 2000). In fact, AVP of parvocellular origin robustly enhances the action of CRH on corticotropes in a synergistic manner, suggesting an important role for AVP during excessive stimulation of the HPA-axis.

ACTH is secreted into the blood in a constant, pulsatile manner, which shows a diurnal variation. During normal, non-stressful situations, the release of ACTH in humans follows a circadian rhythm with the highest levels occurring around 8:00 am in the morning and the lowest levels around midnight. Exposure to acute stressors increases the frequency and the amplitude of hypothalamic hormonal pulses towards the anterior pituitary, to cope with higher demand of circulating glucocorticoids (for review, see Jacobson, 2005).

ACTH enters the systemic circulation and binds to high affinity receptors located on the surface of adrenal cortical cells, thereby triggering the secretion of Cort. Cort is not stored in the adrenal cortex, but is quickly secreted upon production. The adrenal cortex synthesizes Cort to preserve basal serum levels for only few minutes, thus the effect of ACTH on Cort production can be observed in the blood within minutes from its stimulation.

Interestingly, the integrity of the sympathetic innervation is required to maintain normal basal levels of Cort, which persists also in CRH knock-out (KO) mice (Ottenweller & Meier, 1982; Edwards & Jones, 1987; Dijkstra *et al.*, 1996). Therefore, it has been suggested that splanchnic nerve stimulation influences also the activity of the adrenal cortex (Ehrhart-Bornstein *et al.*, 1995). The mechanism by which the SAS promotes glucocorticoid secretion into the blood is still elusive, but the close anatomical localisation of the medulla and the cortex, as well as the presence of chromaffin cells also in the cortical area (Bornstein *et al.*, 1991), and, conversely, cortical cells within the medulla (Bornstein *et al.*, 1994), implies an intensive cross-talk between the two systems. Indeed, there is evidence for a direct influence of catecholamines on cortical function (Bornstein *et al.*, 1990).

## 1.4 The hypothalamic-neurohypophyseal system

A third population of cells that reside within the PVN and are involved in the processing of stress-related information are the magnocellular neurones of the HNS. The HNS is composed of magnocellular neurones of the PVN and of the supraoptic nucleus (SON), which extends atop the optical chiasma. The axons of these neurones project through the internal part of the median eminence to the neurohyphysis, where AVP and OXT are released from their terminals into the bloodstream. These hormones govern body fluid homeostasis (AVP), reproduction and mating behaviour (OXT, for review, see Cunningham & Sawchenko, 1991), but are also released in response to defined stressor exposure (Lang *et al.*, 1983; Wotjak *et al.*, 1996b; Wotjak *et al.*, 1998; Fig. 3).

Since the HNS was the first neuroendocrine system discovered, it was originally believed to modulate ACTH secretion through the release of AVP of magnocellular origin (Bargmann, 1949; Bargmann & Scharrer, 1951; McCann & Brobeck, 1954; Mirsky *et al.*, 1954; Martini & Monpurgo,1955). However, this theory lost importance after the discovery of the parvocellular pathway to the adenohypophysis and after Vale *et al.* (1981) demonstrated that these parvocellular neurones secrete CRH, the most potent secretagogue of ACTH. In the past years, the interaction between AVP/OXT and ACTH release gained renewed interest. Several studies revaluated the HNS with regard to its function in modulating the HPA-axis and the stress response (for review, see Engelmann *et al.*, 2004a).

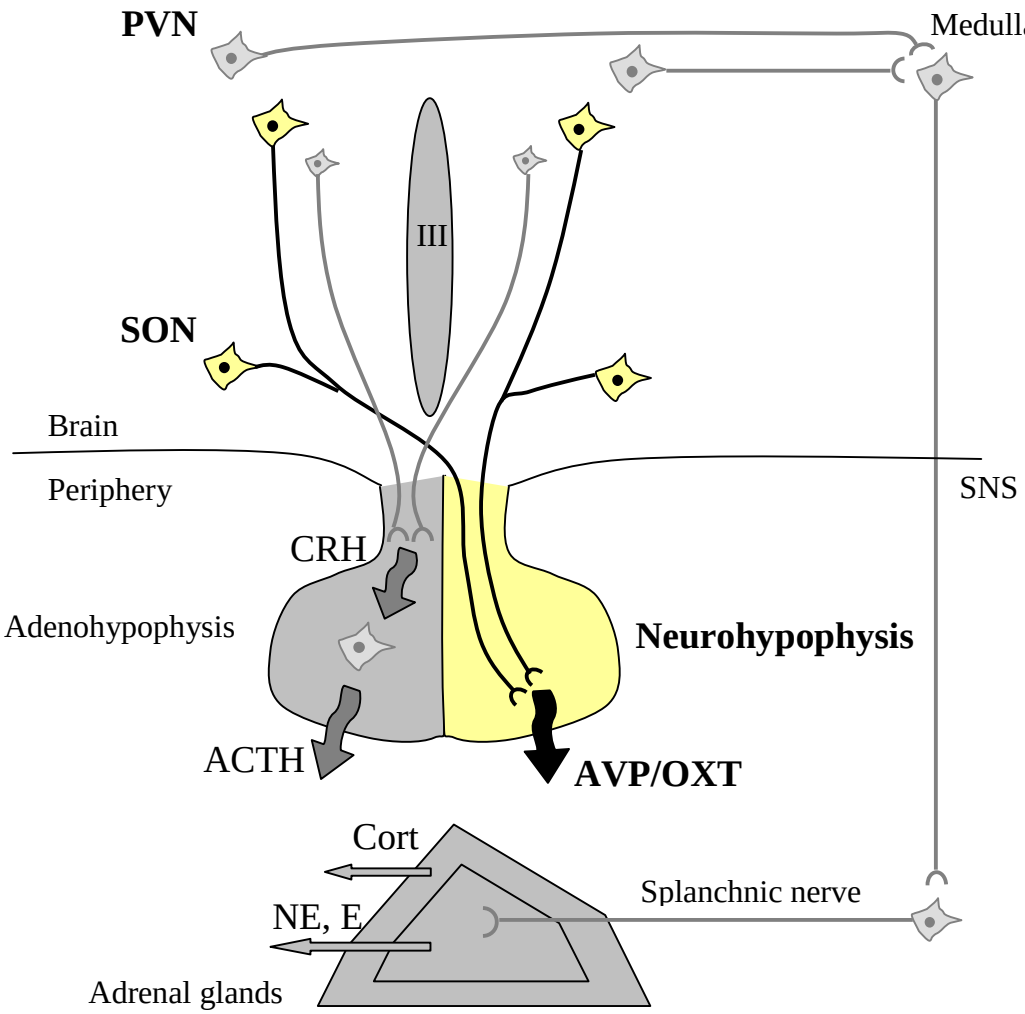


Fig. 3. Schematic representation of the SAS, the HPA-axis and the HNS with the focus on the latter endocrine system. Magnocellular neurones of the PVN and the SON project to the neurohypophysis and release AVP and OXT from their axon terminals into the peripheral blood (black arrow). AVP: vasopressin; OXT: oxytocin; SON: supraoptic nucleus. See Fig. 1 and 2 for more details.

In fact, increasing evidence indicates that the HPA-axis and the HNS closely interact with each other (Wotjak *et al.*, 2001; Engelmann *et al.*, 2004a) to orchestrate a finely tuned stress response. Numerous studies have shown that in the rat, other than in humans, OXT, but not AVP, levels increase in the blood in response to a variety of stressors (Lang *et al.*, 1983; Gibbs, 1984). Moreover, both AVP and OXT may be released from somata and dendrites of magnocellular neurones into the extracellular space of the PVN and the SON (Di Scala-Guenot *et al.*, 1987; Pow & Morris, 1989; Hattori *et al.*, 1990; Landgraf & Ludwig, 1991; Hattori *et al.*, 1992; Ludwig *et al.*, 1994) not only during thirst (Ludwig *et al.*, 1996), suckling

and parturition (Neumann *et al.*, 1993), but also in response to defined stressor exposure, including forced swimming (Wotjak *et al.*, 1998). In the rat, the intra-hypothalamic release of OXT induced by forced swimming is paralleled by an enhanced secretion of this neuropeptide into the blood. In contrast, AVP plasma levels remain unchanged after forced swimming, despite AVP intra-hypothalamic release being significantly increased. The latter dichotomy reflects a peculiar feature of HNS neurones, which is the capability of independently regulating the central, i.e. somato-dendritic, from the peripheral, i.e. axonal, release of AVP and OXT. The peripheral secretion of AVP in the rat, unlike in humans (Kohl, 1992; Dugue *et al.*, 1993), appears therefore to be tightly regulated and limited to situations linked to body hydro-mineral balance. For instance, electrical stimulation of the SON (Makara *et al.*, 1982) evoke a significant rise also in Cort plasma levels. Moreover, animal models characterised by a disrupted magnocellular AVP tone display a pronounced hypo-activity of the HPA-axis (Conte-Devolx *et al.*, 1982; Dohanics *et al.*, 1991).

On the other hand, several studies have pointed out the contribution of AVP of magnocellular origin in modulating the activity of the HPA-axis. More detailed analysis revealed that AVP and OXT synthesised by magnocellular neurones may reach the adenohypophysis through the short portal vessels that connect the posterior and the anterior pituitary. In addition, both neuropeptides can be released *en passant* from axons at the neurohypophysis into the long portal vessels that run from the median eminence to the anterior pituitary (Holmes *et al.*, 1986; Wotjak *et al.*, 1996a). AVP can promote ACTH secretion into the bloodstream by activating the V1b receptor subtype located on corticotropes of the adenohypophysis. In contrast to this action as secretagogue at the level of the pituitary, AVP released from somata and dendrites within the SON and the PVN inhibits the ACTH secretion and the activation of magnocellular neurones (Hermes *et al.*, 2000; Wotjak *et al.*, 2002; Hirasawa *et al.*, 2003). This effect is mediated by the V1a receptor subtype, which is widely expressed throughout the hypothalamus (Ostrowski *et al.*, 1994). Finally, other investigators have reported the existence of a finely tuned mechanism depending on the initial state of activation of AVP neurones, with an excitatory autocrine effect of somato-dendritically released AVP on quasi-silent neurones and an inhibitory effect on highly active neurones (Gouzenes *et al.*, 1998).

The peripheral secretion of OXT following defined stressor exposure, like forced swimming (Wotjak *et al.*, 1998) or shaker stress (Nishioka *et al.*, 1998), is a well characterised feature of the stress response in the rat. However, its biological significance is still unclear. Some investigators have suggested that OXT might be involved in metabolic regulation by acting on the pancreas (Stock *et al.*, 1990) and on adipocytes (Lederis *et al.*, 1985), but whether such

effects explain the increased peripheral secretion of OXT in response to stressor exposure remains speculative. Like AVP, also OXT has been shown to act at the level of the median eminence/neurohypophysis to promote ACTH release from the adenohypophysis, although less efficiently than AVP (Schlosser *et al.*, 1994). Similarly, intra-PVN released OXT appears to reduce ACTH and Cort secretion (Neumann *et al.*, 2000a; Neumann *et al.*, 2000b; for review, see Neumann, 2002), which is seems to be a counterbalancing mechanism most likely aimed at preventing an overshooting of the HPA-axis. Conversely, it remains contentious the role intra-SON released OXT plays, with reports of auto-inhibitory and auto-excitatory actions on magnocellular neurones via modulation of pre- and post-synaptic inputs (Brussaard *et al.*, 1996; Pittman *et al.*, 2000; Kombian *et al.*, 2002; Landgraf & Neumann, 2004). Taken together, the findings collected above indicate that the influence of the HNS on the activity of the HPA axis is an important player in the game of balancing forces that coordinate the stress response.

## 1.5 Nitric oxide modulation of the stress response

The neuroendocrine stress response is modulated by an intricate interplay of various neurotransmitters, among which, not only intrahypothalamically released AVP and OXT, but also nitric oxide (NO) have emerged as significant factors. NO is a highly diffusible free radical gas that is derived through an oxidative reaction catalysed by the nitric oxide synthase (NOS) from L-arginine and oxygen to produce citrulline and NO (Alderton *et al.*, 2001). Due to its diffusible nature and its ability to freely cross cell membranes, NO can act in an autocrine and paracrine manner also on targets relatively distant from the place of its production (Wood & Garthwaite, 1994). To date, three subtypes of NOS have been described: 1) the inducible NOS (iNOS), which may be induced in macrophages, hepatocytes, microglia and other cell types (Bandaletova *et al.*, 1993) upon stimulation with lipopolysaccharides and cytokines (Xie *et al.*, 1992), 2) the endothelial NOS (eNOS), which is mainly found in the endothelium of blood vessels (Marsden *et al.*, 1993) (Xue *et al.*, 1994; Reiling *et al.*, 1996; Wang *et al.*, 1996; Abe *et al.*, 1997; Helfrich *et al.*, 1997; Colasanti *et al.*, 1998), and 3) the neural NOS (nNOS; Bredt *et al.*, 1990), which is almost exclusively expressed in neurones and astrocytes (Arbones *et al.*, 1996; Cork *et al.*, 1998; Asano *et al.*, 1994; Kobzik *et al.*, 1994; Magee *et al.*, 1996; Shimizu *et al.*, 1997; Xu *et al.*, 1999). Interestingly, nNOS expression has been observed also in the adrenal medulla, which is a tissue of ectodermal origin, as are neurones. High levels of nNOS have been demonstrated by biochemical and

immunocytochemical methods in chromaffin cells (Oset-Gasque *et al.*, 1994; Schwarz *et al.*, 1998), and also in fibres closely associated with them (Afework *et al.*, 1994; Heym *et al.*, 1994; Tanaka & Chiba, 1996). Data derived from pharmacological studies suggest that NO inhibits catecholamine release evoked by depolarising stimuli like acetylcholine, nicotine and high potassium chloride (KCl). NO acts by elevating intracellular cyclic guanidine-monophosphate (cGMP) and activating protein kinase G (PKG), which selectively inhibit voltage-dependent $Ca^{++}$ influx and therefore reduce catecholamine exocytosis (Oset-Gasque *et al.*, 1994; Uchiyama *et al.*, 1994; Rodriguez-Pascual *et al.*, 1996; Schwarz *et al.*, 1998). These data suggest that NO controls catecholamine secretion under conditions of high levels of stimulation.

NO has also attracted considerable attention as potential modulator of the HPA-axis (Givalois *et al.*, 2002). Indeed, nNOS is widely present in the HPA-axis or in closely related anatomical structures. In the PVN, the most abundant subtype is nNOS (Bhat *et al.*, 1996), which is expressed in the HPA-axis and in medullary-projecting preautonomic neurones (Rodrigo *et al.*, 1994; Siaud *et al.*, 1994; Nylen *et al.*, 2001a). It is activated by the glutamate-driven opening of the ionotropic N-methyl-D-aspartate (NMDA) receptor, which leads to increased cytosolic levels of free calcium. iNOS is not evident in the PVN under basal conditions but only upon lipopolysacchride stimulation (Lopez-Figueroa *et al.*, 1998), while eNOS is not expressed within the PVN itself, but has been described in endothelial cells of hypophyseal blood vessel (Ceccatelli *et al.*, 1996). The external zone of the median eminence, where parvocellular axonal terminals project towards the anterior pituitary, shows only little nNOS immunoreactivity, whereas at the level of the anterior pituitary nNOS is present in folliculo-stellate cells and gonadotrophs, but not in corticotrophs (Ceccatelli *et al.*, 1993; Wang *et al.*, 1997). Finally, nNOS is expressed in the adrenal cortex, where nNOS mRNA has been shown to increase markedly following immobilisation stress (Kishimoto *et al.*, 1996; Tsuchiya *et al.*, 1996).

Finally, nNOS is widely expressed also throughout the HNS. Several studies have demonstrated the presence of nNOS and nicotinamide dinucleotide phosphate (NADPH)-diaphorase activity, a histochemical marker of NOS, in magnocellular neurones of both the SON and the PVN (Summy-Long *et al.*, 1984; Arevalo *et al.*, 1992; Rodrigo *et al.*, 1994). Co-localisation studies have shown that nNOS is present in a large percentage of oxytocinergic magnocellular cells, and to a lesser extent, also in vasopressinergic neurones (Nylen *et al.*, 2001a; Nylen *et al.*, 2001b). This enzyme is also abundant at the level of the neurohypophysis (Rodrigo *et al.*, 1994; Alm *et al.*, 1997). *In vivo* (Okere *et al.*, 1996) and *in vitro* (Liu *et al.*,

1997b; Ozaki *et al.*, 2000; Stern & Ludwig, 2001) electrophysiological studies speak in favour of an inhibitory action of NO on the electrical activity of vasopressinergic and oxytocinergic neurones, which seems to be exerted through a potentiation of gamma-aminobutyric acid (GABA) innervation (Bains & Ferguson, 1997; Stern & Ludwig, 2001; Li *et al.*, 2004).

Despite there is increasing agreement that NO of nNOS origin is involved in the control of all three neuroendocrine systems that coordinate the stress response, conflicting data have been reported and the influence of NO on these systems remains contentious. For instance, the role of NO on basal catecholamine secretion is still a matter of debate: Some investigators have shown a stimulatory effect (Oset-Gasque *et al.*, 1994; Uchiyama *et al.*, 1994), while some have reported an inhibitory action (Ward *et al.*, 1996) or no effect (Marley *et al.*, 1995; Rodriguez-Pascual *et al.*, 1995) of NO on intracellular $Ca^{++}$ concentration and catecholamine exocytosis under resting conditions. A third group of authors observed a long-term up-regulation by NO of the genes encoding for the catecholamine biosynthetic enzymes (Kim *et al.*, 2003). These contradictory results might be due to the use of dissociated chromaffin cell cultures, which contain a different proportion of adrenergic/noradrenergic cells according to the method of separation. NOS is clearly asymmetrically distributed among chromaffin cells, with noradrenergic cells being the main NOS-immunoreactive subpopulation of the adrenal medulla (Dun *et al.*, 1993; Heym *et al.*, 1994). Thus, the average response, for instance in terms of $Ca^{++}$ influx, observed in a mixed population following pharmacological stimulation might be significantly affected by the proportion of noradrenergic versus adrenergic cells present in culture. The different subcellular localisation of NOS in the adrenal medulla speaks in favour of a functional segregation of this enzyme, with noradrenergic cells specialised in producing NO, whereas adrenergic cells might represent its main target (Oset-Gasque *et al.*, 1998).

As for the SAS, there is little agreement amongst investigators on the influence of NO on the HPA-axis. Earlier investigations addressing this issue yielded conflicting results, which can be ascribed to different experimental approaches, for instance *in vivo* versus *in vitro* experiments, or peripheral versus central pharmacological treatment (Costa *et al.*, 1993; Rivier & Shen, 1994; Giordano *et al.*, 1996; Lee *et al.*, 1999; Riedel, 2000). However, in the past years it has became increasingly apparent that NO may exert mutually opposing influences on the activity of the HPA-axis depending on the nature of the stressor. In particular, in case of stressors inducing both systemic or neurogenic stress, such as immobilisation, electroshocks or water avoidance, NO appears to play a stimulatory role on

the neurones of the PVN and on the peripheral ACTH release (Rivier, 1994; Amir *et al.*, 1997), whereas in response to immune challenges, such as lipopolysaccharide or interleukin-1 injection, it seems to exert an inhibitory effect upon CRH release at the level of the median eminence, thereby blunting ACTH and Cort release. This suggests that, since each type of stressors evokes a unique hypothalamic response, the related activation of brain areas involved in PVN modulation under stress may define the role NO plays (Rivier, 1998).

Finally, also measurements of plasma levels of AVP and OXT after administration of NO donors or NOS inhibitors have provided incongruent results, with reports documenting stimulation (Ota *et al.*, 1993; Raber & Bloom, 1994), inhibition (Goyer *et al.*, 1994; Lutz-Bucher & Koch, 1994; Kadekaro & Summy-Long, 2000) or no change (Srisawat *et al.*, 2000; Yamaguchi & Hama, 2003) in the basal release of AVP and OXT in response to osmotic stimulation or reproductive conditions. The effect of stressor exposure on nNOS expression in magnocellular neurones has been less investigated. Forced swimming was shown to increase the number of NADPH-diaphorase-positive magnocellular cells in the PVN (Sanchez *et al.*, 1999a) as well as the expression of nNOS mRNA (Engelmann *et al.*, 2004b; Salchner *et al.*, 2004). However, the importance of NO with regard to the interaction HNS/HPA-axis in response to stressor exposure remains to be elucidated.

At least some of the reported discrepancies might be attributed not only to different experimental approaches, but also to the fact that in most studies addressing these issues pharmacological administration of NO donors and NOS inhibitors have been employed. In particular, the use of the latter compounds have several limitations (Horn *et al.*, 1994; Alderton *et al.*, 2001), which can account for the conflicting results reported in the literature. Some chemical agents originally used as nNOS inhibitors, for instance 7-nitroindazole, turned out to inhibit also other isoforms of NOS. In addition, the expression of eNOS in blood vessels represents a difficulty *per se* in studies whose target is primarily nNOS, since systemic as well as local administration of inhibitors inevitably affect also eNOS due to the close anatomical vicinity of blood vessels with cells of any tissue.

The availability of nNOS KO mice (for details, see Materials and Methods, chapter 2.1) allows to circumvent some of the problems associated with the administration of pharmacological agents and gives a privileged access to the mechanisms underlying the modulation of the stress response. These animals show no apparent differences with wild type (WT) mice, as they are viable and fertile, with normal locomotor and breeding activity, and overall sensorimotor function. The peripheral nervous systems is also normal, with no evident anatomical or histological anomalies (Huang *et al.*, 1993). Nevertheless, it deserves noticing

that a residual NOS immunoreactivity has been shown to persist in these mice, which, however, could be observed exclusively in skeletal muscle cells and not in the brain (Rothe *et al.*, 2005). Other investigators reported detectable levels of nNOS mRNA due to the up-regulated catalytic activity of the splice isoforms nNOS-beta and, to a lesser extent, nNOS-gamma in several brain regions of these animals, such as olfactory bulb, cerebellum and mesencephalic nuclei (Putzke *et al.*, 2000). However, none of the nNOS mRNA-splice variants were detected in the hypothalamus (Eliasson *et al.*, 1997). A lower number of beta-endorphine producing neurones was observed in the hypothalamic arcuate nucleus in mutant mice, whereas the expression of the precursor proopiomelanocortin as well as of other proopiomelanocortin-derived peptides was found to be unchanged. In addition, fewer beta-endorphine immunoreactive fibers were found in the hypothalamus of KO mice in comparison to WT animals (Bernstein *et al.*, 1998a). In the pituitary, the lack of nNOS affects cellular levels of opioid peptides, since proopiomelanocortin mRNA was shown to be here considerably reduced. However, this reduction was most pronounced in the intermediate lobe, while the anterior lobe was only marginally affected (Keilhoff *et al.*, 2001).

## 1.6 Aim of the study

The critical analysis of the aforementioned findings reveals that the role NO plays in controlling the stress response is still matter of debate. This is predominantly due to the fact that an exhaustive characterisation of the effect of NO on the activity of all three systems is still missing. The present study was designed to comprehensively characterise the influence of NO/nNOS on the basal and the activated state of the SAS, the HPA-axis and the HNS. We decided to focus on *in vivo* experiments in order to avoid misinterpretation that might come from using isolated tissues, as results obtained from these preparations are difficult to extend to the whole animal. In this context, we used genetically modified mice in order to examine in the intact animal the effect that a congenital absence of neural NO might have on the regulation of the three aforementioned systems under resting conditions and in response to defined stressor exposure. In the first set of experiments, we compared the SAS, the HPA-axis and the HNS between WT and nNOS KO animals under basal conditions. By western blot analysis we examined the expression of catecholamine biosynthetic enzymes in both genotypes, to determine whether nNOS KO mice express a normal content of biosynthetic enzymes. We subsequently moved the focus to the hypothalamic level, where all three systems originate. We employed immunohistochemistry to determine whether the absence of

NO of NOS origin induces any change in the number of AVP- and OXT-immunolabelled cells of the PVN and the SON of nNOS KO mice. We examined the same hypothalamic nuclei also by *in situ* hybridisation, using anti-AVP, -OXT and also -CRH radioactive probes, to verify possible alterations at the transcription level. In the second set of experiments, we characterised the effect of a 10-min forced swimming session on the activity of the SAS, the HPA-axis and the HNS with respect to the impact of NO/nNOS on the release of Cort. For this purpose, we monitored the peripheral secretory activity of the three systems at different time points, to investigate if and to what extent NO of neural origin is involved under acute stress conditions in the modulation of AVP, OXT and ACTH secretion from the pituitary, as well as in Cort and catecholamine exocytosis from the adrenal glands.

# 2 Materials and methods

## 2.1 Animals

Adult male WT and nNOS KO mice from our breeding colony were used in this study. The colony was originally established with breeders derived from the Cardiovascular Research Center, General Hospital, Massachusetts, USA. KO animals bear a targeted deletion of the exon 2 of the nNOS gene, which was achieved by homologous recombination. Mutant mice show >95% loss of nNOS production in the brain due to the disruption of the alpha isoform of the nNOS enzyme (Huang *et al.*, 1993). Their genetic background is derived from multiple backcrossings with C57BL/6J mice. Animals were housed in groups of six under standard laboratory conditions (22 ± 1 °C, 60 ± 5% humidity, 12-h light : 12-h dark cycle with lights on at 06:00h, food and water *ad libitum*). Mice were single-housed a week before the experiments to avoid uncontrolled stress reactions. Experimental protocols were approved by the local governmental body (Regierungspräsidium, Halle) and all efforts were made to minimise animal suffering during the experiments.

The status of each nNOS KO and WT mouse was verified by genotyping. Briefly, genomic DNA was isolated from mouse tails (Invisorb Spin Tissue Mini Kit, Invitek, Berlin, Germany). PCRs were carried out with approximately 200 ng genomic DNA in a total volume of 25 µl containing 100 mM Tris–HCl (Sigma, Steinheim, Germany), pH 8.8, 500 mM KCl (Sigma, Steinheim, Germany), 15 mM $MgCl_2$ (Sigma, Steinheim, Germany), 200 µM of each of the four deoxyribonucleotides triphosphate (dNTPs, PeqLab, Erlangen, Germany), 2 U Taq polymerase (PeqLab, Erlangen, Germany), and 0.3 µM each primer, respectively. Primer sequences for nNOS were used as detailed by P.L. Huang, Harvard Medical School, Charlestown, Massachusetts (personal communication): B1 primer 5'-CCTTAGAGAGTAAGG AAGGGGGCGGG-3' and B2 primer 5'-GGGCCGA TCATTGACGGCGAGAATGATG-3', giving raise to a 404bp PCR product. The sequence of the standard Neo primers was 5'-TGCCGAGAAAGTATCCATCATGGCTGATGC-3' and 5'-CAGAAGAACTCGTCA AGAAGGCGATAGAAGG-3' producing a 460bp product (MWG-Biotech, Ebersberg, Germany).

## 2.2 Stressor exposure and behavioural observations

Animals were forced to swim for 10 minutes in a glass cylinder 27 cm high and 15 cm large, which was filled with tap water (20 ± 1°C) to a height of 15 cm. Mice were assigned to one of three different groups and forced to swim for either 5 min ($T_5$) or 10 min ($T_{15}$ and $T_{60}$). The animals of group $T_5$ were immediately sacrificed after the end of a 5 minute-swimming session, whereas those of groups $T_{15}$ and $T_{60}$ were gently dried with a towel after a 10-minute swimming session and returned to their home cages for 5 and 50 minutes respectively before being sacrificed. Fresh water was used in every swimming trial. In order to minimize circadian differences, forced swimming was always carried out early in the morning (between 8:00 and 9:30). Control animals were left undisturbed in an adjacent room while stress experiments were being performed.

The behaviour of the animals during each swimming session was recorded with a camera and later analysed by typing pre-set keys on a computer. Three different behaviours were scored: swimming, struggling and floating. Swimming was defined as movements of both hindlimbs and forelimbs below the surface of the water, struggling when the forelimbs break the surface of the water (for instance by scratching the walls) and floating when the animal simply keeps itself afloat, with little limb and no trunk movements.

## 2.3 Western Blot

Animals (WT = 7, KO = 8) were deeply anaesthetised with 0.15 ml i.p. of Ketavet (Pharmacia, Karlsruhe, Germany) + Domitor (OrionPharma, Finnland) (5:3) and quickly decapitated. Adrenal glands were excised, carefully freed from fat, homogenised in a lysis buffer containing 50 mM K-/Na-phosphate buffer (pH 6.7), 0.2% Triton X-100 and a cocktail of protein inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and finally centrifuged at 4°C 10000 x g for 20 min. Only the supernatant (soluble proteins) was used for Western blot. Protein concentration was determined at a spectrometer (Perkin Elmer, Rodgau-Juegesheim, Germany) using a bicinchoninic acid protein assay kit (BCA kit, Pierce, Rockford, IL). The homogenates were stored at –80°C until use. Samples were thawn, diluted 1:4 with Rotiload 4x (Roth, Karlsruhe, Germany), which contains mercaptoethanol and sodium dodecylsulfate (SDS) to unfold and negatively charge the proteins, and then boiled at 96°C for 4 min. The denaturated samples (5 µg for TH and 10 µg for PNMT) were

electrophoresed on a SDS-polyacrylamide gel (gradient gel from 5% to 20%; Laemmli, 1970) for 2h at 8-10 mA/gel. Samples from WT and KO mice were loaded on the same gel for comparison.

Gels were washed briefly in blot buffer and then the electrophorased protein extracts were transferred onto nitrocellulose membranes (Amersham Biotech, Little Chalfont, UK) by blotting for 1h 30 min at 200 mA.

After blotting, the correct transfer of all proteins from the gels to the nitrocellulose membranes was verified by incubating 5-10 min in Ponceau 0.2% solution at RT. The membranes were then blocked with 5% non-fat dry milk in Tris buffered saline with 0.1% Tween-20 and then incubated with either rabbit anti-TH polyclonal antibody (1:500, Chemicon, Chandlers Ford, UK) or rabbit polyclonal anti-PNMT (1:200, Acris Antibodies GmbH, Hiddenhausen, Germany) at 4°C overnight, washed three times 10 min and then incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:10000, Jackson ImmunoResearch Lab., West Grove, PA) at room temperature (RT) for 2h. To assure equal sample loading, the membrane blots were co-incubated with mouse anti-beta-actin monoclonal antibody (1:2500, Sigma, Steinheim, Germany). After three washing steps, TH and PNMT were visualised by enzymatic chemiluminescence (ECL assay kit, Amersham Biosciences, Little Chalfont, UK). Blots were exposed to hyperfilm ECL autoradiographic film (Amersham Biosciences, Little Chalfont, UK) for 5 sec (TH) or 1 min (PNMT) and bands were quantitated using Kodak 1D Image Analysis Software (Kodak, Rochester, NY). Graphs indicate densitometric analysis normalised to beta-actin values.

Table 5: Electrophoresis and blot buffers composition

| **Components** | **Electrophoresis buffer** | **Blot buffer** |
|---|---|---|
| Tris | 25 mM | 48 mM |
| Glycin (Merck, Darmstadt, Germany) | 250 mM | 39 mM |
| SDS (Serva, Heidelberg, Germany) | 0.1% | 0.0375% |
| Methanol | - | 20% |
| $H_2O$ | + | + |

## 2.4 Immunohistochemistry

### 2.4.1 Tissue processing

Animals were deeply anaesthatised with 0.15 ml i.p. of Ketavet + Domitor (5:3) and transcardially perfused with 0.1 N NaPi phosphate buffer solution followed by 80 ml of phosphate-buffered 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany) + 0.05% glutarhaldehyde (Sigma, Steinheim, Germany). Brains were carefully removed from the skull, post-fixed overnight at 4°C in 4% PFA and then transfered to 30 % sucrose (Roth, Karlsruhe, Germany) as cryoprotectant until they sank (48 h). All the brains were frozen at – 40°C in dry ice-chilled methylbutane (Roth, Karlsruhe, Germany) and stored at – 80°C.
Serial 25-µm coronal frozen sections were cut in a cryostat (model CM3050 S, Leica, Nussloch, Germany) transfered either in cold phosphate buffered saline (PBS) and processed within 24 h, or in ethylenglycol (Merck, Darmstadt, Germany) + 15% sucrose in 0.05M NaPi phosphate buffer as a cryoprotectant solution and stored at –20°C until immunohistochemistry was performed. For each animal, all sections spanning the hypothalamus according to the Atlas of Franklin & Paxinos (1997) were collected (from bregma – 0.10 mm to bregma – 1.34 mm).

### 2.4.2 Immunohistochemical analysis

Sections were processed as free-floating slices. After three initial washing steps in PBS, they were incubated in 1% Na-borohydride (Sigma, Steinheim, Germany) for 15 minutes to reduce the aldehydeic groups of paraformaldehyde and glutaraldehyde in order to obtain a better interaction between antigens and antibodies. After washing again three times, a pre-incubation of 30 minutes at RT with PBS containing normal goat serum (NGS; PAN, Aidenbach, Germany) or horse serum (HS, Gibco, Eggenstein, Germany), in the case of goat anti-rabbit or donkey anti-goat secondary antibodies respectively. This was performed to block unspecific binding sites. The sections were then incubated with primary antibodies as follows: polyclonal guinea-pig anti AVP antibody (Peninsula laboratories, Inc., Belmont, CA) diluted 1:3000; polyclonal rabbit anti OXT antibody (Peninsula Laboratories, Inc., Belmont, CA) diluted 1:10000; goat nNOS antiserum (produced at the Institute of Medical Neurobiology, Magdeburg) diluted 1:100. All the incubations with primary antibodies were performed in PBS with 10% NGS or HS, 0.3% Triton X-100 (Serva, Heidelberg, Germany)

and 0.1% Sodium Azide (Serva, Heidelberg, Germany) for 2 days at 4°C. After washing steps and a pre-incubation of 1h at RT with PBS + 10% bovine serum albumine (BSA, PAA Laboratories, Cölbe, Germany), the slices were incubated 3h at RT with a secondary antibody anti guinea-pig Alexa 488 nm for AVP (Initrogen, Karlsruhe, Germany), donkey anti-goat Cy2 for nNOS (Dianova, Hamburg, Germany) or goat anti-rabbit Cy3 for OXT (Dianova, Hamburg, Germany). Finally the sections were washed in PBS, mounted on gelatine coated glass slides, air-dried and covered with Immunomount (Thermo Shandon, Pittsburg, PA) to prevent fading. Slides were stored at 4°C in darkness. Appropriate negative controls were performed by omission of the primary antibody. All the antibodies were tested before use. Fluorescence images were taken using a Zeiss confocal laser microscope (Axiovert 100 M, Jena, Germany) which is equipped with a 488 nm-argon and a 546 nm-krypton laser. Pictures were obtained by an integrated Zeiss software (LSM 5 Pascal, Jena, Germany) with a 20x lens using a 505-530 nm bandpass filter for the 488 nm excitation wavelenght and a longpass filter for the 546 nm excitation wavelenght.

Cell counting was performed following a blinded protocol. Immunopositive cells were counted bilaterally in two representative SON sections (from Bregma –0.58 to –0.70; Franklin & Paxinos, 1997) and in three representative PVN sections (from Bregma –0.70 to –0.94). The immunofluorescent signal of nNOS in the SON and PVN of KO mice was in the range of background levels in all cases.

## 2.5 In Situ Hybridisation

### 2.5.1 Tissue processing

*In situ* hybridisation was performed using radioactive cRNA probes. Animals were deeply anaesthetised with 0.15 ml i.p. of Ketavet and Domitor (5:3) and killed by decapitation. Brains were quickly removed from the skull, frozen at – 40°C in dry ice-chilled methylbutane and stored at – 80°C. 16 µm-coronal sections were cut in a cryostat spanning the region of the hypothalamus according to the Atlas of Franklin & Paxinos (1997), with every fifth slice being thaw-mounted on the same glass slide. Fat-free glass slides were previously rinsed with alchool and let dry at 180°C for 3 hours to avoid RNAase contamination.

Sections were stored at – 80°C until use.

## 2.5.2 Plasmid preparation

The preparation of cRNA probes for OXT and AVP required a preliminary step to produce bacteria containing plasmids with the DNA of the two peptides. This procedure was not necessary for the CRH and nNOS probes, since these plasmids were already available in our laboratory from previous experiments.

The two vectors containing the sequence of DNA specific either for OXT or AVP, together with the ampicillin resistence as selective agent, have been kindly provided by Dr. Evita Mohr, Institute of Cellular Biochemistry and Clinical Neurobiology, Hamburg-Eppendorf University, Germany. To obtain a large amount of DNA, Escherichia Coli XL-1 Blue were transformed with the vectors as follows. Bacteria were taken from – 80°C, thawn on ice for 5 minutes and incubated 20 minutes on ice with 0.5 µl of either Tris-EDTA buffer-eluted OXT-DNA or AVP-DNA. They were transfered to 42°C for 90 seconds, cooled on ice for 2-3 minutes and then incubated 1 hour at 37°C in standard Luria Bertani buffer (Gibco, Eggenstein, Germany). At the end of the incubation, 50 µl of cell suspension were spread on an agar plate (Gibco, Eggenstein, Germany) containing ampicillin and grown overnight at 37°C. Only the bacteria which have incorporated the vectors survived and were able to give rise to colonies. A single colony was then inoculated into 30 ml of Luria Bertani medium containing ampicillin and grown with vigorous shaking at 37°C for ~12 hours, which is tipically the transition from logarithmic into stationary growth phase. After 15 minutes of centrifugation at 6000 x g at the end of the incubation, the pellet was frozen at –20°C until plasmid purification was performed.

The isolation of the plasmid from the transformed bacteria was carried out using the QIAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany), following the manifacturer's instructions.

The amount of plasmid DNA harvested at the end of the procedure and its purity (expressed as „R", the ratio between the absorbance at 260 nm to the absorbance at 280 nm), were assessed by spectrophotometry (see table 1).

The samples were stored at – 20°C.

Table 1: Concentration and purity of OXT and AVP plasmid DNA

| | **Concentration** | **R** |
|---|---|---|
| **OXT** | 0.542 µg/µl | 1.63 |
| **AVP** | 0.707 µg/µl | 1.83 |

## 2.5.3 DNA linearisation

The plasmid DNA is linearised with a restriction enzyme, which is chosen such that the plasmid is cut only at one site. Antisense and sense probes (as negative controls) corresponding to a total of 15 µg of linearised DNA were prepared as follows:

Table 2: nNOS and CRH antisense probes preparation.

| **Components** | **nNOS antisense** | **CRH antisense** |
|---|---|---|
| Plasmid DNA | 28.8 µl | 65.2 µl |
| Buffer | 10 µl (AGS) | 10 µl (Bol A) |
| Restriction enzyme | 10 µl (PST I) | 10 µl (APA I) |
| DEPC-treated water | 51.2 µl | 14.8 µl |
| Total | 100 µl | 100µl |

Sense probes for nNOS and CRH were already available from previous experiments in our laboratory and therefore were not newly synthesised.

Table 3: AVP and OXT antisense and sense probes preparation.

| Components | AVP antisense | OXT antisense | AVP sense | OXT sense |
|---|---|---|---|---|
| Plasmid DNA | 21.2 µl | 27.7 µl | 21.2 µl | 27.7 µl |
| Buffer | 10 µl (B) | 10 µl (H) | 10 µl (H) | 10 µl (B) |
| Restriction enzyme | 10 µl (Hind III) | 10 µl (Eco RI) | 10 µl (Eco RI) | 10 µl (Hind III) |
| DEPC-treated water | 58.8 µl | 52.3 µl | 58.8 µl | 52.3 µl |
| Total | 100 µl | 100µl | 100 µl | 100 µl |

Plasmid DNAs were incubated for 3 hours at 37°C with different restriction enzymes. The use of specific buffers which were supplied by the manifacture (Qiagen, Hilden, Germany) ensures the optimal ionic concentration necessary to each enzyme.

At the end of the incubation, a 0.8% agarose gel was run for 1h at 70 mV to examine the quality of the linearisation. The gel revealed that the DNA has been properly linearised, so we proceeded to its extraction after having destroyed all the proteins in the mixture with 10 mg/ml of proteinase K (Roche, Mannheim, Germany) for 45 minutes at 37°C. This was done to eliminate all Rnase in the preparation. From now on, only diethyl pyrocarbonate (DEPC)-treated water and material were used.

The linearised DNA mixture was extracted twice with phenol/chlorophorm (Roth, Karlsruhe, Germany). The upper phase was transfered to a clean tube and the extraction was repeated only with chlorophorm for 1 minute at 14000 x g. The upper phase was transfered again in an other clean tube and the DNA was precipitated by adding sodium acetate 3M 1:10 (ph 5.2) and 2.5x of 100% ethanol at –20°C for 1h. After centrifuging at maximum speed twice (30 minutes and 20 minutes) and washing respectively with 70% and 100% ethanol, the pellet was resuspended in 15 µl of water. The quality and the quantity of the preparation was verified by running 1 µl of the solution on a 0.8% agarose gel for 1h at 70 mV.

All the linearised DNA probes were stored at –20°C.

### 2.5.4 cRNA probe labeling

The linearised DNA template was used in a transcription reaction to produce $^{35}$S- uracil tri-phosphate (UTP) cRNA radioactive probes. 25 µl of $^{35}$S-UTP solution (Amersham, Little

Chalfont, UK) was pipetted into a microfuge tube and let dry completely. This volume ensures in solution the minimal amount of UTP (12 µM) which is required for the transcription reaction to proceed, being this nucleotide the rate limiting component of the process. After resuspending the $^{35}$S-UTP in 4 µl of DEPC-treated water, the following reagents were added and incubate at 37°C for 90 minutes: 1 µl of 10x transcription buffer, 1 µl of a NTPs solution without UTP (containing 5 mM ATP, GTP and CTP), 1 µl of RNAase inhibitor (Boehringer Manneheim, Germany), 1 µl of 100 mM dithiothreitol (DTT, Boehringer Manneheim, Germany), 1 µl of linearised DNA, 1 µl of RNA polymerase (Boehringer Mannheim, Germany).

Table 4: Composition of 10x transcription buffer.

| **Components** | **10x Transcription buffer** |
|---|---|
| Tris | 400 mM pH 7.4 |
| $MgCl_2$ | 60 mM |
| DTT | 100 mM |
| Spermidine (Sigma, Steinheim, Germany) | 40 mM |

At the end of the incubation 5 µl yeast tRNA (5mg/ml) (Boehringer Mannheim, Germany), 4 µl 10x transcription buffer, 1 µl RNAse inhibitor (Boehringer Mannheim, Germany), 29 µl of DEPC-treated water and 1 µl DNAase (Boehringer Mannheim, Germany) were added and incubate 15 minutes at 37°C to digest the DNA template.
An extraction with phenol/chlorophorm was performed and then the free $^{35}$S-UTP in solution was separated from the labeled probes on Sephadex G50-50 spin columns (Roche, Mannheim, Germany). 1 µl of each fraction was counted and probes were diluted to appropriate concentration ( $10^6$/30 µl) in the following hybridisation buffer:

75% hybridisation buffer

7.5 ml formamide (Gibco, Eggenstein, Germany)

1.5 ml 20x standard sodium citrate (SSC)

200 µl 50x Denhardts (Sigma, Steinheim, Germany)

400 µl yeast tRNA (5 mg/ml)

500 µl 1 M sodium phosphate buffer pH 7.4
1 g dextran sulfate (Sigma, Steinheim, Germany)

Since probes with $^{35}S$ need to be maintained under reducing conditions, 1/100 volume of DTT was added to the mixture.
Probes were used immediately to avoid high background during hybridisation.

### 2.5.5 Tissue hybridisation

Slides were taken out from – 80°C and soaked in PFA 4% for 1h at RT. After 2 washing steps in PBS, sections were treated with proteinase K (0.1 µg/ml) in 100 ml Tris-HCl pH 8.0, 50 mM EDTA for 10 min at 37°C. Subsequent washing steps were performed in DEPC-treated water for 5 min, 0.1 M triethanolamine (TEA; Sigma, Steinheim, Germany) ph 8.0 for 5 min, 0.1 M TEA pH 8.0 with freshly added acetic anhydride (Roth, Karlsruhe, Germany) for 10 min and finally 2x SSC for 5 min. Sections were dehydrated in graded ethanol 50% to 100% and air dried.
30 µl of cRNA radioactive probe was used for each slide. Hybridisation was carried out at 55°C for 16 hours in humid chambers with 75 % formamide.
The post-hybridisation procedure consists of an initial washing step in 2x SSC, a treatment with RNAase A (40 µg/ml; Roche, Mannheim, Germany) at 37 °C to eliminate all single stranded RNA and several washing steps in 2x SSC, 1x SSC and 0.5x SSC at RT for 10 min, and in 0.1x SSC at 60°C for 45 min. After having been dehydrated in graded ethanol 50% to 100%, slides were air dried.

### 2.5.6 Autoradiography emulsion dipping

To visualise silver grains at the cellular level, the slides were dipped in a photographic emulsion (Integra, Fernwald, Germany) which had been diluted 1:1 in 0.5% glycerol (Merck, Darmstadt, Germany). Slides were dipped manually in the emulsion, let dry at RT for 2 hours and developed at 4°C in the dark. After being exposed, the slides were developed in Kodak D-19 photographic solution (Kodak, Rochester, NY) and fixed in 30% sodium thiosulfate (Kodak, Rochester, NY). In order to observe single cells in the tissue, the sections were counterstained with hematoxylin-eosin and then coverslipped with mounting medium (Serva, Heidelberg, Germany). Grey levels were measured bilaterally in dark-field images

(AxioVision 4.2, Carl Zeiss Vision, Jena, Germany) at two different representative SON and three representative PVN sections. Each image was adjusted for equal background. The hybridisation signal of nNOS in the PVN of KO mice was in the range of background levels in all cases.

## 2.6 Blood sampling and neuroendocrine measurements

### 2.6.1 Blood sampling procedure

Blood sampling (n = 6-8/group, carried on between 8:00 and 11:00 a.m.) was performed by heart puncture from three separate groups of mice as follows: 5 minutes ($T_5$), 15 minutes ($T_{15}$) and 60 minutes ($T_{60}$) after stressor onset. The animals of group $T_5$ were immediately anaesthetised with isofluorane (Abbott GmbH, Wiesbaden, Germany) at the end of a 5 minute-swimming session, whereas those of groups $T_{15}$ and $T_{60}$ were gently dried with a towel after a 10 minute-swimming session and returned to their home cages for 5 and 50 minutes respectively before being anaesthetised. The interval between anaesthesia and blood sampling was less than 1 minute. Blood (0.6-0.8 ml) was collected in ice chilled-EDTA-coated vials (Kabe Labortechnik, Nümbrecht-Elsenroth, Germany) containing a protease inhibitor (10 µl aprotinin; Trasylol, Bayer, Leverkusen, Germany) and centrifuged (3000 x g, 5 minutes at 4°C; Eppendorf Centrifuge 5417R, Leipzig, Germany) to separate plasma from cellular components. Control mice were left undisturbed in their home cages until blood sampling was performed. Aliquots of the supernatants were stored frozen at –80°C until peptide content measurement. Plasma OXT and AVP levels were determined by Radioimmunoassay (RIA) at the Department of Behavioural Neuroendocrinology, Max Plank Institute of Psychiatry, Munich, Germany. Cort and ACTH plasma values were measured using commercially available RIA kits at the Department of Endocrinology and Metabolism, Otto von Guericke University, Magdeburg. NE and E plasma values were measured by Enzyme Immunoassay (2 CAT EIA, LDN, Nordhorn, Germany). See below for all details.

### 2.6.2 AVP and OXT plasma values measurement

AVP and OXT were measured by specific and sensitive RIAs that were established by Landgraf (1981). 160 µl of plasma were used for each measurement. To isolate the peptides

from substances in plasma that may interfere with their quantitation, prior to RIA determination all samples were extracted as follows. 20mg/sample of Vycor glass powder were activated for 8h at 700°C and then, once cooled down to RT, diluted in 1 ml water. Each sample was diluted with 1 ml glass powder-containing water and mixed on a rotating shaker at 4°C for 30 min. After a quick centrifugation at 14000 x g for 5 sec, the supernatant was discarded and the pellet resuspended with 0.5 ml of water. The samples were centrifuged again, the supernatant discarded and the pellet resuspended in 0.5 ml of 0.1 N HCl. After a third centrifugation, the remaining pellet was diluted in 0.5 ml of 60% aceton, the tubes were capped and let stand for 10 min at –20°C. After being mixed on a rotating shaker for 30 min at 4°C, the samples were centrifuged at 14000 x g for 5 sec and the supernatant was transferred into a clean tube. Finally, the tubes were air-dried overnight at 4°C and the rest lyophilised.

To assess the AVP/OXT concentration, 100 µl of assay buffer were added to the lyophilised extract and 50 µl aliquots were used for the determination of both nonapeptides. 50 µl of rabbit antibodies, which were made at the Department of Behavioural Neuroendocrinology, Max Plank Institute of Psychiatry, Munich, Germany, and 10 µl of either AVP-$^{125}$ I or OXT-$^{125}$ I were then added. After 3 days of incubation at 4°C, unbound counts were precipitated by charcoal (Norit A) and samples were measured in a gamma counter. Synthetic AVP and OXT (Ferring Pharmaceuticals, Malmo, Sweden) were used as standard controls. A blank tube without AVP or OXT anti-serum was used to assess non specific binding. The calibrator curve was obtained by plotting the percent bound versus the concentration of AVP and OXT for all the standards. Sample values were then read directly from this curve.

### 2.6.3 ACTH and Cort plasma values measurement

ACTH and Cort plasma values were measured with two different RIA kits (ICN Biomedicals, Inc., Costa Mesa, CA). For ACTH, 50 µl of plasma were used for each measurement. Prior, to assay, all lyophilised reagents were reconstituted with water, mixed gently and let stand for 15 min at 4°C. 50 µl of ACTH-$^{125}$ I, 50 µl of ACTH anti-serum and 50 µl of standard controls (10 pg/ml to 1000 pg/ml) or plasma sample were mixed in each test tube, vortexed thouroughly and incubated at 4°C for 16 h. After adding 500 µl of precipitant solution, all tubes were centrifuged at 1000 x g at 6°C for 15 min. Supernatants were discarded and the precipitates were counted in a gamma counter. A blank tube without ACTH anti-serum was used to assess non specific binding.

For Cort, 10 µl of plasma for each measurement were diluted 1:200 with steroid diluent. 200 µl of Cort-$^{125}$ I, 200 µl of Cort anti-serum and 100 µl of standard controls (25 ng/ml to 1000 ng/ml) or plasma diluted sample were mixed in each test tube and incubated at RT for 2 h. After incubation, 500 µl of precipitant solution was added and all tubes were centrifuged at 1000 x g for 15 min. Supernatants were discarded and the precipitates were counted in a gamma counter. A blank tube without Cort anti-serum was used to assess non specific binding.

Both ACTH and Cort assays were set up in duplicate. The percent bound was calculated by subtracting the blank counts from the average of all duplicate tubes, and then dividing the corrected values by the corrected zero standard value. The calibrator curve was obtained by plotting the percent bound versus the concentration of Cort for all the standards. Sample values were then read directly from this curve. All the solutions were provided by the manifacturer.

### 2.6.4 NE and E plasma values measurement

To extract NE and E from blood, 100 µl of plasma were diluted with 200 µl of distilled water and incubated for 30 min at RT on an orbital shaker with 50 µl of Assay Buffer and 50 µl of Extraction Buffer. After decanting, each well was washed twiced with 1 ml of Wash Buffer Concentrate. 150 µl of Acylation Buffer were then incubated with 25 µl of Acylation Reagent in all wells for 15 min at RT on an orbital shaker. After decanting and washing twice with Wash Buffer Concentrate, NE and E were eluted with 150 µl of hydrochloric acid from all wells.

100 µl of the extracted and acylated samples were used for E EIA and 20 µl for NE EIA. After 30 min of incubation at RT with 25 µl of a freshly prepared Enzyme Solution, 50 µl of Adrenaline or Noradrenaline Antiserum were pipetted into all wells and incubated 2 h at RT. Three washing steps were performed, and then 100 µl of Enzyme Coniugate was added to all wells and incubated for 30 min. After repeated washings, a subsequent incubation with 100 µl of Substrate was carried on for 30 min. The reaction was stopped with 100 µl of Stop Solution and the absorbance of the solution in the wells was read within 10 min using a microplate reader set to 450 nm with a reference wavelenght between 620 nm and 650 nm.

The linear mean absorbance readings of six standards were plotted along the y-axis versus log of the standard concentrations in ng/ml along the x-axis. The concentrations of the samples were determined from this standard curve by matching their mean absorbance readings with

the corresponding analyte concentrations. All the solutions were provided by the manifacturer.

## 2.7 Statistical Analysis

Behavioural data were analysed using the Student's t-test. Neuroendocrine measures data were analysed by two-way analysis of variance (Two-way ANOVA, GraphPad Software, San Diego, California; genotype x time points) followed by Fischer LSD *post-hoc* test (GB-Stat 6.0, Dynamic Microsystems, Silver Spring, MD, U.S.A).

Data from Western blot, *in situ* hybridisation and immunohistochemical analysis were analysed by Mann-Whitney U-test (GraphPad Software, San Diego, California).

All values are reported as mean + SEM. A $p < 0.05$ was considered statistically significant.

# 3 Results

## 3.1 Basal conditions

### 3.1.1 Western Blot analysis

TH. TH signal was seen as a single band at ~60 kDa (Fig. 4), which is consistent with previous studies. Analysis of Western blot data revealed a reduced expression of TH in adrenal gland homogenates of KO mice compared to WT (Mann Whitney U-test, $p < 0.05$, $U = 9$, Fig. 5).

PNMT. PNMT signal was seen at ~27 kDa (Fig. 4). Beta-actin blot is also shown to confirm equal loading of samples. Similarly to TH, quantitative analysis of PNMT-stained immunoblots revealed a significantly lower expression of this enzyme in KO mice compared to WT (Mann Whitney U-test, $p < 0.05$, $U = 7$, Fig. 5).

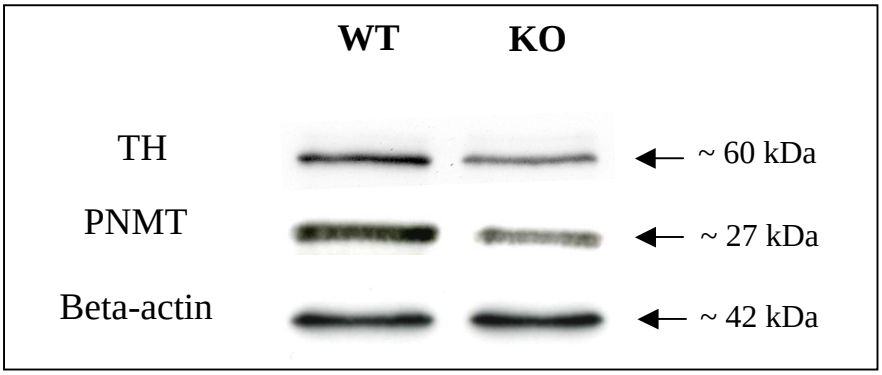


Fig. 4. Western blot gel lanes for tyrosine hydroxylase (TH), phenylethanolamine *N*-methyltransferase (PNMT) and beta-actin from adrenal gland homogenates of WT and KO mice. The positions of the respective molecular weight markers are indicated.

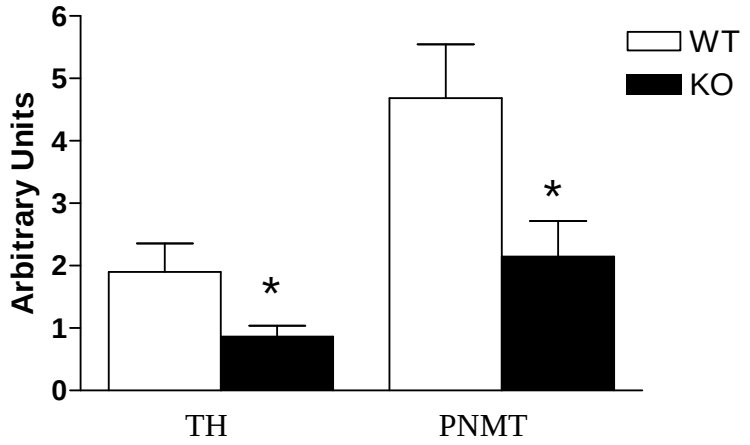


Fig. 5. Semi-quantitative histogram showing tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyltransferase (PNMT) expression in adrenal gland homogenates of WT and KO mice. Data were normalised to the respective beta-actin values and presented as mean + SEM. * = $p < 0.05$ vs WT, Mann Whitney U-test.

### 3.1.2 AVP- and OXT-immunopositive cell count in the PVN

AVP. AVP-immunopositive cell count was performed bilaterally in three PVN sections of each WT and KO mouse. No statistical difference was observed between the genotypes, although KO mice showed a tendency towards a lower number of positively stained cells ($p = 0.15$, $U = 5$, Mann Whitney U-test; Fig. 6A and Fig. 7A and B).

OXT. OXT-immunopositive cells were counted bilaterally in three PVN sections of each WT and KO mouse. The total number of OXT positive cells was similar in both genotypes ($p = 0.54$, $U = 9$, Mann Whitney U-test; Fig. 6B and Fig. 7C and D).

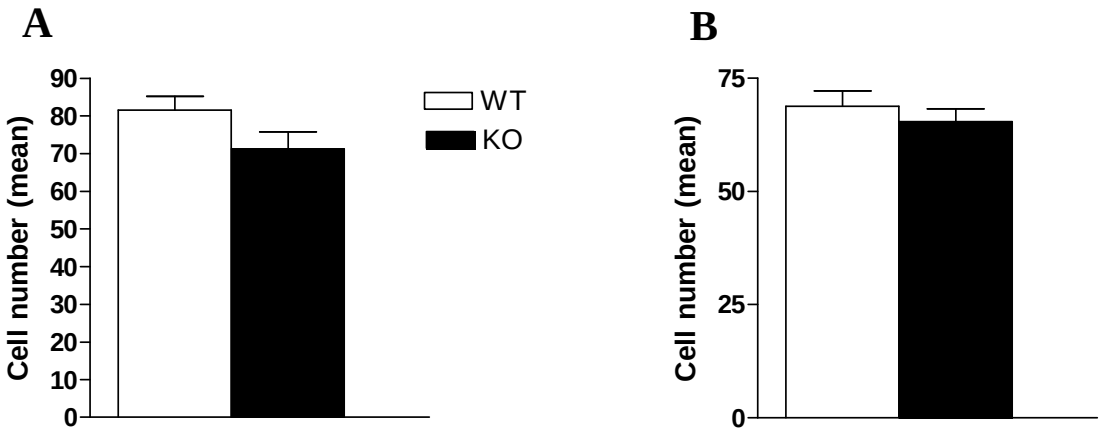


Fig. 6. AVP- (A) and OXT- (B) immunofluorescent cell number in the PVN of KO and WT under basal conditions. Data are expressed as means + SEM (n = 5/genotype). No statistical difference was observed between the genotypes.

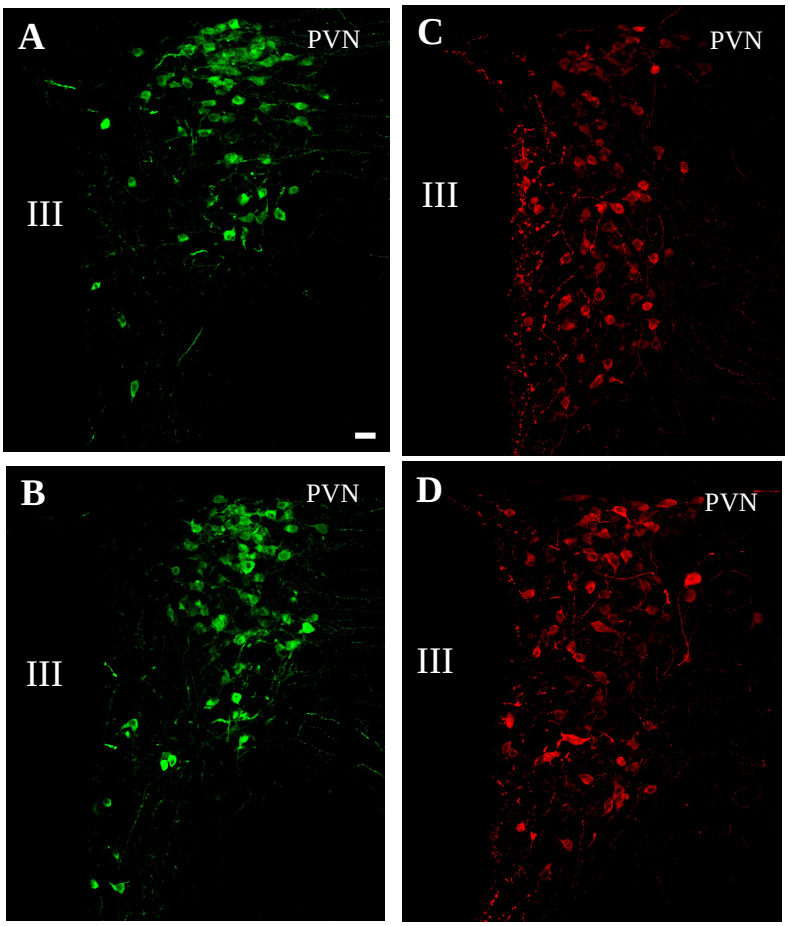


Fig. 7. Representative immunofluorescence pictures of AVP- (green) and OXT- (red) labelled magnocellular neurones in the PVN of WT (A and C) and KO (B and D) animals. III = third ventricle. Scale bar: 20 µm.

### 3.1.3 Expression of AVP, OXT and CRH mRNA in the PVN

AVP. The relatively high intensity of the hybridisation signal did not allow to count individual cells. Quantification of the emulsion-dipped slices was performed bilaterally in three PVN sections, and it revealed that AVP mRNA grey value intensity in the PVN of KO mice was significantly lower than in WT animals ($p < 0.01$, $U = 0$, Mann Whitney U-test; Fig. 8A and $9A_1$ and $A_2$).

OXT. KO mice displayed a tendency to lower OXT mRNA expression if compared to WT, without, however, reaching statistical significance (Mann Whitney U-test, $p = 0.12$, $U = 6$; Fig. 8B and $9B_1$ and $B_2$).

CRH. Figure 8C shows the CRH mRNA grey values in the two groups. The intensity of the hybridisation signal in KO animals was similar to that seen in WT mice (Mann Whitney U-test, $p = 1$, $U = 15$). Representative pictures of CRH mRNA levels in WT and KO mice are shown in Fig. $9C_1$ and $C_2$.

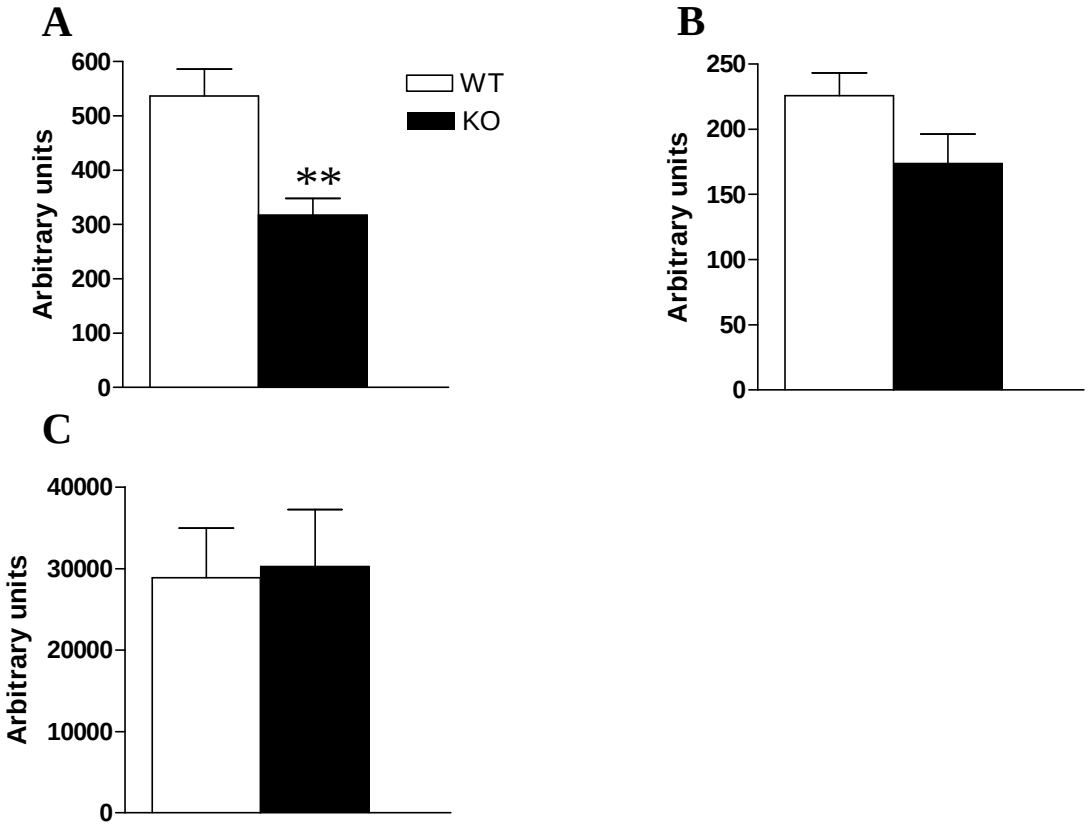

Fig. 8. Hybridisation signal of (A) AVP mRNA, (B) OXT mRNA and (C) CRH mRNA in the PVN of KO and WT mice under resting conditions. Data are expressed as means + SEM (n = 5-6). ** = $p < 0.01$ versus WT. Mann Whitney U-test.

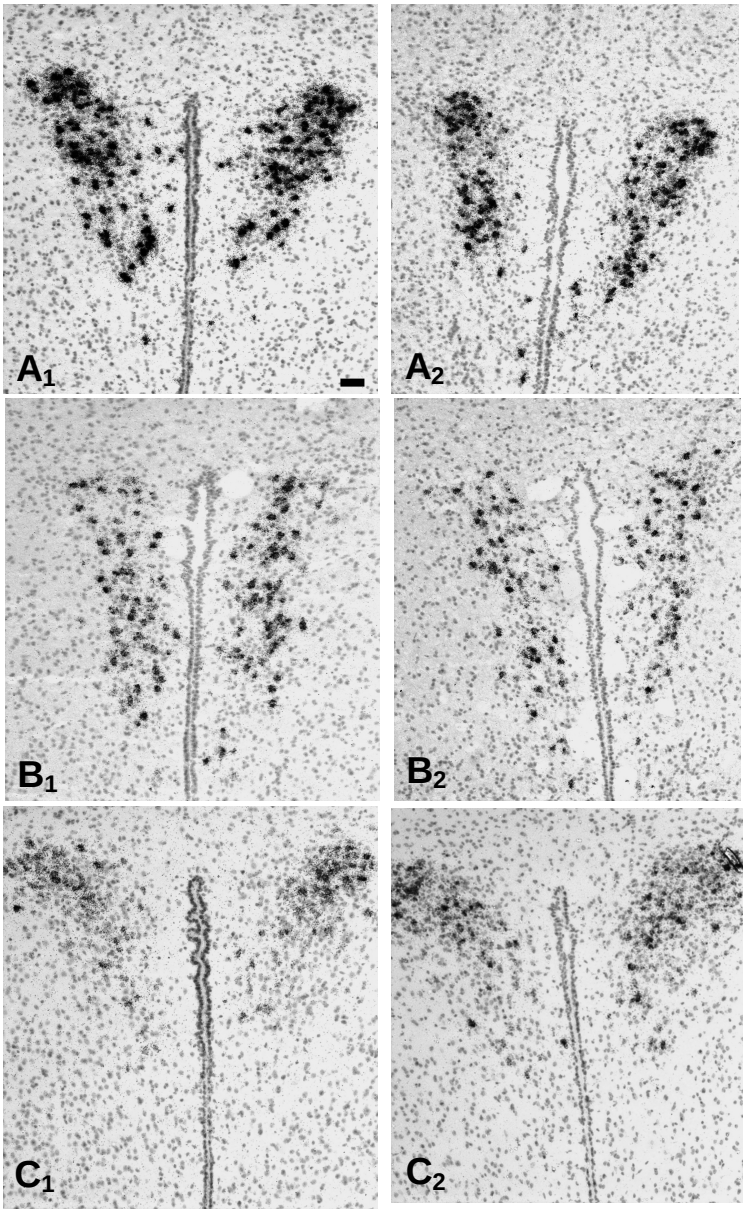


Fig. 9. Representative bright-field microphotographs illustrating the hybridisation signal in the PVN of WT (left panels: $A_1$, $B_1$, $C_1$) and KO (right panels: $A_2$, $B_2$, $C_2$) for (A) AVP mRNA, (B) OXT mRNA and (C) CRH mRNA. Scale bar: 20 µm.

### 3.1.4 AVP- and OXT-immunopositive cell count in the SON

AVP. AVP-immunopositive cells were counted bilaterally in two representative SON sections of each animal. No statistical difference was observed between the genotypes ($p = 0.42$, $U = 8$, Mann Whitney U-test; Fig. 10A and Fig. 11A and B).

OXT. Although the number of OXT-immunopositive cells tended to be higher in KO than in WT, the difference failed to reach statistical significance ($p = 0.09$, $U = 4.5$, Mann Whitney U-test; Fig. 10B and Fig. 11C and D).

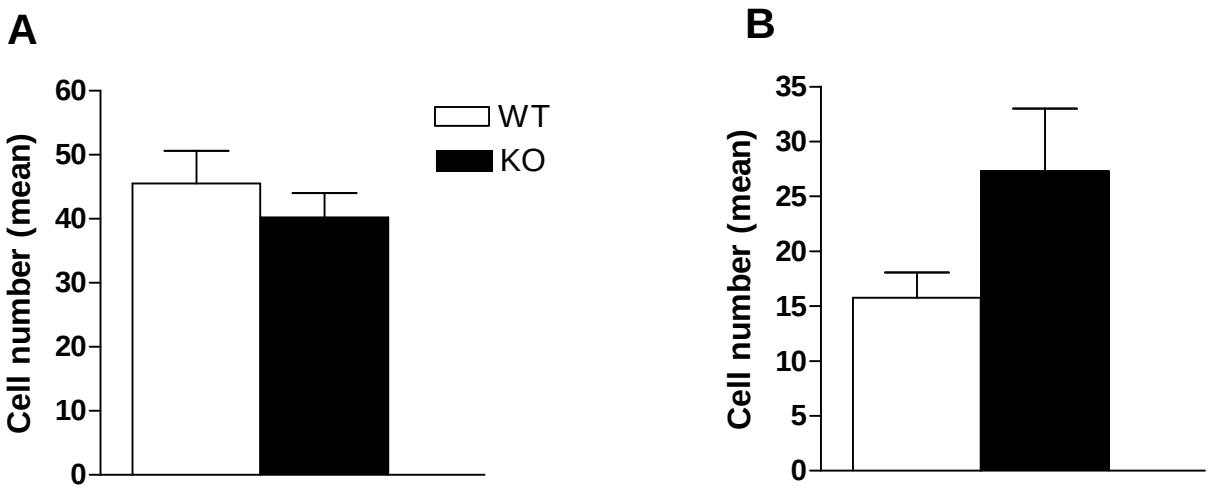


Fig. 10. AVP- (A) and OXT- (B) immunopositive cell number in the SON of KO and WT under basal conditions. Data are expressed as means + SEM (n = 5/genotype). No statistical difference was observed between the groups.

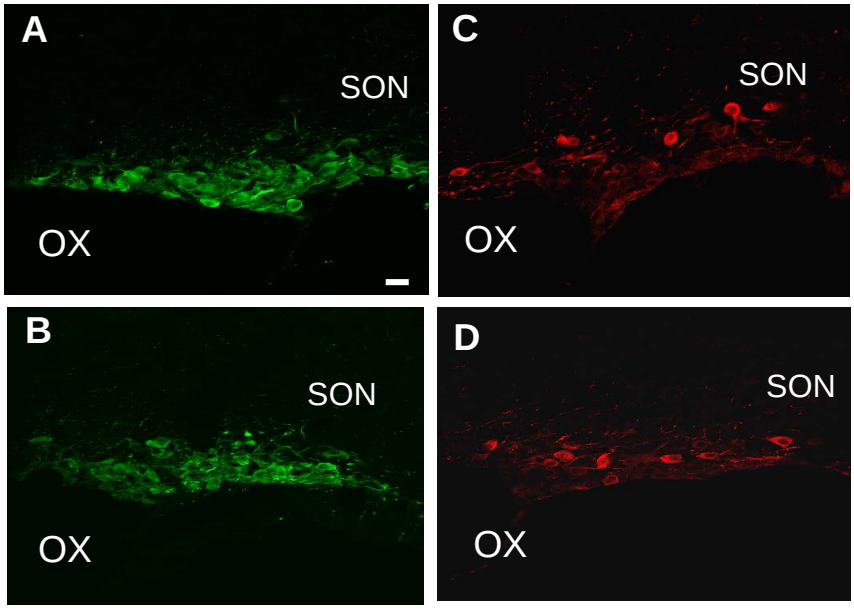


Fig. 11. Representative immunofluorescence pictures of AVP- (green) and OXT- (red) labelled magnocellular neurones in the SON of WT (A and C) and KO (B and D) animals. OX = optical chiasm. Scale bar: 20 µm.

### 3.1.5 Expression of AVP and OXT mRNA in the SON

AVP. We evaluated AVP mRNA expression bilaterally in two different SON sections of each WT and KO mouse by grey value analysis. Quantification of the emulsion-dipped slices revealed that AVP mRNA levels in KO animals were significantly higher than in WT ($p < 0.02$, $U = 3$, Mann Whitney U-test; Fig. 12A and Fig. 13).

OXT. Analysis of emulsion-dipped slices revealed OXT mRNA levels in KO and WT mice were similar ($p = 0.14$, $U = 8$, Mann Whitney U test; Fig. 12B and Fig. 14).

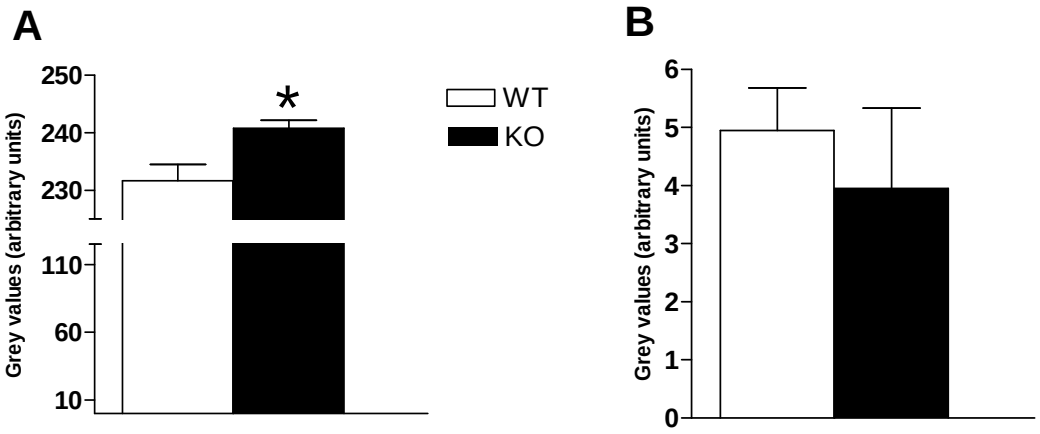


Fig. 12. Hybridisation signal of mRNA coding for AVP (A) and OXT (B) in the SON of KO and WT under basal conditions. Data are expressed as means + SEM (n = 5-6). * = $p < 0.02$ vs WT control. Mann Whitney U-test.

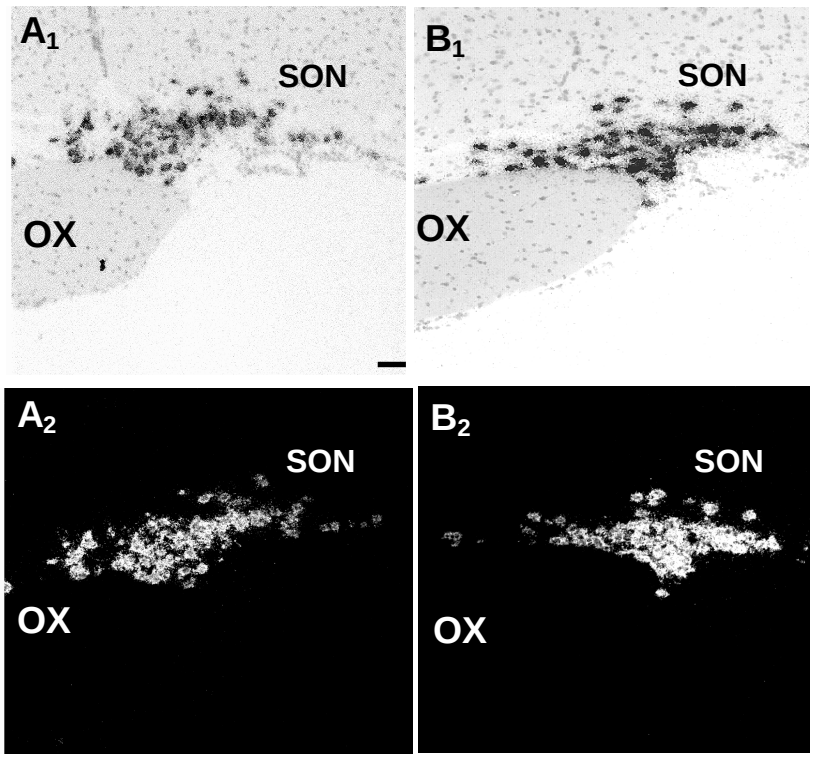


Fig. 13. Representative bright and dark-field microphotographs of AVP mRNA expression in the SON of WT ($A_1$, $A_2$) and KO mice ($B_1$, $B_2$). OX = optical chiasm. Scale bar: 20 µm.

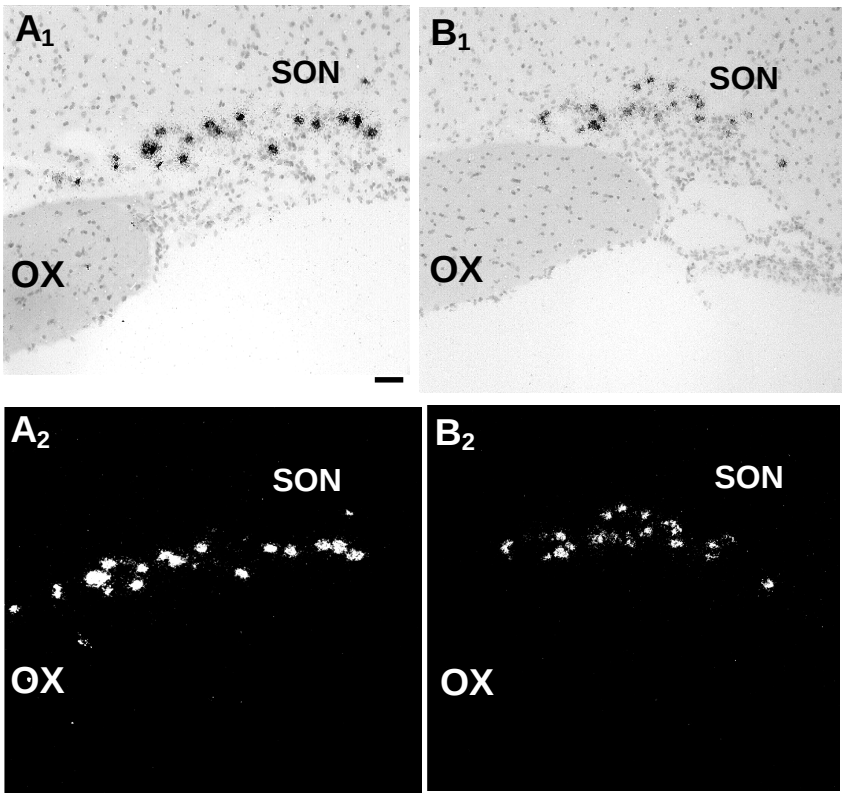


Fig. 14. Representative bright- and dark-field microphotographs of OXT mRNA expression in the SON of WT ($A_1$, $A_2$) and KO mice ($B_1$, $B_2$). OX = optical chiasm. Scale bar: 20 µm.

## 3.2 Stress conditions

### 3.2.1 Behavioural observations

Figure 15 shows the behaviour of the mice during a 5 min- (A) and a 10 min- (B) swimming session in 20°C cold water. No statistically significant difference was observed between the two genotypes in terms of time spent either floating (Student's t-test, p = 0.59 for A and p = 0.14 for B), or swimming (p = 0.26 for A and p = 0.19 for B) or struggling (p = 0.19 for A and p = 0.19 for B).

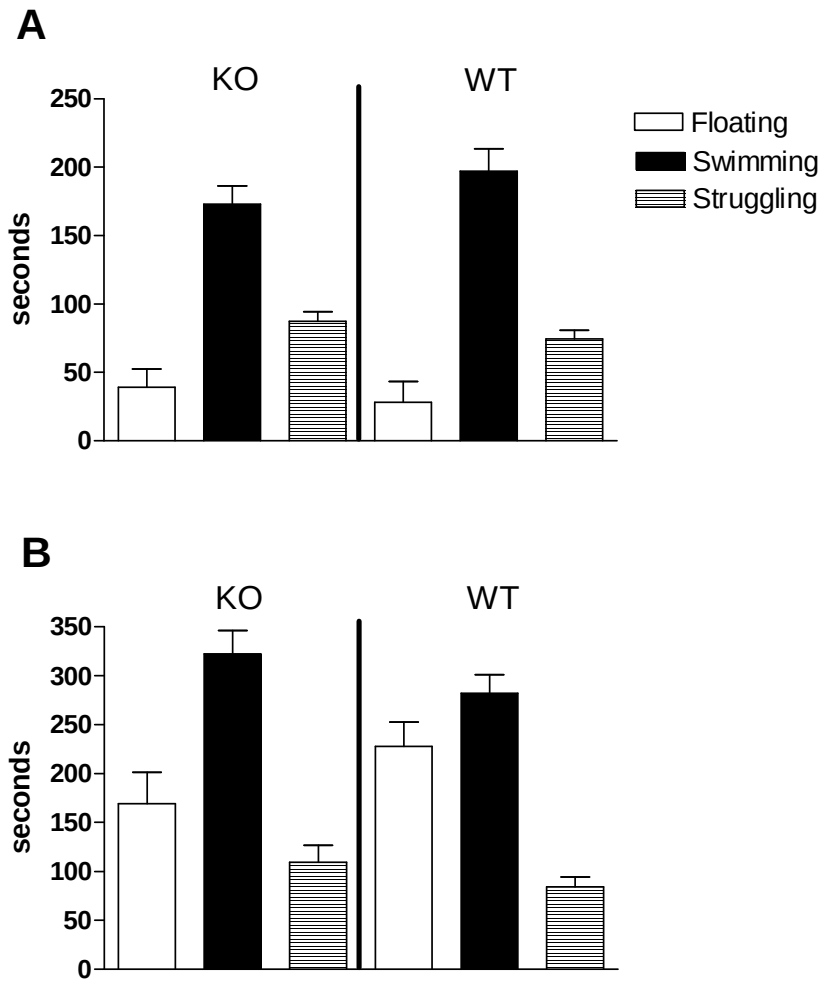


Fig. 15. Behavioural parameters of nNOS KO and WT mice observed during a 5 min- (A) and a 10 min-forced (B) swimming session at 20 °C. Values are means + SEM (in A n = 8 for both genotypes; in B n = 43 for WT and n = 31 for KO). Data were analysed by Student's t-test between the genotypes and the same behavioural parameters.

### 3.2.2 Neuroendocrine measurements

E. Plasma E levels in KO control mice were slightly higher than in WT, but without reaching statistical significance. In WT animals, forced swimming caused an increase in E plasma values which peaked 15 min after stressor onset (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.05$ compared to control, Fig. 16A). A biphasic response was observed in KO animals after forced swimming: at $T_{15}$ the E levels dropped significantly if compared to $T_5$, and rose again at $T_{60}$ (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.05$ compared to $T_5$ and $T_{60}$, Fig. 16A).

NE. NE plasma concentrations in unstressed WT and KO mice were very similar. After stressor exposure, the overall NE release profile of KO animals was slightly higher than that of WT, but it failed to reach statistical significance (Two-way ANOVA, Fischer LSD *post-hoc* test, $p = 0.06$, Fig. 16B).

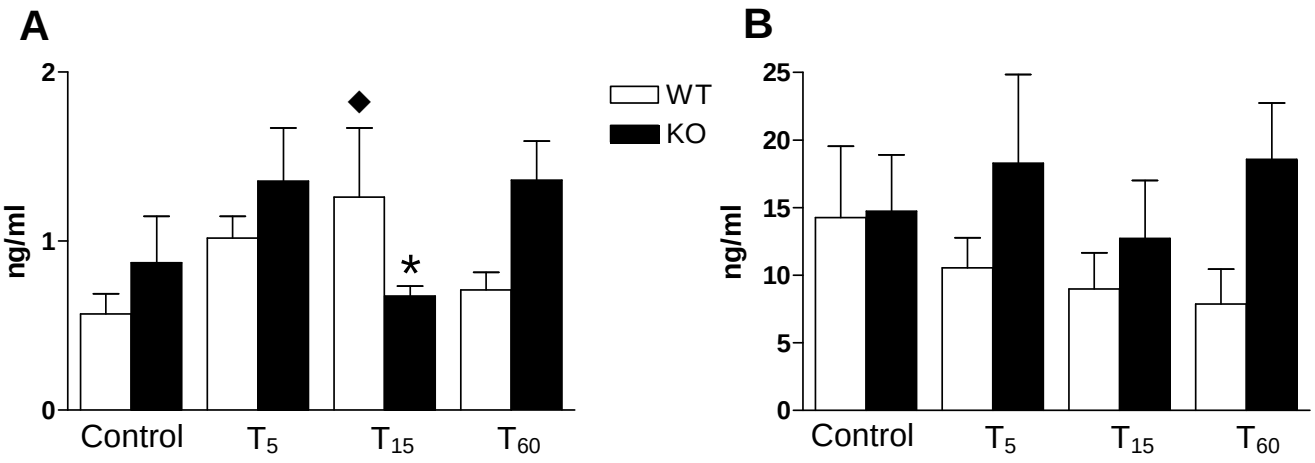


Fig. 16. Plasma concentrations of (A) E and (B) NE in KO and WT mice under resting conditions (control) and 5 min after forced swimming, as well as 5 min and 50 min after a 10-min forced swimming session ($T_5$, $T_{15}$ and $T_{60}$, respectively). Data are expressed as means + SEM (n = 6-8). In (A): ◆ = $p < 0.05$ versus the respective control; * = $p < 0.05$ versus KO $T_5$ and $T_{60}$. Two-way ANOVA followed by Fisher's LSD *post-hoc* analysis.

ACTH. Basal plasma ACTH concentrations did not differ between the genotypes. Upon stressor exposure, we observed at $T_5$ a significant rise of plasma ACTH levels in both groups (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.05$, Fig. 17A). At $T_{15}$ and $T_{60}$ plasma ACTH concentration returned close to basal levels without revealing differences between WT and KO animals.

Cort. Basal plasma Cort concentrations were similar in KO and WT control animals. In WT animals plasma values rose 5 min after stressor onset, peaked at $T_{15}$ (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.01$, Fisher LSD test compared to control, Fig. 17B) and decreased at $T_{60}$. KO animals mounted a faster response to forced swimming, as plasma values at $T_5$ were already markedly different from control (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.01$, Fig. 17B). The overall release profile looked, however, analogous to WT mice, reaching a peak at $T_{15}$ (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.01$, compared to controls, Fig. 17B) and decreasing at $T_{60}$.

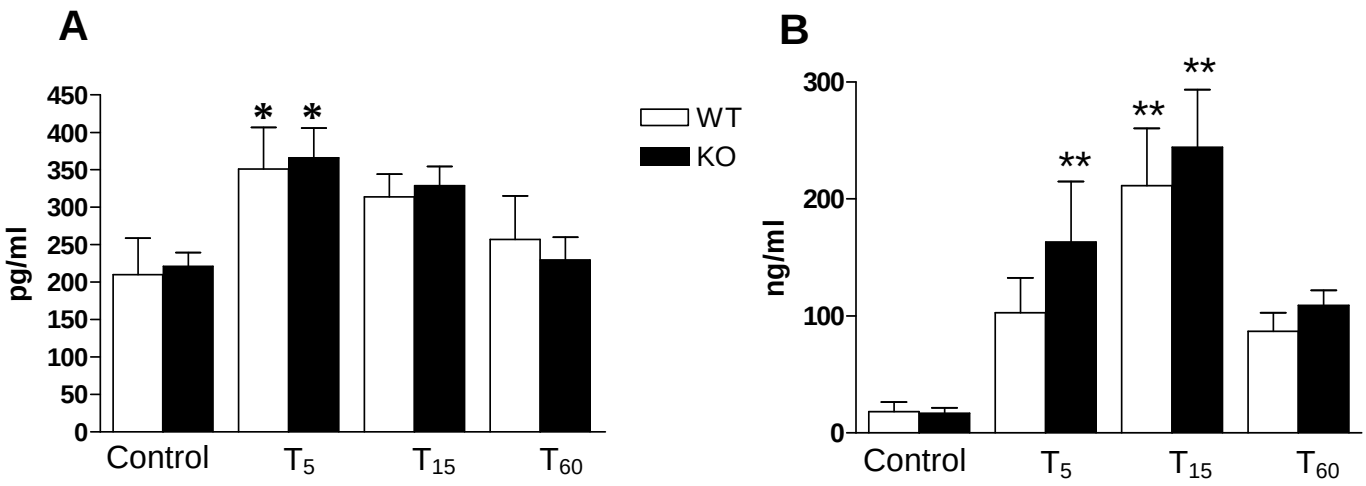


Fig. 17. Plasma concentrations of (A) ACTH and (B) Cort in KO and WT mice under resting conditions (control) and 5 min after forced swimming, as well as 5 min and 50 min after a 10-min forced swimming session ($T_5$, $T_{15}$ and $T_{60}$, respectively). Data are expressed as means + SEM (n = 6-8). In (A): * = $p < 0.05$ versus the respective controls. In (B): ** = $p < 0.01$ versus the respective controls. Two-way ANOVA followed by Fisher's LSD *post-hoc* analysis.

AVP. Basal plasma AVP concentrations did not differ between the genotypes. Forced swimming tended to induce in both stressed groups a subtle increase of AVP plasma values at $T_5$, which however failed to reach statistical significance. At $T_{15}$ plasma values in WT animals remained similar to basal levels, but dropped in KO mice to levels significantly lower than in controls and at $T_5$ (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.05$; Fig. 18A). At $T_{60}$ the AVP concentration markedly rose again if compared to $T_{15}$ (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.01$; Fig. 18A), whereas in WT animals it remained unchanged.

OXT. Basal OXT plasma concentrations were similar in KO and WT control animals. Stressed WT stressed animals showed at all time points measured plasma values indistinguishable from those seen under control conditions. In contrast, KO animals responded to forced swimming with an altered release profile of OXT. Interestingly, OXT plasma concentration was significantly lower in KO mice at $T_{15}$ (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.05$, Fig. 18B), whereas at $T_{60}$ it was robustly increased compared to control, $T_5$ and $T_{15}$ of the KO group as well as $T_{60}$ of the WT group (Two-way ANOVA, Fischer LSD *post-hoc* test, $p < 0.01$, Fig. 18B).

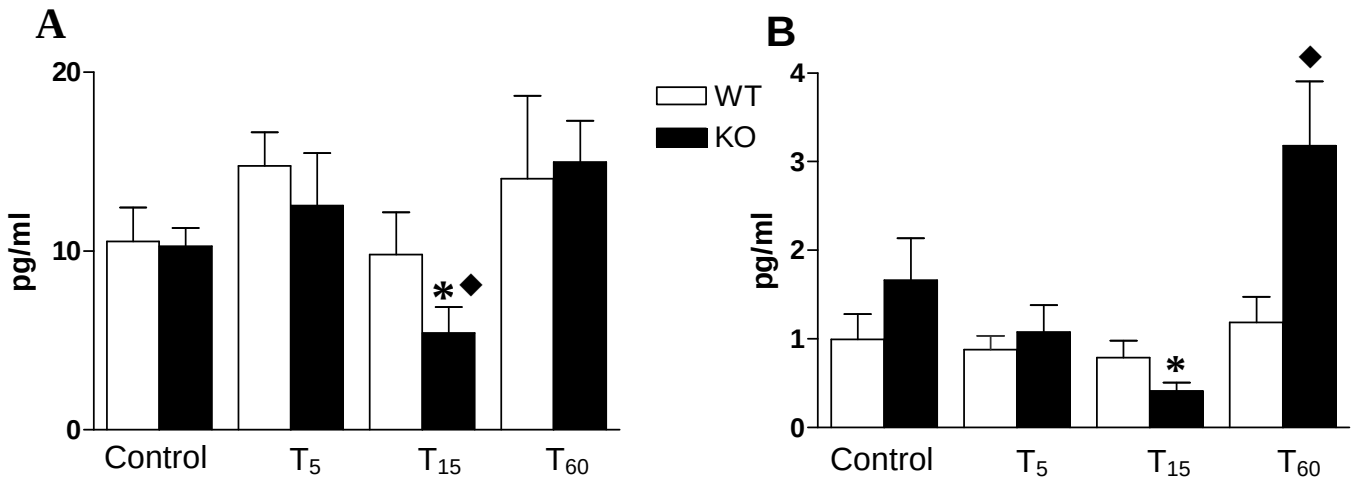


Fig. 18. Plasma concentrations of AVP (A) and OXT (B) in KO and WT mice under untreated conditions (control) as well as 5 min, 15 min and 60 min after forced swimming ($T_5$, $T_{15}$ and $T_{60}$, respectively). Data are expressed as means + SEM (n = 6-8). In (A): * = $p < 0.05$ vs KO control and KO $T_5$; ◆ $p < 0.01$ vs KO $T_{60}$. In (B): * = $p < 0.05$ vs KO control; ◆ $p < 0.01$ vs KO control, $T_5$, $T_{15}$ and $T_{60}$ as well as WT $T_{60}$. Two-way ANOVA followed by Fisher's LSD *post-hoc* analysis.

# 4 Discussion

This study aims at elucidating the role NO of nNOS origin plays in regulating the activity of the SAS, the HPA-axis and the HNS under resting conditions and following acute stressor exposure. We examined in WT and nNOS KO mice the main physiological blood parameters that, in the periphery, are index of the status of the above mentioned systems, and the transcriptional and protein levels of the key molecules that govern the function of each system at the level of the brain and the adrenal gland.

## 4.1 Considerations about transgenic mice

Before discussing in detail the results obtained in this study, some remarks are due regarding the use of mutant mice as a tool to characterize *in vivo* the function of molecules that have previously been studied *in vitro* or by using pharmacological agents. The generation of transgenic animals boosted studies concerning the physiological relevance of genes/proteins at many levels, reaching from biochemistry to cell and system biology up to behaviour. Precious information have already been obtained in the past decade by the use of mutant mice (Picciotto & Wickman, 1998; Ryan & Sigmund, 2002), and the convergence of data from pharmacological studies and from in vivo experiments using KO mice models have undoubtedly deepened our knowledge of nervous system function. However, different variables can influence the phenotype of the mutant mouse. The existence of different subtypes or splicing isoforms, for instance, might cover up the disruption of the target protein. Furthermore, the occurrence of compensatory mechanisms should also be taken into consideration, as the ablation of a single protein, if does not result in a lethal phenotype, might be balanced by other pathways. In addition, one has also to consider the impact of the genetic background of mutant mice that have been develop using cultured embrionic stem cells derived from the mouse strain 129, and backcrossed to mice from a different strain, for example C57Bl/6. The offspring of such matings, even after several backcrossings to the C57Bl/6, still maintain a small amount of genetic material of 129 origin, which extends on either side of the disrupted allele of the targeted gene. As the likelihood of cross-over events occurring precisely on both sides of the targeted gene is infinitesimally small, the 129-derived flanking regions of the mutated allele of the gene of interest will be most probably transmitted

together with the mutation. Conversely, wild-type offspring will not carry the 129-derived genes located close to the disrupted allele, but will carry C57Bl/6-type alleles instead. Therefore, the different genetic background due to the presence of residual material from the 129 strain in mutants might account, at least partially, for phenotypical differences between mutant and control mice (for review, see: Gerlai, 1996; Lathe, 1996). Also epigenetic effects induced by breeding have to be taken into consideration. This seems to be of particular relevance with respect to early life experience on the development of the neuroendocrine response to stress. Maternal care during the first weeks of life can influence profoundly the capacity of pups of mounting an appropriate defensive response to environmental challenges once reached the adult age. Previous studies showed that maternal behaviour is an important epigenetic process whereby the HPA-axis response to stress can be affected (Liu *et al.*, 1997a) (for review, see: Meaney, 2001). Several attempts have been made to overcome both the genetic and the epigenetic problems. The future of gene-targeting technique seems to be oriented to expand and improve existing methods to generate, for instance, inducible KO mice, where the gene of interest can be turned off at particular times. The phenotype of the animals could be then compared within the same genetic background before and after gene inactivation. Another valid alternative might be the generation of conditional KO mice, where the gene disruption can be achieved in a specific subset of cells, leaving the rest of the organism genotypically unchanged. This allows to investigate the role of a molecule in the exact contest it normally works. Regarding breeding-related epigenetic effects, cross-fostering studies using heterozygous parents may help rule out that observed neuroendocrine and behavioural differences in mutant mice bred by mutant parents are not due to impaired parental care, and thus to non-genomic effects, but primarily to the genetic disruption.

In the present study, the influence of epigenetic factors should be considered, as the colony of mice was initially established with WT and KO breeders that were then kept separated and further bred as two independent colonies. Thus, albeit several benefits can come from mutant mice studies and assumptions with respect to normal physiological situation can be made from such studies, conclusions are necessarily restricted. In this context, as to date there is no inducible or conditional KO model for nNOS available, the results of the present work are subjected to the above mentioned limitations that hold true for our conventional KO animals. Nevertheless, the findings obtained in the course of this study provided new insight into the role NO/nNOS plays in neuroendocrine regulation.

## 4.2 Effect of nNOS gene inactivation on behaviour

Previous investigators have reported that nNOS KO mice displayed altered behavioural parameters, such as excessive sexual behaviour and increased aggression (Nelson *et al.*, 1995; Chiavegatto *et al.*, 2001; Chiavegatto & Nelson, 2003). Cerebellar functions such as balance and coordination appeared unchanged in these animals, although some investigators reported abnormalities in balance and motor coordination selectively during the night (rodent active phase; Kriegsfeld *et al.*, 1999). With regard to cognitive functions, KO mice showed impaired cognitive performance in the Morris water maze test under stressful conditions (Weitzdoerfer *et al.*, 2004). It has also been reported that nNOS KO mice showed an altered behavioural response during a 10-min swimming session at a rather warm temperature of 25 °C (Salchner *et al.*, 2004), with a considerably reduced time of immobility than WT mice and an enhanced duration of swimming behaviour. Since the intensity with which the stressor is perceived by the animals determines the behavioural response, we decided to use a lower water temperature (20 °C), as this is perceived more aversive. As can be seen in Fig. 15, our results differ from those of the above mentioned authors as the behaviour of the KO mice in our hands failed to significantly differ from WT animals. This discrepancy is likely to be ascribed to different experimental conditions, in particular the water temperature, which has been reported to be an important parameter in the forced swim test (Peeters, 1991; Jefferys & Funder, 1994; Drugan *et al.*, 2005). The fact that in our hands both genotypes respond to the defined stressor exposure with the same behavioural strategy indicates that the differences in the endocrine parameters observed in response to forced swimming are due to the congenital absence of nNOS, and not to a different interpretation of the stressor within the limbic system.

## 4.3 Effect of nNOS gene inactivation on the adrenal activity under basal conditions and in response to forced swimming

There is growing evidence that NO decreases catecholamine secretion in response to a defined stressor exposure. This paracrine and/or autocrine mechanism is most likely aimed at controlling acetylcholine-evoked adrenal medulla activation under stress (Uchiyama *et al.*, 1994; Rodriguez-Pascual *et al.*, 1996). However, the physiological role of NO on chromaffin cells, in particular under resting conditions, is still a matter of debate. Some studies have shown that NO triggers basal catecholamine secretion and that different NO donors increase intracellular calcium concentrations (Oset-Gasque *et al.*, 1994; Uchiyama *et al.*, 1994),

whereas other authors reported that N-methyl L-arginine, a NOS inhibitor, increased efflux of catecholamine in perfused dog adrenal glands (Ward *et al.*, 1996). Finally, some investigators even failed to observe any effect of NO on chromaffin cells activation and calcium levels (Marley *et al.*, 1995). In KO mice the activity of the sympathetic nervous system, measured in terms of NE and E plasma values, appeared normal under resting conditions (see Fig. 16). After stressor exposure, E plasma levels in WT animals rose and peaked at 15 min, whereas in KO mice the E profile release showed a biphasic nature with a drop at 15 min and a later increase at 60 min. The reduced E plasma values at 15 min speaks in favour of a change in E synthesis, which is then reflected by a lower amount of E stored in vesicles ready to be released upon stimulation. The activity of catecholamine biosynthetic enzymes is regulated by different protein kinases through phosphorylation (Zigmond *et al.*, 1989) and gene expression (Sabban & Kvetnansky, 2001). Previous reports have shown that NO up-regulates activity and transcript levels of these enzymes through a cGMP/PKG-activated pathway (Kim *et al.*, 2003). Our findings correlate well with this view, as in KO animals TH and PNMT protein levels were significantly reduced if compared to WT (see Fig. 5). The lower E plasma levels observed in KO mice 15 min after stressor onset may be ascribed to reduced amounts of both TH and PNMT, which seem be sufficient to ensure normal basal levels of E, but become inadequate in case of higher demand, such as under acute stress conditions. Conversely, the plasma concentration of E in genetically modified mice was robustly increased 60 min after stressor onset. The literature suggests a dual role for cGMP in modulating the function of chromaffin cells. Beyond stimulating catecholamine synthesis through TH activation, cGMP also inhibits catecholamine secretion by reducing calcium influx (O'Sullivan & Burgoyne, 1990; Torres *et al.*, 1994). The latter effect seems to be due to inhibition of voltage-dependent calcium channels through a mechanism that involves PKG. There is evidence that the mechanism that links PKG activation to calcium influx inhibition appears to be rather slow, since long times of incubation with the NO donor sodium nitroprusside are required *in vitro* to observe an inhibition of catecholamine secretion (Rodriguez-Pascual *et al.*, 1996). In agreement with this view, our results confirm the dual effect of the NO/cGMP/ PKG pathway with an initial stimulatory and a later inhibitory role on E release.

Interestingly, the reduced TH levels in KO mice seem to have no significant effect on stressor exposure-induced NE release. NOS is preferentially expressed by noradrenergic chromaffin cells in the human (Heym *et al.*, 1994) and rat adrenal medulla (Dun *et al.*, 1993), while adrenergic cells show a lower expression of this enzyme. However, adrenergic cells are more responsive to NO donors than noradrenergic cells. NO may, therefore, act in a paracrine

manner, diffusing from noradrenergic cells to adrenergic cells, which constitute its main target (Oset-Gasque *et al.*, 1998). It is then plausible that the constitutive absence of NO could affect more prominently the function of adrenergic chromaffin cells rather than that of noradrenergic ones.

## 4.4 Effect of nNOS gene inactivation on stress-related PVN structures under basal conditions and in response to forced swimming

Previous studies suggested a role for nNOS in the ontogeny of AVP and OXT-expressing cells of the hypothalamus. In particular, the time course of nNOS expression in hypothalamic structures has been shown to coincide with the maturation of vasopressinergic and oxytocinergic neurones, which strongly suggests an important role of NO of nNOS origin on the correct postnatal development of these cells (Yuan *et al.*, 2006). We therefore evaluated by cell count the number of AVP- and OXT- expressing neurones in the PVN of mutant mice, as possible variations in the number of cells expressing these two neuropeptides in the PVN might be associated with alterations of the HPA-axis activity, given that both AVP and OXT are known to enhance the effects of CRH. Surprisingly, the number of OXT- and AVP-immunoreactive cells in the PVN of KO mice was not different from that of WT mice (see Fig. 6). This suggests that the chronic absence of nNOS had no impact on the number of immunopositive cells that produce the two neuropeptides in this part of the hypothalamus. This finding is concordant with a previous observation (Bernstein *et al.*, 1998a) that reported no significant changes between WT and mutant mice in the number of neurophysin-positive cell bodies in the PVN. We further evaluated the intensity of the hybridisation signal of mRNA coding for AVP and OXT in the neurones of the PVN, to verify whether the production of these neuropeptides might be affected by the congenital absence of nNOS/NO. Interestingly, KO mice displayed a remarkably reduced AVP mRNA content in the PVN (see Fig. 8A), which suggests NO to promote mRNA synthesis for AVP in this hypothalamic nucleus already at resting conditions. This is in agreement with previous reports showing that AVP transcriptional activity was enhanced after intracerebroventricular injection of the NO donor 3-morpholino-sydnonimine, mostly in the parvocellular division of the PVN in the intact rat (Lee *et al.*, 1999). Our hybridisation protocol did not allow to easily discriminate between parvocellular and magnocellular neurones. This is predominantly due to the peculiar anatomical structure of the murine PVN, where parvocellular and magnocellular neurones are, unlike the rat, intensively intermingled with one another (Schonemann *et al.*, 1995; Dijkstra *et*

*al.*, 1998; Reyes *et al.*, 2003). Nonetheless, the striking difference in AVP mRNA content between the two neuronal populations in naïve animals (Sawchenko, 1987) makes it rather unlikely that parvocellular neurones contribute significantly to the detected AVP mRNA levels of the PVN. Earlier studies in this regard focused mainly on the effect of NO on AVP peripheral release, and reported contradictory results (Ota *et al.*, 1993; Yasin *et al.*, 1993; Lutz-Bucher & Koch, 1994; Cao *et al.*, 1996; Kadekaro *et al.*, 1997). Furthermore, these studies were based almost exclusively on the rat as experimental subject, which differs from the mouse not only in the cyto-architectural organisation of the PVN, but also in the general distribution of NOS-like immunoreactivity (Ng *et al.*, 1999). Our data suggest that, in the mouse, endogenous NO of nNOS origin exerts a stimulatory influence on the activity of AVP magnocellular cells in the PVN.

In contrast to AVP, OXT mRNA levels in KO mice did not significantly differ from WT (see Fig. 8B). The responsiveness of OXT magnocellular neurones to NO seems to be different from that of AVP cells, a functional heterogeneity that has been observed also by other authors (Roberts *et al.*, 1993).

The involvement of NO in the modulation of ACTH secretion may be conceivable due to the subcellular localisation of nNOS in the PVN of rodents (Torres *et al.*, 1993; Siaud *et al.*, 1994; Hatakeyama *et al.*, 1996), which implies NO to participate in an autocrine and/or paracrine manner into the regulation of CRH release into the portal blood. In fact, recent studies conform to the view that NO is involved in the control of the CRH neurosecretory system (Riedel, 2000). However, previous investigations addressing this issue yielded conflicting results, which can be ascribed to different experimental approaches, for instance peripheral versus central pharmacological administrations (Giordano *et al.*, 1996; Lee *et al.*, 1999). Other investigators reported no effect of NO precursors or NOS inhibitors on basal CRH release (Costa *et al.*, 1993). We extend these findings by our data showing in KO mice baseline CRH mRNA levels comparable to WT animals (see Fig. 8C). Consistently with this observation, plasma ACTH levels were equivalent in both genotypes under resting conditions. Also, we observed in WT and KO mice a similar rise in ACTH blood levels 5 min after stressor onset (see Fig. 17A). These results suggest that the responsiveness of the HPA-axis has not been impaired by nNOS gene disruption and that, consequently, NO does not play an important role in CRH gene expression and ACTH release. However, we can not totally rule out the possibility that normal plasma values of ACTH might result from complementary effects of NO at the levels of the median eminence and/or anterior pituitary. In fact, some investigators have reported a stimulatory role of NO at the anterior pituitary (Brunetti *et al.*,

1993), while others have suggested an inhibitory influence at the level of the median eminence (Rivier & Shen, 1994). Thus, the general absence of nNOS in our mice may have caused effects that compensate each other, and this might explain the unaltered ACTH release in defined stressor exposure.

Baseline Cort plasma levels in KO mice were indistinguishable from those seen in WT (see Fig. 17B). This is in disagreement with a previous study reporting significantly higher Cort plasma levels in KO mice under resting conditions (Bilbo *et al.*, 2003). However, the study by Bilbo *et al.* (2003) was performed using mice that were single-housed at weaning, whereas we used group-housed animals, single-housed only during the week antecedent the experiment. Since long-term individual-housing has been shown to lead to chronic stress-like responses *per se* (Sharp *et al.*, 2002), the different husbandry conditions are likely to explain this discrepancy. KO animals displayed a more pronounced increase in Cort release 5 min after the beginning of the swimming session. A recent study (Mohn *et al.*, 2005) suggested a facilitatory role of NO through activation of cyclooxygenase on ACTH-induced Cort release. This study, though, was performed *in vitro* on isolated adrenal glands and did not take in consideration the contribution of the sympathetic nervous system, which may alter Cort release via secretion of catecholamines (Bornstein *et al.*, 1990; Bornstein *et al.*, 1994). Because the response of the sympathetic nervous system precedes that of the HPA axis to stressors, it is plausible that it may control Cort release in a paracrine fashion through NE and/or E secretion from chromaffin cells located in the adrenal medulla (Ehrhart-Bornstein *et al.*, 1998). It was originally believed that the adrenal gland is clearly separated in two different endocrine tissues, an outer Cort-producing cortex and an inner NE- and E-producing medulla (Rittmaster & Cutler, 1990). However, chromaffin cells can be found also in the cortical area (Bornstein *et al.*, 1991), and, conversely, cortical cells are scattered in the medulla (Bornstein *et al.*, 1994). This close intercalation of the two cell types implies a wide cross-talk between the two systems, which, thus, might influence one another.

## 4.5 Effect of nNOS gene inactivation on stress-related SON structures under basal conditions and in response to forced swimming

A previous study (Salchner *et al.*, 2004) showed a modified pattern of protooncogene (c-Fos) expression in the SON in response to forced swimming, which suggests neuronal activation of SON magnocellular neurones and, consequently, their involvement in the response to acute stressor exposure. It is therefore reasonable to expect the increased SON cellular activity to be

mirrored peripherally by altered AVP and OXT plasma levels. As up-regulated levels of c-fos are index of rapid transcriptional activation (Yoshida *et al.*, 2006), increased c-fos levels may indicate ongoing *de novo* synthesis of neuropeptides, which usually occurs upon secretion to refill intracellular stores. AVP of magnocellular origin is released from axon terminals into the peripheral circulation and, in rats, plasma levels can be elevated by ether, haemorrhage, electric shock, hypoglycaemia, hypoxia (for review, see: Gibbs, 1986) and body compression (Husain *et al.*, 1979). Previous studies have reported that AVP plasma values are not affected by forced swimming in the rat, except for a fast and transient increase one min after stressor onset (Engelmann & Ludwig, 2004). The present study shows that this is valid also in the mouse, as forced swimming induced no significant changes in AVP plasma concentration in WT stressed mice at any of the time points investigated (see Fig. 18A). These findings suggest that, unlike in humans (Dugue *et al.*, 1993; Kohl, 1992), AVP blood values in rodents appear to be tightly regulated, and noticeable variations occur mostly in response to stressors that affect body fluid homeostasis. Mutant mice displayed AVP plasma values equal to WT animals under resting conditions, whereas the release profile during stress was equivalent to the corresponding values in WT at $T_5$ and $T_{60}$, but significantly lower at $T_{15}$ (see Fig. 18A). Plasma OXT levels in KO animals were also significantly reduced at $T_{15}$, resembling the release profile of AVP at the same time point, whereas a robust increment of OXT plasma concentration was observed at $T_{60}$. This dichotomy in the peripheral secretion of OXT after stressor exposure suggests the presence of two different mechanisms acting in the same direction, or of a common mechanism,that controls the secretion of both neuropeptides in KO mice in the initial phase of the stress response, and the existence of another mechanism that controls the secretion of oxytocinergic rather than vasopressinergic cells at a later stage. Studies in the rat have demonstrated that the regulation of peripheral AVP release is predominantly accomplished at the level of the hypothalamus by excitatory inputs that primarily originate in other brain regions and act via glutamate (Csaki *et al.*, 2002) and NE (Vacher *et al.*, 2002). Inhibitory signals are perceived by magnocellular neurones either synaptically (GABA originating from local interneurones; Gies & Theodosis, 1994) or in a non-synaptic manner (taurine originating from glial cells; Decavel & Hatton, 1995). Previous investigations have shown that forced swimming triggered the release of glutamate and taurine within the SON in the rat, whereas GABA levels remained unchanged (Engelmann *et al.*, 2001; Engelmann & Ludwig, 2004). Glutamate-mediated activation of NMDA channels located on NOS-containing neurones enhances NOS activity (Esplugues, 2002), thereby promoting NO diffusion in the surrounding area where it has been proposed, among other

mechanisms (Boeckxstaens *et al.*, 1991; Peunova & Enikolopov, 1993; Choi *et al.*, 2000; Kiss & Vizi, 2001), to reversibly inhibit the re-uptake of glutamate (Pogun *et al.*, 1994) and NE (Kaye *et al.*, 1997) from the synaptic cleft. By increasing the half-life of these neurotransmitters in the extracellular space around the synapse, NO amplifies their excitatory effect on target cells (Kiss & Vizi, 2001). Our data, however, imply that in WT animals this stimulatory effect on AVP SON magnocellular neurones after forced swimming is counterbalanced by inhibitory inputs, since AVP plasma values were indistinguishable from those seen under resting conditions. This may be achieved through the non-synaptic action of taurine, which selectively inhibits the firing activity of AVP-containing cells (Engelmann *et al.*, 2001). The absence of NO-mediated inhibition of monoamines transporters in our mutant mice might therefore result in a reduced glutamatergic and noradrenergic stimulation of vasopressinergic magnocellular cells during forced swimming. This finding conforms well with the assumption that NO inhibits the neuronal activity of vasopressinergic magnocellular neurones, as it has been reported by several pharmacological studies (Liu *et al.*, 1997b; Stern & Ludwig, 2001). In addition AVP released from somata/dendrites of magnocellular cells has been proposed to provide a negative feedback on SON neurones themselves (Ludwig & Leng, 1998; Kombian *et al.*, 2000).

Like for the PVN, also the number of vasopressinergic and oxytocinergic magnocellular neurones in the SON was unchanged in mutant animals (see Fig. 10). We, therefore, further evaluated the intensity of the hybridisation signal for AVP and OXT in these cells by grey values analysis. This revealed that in KO mice AVP gene transcription is up-regulated if compared to WT mice (see Fig. 12A). Increased mRNA levels suggest a longer-lasting AVP-mediated autocrine inhibition on magnocellular neurones, which is aimed at preventing an overshoot of the hypothalamic-pituitary-adrenal axis under stress conditions (Wotjak *et al.*, 2002) and is independently controlled from the peripheral release (Di Scala-Guenot *et al.*, 1987). In light of these findings, the reduced AVP plasma levels in the blood of KO mice at 15 min might reflect a disproportion between non-synaptically driven excitatory and inhibitory inputs on SON vasopressinergic cells. However, further experiments are needed to determine whether the small, albeit significant, increase of AVP mRNA levels observed in KO mice is indeed of biological significance. It may well be that this difference is mirrored by altered plasma levels only in case of strong osmotic challenge, when AVP is massively released into the bloodstream.

As alluded to earlier, the profile of OXT release in KO animals after stressor exposure differed from AVP in that it showed a biphasic pattern (see Fig. 18B). Since grey value

analysis of the intensity of OXT hybridisation signal in the SON revealed no difference between KO and WT mice (see Fig. 12B), it is reasonable to hypothesise that the lack of nNOS does not affect OXT gene expression and that the amount of releasable pools of OXT is similar in both genotypes. Other mechanisms must, therefore, come into play in KO mice to elicit a delayed rise in OXT plasma levels. Previous studies carried on in this laboratory (Bernstein *et al.*, 1998a) showed a reduced number of β-endorphine positive cells and fibers in the hypothalamus of mutant animals. Further studies will have to reveal whether this reduced endorphinergic innervation may indeed account for the delayed increase in plasma OXT observed in our mutant mice (Soldo & Moises, 1998; Muller *et al.*, 1999; for review, see Brown *et al.*, 2000).

OXT plasma values in WT mice remained surprisingly unchanged after forced swimming at all time points investigated (see Fig. 18B). Earlier studies have reported a pronounced augment of OXT plasma levels following forced swimming in the rat (Wotjak *et al.*, 1998). Our data indicate that, in the mouse, a different orchestra of modulators is activated at the level of the SON to control the secretory activity of oxytocinergic neurones. It appears likely that the regulatory mechanism that is set in motion to control the peripheral release of OXT under defined stressor exposure is species-specific.

Fig. 19 summarises the data obtained in this study concerning the possible role of NO/nNOS in the regulation of the structures coordinating the stress response.

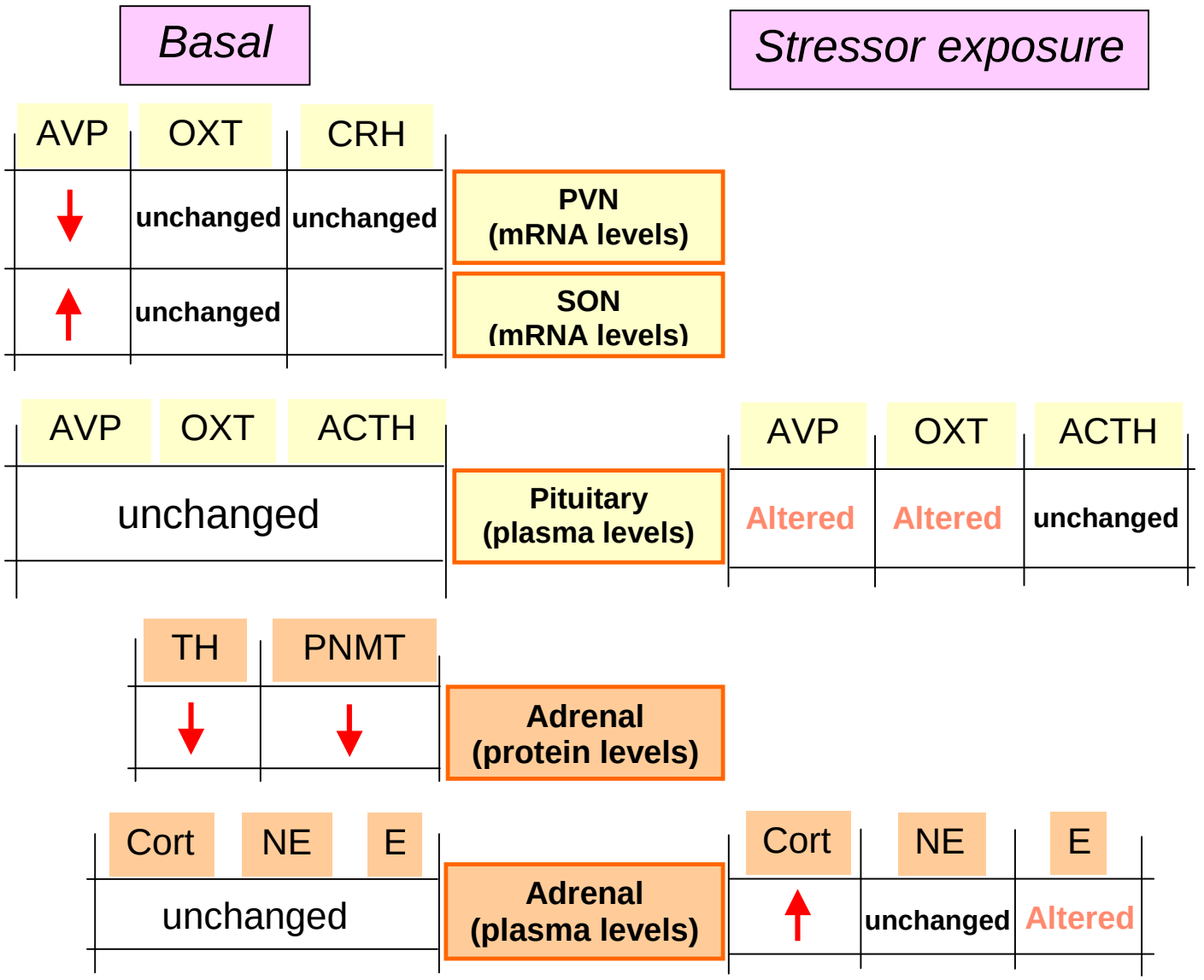


Fig. 19. Impact of the congenital absence of nNOS on the release of AVP and OXT from the PVN, the SON, the pituitary and the adrenal gland either under basal conditions or in response to defined stressor exposure. Arrows indicate an inhibitory (↓) or a stimulatory action (↑).

AVP: vasopressin; Cort: corticosterone; E: epinephrine; NE: norepinephrine; OXT: oxytocin; PNMT: phenylethanolamine *N*-methyltransferase; PVN: paraventricular nucleus; SON: SON: supraoptic nucleus; TH: tyrosine hydroxilase.

## 4.6 Clinical implications

Affective disorders have become a distinctive trait of modern society. Several studies have demonstrated that a dysregulation of the systems deputed to organise the stress response is critically involved in the pathogenesis of these diseases, which include anorexia nervosa (Gold *et al.*, 1986; Kaye *et al.*, 1987), panic disorders (Roy-Byrne *et al.*, 1986; Gold *et al.*, 1988c), obsessive-compulsive disorders, depression, anxiety and post-traumatic stress disorders (for review, see: Yehuda *et al.*, 1991; Scott & Dinan, 1998; Holsboer, 1999; Strohle & Holsboer, 2003; Shelton, 2004). A pathological state may arise from either a decreased stress system activity, like post-traumatic stress disorder (Marshall & Garakani, 2002), or from an increased stress system activity, like melancholic depression, anxiety or anorexia nervosa (for review, see: Licinio *et al.*, 1996; Ninan, 1999; Mello Ade *et al.*, 2003). Our findings suggest that NO is implicated in the control of the systems involved in the pathogenesis of mood disorders, and can therefore be listed among the signals that link the emotional to the endocrine response under stressful conditions. These results gain added significance in view of recent studies concluding that a dysregulation of nNOS activity and, hence, of NO production, is likely to contribute to behavioural abnormalities linked to neuropsychiatric disorders, such as schizophrenia and depression (Bernstein *et al.*, 1998b; Masood *et al.*, 2003; Bernstein *et al.*, 2005; Sevgi *et al.*, 2006). Our data provide evidence that congenital absence of nNOS results in altered endocrine responses associated with stress coping and this may, thus, open new diagnostic and therapeutical perspectives for the treatment of mood disorders.

## 4.7 Perspectives

As our data imply that NO of nNOS origin is called into play primarily in response to stressor exposure, future studies will have to investigate possible alterations of transcriptional and translational activity at the level of the hypothalamus and the adrenal gland in mutant mice following forced swimming. Also the cellular and molecular mechanisms underlie the action of NO in shaping the patterns of neuronal activation under stress conditions still remain elusive. Given that numerous studies conducted in the rat have demonstrated the importance of AVP and OXT somato-dendritic release on the regulation of vasopressinergic and oxytocinergic cells of the SON and the PVN, it is reasonable to infer that similar mechanisms may be of utmost importance also in the mouse. Therefore, future investigations should

implement the microdialysis technique to measure the intranuclear secretion of AVP and OXT as well as of excitatory and inhibitory amino acids, such as glutamate, taurine and GABA, in the extracellular space of the SON and the PVN in KO mice exposed to forced swimming. This would shed light on the role NO has in the intricate interplay of neurotransmitters that act centrally on the hypothalamic nuclei to govern the peripheral release of neurohormones. Additionally, since NO regulates the activity of the HPA-axis and the HNS at multiple levels, with supposingly opposite actions centrally and peripherally, *in vitro* experiments employing posterior and anterior pituitary slices, or hypothalamic organotypic cell slices containing the SON and the PVN of WT and KO animals may clarify mechanisms that are called into play at structures located within and outside the blood-brain barrier. Finally, our findings that KO mice express reduced levels of catecholamine biosynthetic enzymes and an altered release of E into the blood in response to forced swimming seem to indicate that NO acts in the adrenal medulla preferentially on adrenergic chromaffin cells, despite being produced mostly by noradrenergic cells. It could be therefore noteworthy to extend these results by investigating more closely the interaction between the two subtypes of chromaffin cells and the role NO plays in it. This could be done by using dissociated chromaffin cell cultures of WT and KO mice to measure, for instance, the intracellular calcium flow upon stimulation with external agents, such as acethylcoline or nicotine.

# 5 Summary and conclusions

This study was undertaken to elucidate the role nitric oxide (NO) plays in the regulation of the stress response in mammals. As previous reports investigating this issue with the aid of pharmacological agents yielded conflicting results, we decided to use neural nitric oxide synthase (nNOS) KO mice as animal model to examine the activity of the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-neurohypophyseal-system (HNS) and the sympatho-adrenal system (SAS) under physiological conditions and in response to a 10 minute-forced swimming session. Our findings suggest that, under basal conditions, NO inhibits vasopressin (AVP) gene transcription at the level of the supraoptic (SON), whereas it promotes it at the level of the paraventricular (PVN). Seemingly, its action is exercised through two mechanisms that differentially affect the activity of vasopressinergic neurones of the SON and the PVN, which supports the hypothesis of the existence of a functional diversity between the two hypothalamic nuclei. The reason of such a dichotomy might lie in the higher cellular complexity that characterises the PVN, which assembles different neuronal and glial cell types, and coordinates autonomic and neuroendocrine inputs in concert with other stressor-exposure sensitive brain areas, including the amygdala. A similar dichotomy appears evident also between vasopressinergic and oxytocinergic neurones, as mutant mice showed normal oxytocin (OXT) mRNA levels in both the SON and the PVN. Thus, NO of nNOS origin seems to modulate, at hypothalamic level, preferentially AVP production. The peripheral release of both neuropeptides under resting conditions in KO mice is unchanged, indicating that AVP and OXT secretion into the bloodstream from the neurohypophysis occurs as normal. Similarly, corticotropin-releasing hormone (CRH) mRNA at the hypothalamus as well as plasma adrenocorticotropic hormone (ACTH) and corticosterone (Cort) basal values were found to be unaffected by the absence of nNOS. Conversely, nNOS gene inactivation appeared to affect catecholamine biosynthetic enzymes, which are significantly reduced in KO mice, although this did not impair plasma norepinephrine (NE) and epinephrine (E) basal values. Overall, mutant mice manifest under resting conditions a mild phenotype, which is in accordance with previous observations.

However, upon acute stressor exposure NO seems to collaborate in maintaining constant AVP, OXT and E plasma profile release, as KO animals revealed anomalous AVP, OXT and E blood levels in response to forced swimming. The HPA-axis peripheral activity appeared to be affected only with respect to plasma Cort levels, which rose faster in KO than in WT mice following forced swimming. This is, however, not surprising, given the fact that we applied

conditions of acute stressor exposure, and a direct effect of the absence of NO/nNOS on the HPA-axis is probably evident only under conditions of chronic stressor exposure. Further studies are necessary to investigate in mutant mice the response of this system to chronic stressor exposure.

Taken together, our findings suggest that NO may be an important intermediary in the network engaged in modulating the endocrine stress response, and might, therefore, be implicated in the pathophysiology of diseases, such as anxiety and depression, that reflect a dysregulation of the stress response.

# 6 Zusammenfassung und Schlussfolgerung

Ziel dieser Promotionsarbeit war die Untersuchung der Rolle, die nNOS/NO in der Regulation der Stressantwort des Säugers spielt. Angesichts der widersprüchlichen Ergebnisse, die bislang aus pharmakologischen Untersuchungen vorlagen, wurde ein nNOS-KO-Mausmodell gewählt und an diesem die Aktivität der HPA-Achse, des HNS und des SAS unter Basalbedingungen und nach akuter Exposition zu einer 10-minütigen forcierten Schwimmsession analysiert. Die Ergebnisse der vorliegenden Arbeit legen die Vermutung nahe, dass unter Basalbedingungen nNOS/NO die Transkription von AVP im SON inhibiert und im PVN begünstigt. Demzufolge erfolgt die Wirkung von nNOS/NO über Mechanismen, die einen unterschiedlichen Einfluss auf die Aktivität von vasopressinergen Neuronen des SON gegenüber dem PVN. Dieser Befund unterstützt die Hypothese der funktionellen Verschiedenheit der zwei hypothalamischen Kerngebiete. Eine mögliche Ursache dieser Verschiedenheit könnte in der zellulären Komplexität des PVN liegen, welcher sich aus verschiedensten Neuronen- und Gliazelltypen zusammensetzt und in Zusammenspiel mit anderen für die Stressgeneration/-verarbeitung relevanten Hirnarealen (einschließlich Amygdala) autonome und neuroendrokrine Signale koordiniert. Eine vergleichbare Dichotomie besteht auch zwischen vasopressinergen und oxytocinergen Neuronen, da KO-Mäuse in SON und PVN OXT-mRNA-Expressionsprofile aufweisen, die denen der WT entsprechen. Demnach scheint von der nNOS produziertes NO auf Ebene des Hypothalamus vorzugsweise die Expression von AVP zu modulieren. Die periphere Freisetzung von AVP und OXT unter Basalbedingungen ist in KO-Mäusen unverändert. Das weißt daraufhin hin, dass die basale AVP- und OXT-Sekretion aus den Axonenterminalen magnozellulärer HNS-Neuronen in der Neurohypophyse vom Fehlen der nNOS unbeeinflusst bleibt. Das gilt auch für die basalen Konzentrationen für CRH-mRNA im Hypothalamus, sowie die vom Plasma ACTH und Cort. Demgegenüber reduziert die Inaktivierung des nNOS-Gens die Synthese der Katecholaminbiosynthese-Enzyme signifikant, jedoch ohne die basalen NE- und E-Plasmawerte zu verändern. Insgesamt stimmen unsere Befunde mit den Literaturbefunden überein, die einen milden Phänotyp für nNOS-KO-Mäuse unter Ruhebedingungen postulieren.

Unter akuten Stressbedingungen (forciertes Schwimmen) ist nNOS/NO offensichtlich an der adäquaten Freisetzung von AVP, OXT und E ins Plasma beteiligt. Die Befunde suggerieren, dass die Unterschiede in der Plasma-Cort-Konzentration zwischen WT-und KO-Mäusen, die in letzteren nach forciertem Schwimmen schneller ansteigt, ein sekundärer Effekt auf die

HPA-Achse sein könnte. Ein direkter Effekt von nNOS/NO auf die HPA-Achse ist möglicherweise nur unter chronischen Stressbedingungen nachweisbar, was diesbezügliche, weitergehende Untersuchungen mit Mutantenmäusen impliziert.

Unsere Ergebnisse suggerieren, dass NO ein wichtiger Modulator im Netzwerk ist, das die endokrine Stressantwort kontrolliert. Unsere Daten lassen zudem die Vermutung zu, dass die modulierende Funktion von nNOS produziertem NO erst in Antwort auf akute Stressorexposition, nicht aber unter Basalbedingungen, zur Geltung kommt. Allerdings sind weitere Studien hinsichtlich der Veränderung der Transkription und der Translation im Hypothalamus bzw. der Nebenniere bei KO-Mäusen erforderlich, um diesbezüglich detaillierte Aussagen treffen zu können.

Nimmt man die hier vorgelegten Befunde zusammen, so scheint es nicht ausgeschlossen, dass nNOS/NO eine Rolle beim Zustandekommen psychiatrischer Erkrankungen wie Angsterkrankungen und Depression spielen, die mir einer Änderung der HPA-Achsen-Aktivität einhergehen.

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# Abbreviations

ACTH: adrenocorticotropin hormone

ANOVA: analysis of variance

ATP: adenine tri-phosphate

AVP: arginine Vasopressin

BSA: bovine serum albumine

cGMP: cyclic guanidine-monophosphate

Cort: corticosterone

CRH: corticotropin-releasing hormone

cRNA: copy ribonucleic acid

CTP: cytidine tri-phosphate

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide tri-phosphate

DTT: dithiothreitol

E: epinephrine

ECL: enzymatic chemiluminescence

EDTA: ethylenediaminetetracetic acid

EIA: enzyme immunoassay

eNOS: endothelial nitric oxide synthase

GABA: gamma-aminobutyric acid

GTP: guanine tri-phosphate

HNS: hypothalamic neurohypophyseal system

HPA-axis: hypothalamic-pituitary-adrenal axis

HS: horse serum

iNOS: inducible nitric oxide synthase

KCl: potassium chloride

kDa: kilo Dalton

KO: knock-out

mRNA: messanger ribonucleic acid

NADPH: nicotinamide dinucleotide phosphate

NE: norepinephrine

NGS: normal goat serum

NMDA: N-methyl-D-aspartate

nNOS: neural nitric oxid synthase

NO: nitric oxide

NOS: nitric oxide synthase

OXT: oxytocin

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PKG: protein kinase G

PNMT: phenylethanolamine N-methyltransferase

PVN: paraventricular nucleus

RIA: radioimmunoassay

RNA: ribonucleic acid

RT: room temperature

SAS: sympatho-adrenal system

SDS: sodium dodecylsulfate

SEM: standard error of the mean

SON: supraoptic nucleus

SSC: sodium citrate buffer

TEA: triethanolamine

TH: tyrosine hydroxilase

tRNA: transfer ribonucleic acid

UTP: uracil tri-phosphate

WT: wild type

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**Academic Qualification**

1994-2000 Graduate degree in Molecular Biology (Università dell'Insubria, Varese, Italy)

(Sept. 1998-Jan. 2000) Training time as undergraduate student at the Laboratory for Experimental Pathology in Locarno (Switzerland)**.**
**Title of Diploma dissertation**: "The alpha-1 adrenergic receptors within the immune system"

1989-94 Higher secondary school (Gymnasyum) at "Liceo Alessandro Volta" in Como (Italy)

1986-89 Secondary school in Osimo (Italy)

1981-86 Primary school in Osimo (Italy)

**Research experience:**

- Undergraduate student at the Laboratory for Experimental Pathology in Locarno (Switzerland) from September 1998 to January 2000.
- Research fellow at the Institute of Medical Neurobiology, Dept. of Neuroimmunology, Otto-von-Guericke University, Magdeburg, Germany, from April 2001 to September 2002. The research focused on proteomic fingerprints of umbilical cord-derived stem cells.
- Ph.D. student at the Institute of Medical Neurobiology, Otto-von-Guericke University, Magdeburg, Germany from October 2002 to present. Research focuses on the role of nitric oxide on the murine hypothalamic-neurohypophyseal system and hypothalamic-pituitary-adrenal axis under resting conditions and upon stress.

**Areas of interest:**

- Neuroendocrinology
- Regulation of the stress response
- Nitric oxide and neuropeptides
- Neuroimmunology

**Area of specialization:** Neuroendocrinology / Stress response

# Publications:

1) P. Salchner, G. Lubec, M. Engelmann, **G.F. Orlando**, G. Wolf, S.B. Sartori, H. Hoeger and N. Singewald. Genetic functional inactivation of neuronal nitric oxide synthase affects stress-related Fos expression in specific brain regions. CMLS 61 (2004) 1498-1506

2) M.G. Spina, K. Langnaese, **G.F. Orlando**, T.F. W. Horn, J. Rivier, W.W. Vale, G. Wolf and M. Engelmann. Co-localization of urocortin and neuronal nitric oxide synthase in the hypothalamus and Edinger-Westphal nucleus of the rat. J Comp Neurol (2004) Nov 15;479(3):271-86

3) **G.F. Orlando**, K. Langnaese, R. Landgraf, M.G. Spina, G. Wolf and M. Engelmann. Neural nitric oxide gene inactivation affects the release profile of oxytocin into the blood in response to forced swimming. (Nitric Oxide, 2007 Feb;16(1):64-70).

4) **G.F. Orlando**, K. Langnaese, C. Schulz, G. Wolf and M. Engelmann. Neural nitric oxide synthase gene inactivation reduces the expression of vasopressin in the hypothalamic paraventricular nucleus and catecholamine biosynthetic enzymes in the adrenal glands of the mouse. (currently under review).

## Abstracts:

M.G. Spina, K. Langnaese, **G.F. Orlando**, S.A. Andrabi, T.F.W. Horn, J. Rivier, W. Vale, M. Engelmann and G. Wolf. Urocortin is coexpressed with neuronal nitric oxide synthase in the supraoptic nucleus but not in the Edinger westphal nucleus of rats.
1st Joint French-German NO Meeting, Strassbourg, France (1-4 October, 2003)

M.G. Spina, K. Langnaese, **G.F. Orlando**, S.A. Andrabi, T.F.W. Horn, J. Rivier, W. Vale, M. Engelmann and G. Wolf. Urocortin and neuronal nitric oxide synthase are colocalized in the supraoptic nucleus of the rat.
American Society for Neuroscience Meeting, New Orleans, USA (8-12 November, 2003)

**G.F. Orlando**, M.G. Spina, R. Murau, R. Landgraf, G. Wolf and M. Engelmann. No evidence for a specific contribution of neural nitric oxide synthase to the regulation of the hypothalamic-neurohypophyseal system in response to forced swimming.
4th Forum of European Neuroscience (FENS), Lisbon, Portugal (10-14 July 2004)
FENS Abstr. Vol.2, Abstract A163.19, 2004

M.G. Spina Horn, **G.F. Orlando**, K. Langnaese, T.F.W. Horn, W. Vale, M. Engelmann and G. Wolf. Mapping hypothalamic urocortin in relation with nitric oxide synthase, arginine vasopressin and oxytocin.
4th Forum of European Neuroscience (FENS), Lisbon, Portugal (10-14 July 2004)
FENS Abstr. Vol.2, Abstract A060.7, 2004

**G.F. Orlando**, K. Langnaese, M.G. Spina, R. Landgraf, G. Wolf and M. Engelmann. Hypothalamic-neurohypophyseal system response to forced swim-stress in WT and nNOS knock out mice.
9th Nitric oxide-Forum der deutschsprachigen Länder, Mainz, Germany (4-6 October 2004)

M.G. Spina, **G.F. Orlando**, T.F.W. Horn, W. Vale, M. Engelmann and G. Wolf. Mapping urocortin 1 in relation to arginine vasopressin and oxytocin in the supraoptic nucleus of the rat hypothalamus.
American Society for Neuroscience Meeting, San Diego, CA, USA (23-28 October, 2004).

**G.F. Orlando**, K. Langnaese, M.G. Spina, R. Landgraf, G. Wolf and M. Engelmann.
Constitutive lack of neuronal nitric oxide synthase affects the hypothalamic vasopressinergic system under resting conditions and upon swim stress in mice.
9th International Congress on amino Acids and Proteins, Vienna, Austria (8-12 August 2005).

**G.F. Orlando**, K. Langnäse, M.G. Spina, R. Landgraf, G. Wolf and M. Engelmann.
Nitric oxide modulation of oxytocinergic and vasopressinergic magnocellular neurones of the hypothalamic supraoptic nucleus.
10th Nitric oxide-Forum der deutschsprachigen Länder, Colon, Germany (6-7 October 2005).

**G.F. Orlando**, K. Langnäse, G. Wolf and M. Engelmann
Adrenaline biosynthetic enzyme expression and adrenaline release into the blood after stress are affected by neural nitric oxide gene inactivation.
5th Forum of European Neuroscience (FENS), Vienna, Austria (8-12 July 2006)
FENS Abstr. Vol.3, Abstract A219.13, 2006

**G.F. Orlando**, K. Langnäse, G. Wolf and M. Engelmann
Catecholamine biosynthetic enzymes are altered in neural nitric oxide synthase knockout mice.
2nd Joint French-German NO Meeting, Hamburg, Germany (5-7 October 2006)