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# The absence of a functional thymus is associated with alterations in peripheral and central neurotransmitters and neurotrophins

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## Summary

It is at present well established that there are physiological interactions between the nervous, endocrine, and immune systems. This work focuses on the impact that the lack of a functional thymus has on neurotransmitter and neurotrophin concentrations in the spleen and in defined brain regions, using as a model homozygous *Foxn1*$^{nu}$ mice. This spontaneous mutation results in defective development of the thymus anlage, and therefore, in lack of mature T cells. The first part of this work shows that *Foxn1*$^{nu}$ mice have: 1) an increased splenic sympathetic innervation that is maintained during adult life; 2) alterations in noradrenergic and serotonergic neurotransmitters in defined brain regions comparable to those in the spleen; 3) increased density of noradrenergic fibers in the spleen and hypothalamus; 4) increased brain-derived nerve growth factor (BDNF) and neurotrophin-3 (NT-3) concentrations, and BDNF signals in the spleen and hippocampus; 5) marked alterations in the anatomy of the hippocampus; and 6) increased corticosterone blood levels. All these alterations are abolished in *Foxn1*$^{nu}$ mice reconstituted by thymus transplantation at birth.

The second part studied whether the alterations detected in nude mice reflect a more general condition that causally relates the catecholaminergic system with the expression of neurotrophins. The results demonstrate that destruction of noradrenergic neurons by administration of a neurotoxin, results in a permanent or transient denervation that is paralleled by increased or decreased neurotrophin concentrations in the spleen and in the brain and in corticosterone blood levels, depending on the age at which mice are denervated.

Taken together, the results reported here indicate that the alterations in neurotransmitters and neurotrophins observed in homozygous *Foxn1*$^{nu}$ mice are not just an epiphenomenon fortuitously associated with the absence of a functional thymus, but that most likely mature T cells, by acting either directly or indirectly, exert an inhibitory influence on the development of splenic sympathetic innervation and of catecholaminergic and serotonergic mechanisms in the central nervous system. In more general terms, these results provide new evidence that the immune system can affect the nervous and endocrine systems.

## Zusammenfassung

Physiologische Wechselwirkungen zwischen neuronalem, endokrinem und Immunsystem sind inzwischen gut belegt. Diese Arbeit befasst sich mit Auswirkungen des Fehlens von funktionsfähigem Thymus auf Neurotransmitter- und Neurotrophin-Konzentrationen in der Milz sowie bestimmten Hirnregionen unter Verwendung homozygoter *Foxn1*$^{nu}$ Mäuse als Modell. Diese Spontanmutation führt zu einer fehlerhaften Entwicklung der Thymusanlage mit Ausbleiben der Entwicklung reifer T-Zellen.

Der erste Teil der Arbeit zeigt bei *Foxn1*$^{nu}$ Mäusen 1) eine verstärkte sympathische Innervation der Milz, die auch im Erwachsenalter anhält; 2) Änderungen noradrenerger und serotonerger Neurotransmitter in bestimmten Hirnregionen, vergleichbar denen in der Milz; 3) eine erhöhte Dichte noradrenerger Fasern in Milz und Hypothalamus; 4) eine Erhöhung der Konzentrationen des Wachstumsfaktors „brain-derived nerve growth factor“ (BDNF) und von Neurotrophin-3 (NT-3) sowie von BDNF-Signalen in Milz und Hippocampus; 5) ausgeprägte anatomische Änderungen des Hippocampus und 6) erhöhte Kortikosteron-Blutwerte. Diese Änderungen verschwinden nach Rekonstitution der *Foxn1*$^{nu}$ Mäuse durch Thymustransplantation bei Geburt.

Der zweite Teil geht der Frage nach, ob diese Änderungen in athymischen Mäusen grundsätzlicher bedeuten, dass das catecholaminerge System ursächlich mit der Neurotrophinbildung in Verbindung steht. Die Befunde zeigen, dass die Zerstörung noradrenerger Neurone nach Neurotoxinapplikation zu einer dauerhaften oder vorübergehenden Denervierung führt, begleitet von zu- oder abnehmenden Kortikosteron-Blutspiegeln sowie Neurotrophin-Konzentrationen in Milz und Gehirn, abhängig vom Alter der Denervierung.

Zusammengenommen weisen die hier vorgelegten Ergebnisse darauf hin, dass die bei *Foxn1*$^{nu}$ Mäusen gefundenen Änderungen an Neurotransmittern und Neurotrophinen kein Epiphänomen darstellen, das zufällig mit dem Fehlen der Thymusfunktion einhergeht, sondern dass wahrscheinlich reife T-Zellen direkt oder indirekt eine inhibitorische Wirkung auf die Entwicklung der sympathischen Milzinnervation sowie auf catecholaminerge und serotonerge Mechanismen des zentralen Nervensystems entfalten. Die Ergebnisse liefern somit neue Belege, dass das Immunsystem nervale und endokrine Systeme beeinflussen kann.

# 1. Introduction

## 1.1. Interactions between the immune, endocrine, and nervous systems

The nervous, endocrine, and immune systems are the major adaptive systems of the body. An appropriate communication between these systems is essential to maintain homeostasis and health. So far, the best studied neural and endocrine pathways involved in this cross-talk are the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Elenkov et al., 2000).

The general immunosuppressive and anti-inflammatory effects of cortisol, the end product of the HPA axis, have been known for over 60 years, and it is now known that changes in endogenous levels of this hormone can affect the immune response (Besedovsky and del Rey, 1996). Evidence accumulated over the last three decades indicates that the SNS, a component of the autonomic nervous system (ANS), innervates all lymphoid organs, and that noradrenaline (NA), the main sympathetic neurotransmitter, can modulate several immune parameters. Therefore, the SNS provides another major integrative and regulatory pathway between the brain and the immune system (Elenkov et al., 2000).

This work focuses on interactions between these systems, in particular on the effect that the thymus, and as a consequence, the presence of T cells, has on neurotransmitter and neurotrophin concentrations in one lymphoid organ, the spleen, and in certain brain regions, and on corticosterone blood levels in mice.

## 1.2. Brief historical background

Evidence that lymphoid organs are innervated dates back to the end of the 19$^{th}$ century, when nerves were found to enter into lymph nodes independently from blood vessels (Elenkov et al., 2000). Between 1880 and 1920, Langley, in collaboration with Anderson, defined the functional features of the sympathetic and parasympathetic systems, showing how differently effector target tissues and organs were affected by segmental ventral root stimulation (Janig and McLachlan, 1992). In 1898, Otto von Fürth isolated a bioactive compound from

animal tissue and called this partly purified product “suprarenin“. Three years later, Takamine and Aldrich isolated the responsible component in crystalline form (Benschop et al., 1996). Takamine named the substance adrenaline, and Aldrich described the correct formula ($C_9H_{18}NO_3$). Thus, adrenaline (A) was the first hormone isolated from tissue more than 100 years ago. In 1907, a by-product in the synthesis of A (or epinephrine) was identified. This substance, which became commercially available as Arterenol in 1908, was in fact NA (or norepinephrine), and was formally discovered and isolated from tissue 40 years later (Benschop et al., 1996).

At the end of the 19$^{th}$ century and at the beginning of the last century, while Sherrington introduced the concept of chemical neurotransmission (Vizi and Labos, 1991), Metchnikoff and Ehrlich developed the concept of cellular and humoral immunity (Elenkov et al., 2000). The pronounced leukocytosis observed after injection of A into humans was first described by Loeper and Crouzon in 1904 (Elenkov et al., 2000). In 1919, the observation of Ishigami that the phagocytic activity of leukocytes was decreased during periods of marked psychological stress in humans suffering from chronic tuberculosis, was probably the first finding to indicate that stress can affect the immune system (Elenkov et al., 2000). In 1920s, Metalnikov and Chorine showed that immune reactions could be conditioned by a classical Pavlovian paradigm (Elenkov et al., 2000). In 1930s, anatomists demonstrated that the thymus gland is innervated (for review see Kendall and al-Shawaf, 1991). At this time, the thymus was regarded as a rudimentary organ, but, after about 30 years, it was discovered that it functions as a primary lymphoid organ. In the 1930s, the concept of “stress response” was developed by Hans Selye, who described the involution of the thymus in animals exposed to stressors (Elenkov et al., 2000). The stress response was called “fight or flight” reaction by the physiologist Cannon, who linked the adaptive response to stress with catecholamine secretion and actions. Cannon also emphasized the sympathetic “generalized” response, or the “wisdom of the body” that occurs during stress, contrasting with more “discrete” functions of parasympathetic pathways (Chrousos and Gold, 1992; Janig and McLachlan, 1992). At about the same time, Loewi and Dale, in pursuing the concept of chemical synaptic transmission, mimicked the response of peripheral organs to autonomic nerve stimulation by applying

substances that they extracted from the same or other peripheral organs (Elenkov et al., 2000).

In the 1940s, Euler isolated NA from a lymphoid organ, the spleen, and later provided evidence that NA is the major neurotransmitter released by sympathetic nerves (Axelrod and Kopin, 1969). However, in the following two decades, the spleen was often considered only as a “blood reservoir“, and studies focused on the role of sympathetic innervation in the regulation of the contraction of the splenic capsule in certain mammals, and of vascular resistance and blood flow. This led to the assumption at that time, that NA-containing nerve fibers in the spleen have no other functions. Interestingly, in the 1950s, Dougherty and Frank noticed an about 400% increase in blood lymphocyte numbers within 10 min after subcutaneous injection of A, and they called them “stress-lymphocytes” (Dougherty and Frank, 1953). These cells had the morphology of large granular lymphocytes or natural killer (NK) cells, whose function and characteristics were described in the late seventies (Benschop et al., 1996).

Only in the 1970s and the 1980s, however, due to the pioneering work of Besedovsky and coworkers, it became clear that classic hormones and newly described cytokines are involved in a functionally relevant cross-talk between the nervous, endocrine, and immune systems (Besedovsky et al., 1986; Besedovsky et al., 1975; Besedovsky et al., 1979). They showed that the immune response to innocuous antigens induces an increase in plasma corticosteroid levels (Besedovsky et al., 1986; Besedovsky et al., 1975; Besedovsky et al., 1981), alters the activity of hypothalamic noradrenergic neurons (Besedovsky et al., 1983), and the content and turnover rate of NA in the spleen (Besedovsky et al., 1979). Also in the 1970s, the presence of functional adrenergic receptors on lymphocytes were first described, when it was reported that adrenergic agents modulate lymphocyte proliferation (Hadden et al., 1970). In the 1970s and 1980s, comprehensive morphological studies provided evidence that both primary and secondary lymphoid organs are innervated by sympathetic noradrenergic nerve fibers (Bulloch and Pomerantz, 1984; Felten and Olschowka, 1987; Fink and Weihe, 1988). Furthermore, it was shown that classical behavioral conditioning (Ader and Cohen, 1982), stressful stimuli (Chrousos, 1995; Cohen et al., 1991; Keller et

al., 1983), or by lesions in specific brain regions (Elenkov et al., 2000) can result in alteration of immune functions. Finally, evidence was obtained in experimental animals that the susceptibility to autoimmune diseases can be modulated by the activity of the HPA axis and the SNS (Sternberg et al., 1989; Wilder, 1995) and that stress mediators may exert both pro- and anti-inflammatory effects (Chrousos, 1995; Karalis et al., 1991). Thus, an explosive growth of the research area that studies neuro-endocrine-immune communication has occurred in the last two decades (Besedovsky et al., 1983; Besedovsky and del Rey, 1996; Besedovsky et al., 1987; Besedovsky et al., 1979; del Rey et al., 2006).

## 1.3. The nervous system

The nervous system, which controls and integrates the functional activity of the organs and bodily systems, enables the body to respond to changes in its external and internal environment. Anatomically, the nervous system is divided into the central nervous system (CNS), composed by the brain and the spinal cord, and the peripheral nervous system (PNS), which consists of cranial, spinal, and peripheral nerves. Functionally, it is divided into the somatic nervous system, which consists of somatic parts of the CNS and PNS, and the ANS, composed by autonomic parts of the CNS and the PNS (Ross, 2011).

### 1.3.1. The brain

The brain has the consistency of firm jelly, and therefore is protectively encased in a thick bony skull. The brain literally floats in cerebrospinal fluid (CSF) secreted by the choroid plexus, which slowly circulates down through the four ventricles, up through the subarachnoid space and exits into the cerebral veins through the arachnoid villi. The brain has no lymphatic system, so the CSF serves as a partial substitute (Zakharov et al., 2003). The dura mater is a tough, protective connective tissue that is tightly bound to the skull, and encases the cerebral veins. The subarachnoid space, containing CSF, arteries, and web-like strands of connective/supportive tissue called the arachnoid mater, is found under the dura mater. The pia mater is a permeable membrane of collagen, elastin fibers, and fibroblasts on the floor of the subarachnoid space, which allows diffusion between the CSF and the interstitial fluid of the brain tissue. The pia mater lies on a membrane that is infiltrated with astrocyte

processes. The dura mater, the arachnoid mater, and the pia mater are collectively referred to as the meninges (Ross, 2011).

While the brain and CSF are separated by the somewhat permeable pia mater, the blood-cerebrospinal fluid barrier and the blood-brain barrier (BBB) represent substantial protection for the brain against undesirable substances present in the blood. These barriers are very permeable to water, oxygen, carbon dioxide, and small lipid-soluble substances. They are also somewhat permeable to small electrolytes, and special transport systems exist for some specific molecules, such as essential amino acids. The barriers are largely constituted by elaborated tight junctions among endothelial cells, which form continuous-type capillaries, and their integrity depends on astrocytes. The BBB creates a protected environment for the brain, wherein certain molecules can perform functions independent of those they perform in the rest of the body. This is particularly important for the neurotransmitters serotonin (5-HT) (which is highly concentrated in platelets and the intestine) and NA (which affects blood pressure and metabolism). All the amino acids that function as neurotransmitters are non-essential. This means that they can be manufactured in the brain, without the need of being supplied from outside the brain.

The brain is subdivided into the cerebrum, cerebellum, and brainstem, which is connected with the spinal cord. The hypothalamus, hippocampus, and brainstem are important brain parts in vertebrates. Only these structures are briefly mentioned below since these are the brain regions studied in this work.

The hypothalamus is a very small and complex region at the base of the forebrain, located below the thalamus, and just above the brainstem. It is composed of numerous small nuclei with a variety of functions. One of the most important functions of the hypothalamus is to link the nervous system to the endocrine system via the pituitary gland. The hypothalamus is responsible for certain metabolic processes and other activities of ANS. It synthesizes and secretes releasing hormones, which in turn stimulate or inhibit the secretion of pituitary hormones. Furthermore, it regulates sleep and wake cycles, eating and drinking, and circadian rhythms.

The hippocampus is found only in mammals. However, the medial pallium, the area from which it derives, has counterparts in all vertebrates. Humans and

other mammals have two hippocampi, one on each side of the brain. It contains two main interlocking parts: Ammon's horn and the dentate gyru (DG). There is evidence that this part of the brain is involved in learning and memory, and plays important roles in the consolidation of information, from short-term to long-term memory, and spatial navigation.

The brainstem is the posterior part of the brain, continuous with the spinal cord. It is usually described as including the medulla oblongata, pons, and midbrain. It provides the main motor and sensory innervation to the face and neck via the cranial nerves. The brainstem also plays an important role in the regulation of cardiac and respiratory functions, and eating. It is pivotal in maintaining consciousness and regulating the sleep cycle.

### 1.3.2. The autonomic nervous system

The ANS forms the major efferent component of the PNS and regulates the function of most tissues and organs, with the exception of skeletal muscles (Tsigos and Chrousos, 2002). The ANS has three divisions: the SNS and the parasympathetic nerve system, which originate in the CNS (Tsigos and Chrousos, 2002), and the enteric system, which lies within the wall of the gastrointestinal tract. While the functioning of most body systems is modulated by the SNS and the parasympathetic nerve systems (Gilbey and Spyer, 1993), the enteric system regulates intestinal functions, although it is also affected by projections from the SNS and the parasympathetic nerve system (Elenkov et al., 2000).

Most sympathetic preganglionic fibers terminate in ganglia located in the paravertebral chains on either side of the spinal column, and the remaining in prevertebral ganglia, such as the superior cervical, the celiac, and the superior and inferior mesenteric ganglia. Post-ganglionic sympathetic fibers, originating in the paravertebral and prevertebral ganglia, run to the organs target of this type of innervation. The preganglionic neurons are cholinergic, whereas the postganglionic neurons are mostly noradrenergic and release NA. The adrenal medulla can be considered as a modified sympathetic ganglion, in which the postganglionic nerve cells do not leave the medulla. They release A and NA at a ratio of approximately 4:1. Thus, the principal end products of the SNS are catecholamines (Elenkov et al., 2000).

### 1.3.3. The hypothalamus-pituitary-adrenal axis

Interactions between the hypothalamus, the pituitary gland, and the adrenal glands constitute a complex neuro-endocrine axis that is critical for the integration and coordination of important physiological functions and for the maintenance of homeostasis (Fig. 1). The hypothalamus controls the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary by secreting corticotrophin-releasing hormone (CRH), a 41 amino acid peptide first isolated in 1981 by W. Vale (Vale et al., 1981). Arginine vasopressin (AVP), also synthetized in the hypothalamus, acts as a synergistic factor with CRH in stimulating ACTH secretion (Lamberts et al., 1984). In turn, ACTH stimulates the secretion of glucocorticoid hormones by the adrenal cortex (Vale et al., 1981).

Under basal conditions, both CRH and AVP synthetized by parvocellular neurosecretory neurons are secreted in the portal system with a frequency of about two to three episodes per hour (Engler et al., 1989). In humans, the secretion of CRH and AVP increases in the early morning, resulting finally in increased ACTH and cortisol secretary bursts in the general circulation (Chrousos and Gold, 1998; Horrocks et al., 1990). CRH and AVP secretion markedly increases during acute stress, resulting in increased ACTH and cortisol secretion (Tsigos and Chrousos, 1994). Depending on the type of stress, other factors such as AVP of magnocellular neuron origin, angiotensin II, and various cytokines and lipid mediators of inflammation act on the hypothalamus, pituitary or adrenal glands, potentiating the activity of the HPA axis (Holmes et al., 1986; Phillips, 1987). Other factors may also participate in the regulation of cortisol secretion, such as hormones and cytokines, either originating from the adrenal medulla or from the systemic circulation, as well as neuronal information from the autonomic innervation of the adrenal cortex (Hinson, 1990; Ottenweller and Meier, 1982).

The final effectors of the HPA axis are glucocorticoids, which contribute to control body homeostasis and the response of the organism to stressful situations. In turn, they play a key regulatory role on the termination of the stress response by exerting a negative feedback at hypothalamic and pituitary gland levels, thus inhibiting further ACTH release. This feedback limits the duration of the tissue exposure to glucocorticoids, thus minimizing the

catabolic, anti-reproductive, and immunosuppressive effects of these hormones (Tsigos and Chrousos, 2002).

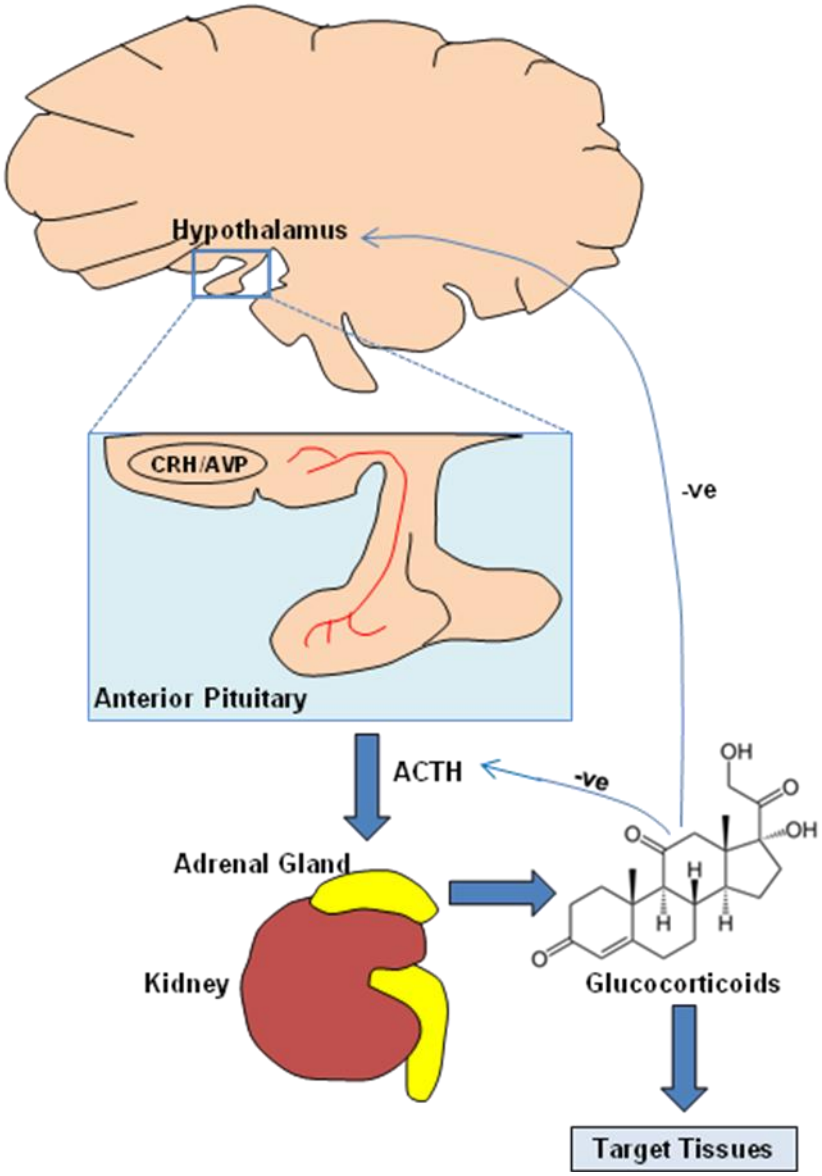


**Fig. 1**. **The hypothalamus-pituitary- adrenal axis.** CRH and AVP are secreted in the portal system, resulting finally in ACTH and cortisol secretory bursts in the general circulation. Glucocorticoids, in turn, exert a negative feedback (-ve) and inhibit further CRH, AVP, and ACTH release.

Noradrenergic neuronal fibers in the hypothalamus, which mainly arise from cell bodies in brainstem nuclei, modulate the activity of efferent pathways to the pituitary and to descending brainstem and spinal cord regions associated with the ANS. Since cell bodies producing CRH are localized in the paraventricular nucleus (PVN) of the hypothalamus, NA changes in the PVN during immune response (Carlson et al., 1987) are important for the activation of the neuro-

endocrine axis (Besedovsky et al., 1986). In general, the activation of the HPA axis inhibits inflammatory mechanisms and regulates both the extent and the specificity of an ongoing immune response (Sapolsky et al., 2000). On the other hand, immune cells release products that have the capacity to affect central and peripheral noradrenergic mechanisms (Besedovsky et al., 1983; Kabiersch et al., 1988), and to induce endocrine changes (Besedovsky et al., 1986; Malarkey and Mills, 2007) that are relevant for immunoregulation (Elenkov et al., 2000; Sapolsky et al., 2000).

### 1.3.4. Neurotransmitters

Neurons communicate with each other by releasing chemical messengers, termed neurotransmitters. More than 100 different neurotransmitters are known today, which result in a large diversity in chemical signaling between neurons. Neurotransmitters are divided into large transmitter molecules composed of 3 to 36 amino acids, and small transmitter molecules. Large transmitter molecules include neuropeptide neurotransmitters, such as CRH, β-endorphin, Substance P, neurotensin, somatostatin, and AVP. Acetylcholine, amino acid neurotransmitters, such as glutamate, GABA, glycine, and aspartate, the biogenic amines, such as dopamine (DA), NA, 5-HT, and histamine, are among the small molecule neurotransmitters (Purves D, 2008).

Only the biogenic amines NA, DA and 5-HT, which are active in the brain and in the PNS, are briefly mentioned below since these are the neurotransmitters studied in this work.

All catecholamines derive from tyrosine (Tyr), a non-essential amino acid that can be synthesized in the liver from phenylalanine by the enzyme phenylalanine hydroxylase. However, Tyr cannot be synthesized in the brain, and it must therefore enter the brain by a large neutral amino acid transporter, which also transports phenylalanine, tryptophan (Trp), methionine, and the branch-chained amino acids. All these amino acids compete for the transporter, so the amount of Tyr entering the brain can be limited by the presence of a large quantity of one of the other amino acids in the blood stream. Tyr can be converted to dihydroxyphenylalanine (DOPA) by a reaction requiring tetrahydrobiopterin as cofactor, oxygen as co-substrate, and the enzyme tyrosine hydroxylase (TH), the first and rate-limiting step in catecholamine synthesis (Purves D, 2008). DOPA is further converted to DA, which is finally converted to NA by dopamine-

β-hydroxylase (DBH) (Elenkov et al., 2000). DA and NA are transported into synaptic vesicles by the vesicular monoamine transporter (VMAT). In the adrenal medulla, NA can be converted to A by the enzyme phenylethanolamine N-methyltransferase (PNMT) (Purves D, 2008) (Fig. 2). TH- and DBH-immunostaining are often used as specific markers of noradrenergic innervation in various organs.

DA is found in several brain regions, and the corpus striatum is the major DA-containing area. This area receives major inputs from the substantia nigra and plays an essential role in the coordination of body movements (Purves D, 2008). Small amounts of DA are found in peripheral organs, for example in mast cells and secretory cells of the gastrointestinal tract, and in the fluid-filled spaces of organs, *e.g.,* the lumen of the small intestine. DA is also found in peripheral organs that receive sympathetic innervation. This DA may serve a neurotransmitter function, activating DA-receptor-mediated events in the organ, and could be released into the extracellular space and potentially into the bloodstream during sympathetic activation (Snider and Kuchel, 1983).

NA in the brain is found mostly in the locus coeruleus, a nucleus in the pons that projects diffusely to a variety of forebrain targets. This brainstem nucleus influences sleep and wakefulness, attention, and feeding behavior. NA is also found in the neocortex, hippocampus, hypothalamus, and cerebellum (Purves D, 2008). In the periphery, NA is released from postganglionic neurons of the SNS, and act as transmitter in the tissues. As already mentioned, the adrenal medulla can also release NA into the blood, thus acting as a hormone.

A is present in the brain at lower levels than the other catecholamines. A-containing neurons in the CNS are present in the lateral segmental and in the medulla, and project to the hypothalamus and thalamus. The function of these neurons is not known (Fuller, 1982; Mefford, 1987; Purves D, 2008). In the periphery, it is produced in the chromaffin cells of the adrenal medulla from NA and is also release into the blood.

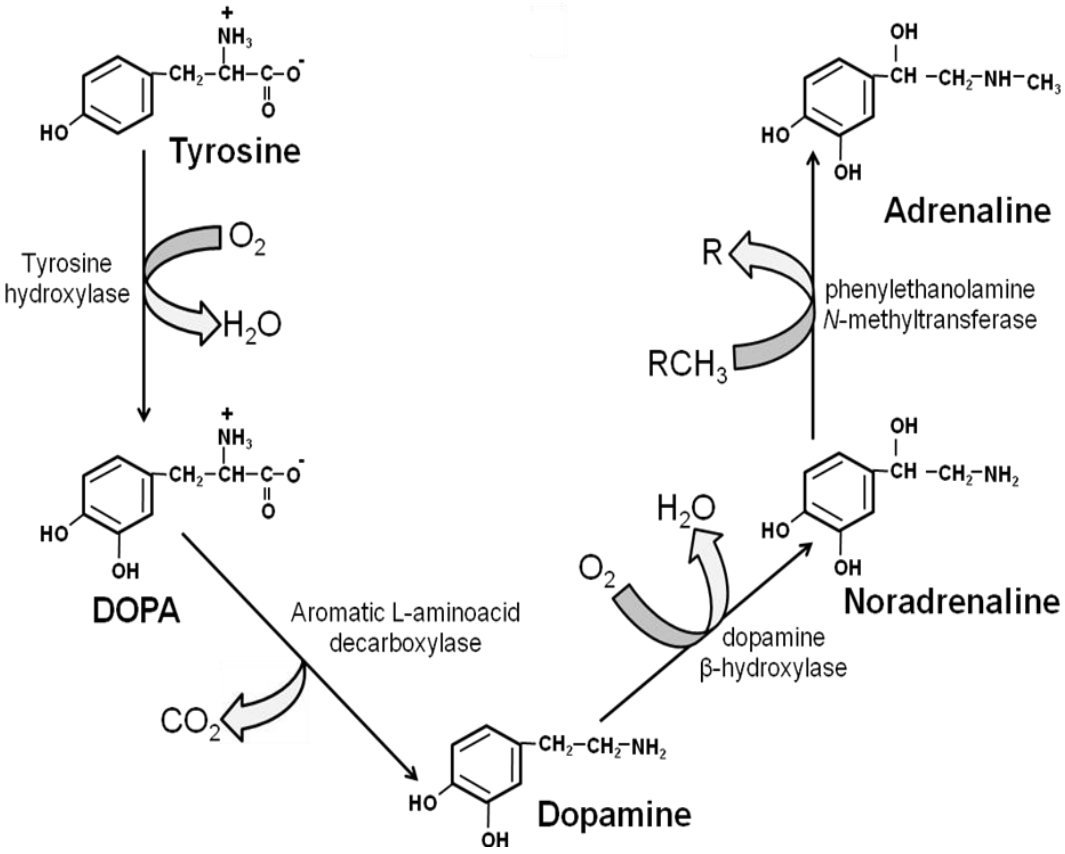


**Fig. 2. Catecholamine biosynthetic pathway.** The amino acid tyrosine is the precursor for all three catecholamines. The first step in this pathway is catalyzed by tyrosine hydroxylase, the rate-limiting step.

Serotonin is an indolamine, and its name derives from “serum” and “tonic” since it was first isolated from the serum as a vasoconstrictory substance. Later, it was found primarily in groups of neurons in the raphe region of the pons and upper brainstem, which have widespread projections to the forebrain. 5-HT is synthesized from the essential amino acid Trp. The first and rate-limiting step in 5-HT synthesis is catalyzed by the enzyme tryptophan hydroxylase (Purves D, 2008). 5-HT in the brain is involved in the control of sleep, thermoregulation, appetite, addictive behaviors, cognitive abilities, learning, memory, mood, aggression and anxiety. In the periphery, 5-HT is mainly located in the enterochromaffin cells in the gut, where it regulates intestinal movement. When it is secreted from these cells, it can reach other tissues via the blood, where it is taken up by platelets and stored, and contributes to hemostasis.

### 1.3.5. Neurotrophins and their receptors

Neurotrophic factors are a group of proteins that play an important role in the development and maintenance of the nervous system (Barde, 1989). The first neurotrophic factor to be characterized was nerve growth factor (NGF), which is essential for neuronal growth and differentiation during development, and also supports the survival and maintenance of sympathetic, neural crest-derived sensory and septal cholinergic neurons during adulthood (Leibrock et al., 1989; Levi-Montalcini, 1987). The second factor of the NGF family described was brain-derived neurotrophic factor (BDNF), which was discovered about two decades ago (Barde, 1989). BDNF supports placode-derived sensory, retinal ganglion, dopaminergic and cholinergic neurons of the substantia nigra and forebrain (Alderson et al., 1990; Hyman et al., 1991; Lindsay et al., 1985; Rodriguez-Tebar et al., 1989). Neurotrophins-3 (NT-3), and NT-4/5 are other NGF homologues, structurally related to NGF with 55%-65% amino acid sequence homology (Berkemeier et al., 1991; Ernfors et al., 1990; Hallbook et al., 1991; Hohn et al., 1990; Ip et al., 1992). It has also been reported that the NT-3 knockout mice develop severe deficits in the peripheral sensory and SNS (Ernfors et al., 1995). Studies performed on adult lung biopsies indicate that neurotrophins and their receptors are expressed by a variety of lung resident cells. Constitutive expression of NGF, BDNF and NT3 on airway epithelial cells has been documented (for review see Renz and Kilic, 2012)

Neurotrophins also play a crucial role in the survival and differentiation of visceral neurons during development (Huang and Reichardt, 2001; Snider, 1994). They are also expressed in visceral targets of adult rodents and humans (Katoh-Semba et al., 1996; Katoh-Semba et al., 1989; Lommatzsch et al., 1999; Timmusk et al., 1993; Yamamoto et al., 1996; Zhou and Rush, 1993), and retrogradely transported in adult visceral sensory and motor neurons (Helke et al., 1998). There is evidence that the levels of NGF in several tissues that are target of sympathetic neurons correlate with the density of sympathetic innervation (Korsching and Thoenen, 1983), and that these tissues are the major source of NGF required by sympathetic neuron survival and functioning (Korsching and Thoenen, 1985). Although originally isolated from the submaxillary gland, NGF is synthesized by several cell types, including smooth

muscle cells, fibroblasts, and neurons, and it was the first neurotrophin shown to be expressed also by immune cells (T and B lymphocytes, macrophages, and mast cells) (Besser and Wank, 1999; Kerschensteiner et al., 1999; Kerschensteiner et al., 2003). BDNF, which was previously thought to be primarily present in neurons in the CNS, is also produced by muscle cells and by developing and mature sympathetic neurons (Causing et al., 1997). More recently, it has been shown that BDNF is also expressed in immune cells (Besser and Wank, 1999; Kerschensteiner et al., 1999; Kerschensteiner et al., 2003), and that it can be produced *in vitro* by all major immune cell types, including $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and monocytes (Kerschensteiner et al., 2003). Thus, neurotrophins can act as growth factors with a wide spectrum of functions outside the nervous system, such as modulation and regulation of immune functions (Aloe, 2001; Nockher and Renz, 2003; Vega et al., 2003). There is evidence suggesting that NGF acts as a cytokine-like factor in the development and function of the immune system. It has already been described that NGF affects mast cell, B cell, T cell, macrophage and eosinophil functions (Braun et al., 1998; Nassenstein et al., 2003; Nilsson et al., 1997; Torcia et al., 2001), but there is much less information available regarding the role of BDNF in the immune system (Schuhmann et al., 2005).

Two classes of receptors for neurotrophins have been described: low affinity receptors with similar characteristics on target cells (p75) (Chao et al., 1986; Hallbook et al., 1991; Hempstead et al., 1989), and high affinity receptors, which are members of the *Trk* proto-oncogene family and have tyrosine kinase activity. *Trk A* binds NGF, NT-3 and NT-4/5 with high affinity (Berkemeier et al., 1991; Cordon-Cardo et al., 1991; Hempstead et al., 1991; Klein et al., 1991) and is expressed on neural crest-derived cells (Martin-Zanca et al., 1990). *Trk B* binds BDNF, NT-3, NT-4/5 (Berkemeier et al., 1991; Soppet et al., 1991; Squinto et al., 1991) and is expressed in the embryonic and adult nervous system (Klein et al., 1990; Klein et al., 1989). Finally, *Trk C* binds only NT-3 (Lamballe et al., 1991; Zhou and Rush, 1993). T lymphocytes express both types of *Trk B* receptors (Besser and Wank, 1999), the full length *Trk* $B_{gp145}$ receptor and the truncated *Trk* $B_{gp65}$ receptor, which lacks the intracellular signal transduction domain (Huang and Reichardt, 2003). Within the context of

this work, it is interesting to mention that the pattern of *Trk B* expression depends on the developmental stage of T cells (Maroder et al., 1996; Maroder et al., 2000; Schuhmann et al., 2005).

## 1.4. Lymphatic tissues and organs

The bone marrow, where B cells are produced in most mammals, and the thymus, where hematopoietic progenitors derived from the bone marrow mature and develop into T cells, are the main primary lymphoid organs in mammals. The thymus is composed of two lobes located in the superior mediastinum and anterior to the heart and great vessels. The thymus is fully formed and functional at birth, but most of the lymphatic tissue is replaced by adipose tissue at the time of puberty, when T cell differentiation and proliferation decrease. This processes is called thymic involution (Ross, 2011), but T lymphopoiesis continues throughout adult life.

The lymph nodes, the spleen, and the mucosa-associated lymphoid tissue (MALT) constitute the main secondary lymphoid organs, where specific immune responses to antigens derived from the tissues, blood, and the mucosa, respectively, take place.

Lymph nodes are small, bean-shaped, and encapsulated lymphatic organs. They are located along lymphatic vessels and filter the lymph, which percolates on its way to the blood vascular system (Ross, 2011). The spleen is the largest lymphatic organ. It is located in the upper left quadrant of the abdominal cavity and has a rich blood supply. It contains large numbers of lymphocytes, macrophages and dendritic cells, and a meshwork of reticular cells and fibers and specialized vascular spaces or channels, which allow the spleen to monitor the blood immunologically (Ross, 2011).

The spleen consists of two functionally and morphologically different regions: the white and the red pulp. The white pulp contains mostly lymphocytes, while the red pulp is made up of several cells types, but mainly red blood cells.

The spleen is encapsulated by a dense connective tissue. Trabeculae extend from the capsule into the parenchyma of the organ. On the medial surface of the spleen is located the hilum, the site for the passage of the splenic artery and vein, nerves, and lymphatic vessels. Lymphatic vessels

originate in the white pulp near the trabeculae, and they constitute a route for lymphocytes to leave the spleen. The branches of the splenic central artery course through the capsule and trabeculae and enter the white pulp. The periarteriolar lymphatic sheath (PALS) is formed by lymphocytes aggregated around the central artery. PALS resemble a lymphatic nodule and consist of B lymphocytes surrounded by T lymphocytes, which are the main cell type in the PALS (Ross, 2011) .

## 1.5. Sympathetic innervation of lymphoid organs

The ANS innervates most organs of the body, although lymphoid organs are predominantly innervated by the SNS (Madden et al., 1995). Histofluorescense studies in the 1960s demonstrated that noradrenergic nerves fibers are present in lymphoid organs (Bulloch and Pomerantz, 1984; Dahlstroem and Zetterstroem, 1965; Felten et al., 1985; Giron et al., 1980; Kendall et al., 1988; Reilly et al., 1979; Williams and Felten, 1981; Zetterstrom et al., 1973). More recently, specific immunohistochemistry for TH and DBH were used to detect and confirm the presence of noradrenergic innervation in lymphoid tissues (Felten and Olschowka, 1987; Vizi et al., 1995; Weihe et al., 1991). The available data about the innervation of lymphoid organs in humans is relatively scarce, since most of the current knowledge is based on studies in rodents (Elenkov et al., 2000; Felten et al., 1985; Felten et al., 1988; Weihe et al., 1991).

The SNS innervates both the smooth muscle of the vasculature and the parenchyma of specific compartments in primary and secondary lymphoid organs (Felten et al., 1985). Sympathetic nerve fibers and their varicosities travel in plexuses that run adjacent to smooth muscle cells of the blood vessels in lymphoid organs (Felten et al., 1985), and can therefore control blood flow and influence lymphocyte traffic (Elenkov et al., 2000). However, noradrenergic fibers are not only associated with blood vessels, but are also present in the parenchyma of lymphoid organs (Felten et al., 1985; Vizi et al., 1995). Thus, perivascular and parenchymal noradrenergic nerve fibers release NA, which can affect lymphoid cell functions and exert an immunomodulatory role (Elenkov et al., 2000). Zones containing T cells, macrophages and plasma cells are richly innervated by noradrenergic fibers, while the nodular and follicular zones in

which mainly developing and mature B cells are found, are poorly innervated (Felten et al., 1985). Thus, immature and mature thymocytes, T lymphocytes, macrophages, mast cells (Blennerhassett and Bienenstock, 1998), plasma cells, and enterochromaffin cells appear to be the main targets of the noradrenergic innervation. Noradrenergic innervation of both perivascular and parenchymal zones of lymphoid organs, particularly in the thymus, are closely associated with mast cells, suggesting that there is a possible humoral role for NA in the development of T cells in the thymus. Since noradrenergic fibers appear early in development, and their arrival generally precedes the development of the cellular compartment of the immune system, it has been proposed that NA plays a role in the maturation of the immune system (Elenkov et al., 2000). The innervation of the spleen is described in more detail below, since this secondary lymphoid organ was studied in this work.

Approximately 98% of the splenic nerve fibers are sympathetic (Klein et al., 1982). These fibers enter the spleen together with the splenic artery and travel with the vasculature in the plexuses (Williams and Felten, 1981). They reach the white pup from both the vascular and trabecular plexuses and continue mainly along the central artery and its branches. From these plexuses, noradrenergic varicosities radiate into the PALS (Williams and Felten, 1981). The highest density of noradrenergic fibers in the spleen is associated with the central artery in the white pulp and the PALS; dense linear arrays of varicosities extend away from the periarteriolar plexus and travel into the parenchyma (Felten et al., 1985; Williams and Felten, 1981). Sympathetic nerve fibers are mainly present in the T-dependent areas, but also in the marginal zone and marginal sinus, where macrophages and B cells reside. These are the sites where lymphocytes can directly contact noradrenergic fibers (Felten et al., 1985; Felten and Olschowka, 1987). Sympathetic nerve fibers sparsely innervate the B cell-containing follicles (Williams and Felten, 1981). The red pulp also contains scattered sympathetic fibers associated with the plexuses along trabecular and surrounding tissues (Elenkov et al., 2000). Activated B cells migrate to the red pulp of spleen although some exit the lymphoid tissue to migrate to the bone marrow or epithelial surfaces. A small number of activated B cells are also present in the T-dependent zone. These B cells can migrate back to the follicle to differentiate into germinal center cells.

Further migration events occur within the germinal center during the processes of selection and affinity maturation (Cyster, 2005). It is worth noting that the spleen is devoid of parasympathetic innervation (Bellinger et al., 1993)

## 1.6. Effects of the immune response on the nervous system

It is at present well established that immune cells produce soluble mediators that can influence the CNS and modify its activity in different ways. For example, these mediators can affect the local release of neurotransmitters and change neuronal plasticity, sleep patterns, thermoregulation, neurogenesis, and behavior (Kin and Sanders, 2006; Ziv et al., 2006).

Although the brain was originally considered as an immunologically privileged site to which immune cells do not have access under physiological conditions, some researchers believe now that the arm of the immune system to the CNS may be not completely blocked by the endothelial BBB (Engelhardt and Coisne, 2011; Kleine and Benes, 2006). Others groups reported T cells trafficking within the meninges and choroid plexus epithelia but not into the brain parenchyma (Carrithers et al., 2000; Carrithers et al., 2002), although T cells can cross BBB and migrate into brain under pathological conditions (Prendergast and Anderton, 2009; Wilson et al., 2010). A well-established fact is that immune-derived cytokines can influence the CNS directly or indirectly. Although it is still not completely clear how cytokines can reach the brain, several mechanisms have been proposed, including 1) a saturable transport mechanism or direct entry via the circumventricular organs, which lack the BBB, 2) stimulation of the release of inflammatory mediators at the BBB, and 3) activation of afferent neurons, mainly of the vagus nerve (Banks et al., 2002; Dantzer et al., 2000; Guyon et al., 2008; Hosoi et al., 2002; Matsumura and Kobayashi, 2004) (for review see Kin and Sanders, 2006). A very interesting find also is that cytokines can induce their own production in several organs, including the brain (Besedovsky and del Rey, 2011).

Cytokines in the CNS exert a variety of neuromodulatory functions. Noteworthy is the effect of pro-inflammatory mediators, especially interleukin-1 (IL-1), on neuronal activity in the hypothalamus, which can be both stimulatory and inhibitory. Through the stimulation of the paraventricular nucleus in the hypothalamus, this cytokine can, for example, affect thermoregulation, food

intake, and the functioning of the HPA axis (Besedovsky and del Rey, 1996, 2011). Furthermore, immune mediators can also influence synaptic plasticity. IL-1β and interleukin-6 (IL-6), for example, can modulate long-term potentiation in the hippocampus in an opposite way, and also affect learning and memory (Balschun et al., 2004; Schneider et al., 1998; Yirmiya and Goshen, 2011). Cytokines can also affect the metabolism of different neurotransmitters in the CNS. For example, IL-1β induces a reduction in NA content in the hypothalamus, hippocampus, brainstem and spinal cord. IL-1β and IL-6 can stimulate the metabolism of DA in the striatum, hippocampus and prefrontal cortex, and reduce of 5-HT in the hippocampus (Besedovsky and del Rey, 1996).

Pro-inflammatory cytokines, mainly IL-1β and tumor necrosis factor α (TNFα), activate autonomic centers in the hypothalamus and brainstem, increasing the sympathetic tone and NA concentration in the systemic circulation, which influence different vegetative functions. However, this cytokine has different effects on the SNS in the peripheral organs (Rogausch et al., 2004; Rogausch et al., 1995; Rogausch et al., 2003). Since, acting at post-ganglionic levels, it can directly inhibit the local release of NA from the nerve endings (Rogausch et al., 1997). IL-6, IL-2 and TNFα can also inhibit NA release. This effect, which can be achieved through the effect on autoreceptors, serves to fine-tuning neurotransmitter release (Straub et al., 1998).

The initial activation of the SNS during the immune response to innocuous antigens is followed by a marked decrease in splenic NA concentration. Conversely, reduced immune activity caused either by the lack of mature T cells in athymic mice or by reduced antigenic challenge in germ-free rats results in increased sympathetic activity in lymphoid organs (for review see del Rey and Besedovsky, 2008). Within the context of this work, it is relevant to mention that it has been shown that T cells or their products can affect the sympathetic innervation of the spleen. Sympathetic innervation is increased in the spleen of young mice that genetically lack a thymus, as evaluated by increased NA concentration and density of noradrenergic nerve fibers, and that these alterations can be normalized by thymus transplantation or T cell inoculation at the birth (Besedovsky et al., 1987).

The large amount of reports available showing *in vitro* effects of immune-derived products on neural cells are not included in these comments. Only *in vivo* work is included here since it may represent a more physiological approach to study immune-neural interactions.

## 1.7. Effects of the nervous system on the immune response

The SNS exerts numerous immunomodulatory functions in lymphoid organs. Catecholamines are potent immune modulators that can both enhance and inhibit the activity of immune cells (for review see Elenkov et al., 2000). The immunoregulatory effects of catecholamines depend on different factors such as the type of immune response in course, the strength and duration of the adrenergic stimulus, and the subtype of stimulated immune cells and its degree of activation and differentiation (Elenkov et al., 2000; Kin and Sanders, 2006). The immunomodulatory effect of SNS mediators is mainly exerted by stimulation of $\beta_2$-adrenoreceptors, which are expressed by B lymphocytes, $CD4^+$ and $CD8^+$ T cells, innate immunity cells, and Th1 helper cells (Nance and Sanders, 2007). It is important to mention that stimulated cells express more $\beta$-receptors than resting cells, also that there is a difference in the number of receptors expressed by $CD4^+$ and $CD8^+$ T cells. Furthermore, there is also functional evidence of the presence of $\alpha$-receptors in lymphoid cells (for review see Elenkov et al., 2000).

Sympathetic neurotransmitters, and in particular NA, can affect nearly all immune parameters evaluated so far, including the production of cytokines and immunoglobulins, antigen presentation, the expression co-stimulatory and adhesion molecules, and the activation, clonal expansion and deletion of lymphocytes (Besedovsky and del Rey, 1996; Kohm and Sanders, 2000). NA can suppress cellular immunity by inhibiting the activity of Th1-cells, macrophages, and NK cells, and inhibiting the production pro-inflammatory cytokines, such as TNFα, IL-1β and interferon γ (INFγ). On the other hand, NA can promote humoral immune functions by increasing the activity of B lymphocytes and, indirectly, the release of anti-inflammatory cytokines, such as IL-10 and transforming growth factor β (TGFβ) (Besedovsky and del Rey, 1996). NA can also inhibit the expansion of antigen-activated T cells. The stimulation of $\beta_2$-adrenoreceptors and consequent increased levels of cAMP,

reduce the production and secretion of IL-2 and the expression of IL-2 receptors (Feldman et al., 1987; Ramer-Quinn et al., 2000). It has also been shown that NA can induce lymphoid cells apoptosis (Del Rey et al., 2003). This evidence indicates that sympathetic neurotransmitters can influence the immune response. Another type of evidence also supports this possibility. For example, chemical denervation at birth results in an increased number of immunoglobulin-secreting cells in the spleen of adult, non-overtly immunized mice. These results strongly suggest that there is a permanent increase in the activity of splenic B lymphocytes in mice deprived of sympathetic innervation, since neonatal administration of 6-hydroxydopamine (6-OH-DA) results in permanent destruction of sympathetic nerve endings. Furthermore, other results show enhancing effects of sympathectomy during adulthood on antibody-forming cells in the spleen during a specific immune response. Since 6-OH-DA administered at birth not only interferes with the sympathetic innervation of peripheral organs but also with central noradrenergic neurons, these studies reveal the relevance of central and autonomic mechanisms in immunoregulation under basal and activated conditions (for review see Besedovsky and del Rey, 1996).

## 1.8. Neurotrophins and the immune response

Neurotrophins, which play a crucial role in neuronal development, survival and function in the peripheral and CNS (Snider, 1994), can also affect the immune system. As mentioned, NGF was the first neurotrophin shown to be expressed by immune cells (T and B lymphocytes, macrophages, and mast cells). B lymphocytes express the two types of NGF receptors, $P75^{NTR}$ and *Trk A*, and can therefore respond to NGF stimulation (Besser and Wank, 1999). BDNF, which was previously thought to be primarily expressed in the nervous system, can be produced in vitro by essentially all major types of immune cells, including $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and monocytes (Kerschensteiner et al., 2003). Comparison of mRNA splice variants in the immune system and the CNS revealed that whereas all splice variants are expressed in the CNS, only mBDNF3 mRNA is detectable in immune cells, in primary and secondary lymphoid organs, and in purified T cells and macrophages. mBDNF 3 mRNA seems to be differentially regulated in the CNS

and the immune system after activation, opening perspectives for selective therapeutic manipulation (Kruse et al., 2007). There are also reports indicating the presence of NT3 and NT4/5 on immune cells (Besser and Wank, 1999; Moalem et al., 2000). It therefore appears likely that neurotrophins can also be involved in the bidirectional crosstalk between the nervous and the immune systems. It has been recently reported that immunoreactive NGF is strongly and widely distributed around the arteries and PALS in the spleen of severe combined immunodeficiency (SCID) mice, which are deficient in functional T and B cells. These results suggest that NGF production is inhibited by immune cells in normal mice (Kannan-Hayashi et al., 2008). On the other hand, activated CD4+ T cell clones express Trk, and also synthesize and release biologically active NGF. These results suggest that NGF, as an autocrine and/or paracrine factor, may be involved in the development and regulation of immune responses (Ehrhard et al., 1993). There are several *in vivo* studies indicating that NGF synthesis is up-regulated during an inflammatory process, and that inflammation and tissue damage generate mediators that control the local concentration of NGF (De Simone et al., 1996; Safieh-Garabedian et al., 1995; Stanzel et al., 2008). For example, inflammatory cytokines such as IL-1β, TNFα and IL-6 are able to modify the basal production of NGF and induce its synthesis in a variety of cell types and tissues (Bandtlow et al., 1990; Manni and Aloe, 1998; Marz et al., 1999; Takaoka et al., 2009).

The expression of BDNF is decreased in the hippocampus of SCID mice and of transgenic mice expressing a T cell receptor that recognizes ovalbumin, but increased in the hippocampus of transgenic mice that recognize myelin basic protein. These results suggest that immune cells play a very important role in the expression of BDNF in the hippocampus (Ziv et al., 2006). On the other hand, BDNF participates in several hippocampal functions, including spatial learning and memory (Mizuno et al., 2000), and adult neurogenesis (Scharfman et al., 2005). It is also interesting to mention that B cells numbers are reduced in $BDNF^{-/-}$ mice, suggesting that this neurotrophin may play an important role in B cell development (Schuhmann et al., 2005). Using a conditional knockout model with inducible deletion of BDNF, it has been shown that clinical symptoms and structural damage are increased when BDNF is absent during the initial phase of clinical EAE (Lee et al., 2012).

## 1.9. Athymic (nude) mice as an animal model

In 1966, Flanagan first described a spontaneous mutation resulting in “nude” mice that was found in the animal colony of the Ruchill Hospital, Glasgow, United Kingdom (Flanagan, 1966; Segre et al., 1995). Mutations in the nude locus produce the remarkable pleiotropic phenotype of hairlessness and athymia (Flanagan, 1966; Pantelouris, 1968). Genetic studies demonstrated that the nude mutation segregates as a single autosomal locus on mouse chromosome 1L (Flanagan, 1966), and is inherited in a classical Mendelian recessive manner (Flanagan, 1966; Pantelouris, 1973). The nomenclature to design nude mice has changed several times since its discovery. The most actual one was introduced in 2000 by Kaestner and colleagues when the gene responsible for the mutation was identified as a member of the Fox gene family, and the nomenclature was updated to *Foxn1*$^{n}$ (Kaestner et al., 2000).

Animals homozygous for *Foxn1*$^{n}$ mutation remain hairless throughout life, have a subnormal growth rate, and reduced fertility (Pantelouris, 1973), and it has been shown that the lack of the thymus affects sexual maturation (Besedovsky and Sorkin, 1974). Because of the athymia, nude mice lack T lymphocyte functions (Loor and Kindred, 1973; Raff, 1973), and it has been shown that IL-2, a product of activated T cells, induces specific cytotoxic T lymphocytes and helper T cells in these mice (Gillis et al., 1979; Wagner et al., 1980). Later, other studies suggested that athymic nude mice have a small number of functional post-thymic T cells (Ikehara et al., 1984).

Athymic nude mice have a highly impaired immune system, and they are extensively used in cancer research for the transplant of tumors and tissues from other species (Segre et al., 1995). These mice offer an excellent model for the purpose of the work reported here, namely to study the relevance of the thymus, and as consequence of T lymphocytes, for the development of the central and peripheral nervous systems because immunocompetence can be reversed by thymus implantation (Loor and Kindred, 1974).

## 1.10. Aims

As described above, it is at present well established that there are physiological interactions between the nervous, endocrine and immune systems. These interactions are based on a complex network, in which immune-derived products, neurotransmitters, neurotrophins, and hormones function as mediators between these systems and influence each other. In the Research Group in which this work has been performed, it has been reported long ago that the sympathetic innervation of the spleen is increased in young athymic mice (Besedovsky et al., 1987). The fact that this alteration can be normalized by thymus transplantation or T cell inoculation showed that this defect is not genetically programmed, but rather a phenotypical manifestation related to the absence of mature T cells. On this basis, the first aim of this work was to study if the absence of a functional thymus in a host can affect noradrenergic neurons in the brain in a comparable way as it does in the spleen, and, if so, whether alterations can be reversed by thymus implantation at birth. For this purpose, mice that congenitally lack a thymus have been used as model. A further aspect that needed investigation was if the alterations in splenic sympathetic innervation observed in young nude mice are still present during adult life. It was also unknown whether the serotonergic system is affected by the absence of the thymus. A systematic study of the effects that the lack of a thymus may have on corticosterone blood levels was also missing. Thus, the first aim of this work was to address these aspects.

The second part aimed at studying whether there are alterations in the concentration of the main neurotrophins in the spleen and brain of athymic mice, since these proteins are essential for neural development and functioning.

Finally, the aim of the last part of this work was to study whether the alterations detected in nude mice reflect a more general condition that causally relates the catecholaminergic system with the expression of neurotrophins. As a first approach to this question, sympathetic innervation was decreased by chemical destruction of noradrenergic neurons at different stages of development, and neurotrophin concentrations in the spleen and brain were determined in parallel.

# 2. Materials

## 2.1 Animals

Original breeding pairs from heterozygous *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice on Balb/c background were kindly provided by Dr. C. Johner, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, and wild type Balb/c were obtained from the colony originally established by Dr. K. Hartmann at the Institute for Experimental Immunology, Marburg. A parallel colony derived from these mice was also maintained at the Department of Physiology, Interamerican Open University, Argentina, and some of the samples used in this work have been obtained there.

Only male mice were used in this study and they were derived from crossing Balb/c *Foxn1*$^{n}$ males and Balb/c *Foxn1*$^{n}$/*Foxn1*$^{+}$ females. Animals were bred under conventional conditions under constant temperature, humidity, and 12h light-dark cycles, and fed *ad libitum.* Newborn homozygous *Foxn1*$^{n}$ pups can be easily identified by the lack, or poorly developed, whiskers. They were separated from the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates when they were less than 1 day-old, and re-distributed so that each *Foxn1*$^{n}$/ *Foxn1*$^{+}$ mother was left with one genotype only and with a maximum of 5 pups.

The *Foxn1*$^{+}$/*Foxn1*$^{+}$ Balb/c mice used for the denervation experiments were derived from *Foxn1*$^{+}$/*Foxn1*$^{+}$ Balb/c breedings.

## 2.2 Chemicals

| **Chemical (continuation)** | **Source** |
|---|---|
| 6-amino caproic acid | Sigma-Aldrich, Germany |
| Acetic acid | Merck, Germany |
| Acetonitrile | Carl Roth, Germany |
| Aluminium oxide | Merck, Germany |
| Ascorbic acid | Sigma-Aldrich, Germany |
| Benzamidin- HCl | Applichem GmbH, Germany |
| Citric acid | Merck, Germany |
| Copper (Ⅱ) sulfate (98%) | Acros Organics, USA |
| Copper (Ⅱ) acetat (monohydrate) | Fisher Scientific, United Kingdom |
| Disodium hydrogen phosphat dihydrate | Marck, Germany |

| Chemical | Source |
|---|---|
| Dulbecco's phosphate buffer saline (PBS) | PAA, Austria |
| Ethanol (96% and 99,8%) | Otto Fischer, Germany |
| Formaldehyd | Merck, Germany |
| Hydrochloric acid | Merck, Germany |
| Isopropanol (99,98%) | Carl Roth, Germany |
| Octyl- sulfat | Merck, Germany |
| Perchloric acid | Merck, Germany |
| Phenylmethyl sulfanyl fluoride | Sigma-Aldrich, Germany |
| Picric acid | Fluka, Switzerland |
| Sodium chloride | Merck, Germany |
| Sodium citrate (dihydrate) | Merck, Germany |
| Sodium dihydrogen phosphate | Merck, Germany |
| Sulfuric acid (2M) | Carl Roth, Germany |

## 2.3 Kits or antibodies

| Kit or antibody | Source |
|---|---|
| Alexa Fluor® 488 conjugated streptavidin | Molecular Probes, Holland |
| Avidin/Biotin blocking Kit | Vector Laboratories, USA |
| Bicinchoninic acid protein assay kit | Pierce, USA |
| Biotinylated donkey anti-sheep IgG polyclonal antibody | Dianova, Germany |
| Corticosterone ELISA Kit | IBL international GmbH, Germany |
| Human BDNF ELISA Kit | R&D Systems Inc., USA |
| Human NT-3 ELISA Kit | R&D Systems Inc., USA |
| Human ß-NGF ELISA Kit | R&D Systems Inc., USA |
| Indocarbocyanine conjugated mouse anti-rabbit IgG polyclonal antibody | Abcam PLC, United Kingdom |
| Rabbit monoclonal anti-BDNF | Epitomics Inc., USA |
| Sheep anti-tyrosine hydroxylase polyclonal antibody | Chemicon, USA |

## 2.4 Reagents

| Reagent | Source |
|---|---|
| 4-hydroxy-3-methoxyphenylglycol-hemipiperazinium salt | Sigma-Aldrich, Germany |
| 5-hydroxy-Indol-acetic acid | Sigma-Aldrich, Germany |
| 5-hydroxytryptamin (serotonin) | Sigma-Aldrich, Germany |
| 6-hydroxydopamin | Sigma-Aldrich, Germany |
| Adrenalin (L-Adrenalin-D-Hydrogentartrate) | Sigma-Aldrich, Germany |
| Bovine serum albumin | Sigma-Aldrich, Germany |
| Cresyl violet acetate | Acros organics, USA |
| Dihydroxyphenylacetic acid | Sigma-Aldrich, Germany |
| Dopamine | Sigma-Aldrich, Germany |
| EDTA disodium salt dihydrate | Carl Roth, Germany |
| Nerve growth factor, 2.5s, murine | Promega, Germany |
| Noradrenaline | Sigma-Aldrich, Germany |
| Tryptophan | Sigma-Aldrich, Germany |
| TWEEN 20 | Sigma-Aldrich, Germany |
| Tyrosine | Sigma-Aldrich, Germany |

## 2.5 Tools

| Tool (continuation) | Source |
|---|---|
| 14ml polypropylene round-bottom tubes | Becton Dickinson Labware, USA |
| 15ml and 25ml conical tubes | Becton Dickinson Labware, USA |
| 96-well plates | Nunc, Denmark |
| Combitips | Eppendorf, Germany |
| Petri dishes (3,5mm) | Iwaki, Japan |
| Serologic pipette (2ml, 5ml, 10ml) | Becton Dickinson Labware, USA |
| Disposable syringes (1ml, 20ml) | Braun, Germany |
| Sterile filters | Sartorius, Germany |
| Sterile needles (27 G) | Becton Dickinson Labware, USA |
| Plastic tubes (1,5ml, 0,5ml) | Eppendorf, Germany |
| EDTA-coated tubes (1,5ml) | Roland Vetter, Germany |

| Tool | Source |
|---|---|
| HPLC-Septen | Wicom, Germany |
| HPLC-Vials | Wicom, Germany |

## 2.6 Equipment

| Equipment | Source |
|---|---|
| High accuracy balances (AJ150, PM400 and PM300) | Mettler, Germany |
| Autosampler AS-2000A | Hitachi, Japan |
| Centrifuge Biofuge® Fresco | Heraeus®, Germany |
| HPLC-Electrochemical detector | Antec Leyden, Holland |
| HPLC-Pump P580 | Gynkotek, Germany |
| Incubator | Heraeus®, Germany |
| Micropipette, 10µl, 100µl, and 1000µl | Eppendorf, Germany |
| Microplate Reader Sunrise Remote | Tecan, Switzerland |
| Microtome HM 325 | Thermo Fisher Scientific Inc., Germany |
| Multi pipette® Plus | Eppendorf, Germany |
| Olympus Fluoview laser scanning microscope | Olympus Optical Co., Germany |
| Oven | Heraeus®, Germany |
| Pipette boy | Integra Biosciences, Germany |
| Sterile bank | Nuaire, USA |
| Vortex | IKA® Labortechnik, Germany |

## 2.7 Software

| Software | Source |
|---|---|
| Endnote (version X4.0.2) | Thomson Reuters, USA |
| Magellan (version 3.11) | Tecan, Switzerland |
| Microsoft office (2007) | Microsoft, USA |
| Statview (version 5.0) | SAS Institute Inc., USA |
| Motic Image plus (version 2.0) | Motic, Germany |
| Chromeleon (version 6.01) | Dionex Corp., USA |
| Paint (version 6.1) | Microsoft, USA |

## 2.8 Various

| Various | Source |
|---|---|
| Dry-ice | FB Chemistry, Uni-Marburg |
| Eukitt quick-hardening mounting medium | Sigma-Aldrich, Germany |
| Fluorescent mounting medium | Dako, USA |
| Histoacryl | Braun, Germany |
| Paraplast plus | Carl Roth, Germany |
| Xylol | Carl Roth, Germany |

# 3. Methods

## 3.1 Preparation of buffers and solutions

### 3.1.1. Phosphate buffer saline (50 mM)

6,55g $NaH_2PO_4.H_2O$, 36,05g $Na_2HPO_4.2H_2O$, and 45g NaCl are dissolved in 4500ml distilled water (dist.W.).

### 3.1.2. Bovine serum albumin (1%)

1g Bovine serum albumin (BSA) is dissolved in 100ml 50mM PBS.

### 3.1.3. Neurotrophin-ELISA wash buffer

500µl TWEEN 20 are added to 1000ml 50mM phosphate buffer saline (PBS) and the pH of solution is adjusted to 7,4 with HCl (1M).

### 3.1.4. Protease inhibitor solution

*Solution A: 10M 6-amino caproic acid: (M.W.=131,18)*

6, 56g 6-amino caproic acid are dissolved in 5ml 50mM PBS

*Solution B: 1M ethylenediaminetetraacetic acid (EDTA): (M.W.=372,24)*

1,861g EDTA are dissolved in 5ml 50mM PBS.

*Solution C: 0.5M benzamidine-HCl: (M.W.= 156,61)*

0,3914g benzamidine-HCl are dissolved in 5ml 50mM PBS.

*Solution D: 20mM phenylmethylsulfonyl fluoride: (M.W.= 174,19)*

17,42g phenylmethylsulfonyl fluoride are dissolved in 5ml 100% ethanol

*Working solution:* 0,5ml from solutions A, B, C, and D each are mixed, the volume is completed to 50ml with 50mM PBS, and the mixture used immediately. Thus, the final working solution contains 100mM 6-amino caproic acid, 10mM EDTA, 5mM benzamidine-HCl and 0,2mM phenylmethylsulfonyl fluoride.

### 3.1.5. Bouin-Hollande fixative

*A stock solution* is prepared by dissolving 50g copper (Ⅱ) acetate (monohydrate) and 60g picric acid (≈ 40% in H2O) in 1L dist.w.

*The working solution* is prepared by mixing 100 parts of the stock solution, 10 parts of formaldehyde, and 1 part of glacial acetic acid, and immediately used.

### 3.1.6. Citrate buffer

*Solution A* is prepared by dissolving 21,01g citric acid (monohydrate) in 1000ml dist.w.

*Solution B* is prepared by dissolving 29,41g sodium citrate (dihydrate) in 1000ml dist.w.

*The working solution* is prepared by mixing 54ml solution A and 246ml solution B, and completing the volume to 3000ml with dist.w. The pH is adjusted to 6,0 with HCl (1M).

### 3.1.7. Eluent solution for high performance liquid chromatography

*Solution A* is prepared by dissolving 9,79g $Na_2HPO4$ in 1100ml dist.w.

*Solution B* is prepared by dissolving 10,5g citric acid in 1000ml dist.w.

The above indicated volumes of solutions A and B are mixed. 0,45g octyl-sulfate and 0,075g EDTA are dissolved in 1800 ml of this mixture, the solution is filtered, and 10% Acetonitril is added. Finally, the solution is degassed for 10min.

### 3.1.8. Cresyl violet solution (0,1%)

0,1g Cresyl violet acetate are dissolved in 100ml dist.w., and 300µl glacial acetic acid are added to the solution before use. The mixture is filtered and immediately used.

## 3.2. Thymus reconstitution

Thymi were obtained from newborn Balb/c *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice and placed in cold PBS. Another group of newborn (less than 24 h-old) athymic Balb/c *Foxn1*$^{n}$ male mice were anesthetized by hypothermia by placing them at -18Cº for 2-3min. A small (about 1mm) incision was done in the skin in each axillary region, forming a small pocket. One thymus per axillary region was carefully placed inside and the skin closed using Histoacryl (Besedovsky et al., 1987).

## 3.3. Chemical denervation

6-HO-DA was dissolved in 0.01% ascorbic acid and injected i.p. at a dose of 150mg/kg body weight. Ascorbic acid is used to protect 6-OH-DA from oxidation. Control mice received 0.01% ascorbic acid alone. Newborn Balb/c male mice received 6-OH-DA or the vehicle on 5 consecutive days and were killed when they were 21 day-old (Thoenen and Tranzer, 1968). Fourteen day-old mice received two injections of 6-OH-DA or vehicle on two consecutive days. One group of mice was killed when they were 21 day-old and another when they were 67 day-old. Adult mice (60 day-old) also received two injections of 6-OH-DA or vehicle on two consecutive days, and were killed one week later (del Rey et al., 2002).

## 3.4. Organ and blood collection

Groups of athymic (Balb/c *Foxn1*$^{n}$) male mice and their heterozygous thymus- bearing littermates (Balb/c *Foxn*1$^{n}$/*Foxn1*$^{+}$) were killed by cervical dislocation at different times after birth. Groups of athymic Balb/c *Foxn1*$^{n}$ male mice that had been implanted with two thymi at birth were killed when they were 21, or 60 day-old. Blood was collected in EDTA-coated tubes. The plasma was aliquot and frozen at -80$^{o}$C until used for corticosterone determinations.

The spleen of 7, 14, 21, and 60 day-old mice was cut into three sections: S1, S2 and S3 (Fig. 3). S1 and S3 were immediately frozen and kept at -80C$^{o}$ until use for neurotransmitter and neurotrophin determinations. S2 sections were fixed by immersion in Bouin-Hollande (see 3.1.5). The spleen of newborn (< 24 hour-old) mice was not divided, but was collected from different mice for histology, neurotransmitter and neurotrophin determinations

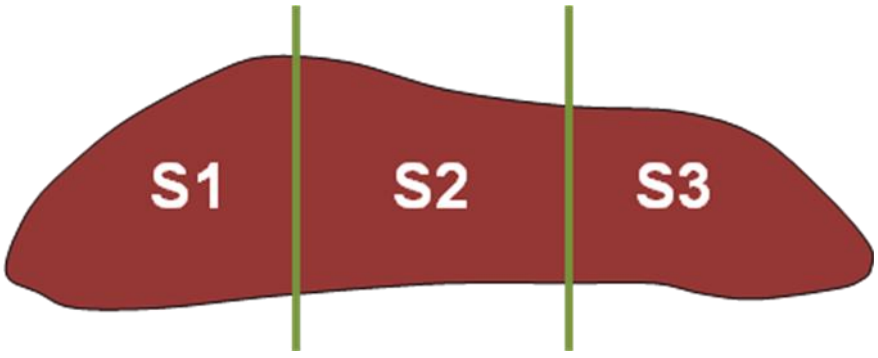


**Fig. 3.** Spleens were divided into three sections, denominated S1, S2 and S3

The left and right kidneys and hippocampi of the same mice were collected, immediately frozen, and kept at -80Cº until use for neurotransmitter and neurotrophin determinations. The hypothalamus and brainstem were collected and divided into right and left parts (Fig. 4), immediately frozen and kept at -80Cº until use for neurotransmitter and neurotrophin determinations. Respectively, some regions were fixed and used for histological studies. In the case of newborn mice, the brain was taken as a whole and not subdivided into different regions.

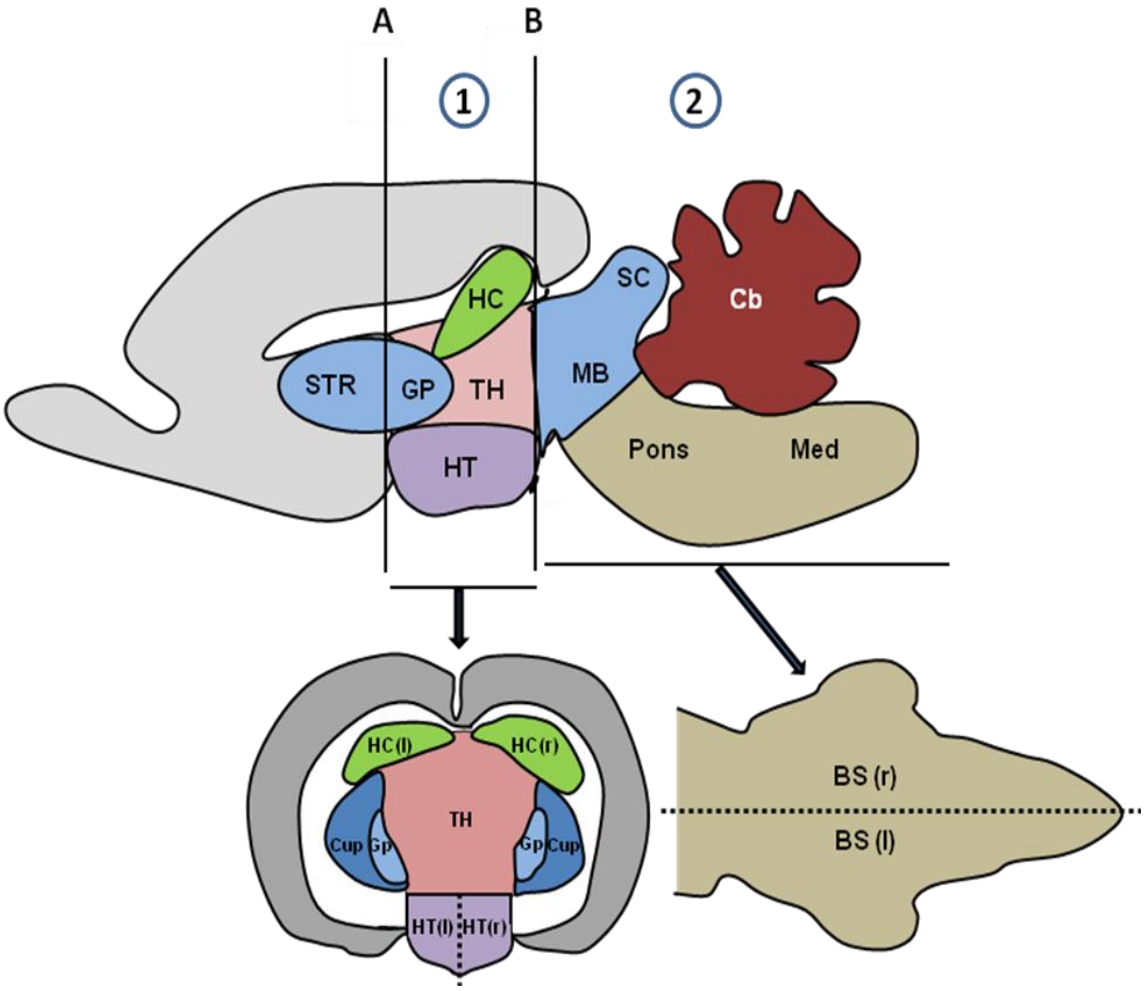


**Fig. 4. Collection of brain regions.** The brains were processed using a Mouse Brain AID, designed by Dr. J. Wildmann at the Research Group Immunophysiology. This system allows transverse cuts at the defined positions A and B. From the resulting slab (1) the HT was punched out and divided into the right and left halves. The right and left parts of hippocampus were also collected separately. The cerebellum (Cb) was removed from the caudal section of the brain (2). The brainstem was collected from the remaining tissue and divided into right and left halves.

The same procedure of organ collection and processing was followed with the spleen and brain of mice that were sympathetically denervated and the corresponding controls.

## 3.5. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a chromatographic technique that allows separating a mixture of compounds based on the competitive interaction between the components of the mixture and the mobile phase (eluent) with the stationary phase. It is used in biochemistry and analytical chemistry to identify, quantify, or purify the individual components of the mixture.

HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase and analytes through the column, and one or more detectors. The detector may also provide other characteristic information (i.e. UV/Vis spectroscopic data for the analytes). Analyte retention time varies depending on the extent of its interaction with the stationary phase, the ratio/composition of solvents used, and the flow rate of the mobile phase. A pump provides the high pressure required to propel the mobile phase and analyte through the densely packed column (Fig.5). The increased density of packed column arises from smaller particle sizes, which allows for a better separation because of highly increased surface area. This allows the use of columns of shorter length when compared to ordinary column chromatography.

The HPLC system used in this work is a reversed-phase chromatography (RPC). RP-HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica treated with RMe2SiCl, where R is a straight chain alkyl group such as C18H37 or C8H17. Substances are pumped by the mobile phase through the stationary phase. The eluted substances are detected electrochemically at the end of the stationary phase. The height and area of the peaks obtained are proportional to the electrical signal generated at the working electrode. The time that elapses from injecting the sample to the appearance of the peak maximum is characteristic for a given substance and is known as its retention time. The use of standards with known concentrations allows identification and quantification

of the individual components of the mixture. A representative chromatogram of the standard mixture used for the HPLC analysis is shown in Fig. 6.

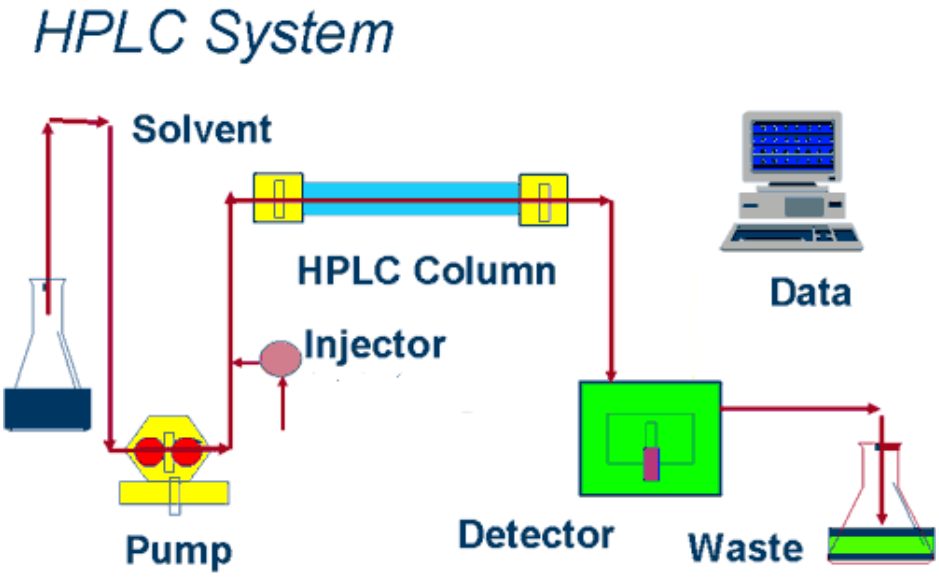


**Fig. 5. Representation of a HPLC system with mobile phase (solvent).** The system is connected to a computer for data analysis.
(From: http://www.chemistry.nmsu.edu/Instrumentation/Waters_HPLCSystem.gif).

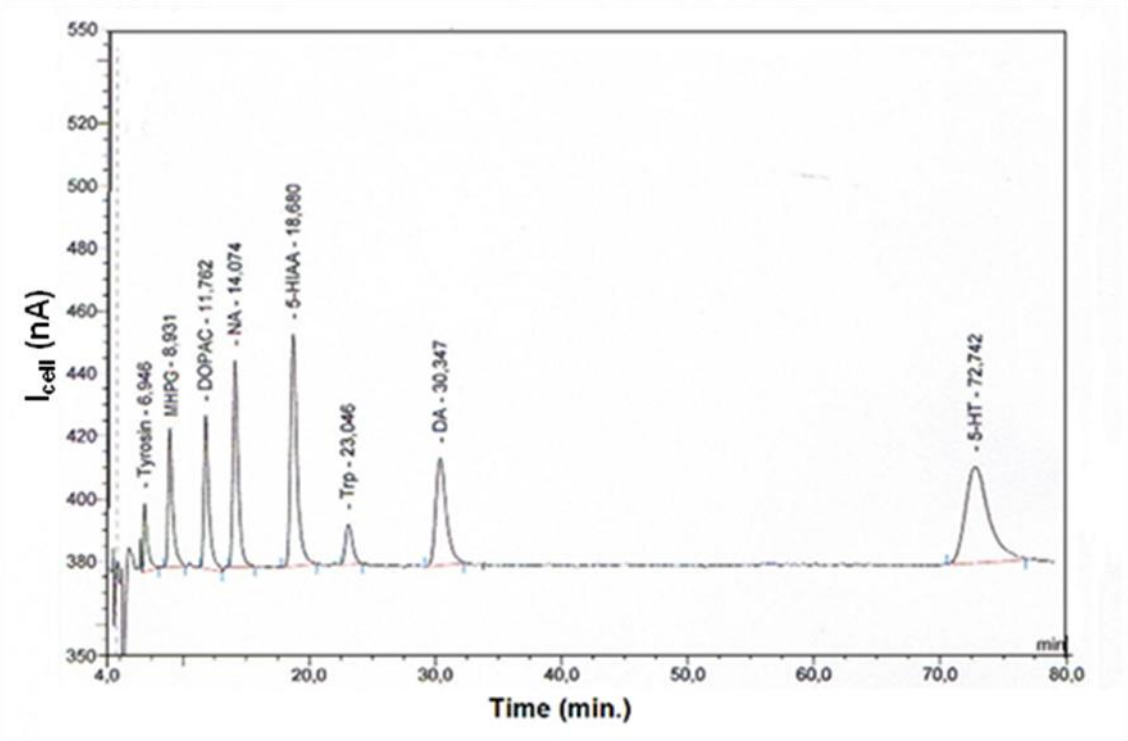


**Fig. 6. Chromatogram of a standard mixture.** The figure shows a representative chromatogram obtained by injecting 20µl of a mixture of 8 standard compounds ($10^{-6}$M Tyr, $10^{-7}$M MHPG, $10^{-7}$M DOPAC, $10^{-7}$M NA, $10^{-7}$M 5-HIAA, $10^{-7}$M Trp, $10^{-7}$M DA, and $10^{-7}$M 5-HT). Y-axis: $I_{cell}$ (nA) (current); x-axis: retention time.

### 3.5.1. Sample preparation

The left kidney and hippocampus, the left part of the hypothalamus and brain stem, and a piece of the spleen (S1) were used for neurotransmitter determination by HPLC. Perchloric acid (PCA) (0,4M) was added to samples of peripheral tissues and eluent (see 3.1.7) to brain tissues, at a proportion 1/20 or 1/40 weight to volume, respectively. The tissue was disrupted using an ultrasonic cell disruptor for 5-10 sec set at maximal power. Sonicates were centrifuged at 20.000 x g at 4Cº for 15min. Supernatants were removed and centrifuged again at the same speed and temperature for 5min.

### 3.5.2. Standards

A mixture containing A, NA, DOPAC, DA, 5-HT, 5-hydroxy-indol-acetic acid (5-HIAA), Trp, and 4-hydroxy-3-methoxy-phenylglycol hemipiperazinium salt (MHPG) at a final concentration of $10^{-7}$M and Tyr at a final concentration of $10^{-6}$ M was used as standard. The mixture was prepared in 0,4 M PCA or in eluent, when used as standard for determinations in peripheral tissue or brain samples, respectively.

### 3.5.3. Procedure

100µl of each sample or 200µl of the standard mixture were filled into glass vials and placed in the autosampler. 10µl of the samples were injected for the analysis. The data were collected and integrated using Chromeleon software (Dionex Corp., version 6.01).

## 3.6. Determination of neurotrophin concentration

The right kidney and hippocampus, the right part of the hypothalamus and brain stem and part S3 of the spleen were used for neurotrophin determinations, and processed as described below.

### 3.6.1. Tissue processing

The frozen tissue was weighed just before homogenization, and transferred to 1,5ml Eppendorf cups. Protease inhibitor solution (see 3.1.4.) was added to the samples at a relation 1/10-1/20 weight to volume for NGF determination, and 1/5-1/10 for BDNF and NT-3 determinations. The tissues were disrupted using an ultrasonic cell disruptor for 5-10sec set at maximal power. Sonicated

samples were centrifuged at 20.000xg at 4Cº for 10min. Supernatants were removed, aliquoted, and stored at -80Cº until used for neurotrophin and protein determinations.

### 3.6.2. Protein determination

Protein concentration in the samples was determined by the Bradford method using a commercially available kit. This assay is based on the use of bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the reduction of $Cu^{+2}$ to $Cu^{+1}$ by proteins in an alkaline medium (the biuret reaction) with a highly sensitive and selective colorimetric detection of the cuprous ion ($Cu^{+1}$) using BCA (Smith et al., 1985). The purple-colored reaction product is formed by the chelation of two molecules of BCA with one $Cu^{+1}$. This water-soluble complex exhibits a strong absorbance at 570nm that is nearly linear with increasing protein concentrations over a broad working range (25-2.000 µg/ml). The macromolecular structure of proteins, the number of peptide bonds, and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA (Wiechelman et al., 1988).

*Assay Procedure*

The test was performed according to the indications of the manufacturer. Bovine serum albumin (BSA) is used as reference standard. Samples are diluted 1/200 with 50mM PBS. A series of standards containing 2.000, 1.500, 1.000, 750, 500, 250, 125 and 25µg/ml BSA/ml is prepared in 50mM PBS. The detection reagent is prepared by mixing 50 parts of BCA (reagent A), consisting of a sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in sodium hydroxide, with 1 part of BCA (reagent B), consisting of a 4% cupric sulfate solution. 15µl of each standard, sample and blank (50mM PBS alone) are distributed per well in 96-well plates, and 200µl of the detection reagent are added. The plate is incubated at 37Cº for 30min, and cooled at room temperature for 10min. The optical density is determined at 570nm immediately after cooling the plate. The concentration of total protein in the samples is calculated with reference to the standard curve.

### 3.6.3. Neurotrophin determination

NGF, BDNF and NT3 concentrations were determined by an enzyme-linked immunosorbent assay (ELISA), using commercially available kits.

In brief, the principle of the sandwich ELISA used for these determinations consists in a capture antibody bound to a 96-well plate, which binds the antigen present in a sample, and a detection antibody that binds to another epitope of the same antigen. The detection antibody is directly linked to biotin, which has a high binding specificity for streptavidin. Streptavidin conjugated to horseradish peroxidase (HRP) is used as detection system. In the presence of hydrogen peroxide, HRP catalyzes the oxidation of the chromogenic substrate tetramethylbenzidin (TMB), which yields a first charge-transfer blue complex (Fig.7). The reaction is stopped by sulfuric acid, resulting in a stable yellow color complex that can be detected spectrophotometrically by determining the optical density at 450 nm.

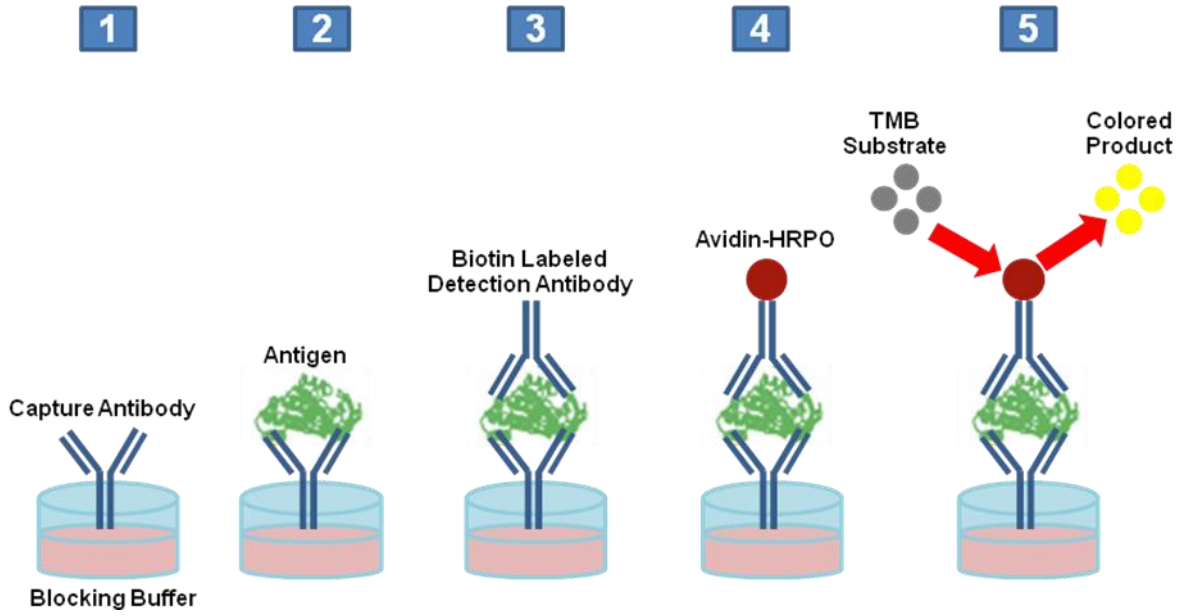


**Fig. 7**. **Sandwich *ELISA*.** (1) A plate is coated with the capture antibody, and remaining protein-binding sites on the plate are blocked by addition of blocking buffer; (2) sample is added, and the antigen present binds to the capture antibody; (3) detecting antibody is added; (4) enzyme-linked secondary antibody is added, which binds to the detecting antibody; (5) substrate is added, and converted by the enzyme to a detectable form.

*Neurotrophin kits*

Human β-NGF, human BDNF and human NT-3 DuoSets from R&D Systems were used for the determination of these neurotrophins. These kits contain the

basic components required for the development of sandwich ELISAs to determine the concentration of the human neurotrophins. Due to the high homology between the murine and human forms of NGF (82-98%) (Lommatzsch et al., 2005; Ullrich et al., 1983), BDNF (>98%) and NT3 (>98%) (Lommatzsch et al., 2005), and antibody cross-reactivity between these species, these kits are suitable to evaluate the concentration of these neurotrophins in the mouse samples.

For all neurotrophins, the capture antibody was the corresponding mouse anti-human neurotrophin. The concentration of these antibodies was 360µg/ml in 50mM PBS. As detection antibody, a biotinylated goat anti-human neurotrophin was used for NGF and NT3, and a biotinylated mouse anti-human was used for BDNF. The concentration of the detection antibodies was 9µg/ml for NGF; 4,5µg/ml for BDNF; and 36µg/ml for NT3, all in 50mM PBS containing 1%BSA (see 3.1.2.). Streptavidin conjugated to horseradish-peroxidase was used as detection system. The standards used derived from stock solutions of murine NGF (mNGF; 0,4µl/ml), hBDNF (110ng/ml), and hNT3 (80ng/ml), all in 50mM PBS containing 1%BSA.

*Plate coating*

The capture antibodies were diluted with 50mM PBS to working concentration of 2µg/ml for NGF and BDNF, and 5µg/ml for NT-3. 100µl/well of the capture antibody were distributed into 96-well plates. The plates were sealed and incubate overnight at RT.

*Blocking step*

On the following day, the content of the plates was aspirated and the wells washed three times with wash buffer (see 3.1.3). After the last wash, the buffer was completely removed by inverting the plates and blotting on clean paper towels. Finally, blockade was performed by 50mM PBS containing 1%BSA (300µl/well) and incubated at RT for 1hr.

*Assay Procedure*

The test was performed according to the indications of the manufacturer, with few modifications. A series of standards containing 25, 125, 250, 500, 750, 1.000, and 1.500pg/ml of the corresponding neurotrophin were prepared.

Based on preliminary experiments to determine the optimal protein concentration to evaluate neurotrophin levels in tissue, supernatants from homogenates were diluted with PBS containing 2% BSA for NGF and 5% BSA for BDNF and NT-3. The standards were prepared using the corresponding BSA concentration. 100µl per well of each standard, samples and blank were distributed in antibody-coated plates. The plates were covered and incubated 2hr at RT. The detection antibodies were diluted with PBS containing 1%BSA to final concentrations of 50ng/ml for NGF, 25ng/ml for BDNF, and 200ng/ml for NT3, and 100µl distributed per well after the washing steps. The plates were covered and incubate 2hr at RT. 100µl of the working dilution (1:200) streptavidin-HRP 1% BSA were distributed per well after the washing step. The plates were covered and incubate 20min at RT in the dark. Finally, after a further wash, 100µl substrate solution, consisting in equal parts of TMB and peroxidase ($H_2O_2$), were distributed per well. The plates were covered and incubate 20min at RT in the dark. The reactions were stopped by addition of 1M $H_2SO_4$ (50µl/well) and the optical density at 450nm was determined immediately after, using 570nm as reference wavelength. The concentration of the neurotrophins in the samples were calculated from the corresponding standard curve

## 3.7. Corticosterone determination

A competitive ELISA was used to evaluate corticosterone levels in plasma. Competitive ELISAs differ from the sandwich ELISAs (as used for neurotrophin determination) in that the antigen to be evaluated in the sample is mixed with a fix amount of an unlabeled antigen-enzyme conjugate. Enzyme-antigen complexes and the endogenous antigen present in the sample compete for binding to an antibody coated to a well. Thus, as more antigen is present in the sample, less enzyme-antigen complexes are able to bind to the antibody attached to the well (hence “competition”). After removal of the unbound enzyme, a substrate is added and converted to a chromogenic signal by the bound enzyme complex. Therefore, higher antigen concentrations in the sample result in the production of weaker signals.

The corticosterone ELISA kit used in this work is a solid phase enzyme-linked immunosorbent assay, based on this principle of competitive binding.

Microtiter wells are coated with a polyclonal antibody directed towards an antigenic site on the corticosterone molecule. The corticosterone present in a sample competes with a corticosterone-HRP conjugates for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of corticosterone in the sample. After addition of the substrate solution, the intensity of the color developed is inversely proportional to the concentration of corticosterone in the sample.

*Corticosterone ELISA kit*

The kit from IBL international used in this work includes a 96-well plate coated with a polyclonal anti-corticosterone antibody, a series of standards containing 0, 5, 15, 30, 60, 120, 140nmol corticosterone/l; the enzyme conjugate (corticosterone conjugated to HRP), conjugate diluent, TMB substrate solution, stop solution (0,5M $H_2SO_4$), and concentrated washing solution.

*Assay Procedure*

The test was performed according to the indications of the manufacturer. 20µl/well of each standard, sample (appropriated diluted with standard 0) or blank (standard 0) were distributed in the anti-corticosterone antibody coated plates, and 200µl enzyme conjugate added. After mixing, the plate was covered and incubated for 1hr at RT. The wells were rinsed three times with wash solution, and residual droplets were removed by striking the wells sharply on absorbent paper. 100µl substrate solution were distributed per well, and the plate was covered and incubated for 15min at RT. The reaction was stopped by addition of 50µl/well of the stop solution. The optical density was determined immediately after at 450nm with a microtiter plate reader. The concentration of corticosterone in the samples was calculated with reference to the standard curve.

## 3.8. Immunohistochemical staining

In principle, the methods used for immunohistochemistry can be classified as direct and indirect. The direct method is a one-step procedure based on the

direct reaction between an antigen and an appropriately labeled antibody. The indirect method requires the use of two antibodies, a primary unlabeled antibody and a secondary labeled antibody. The key step responsible for good quality immunohistochemical staining is the binding of the primary antibody to its antigen and depends on many factors, including fixation, washing, and incubation conditions as well as the appropriate mounting of specimens onto slides (Buchwalow and Böcker, 2010). Indirect immunostaining was used in this work to detect TH and BDNF.

### 3.8.1. Fixation and washing

The choice of an appropriate fixation method for biological probes is crucial, since it may affect the quality of immunohistochemical staining. The morphology of the cells and tissues must be preserved to characterize the localization of the component under study, and the antigenicity of the component of interest must still be present after fixation and accessible to the antibody (Hyatt, 2002).

Formaldehyde has several advantages over alcohols and acetone. It can preserve the morphological detail of the tissues due to inducing molecular cross-links in proteins, but on the other hand, it can change the native three-dimensional protein conformation, which makes it more difficult for the antibody to bind to its target (Boenisch, 2005). In order to avoid this problem, the tissues should be placed in 70% alcohol for long-term storage, which can minimize adverse effects of antigen damage (Buchwalow and Böcker, 2010).

A better fixative is Bouin-Hollande (see 3.1.5.), which combines the advantages of picric acid and formaldehyde (Treilhou-Lahille et al., 1981).

The S2 section of the spleen, selected left kidneys and hippocampi, and left parts of the hypothalamus and brainstem were fixed by immersion in Bouin-Hollande solution for 48hr with permanent shaking. The volume of the fixation solution used was 10 times the weight of the tissue. After fixation, samples were washed in 70% isopropanol until the alcohol became colorless.

### 3.8.2. Embedding and cutting

Dehydration is the first step in the processing of fixed tissues. The water in the specimen is replaced first with alcohol and then with a paraffin solvent

(clearing agent) such as xylol. After fixation, dehydration and clearing, tissues blocks are impregnated by paraffin wax (Buchwalow and Böcker, 2010).

*Procedure*

The fixed tissues were dehydrated by immersion in a graded series of isopropanol at RT (once in 80% isopropanol, twice in 95% isopropanol, and three times in 100% isopropanol, 15min each time). The dehydrated tissues were then cleared by immersion in xylol (twice for 15min at RT) and impregnated by immersion in Paraplast plus (twice for 15min in a 62ºC oven). Finally, the tissues were embedded in Parapast plus. Adjacent sections (7µm thick) were cut using a microtome.

### 3.8.3. Mounting paraffin sections onto slides

To promote adherence to the glass and to decrease the chance that the specimens dissociate from the slides, paraffin tissue sections were mounted on “double dipped” salinized slides by treating them with amino-propyl-tri-ethoxy-saline (APTES). Treated slides can be kept indefinitely (Buchwalow and Böcker, 2010). These slides were prepared by washing them in detergent for 1hr, followed by washing with running tap water for 10min, three times with dist.w. for 5min, and 70% isopropanol for 45min, and kept at 60ºC overnight. On the following day, slides were immersed in a freshly prepared 2% APTES solution in dry acetone for 30sec, and then in dry acetone twice for 30sec. Slides were quickly washed twice in dist.w., finally dried overnight at 42ºC, and store at R.T until used.

*Mounting procedure*

The paraffin sections were mounted onto treated slides using a water bath at 42ºC filled with deionized water. The slides were dried on a 56ºC warm plate during cutting and then overnight in an oven at 60ºC to increase adhesion of the tissue sections to the surface of the glass slide.

### 3.8.4. Deparaffinization and rehydration

Sections were deparaffinized by placing the slides in a cuvette containing sufficient xylol to cover the tissue completely, twice for 10min at RT, and rehydrated by treatment in 100% isopropanol for 10min, methanol/$H_2O_2$ (600ml 100% methanol mixed with 3ml 30% $H_2O_2$) for 30min, and a graded series of

isopropanol at RT (100% for 10min, and 96%, 80%, and 70% for 5min each). Finally, slides were washed twice in dist.w. for 5min.

### 3.8.5. Heat-induced antigen retrieval

The previous steps, especially fixation and embedding, may alter the conformation of the protein, negatively affecting antigen-antibody interactions and decreasing the intensity of the final reaction in the immunohistochemical staining (Boenisch, 2005; Buchwalow and Böcker, 2010; Shi et al., 2001). Antigen retrieval, which is important to increase the sensitivity for immunodetection, was performed by heating. The slides were placed in a container with citrate buffer (see 3.1.6) and heated at 92-95ºC for 15min. The slides were then cooled at RT, and the tissue sections were circled with a hydrophobic barrier pen. After heating, the sections were immediately processed for immunohistochemistry and they were not allowed to dry during the whole procedure.

### 3.8.6. Indirect immunostaining method

Indirect immunostaining methods are more commonly used than direct ones, due to higher sensitivity as a result of signal amplification by several secondary antibodies binding to different antigenic sites on Fc and Fab (antigen binding) fragments of the primary antibody. In indirect immunostainig, the bound primary unlabeled antibody (first layer) is visualized by using a secondary antibody (second layer). The secondary antibody is directly labeled with a fluorescent dye, for example, indocarbocyanine (CY3). Alternatively, the secondary antibody is biotinylated and detected by streptavidin conjugated with Alexa flour 488. This method was used in this work to detect TH and BDNF.

***TH detection***

*Blocking step:* sections were sequentially incubated in PBS containing 5% BSA (30 min) and 1% BSA (15 min), followed by treatment with 30% avidin in 1% BSA (20 min) and 30% biotin in 1% BSA (20 min) at RT, using the Avidin/Biotin blocking kit from Vector Laboratories. Slides were washed in dist.w. and rinsed in PBS containing 1%BSA.

*Primary antibody:* sections were incubated with a polyclonal anti-TH antibody raised in sheep at a 1/200 dilution overnight at 4°C, and then for 2h at

37°C. Slides were washed three times in dist.w. for 5min, then in 50mM PBS for 10min, and finally rinsed with 50mM PBS containing 0,5% BSA.

*Secondary antibody and visualization:* following treatment with the primary antibody, sections were incubated with the corresponding dilution of the biotinylated donkey anti-sheep IgG polyclonal antibody at a 1/200 dilution, for 45 min at 37°C. The slides were washed three times in dist.w. for 5min, followed by 50mM PBS for 10min. After rinsing with 50mM PBS containing 0,5% BSA, the sections were incubated with Alexa Fluor®488 steptavidin conjugate (which binds to the biotinylated secondary antibody) at a 1/200 dilution for 2h at 37°C, and the washing steps in dist.w. and PBS were repeated.

*Mounting:* The stained sections were mounted in fluorescent mounting medium and incubated overnight at 4°C in the dark. Green positive signals were visualized with a fluorescence microscope.

**Table 1.** Antibodies and fluorescence label used to detect TH-containing fibers

| | Detection | Produced in | Conjugation | Isotypic |
|---|---|---|---|---|
| Primary Ab. | Anti-TH | sheep | ---------- | polyclonal |
| Secondary Ab. | Anti-sheep IgG | donkey | biotinylated | polyclonal |
| Fluorescence label | Alexa Fluor®488 | ---------- | streptavidin | ---------- |

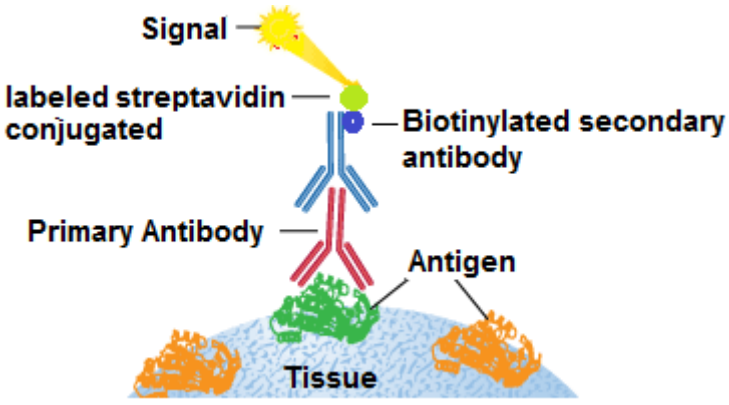


**Fig. 8. TH detection.** The indirect method of immunohistochemical staining used to detect TH, uses a first (primary) antibody against the antigen, a biotinylated secondary antibody against the first antibody, and a fluorescent-labeled streptavidin-conjugate.

***BDNF detection***

The blocking steps and the treatment with the primary antibody were exactly as described for TH detection. In this case, the primary antibody was a rabbit anti-BDNF monoclonal antibody, used at a dilution 1/200. The sections were then incubated with a secondary mouse anti-rabbit IgG polyclonal antibody conjugated with indocarbocyanine (CY3) for 2h at 37°C. The sections were shortly treated (1-3 min) with 4% formaldehyde in 50mM PBS. This step is recommended to block the detachment of the fluorophore from the antibody and to preserve the staining pattern for a longer storage. The washing step was repeated. Sections were mounted as described above for the method used to detect TH, and red positive signals were visualized using a fluorescent microscope.

**Table 2.** Antibodies used for BDNF detection

| | Antibody | Produced in | Conjugation | Isotype |
|---|---|---|---|---|
| Primary Ab. | Anti-BDNF | rabbit | ---------- | monoclonal |
| Secondary Ab. | Anti-Rabbit IgG | mouse | indocarbocyanine (CY3) | polyclonal |

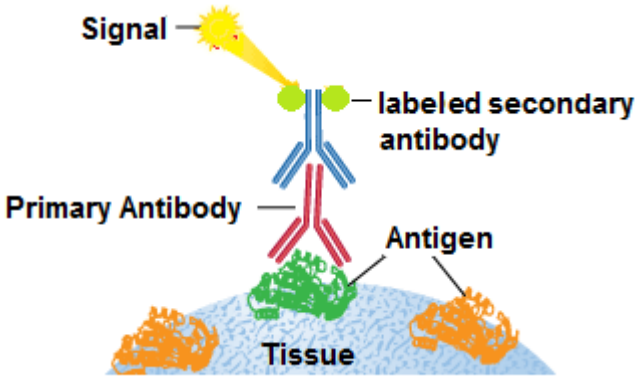


**Fig. 9. BDNF detection.** The indirect method of immunohistochemical staining used to detect BDNF uses a first (primary) antibody against the antigen, and a directly labeled secondary antibody against the first antibody.

## 3.9. Histological staining (Nissl staining)

This staining is commonly used to identify the basic neuronal structure in brain and spinal cord tissue, and serves to detect Nissl bodies in the cytoplasm

of neurons on paraffin embedded tissue sections. Nissl bodies are stained purple-blue.

*Procedure*

Fixation, washing, embedding, cutting, and mounting of paraffin sections onto slides were done exactly as described above for immunohistochemistry. The sections were deparaffinized in xylol three times for 10min, rehydrated in isopropanol (100% twice for 5 min, and 95% and in 70% for 3min each), rinsed in tap water, and then in dist.w. Sections were stained with 0,1% cresyl violet solution for 10min, quickly rinsed in dist.w., and differentiated in 95% isopropanol for 30 min. The sections were dehydrated in 100% isoproponal twice for 5min, cleared in xylol twice for 5 min, and finally, mounted with permanent mounting medium.

## 3.10. Statistical analysis

Results are expressed as mean ± SE. Data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher`s test for multiple comparisons, using Statview version 5.0. Differences were considered significant when $p<0.05$. Regression analysis was performed by analysis of covariance (ANCOVA) also using Statview version 5.0.

# 4. Results

The results are divided into 5 main sections. The first four sections deal with the effect that the lack of a functional thymus has on the concentration of: 1) catecholamines, 2) indolamines, and 3) neurotrophins in the periphery and in defined brain regions, and 4) corticosterone blood levels. The fifth section addresses the effect that chemical sympathectomy has on neurotrophin and corticosterone concentration in tissues and blood, respectively.

The animals used in the first four sections of this work were congenitally athymic *Foxn1*$^{n}$ mice, their heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$), and *Foxn1*$^{n}$ mice that had been transplanted with a thymus at birth. The terms *Foxn1*$^{n}$, nude, or athymic mice are used indistinctly in the text to refer to the homozygous mutated animals.

Since it has been reported that athymic mice have an abnormal growth rate as compared to normal mice, the body weight was recorded before sacrifice. The organs were weighted before homogenization, and the corresponding organ/body weight index was calculated. The results of these determinations are shown in Table 3. The body weight was significantly lower in athymic mice than in their heterozygous thymus-bearing littermates at nearly all ages studied. Thymus transplantation into newborn nude mice resulted in normalization of the body weight in 21 day-old mice, although it was still lower in 60 day-old mice when compared to the heterozygous littermates. The only statistically significant difference between athymic and heterozygous mice in the spleen weight was observed on 1 and 7 day-old animals**.** Thymus implantation at birth resulted in a spleen weight that was even bigger than that of heterozygous mice on day 21. The weight of the kidney of *Foxn1*$^{n}$ mice was lower than that of the *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals on days 14, 21, and 60; and it was completely normalized by thymus implantation. When the weight of the organs was refered to the body weight, the spleen index in athymic mice was relatively smaller or larger than in the heterozygous littermates on days 7 and 21, respectively. Thymus transplantation resulted in a significant higher splenic index than in heterozygous mice at this age. The kidney/body weight index was only larger in 14 day-old athymic mice than in the thymus-bearing animals. Thymus transplantation did not affect this index in 21- and 60-day-old mice.

**Table 3.** Body, spleen, and kidney weight of athymic *Foxn1$^n$*, *Foxn1$^n$*/*Foxn1*+, and *Foxn1$^n$* transplanted with a thymus at birth

**A. Body weight (g)**

| | Body weight (g) | | |
|---|---|---|---|
| **Age (days)** | ***Foxn1$^n$* (athymic)** | ***Foxn1$^n$* (athymic)** | ***Foxn1$^n$*+ thymus** |
| **1** | 1,29 ± 0,03* | 1,48 ± 0,05 | n.d. |
| **7** | 5,11 ± 0,20 | 5,36 ± 0,36 | n.d. |
| **14** | 6,08 ± 0,45* | 7,91 ± 0,52 | n.d |
| **21** | 7,85 ± 0,50 * # | 10,11 ± 0,79 | 9,72 ± 0,20 |
| **60** | 21,31 ± 0,27* | 23,76 ± 0,33 | 21,27 ± 0,65§ |

**B. Organ weights**

| | Spleen weight (mg) | | |
|---|---|---|---|
| **Age (days)** | ***Foxn1$^n$* (athymic)** | ***Foxn1$^n$*/*Foxn1$^+$*** | ***Foxn1$^n$* + thymus** |
| **1** | 2, 99 ± 0,18* | 3, 49± 0,23 | n.d. |
| **7** | 36,18 ± 2,11* | 45,89± 1,61 | n.d. |
| **14** | 44,42 ± 5,93 | 55,03 ± 5,23 | n.d. |
| **21** | 68,83 ± 7,13 # | 63,10 ± 5,63 | 111,38 ± 9,77§ |
| **60** | 118,56 ± 8,93 | 99,73 ± 6,03 | 116,40 ± 10,96 |

| | Kidney weight (mg) | | |
|---|---|---|---|
| **Age (days)** | ***Foxn1$^n$* (athymic)** | ***Foxn1$^n$*/*Foxn1$^+$*** | ***Foxn1$^n$* + thymus** |
| **1** | 7,40 ± 0,41 | 8,57 ± 0,56 | n.d. |
| **7** | 29,65 ± 1,43 | 31,04 ± 2,16 | n.d. |
| **14** | 43,50 ± 2,20* | 52,20 ± 3,61 | n.d. |
| **21** | 55,96 ± 3,24*# | 67,20 ± 4,23 | 65,71 ± 1,54 |
| **60** | 182,90 ± 4,23* | 195,80 ± 4,08 | 178,94 ± 8,06 |

**C. Organ/body weight index**

| | Spleen mg/g body weight | | |
|---|---|---|---|
| **Age (days)** | ***Foxn1$^n$* (athymic)** | ***Foxn1$^n$*/*Foxn1$^+$*** | ***Foxn1$^n$* + thymus** |
| **1** | 2,21 ± 0,18 | 2,33 ± 0,10 | n.d. |
| **7** | 7,08 ± 0,34* | 8,77 ± 0,58 | n.d. |
| **14** | 6,89 ± 0,57 | 6,87 ± 0,27 | n.d. |
| **21** | 8,63 ± 0,66* # | 6,25 ± 0,25 | 11,46 ± 1,02 § |
| **60** | 5,61 ±0,42 | 4,21 ± 0,28 | 5,57 ± 0,67 |

| | Kidney mg/g body weight | | |
|---|---|---|---|
| **Age (days)** | ***Foxn1$^n$* (athymic)** | ***Foxn1$^n$*/*Foxn1$^+$*** | ***Foxn1$^n$* + thymus** |
| **1** | 5,448 ± 0,278 | 5,536 ± 0,171 | n.d. |
| **7** | 5,798 ± 0,142 | 5,817 ± 0,199 | n.d. |
| **14** | 7,354 ± 0,290* | 6,550 ± 0,13 | n.d. |
| **21** | 7,160 ± 0,165 | 6,720 ± 0,204 | 6,770 ± 0,139 |
| **60** | 8,543 ± 0,148 | 8,186 ± 0,162 | 8,411 ± 0,221 |

n.s. = not statistically significantly different. Statistically significant differences: * *Foxn1$^n$* vs *Foxn1$^n$*/*Foxn1$^+$*; # *Foxn1$^n$* vs *Fox1$^n$* with thymus; § *Fox1$^n$* with thymus vs *Foxn1$^n$*/*Foxn1$^+$*.

## 4.1. The absence of the thymus affects catecholamine, precursor, and metabolite concentrations in the spleen and brain

### 4.1.1. Effects on the spleen

Changes in NA concentration in organs during ontogeny are considered an expression of the development of sympathetic innervation (De Champlain and Smith, 1974). Since it has been previously shown that T cells or their products can affect the sympathetic innervation of the spleen (Besedovsky et al., 1987), the first part of the studies reported in this work were performed to confirm these results, and to study whether the increase in sympathetic innervation detected in young athymic mice is also observed during adulthood.

NA concentration was determined by HPLC in the spleen of 1, 7, 14, 21 and 60 day-old athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$). The same determinations were performed in 21 and 60 day-old athymic mice implanted with a thymus at birth. The kidney was used as an abdominal control organ.

While no differences were detected at the birth, higher NA concentrations were found in the spleen of 7, 14, 21, and 60 day-old *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates (Fig. 10A). The difference in splenic NA concentration between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice was statistically significant in 7 ($p<0,02$), 14 ($p<0,007$), and 21 ($p<0,008$) day-old mice, confirming the results obtained by Besedovsky et al.(Besedovsky et al., 1987), and further showing that the same tendency is observed in adult mice. The results also confirmed that NA concentration in the spleen of 7 day-old thymus-bearing mice (113,9 ± 35,3 ng/g spleen) dropped to values of about 40% of those observed at birth (189,1 ± 32,7ng/g spleen). Such a drop was not detected in the spleen of Foxn1$^{n}$ mice (1 day-old: 188,1 ± 60,2 ng/g spleen; 7 day-old: 165,3 ± 37,1 ng/g spleen). It is remarkable that T cells effector functions, such as cytotoxicity, are also first detected in the normal mouse spleen during the first week of life (Wu et al., 1975).

To assert whether the high splenic NA concentration in athymic mice was restricted to this lymphoid organ or it reflects a general hyperactivity of the sympathetic system, the concentration of NA in another abdominal organ, the kidney, was determined. No differences between NA levels in the kidney of

*Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice were detected (Fig. 10B), confirming previous results (Besedovsky et al., 1987). The results obtained in the kidney were of additional significance as they allowed to discard the possibility that the differences observed between the splenic NA concentration of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice may have been a consequence of endocrine disturbances known to occur in athymic mice (Besedovsky and Sorkin, 1974).

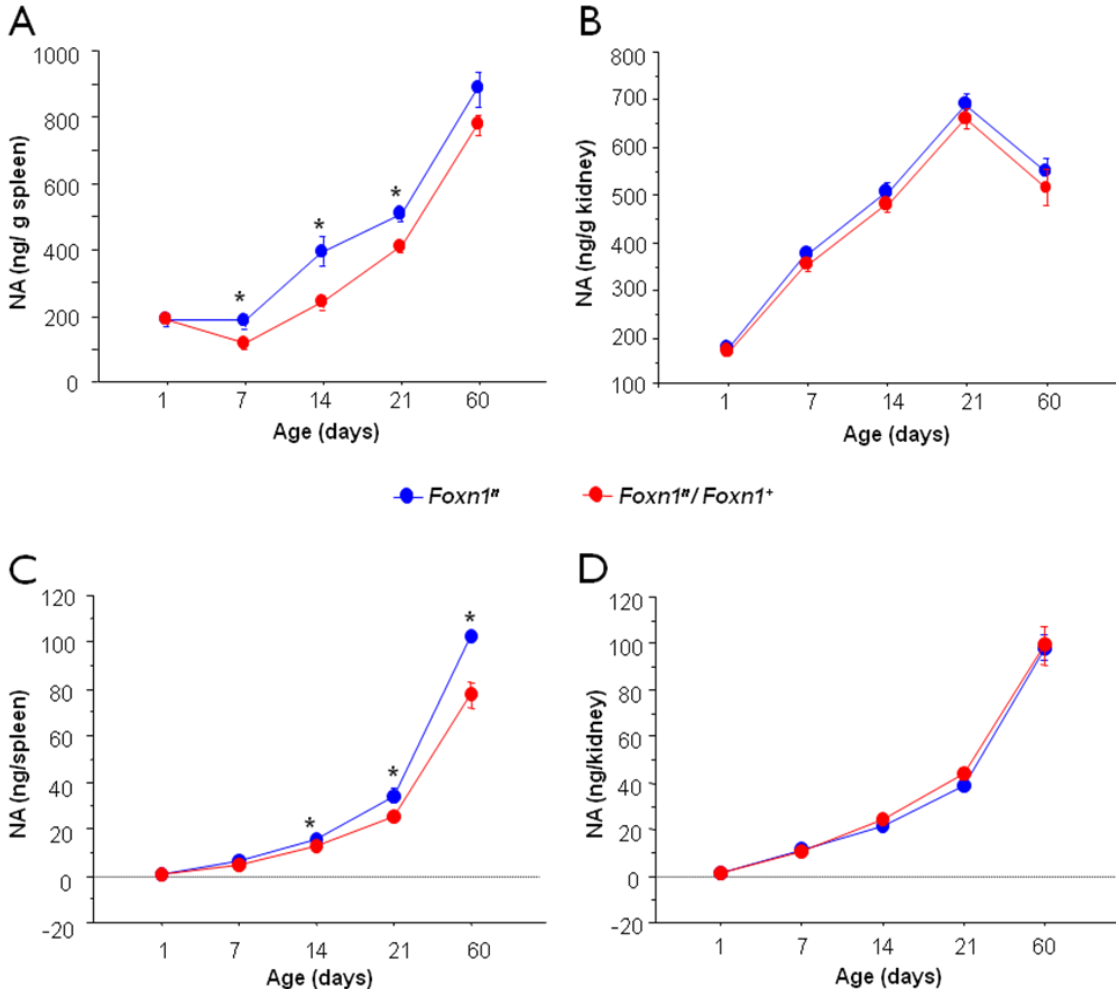


**Fig. 10. Increased splenic NA concentration and content in athymic mice during ontogeny.** NA concentration was determined in the spleen and left kidney of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/*Foxn1*$^{+}$) littermates at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice. **(A, C)** spleen; **(B, D)** kidney. **A, B:** NA concentration; **C, D:** total NA content in the organ. ***** Statistically significantly different from *Foxn1*$^{n}$/*Foxn1*$^{+}$.

Since *Foxn1*$^{n}$ mice have smaller spleen and kidneys than *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice, the results were also expressed as total NA content in the organs studied. The total NA content in the spleen of *Foxn1*$^{n}$ mice was also significantly higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (14 day-old: $p<0,04$; 21 day-

old: p<0,0006; 60 day-old:p<0,0003). The same tendency was observed in 7 day-old mice (Fig. 10C). No significant differences were detected in the kidneys (Fig. 10D).

Following neonatal thymus grafting to nude mice, lymphoid organs are populated by T cells (Loor and Kindred, 1974), and reconstituted mice develop T cell-mediated immune mechanisms (Loor and Kindred, 1974). Furthermore, some endocrine functions are normalized (Besedovsky and Sorkin, 1974). Thus, the concentration of NA in the spleen of 21 and 60 day-old athymic mice implanted with thymus at birth was determined. Splenic NA concentration in these mice reached levels comparable to those of normal thymus-bearing littermates (Fig. 11A), confirming previously reported results (Besedovsky et al., 1987). The differences between athymic mice and athymic mice implanted with thymus at birth were statistically significant in 21 day-old animals (p<0,0001), and the same tendency was observed in 60 day-old mice. Splenic NA concentration in 21 day-old athymic implanted with thymus at birth was also significantly lower than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (p<0,03).

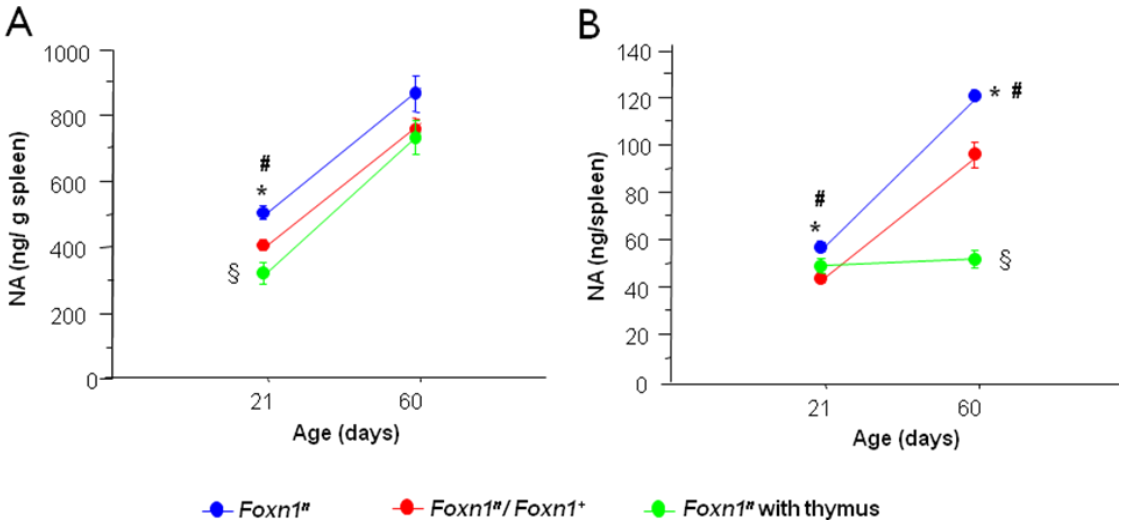


**Fig. 11. Thymus implantation into athymic newborn mice results in decreased NA concentration and content in the spleen.** Two thymi from newborn *Foxn1*$^{n}$/*Foxn1*$^{+}$ donors were grafted into less than 24h-old male *Foxn1*$^{n}$ mice (*Foxn1*$^{n}$ with thymus). NA concentration in the spleen was determined when mice were 21 (n=9) or 60 (n=10) day-old mice. (**A)** NA concentration; **(B)** total NA content. Statistically significant difference: ⋆ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The results were also expressed as total NA content in the spleen. Total NA content in the spleen of 21 and 60 day-old athymic mice implanted with thymus

at birth was statistically significantly lower than in athymic mice (p<0,04 and p<0,0001, respectively). Splenic NA content in 60 day-old athymic implanted with thymus at birth was also significantly lower than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (p<0,0001) (Fig. 11B).

To study whether the alteration in NA observed in the spleen of athymic mice was restricted to this catecholamine, the concentration of DA, another catecholamine that is the direct precursor in the synthesis of NA, was determined by HPLC in the spleen and kidney of the same animals used above.

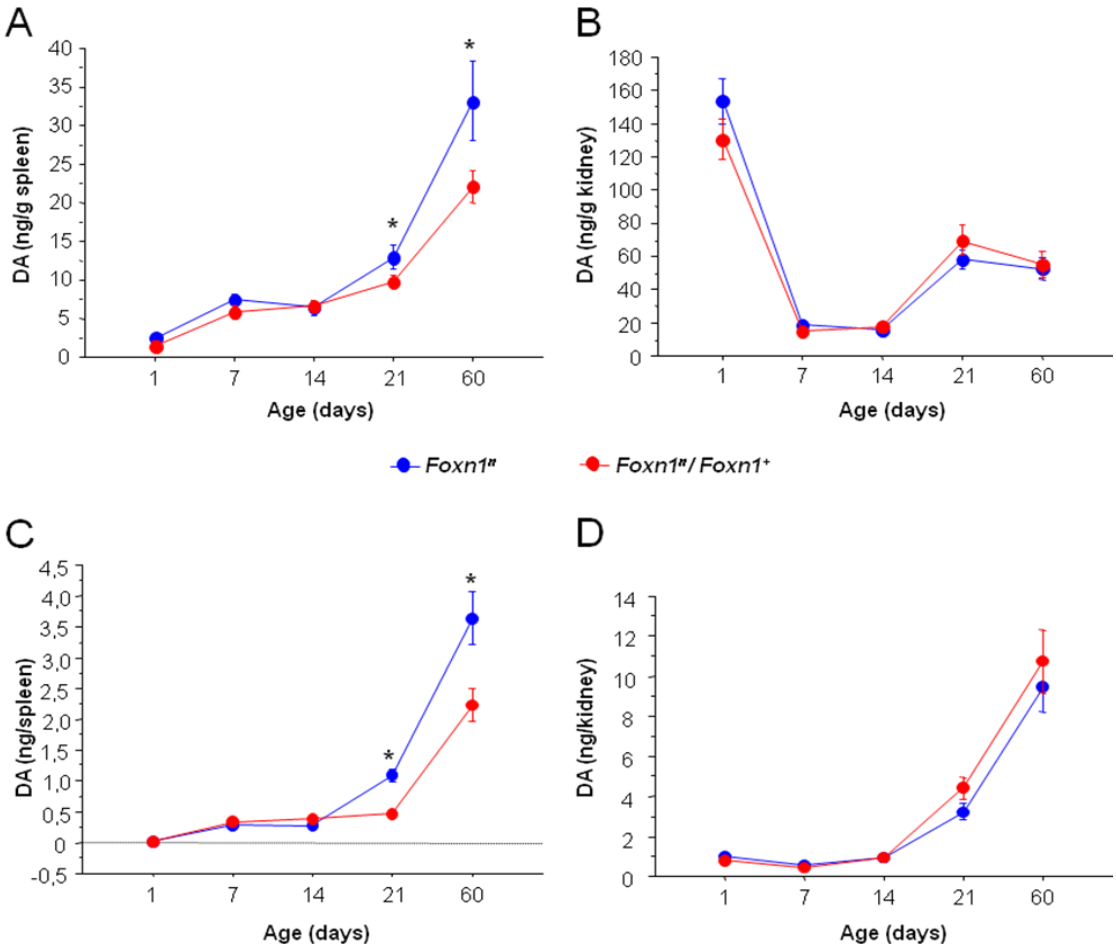


**Fig. 12. Increased splenic DA concentration and content in athymic mice during ontogeny.** DA concentration was determined in the spleen and left kidney of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/ *Foxn1*$^{+}$) littermates at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice. **(A, C)** spleen; **(B, D)** kidney. **A, B:** DA concentration; **C, D:** total DA content in the organ.

* Statistically significantly different from *Foxn1*$^{n}$/*Foxn1*$^{+}$.

While no differences were detected at the birth, and on 7 and 14 day-old mice, statistically significantly higher DA concentrations were found in the spleen of 21 and 60 day-old *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates, ($p<0,04$ and $p<0,02$, respectively) (Fig. 12A). No differences in DA concentration in the kidney of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice were detected (Fig. 12B).

The results were also expressed as total DA content in the organs studied. While no statistically differences were detected at the birth and in 7 and 14 day-old mice, DA content was statistically significantly higher in the spleen of 21 and 60 day-old *Foxn1*$^{n}$ mice as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice ($p<0,0001$ and $p<0,005$, respectively) (Fig. 12C). No significantly differences were detected in the kidney (Fig. 12D).

The concentration of DA in the spleen of 21 and 60 day-old athymic mice implanted with thymus at birth was also determined. Splenic DA concentration in these mice reached levels comparable to those of normal thymus-bearing littermates (Fig. 13 A). The differences between athymic and athymic mice implanted with thymus at birth were statistically significant (21 day-old: $p<0,001$; 60 day-old: $p<0,004$).

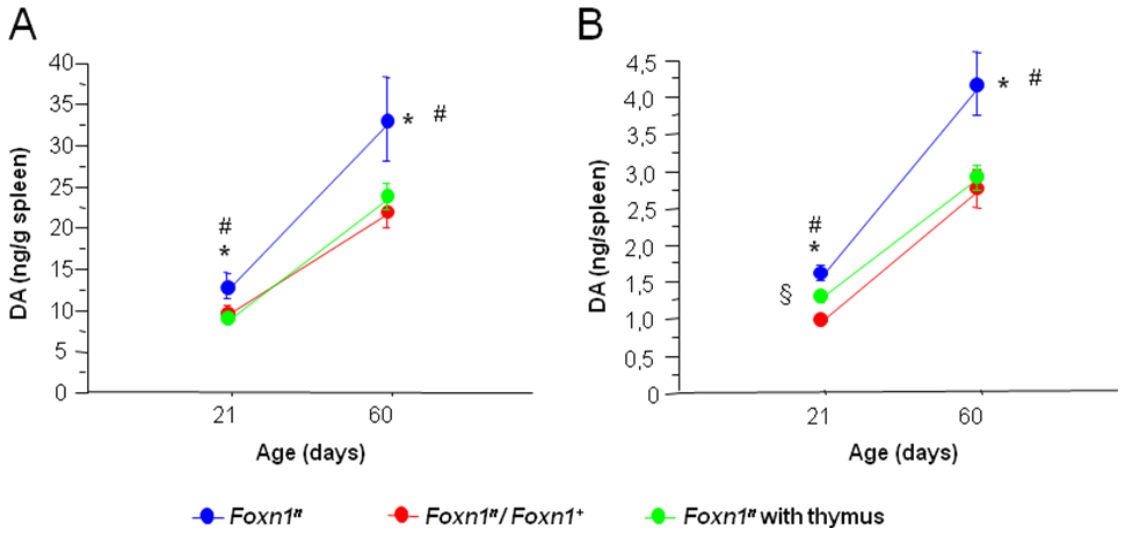


**Fig. 13. Thymus implantation into athymic newborn mice results in decreased DA concentration and content in the spleen.** Two thymi from newborn Balb/c *Foxn1*$^{n}$/*Foxn1*$^{+}$ donors were grafted into less than 24 h-old athymic male nude Balb/c mice (*Foxn1*$^{n}$ with thymus). DA concentration in the spleen was determined when mice were 21 (n=9) or 60 (n=10) day-old (*Foxn1*$^{n}$ with thymus). (**A)** DA concentration. **(B)** Total DA content. Statistically significantly different between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ **(*)**; *Foxn1*$^{n}$ and *Fox1*$^{n}$ with thymus **(#)**; and between *Fox1*$^{n}$ with thymus and *Foxn1*$^{n}$/*Foxn1*$^{+}$ **(§).**

Total splenic DA content in 21 and 60 day-old athymic mice implanted with thymus at birth was statistically significantly lower than in athymic mice ($p<0,006$; and $p<0,009$, respectively) (Fig. 13B).

Tyr, the precursor of NA, and DOPAC, the metabolite of DA, were determined by HPLC in the spleen and kidney of the same animals used above. While no differences were detected at the birth, higher Tyr concentrations were found in the spleen of 7, 14, 21, and 60 day-old *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates. These differences reached statistically significance in 7 ($p<0,007$) and 60 ($p<0,03$) day-old mice, and the same tendency was observed in 14 and 21 day-old mice (Fig. 14 A). No significantly differences were detected in the kidney (Fig 14 B).

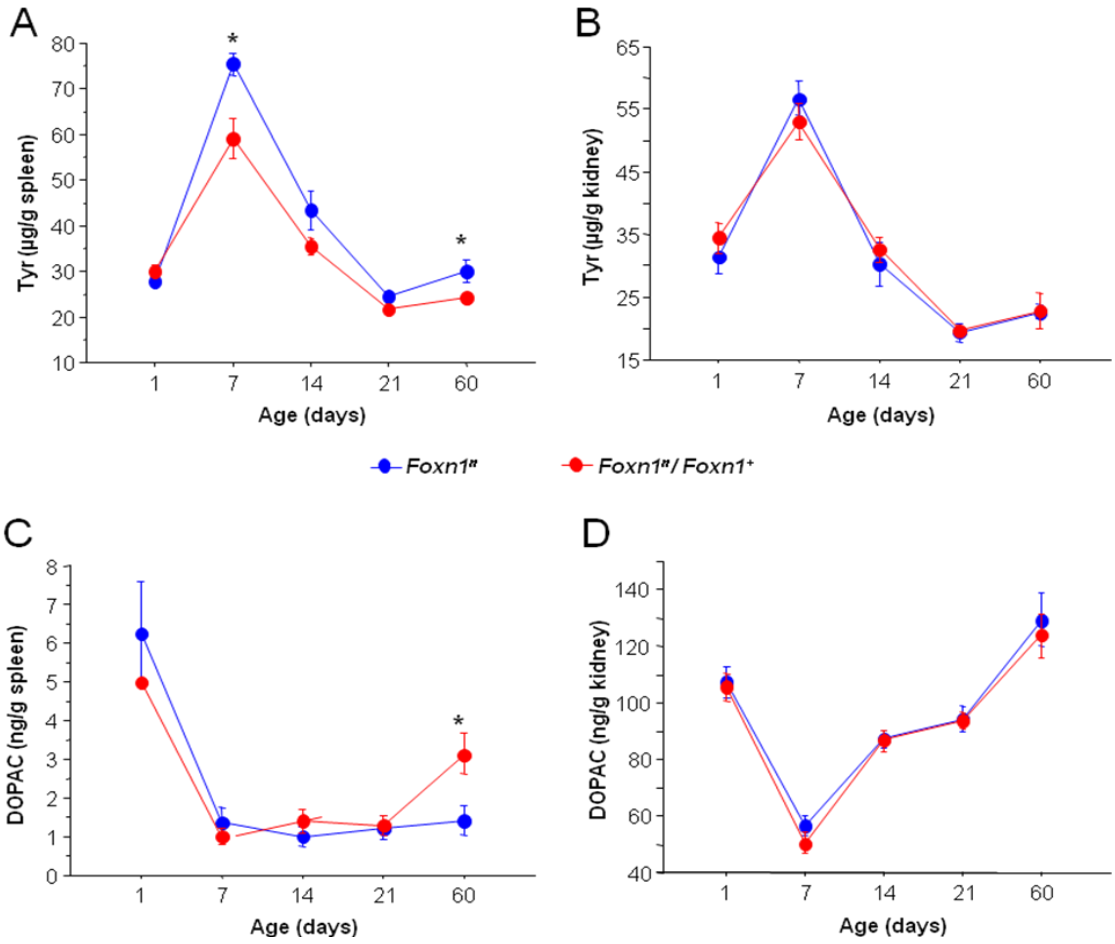


**Fig. 14. Increased splenic Tyr, but not DOPAC concentrations in athymic mice.** Tyr and DOPAC concentrations were determined in the spleen and left kidney of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/ *Foxn1*$^{+}$) littermates at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice. **(A, C)** spleen; **(B, D)** kidney. **A**, **B:** Tyr concentration; **C, D:** DOPAC concentration. * Statistically significantly different from *Foxn1*$^{n}$/*Foxn1*$^{+}$.

Significantly higher DOPAC concentrations were detected only in the spleen of 60 day-old *Foxn1*$^{n}$/*Foxn1*$^{+}$ as compared to *Foxn1*$^{n}$ (p<0,006) (Fig. 14 C). No significantly differences were detected in the kidney (Fig. 14D).

Thymus implantation into newborn *Foxn1*$^{n}$ resulted in splenic Tyr concentrations comparable to those of normal thymus-bearing littermates, and statistically significantly different from those of *Foxn1*$^{n}$ mice (21 day-old: p<0,004; 60 day-old: p<0,002) (Fig. 15 A). However, thymus grafting did not normalize DOPAC concentration in the spleen of 60 day-old mice, since the values were still significantly lower in the reconstituted mice when compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (p<0,03) (Fig. 15 B).

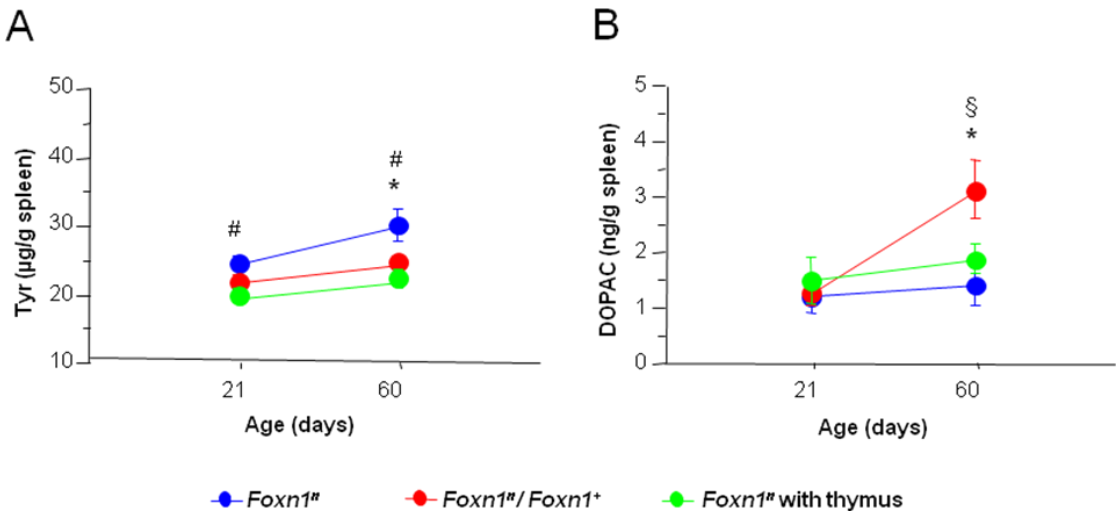


**Fig. 15. Thymus implantation into athymic newborn mice decreases Tyr, but not DOPAC, concentration in the spleen.** Thymus implantation into athymic newborn mice decreases Tyr, but not DOPAC, concentration in the spleen. Two thymi from newborn Balb/c *Foxn1*$^{n}$/*Foxn1*$^{+}$ donors were grafted into less than 24 h-old athymic male nude Balb/c mice (*Foxn1*$^{n}$ with thymus). Tyr **(A)** and DOPAC **(B)** concentrations were determined when mice were 21 (n=9) or 60 (n=10) day-old mice. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

### 4.1.2. Effects on the brain

Since administration of supernatants from T cell cultures to normal mice evokes changes in central noradrenergic mechanisms (Besedovsky et al., 1983; Besedovsky et al., 1981), it was likely that mature T cells would have also an impact on brain neurotransmitters. Thus, the concentration of NA, its precursor and main metabolite were determined in several brain regions of

athymic male mice, and their heterozygous thymus-bearing littermates at different times in ontogeny. A third group of athymic mice implanted with thymus at birth was also included. The brain tissue used in these studies derives from the same mice used for the determinations in the spleen and kidney shown above.

It was not possible to divide the small brain of newborn mice into well-defined regions with the technology available. Thus, at this time-point, the brain was taken as a whole and divided only into right and left hemispheres. The left part was used for neurotransmitter determinations. No differences in NA concentration between newborn athymic mice and thymus-bearing heterozygous littermates were detected in the whole brain (Fig. 16).

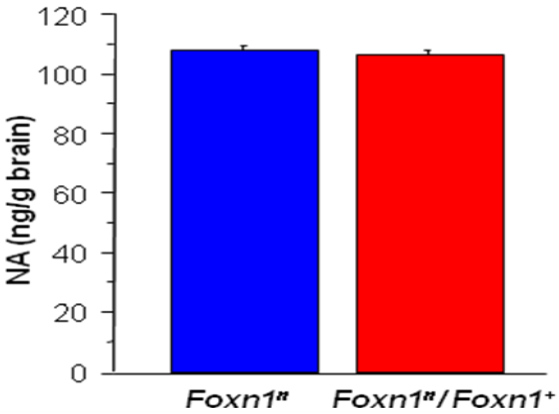


**Fig. 16.** No differences in NA concentration in the whole brain of newborn athymic mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$); n=7/ group.

Higher NA concentrations were found in the hypothalamus, brainstem, and hippocampus of *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates at later stages of development.

The differences between hypothalamic NA levels in *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant at all ages studied (7 day-old: $p<0,02$; 14 day-old: $p<0,04$; 21 day-old: $p<0,0002$; 60 day-old: $p<0,0005$). Thymus implantation at birth normalized hypothalamic NA concentration in athymic mice. The differences between athymic and athymic mice implanted with thymus at birth were statistically significant in 21 ($p<0,01$) and in 60 ($p<0,0002$) day-old mice (Fig 17 A).

Comparable results were obtained in the brainstem and in the hippocampus at the same time-points. The differences between NA levels in the brainstem of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant in 7 ($p<0,02$), 14 ($p<0,0001$), 21 ($p<0,0001$), and 60 ($p<0,0001$) day-old mice. The differences

between athymic and athymic mice implanted with thymus at birth were statistically significant in 21 (p<0,008) and 60 (p<0,0001) day-old mice (Fig 17 B).

NA concentration in the hippocampus of 7 (p<0,01), 21 (p<0,04) and 60 (p<0,005) day-old *Foxn1*$^{n}$ mice was higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals and the same tendency was observed in 14 day-old mice. Although not reaching statistical significance, thymus implantation at birth tended to abrogate these differences (Fig 17 C).

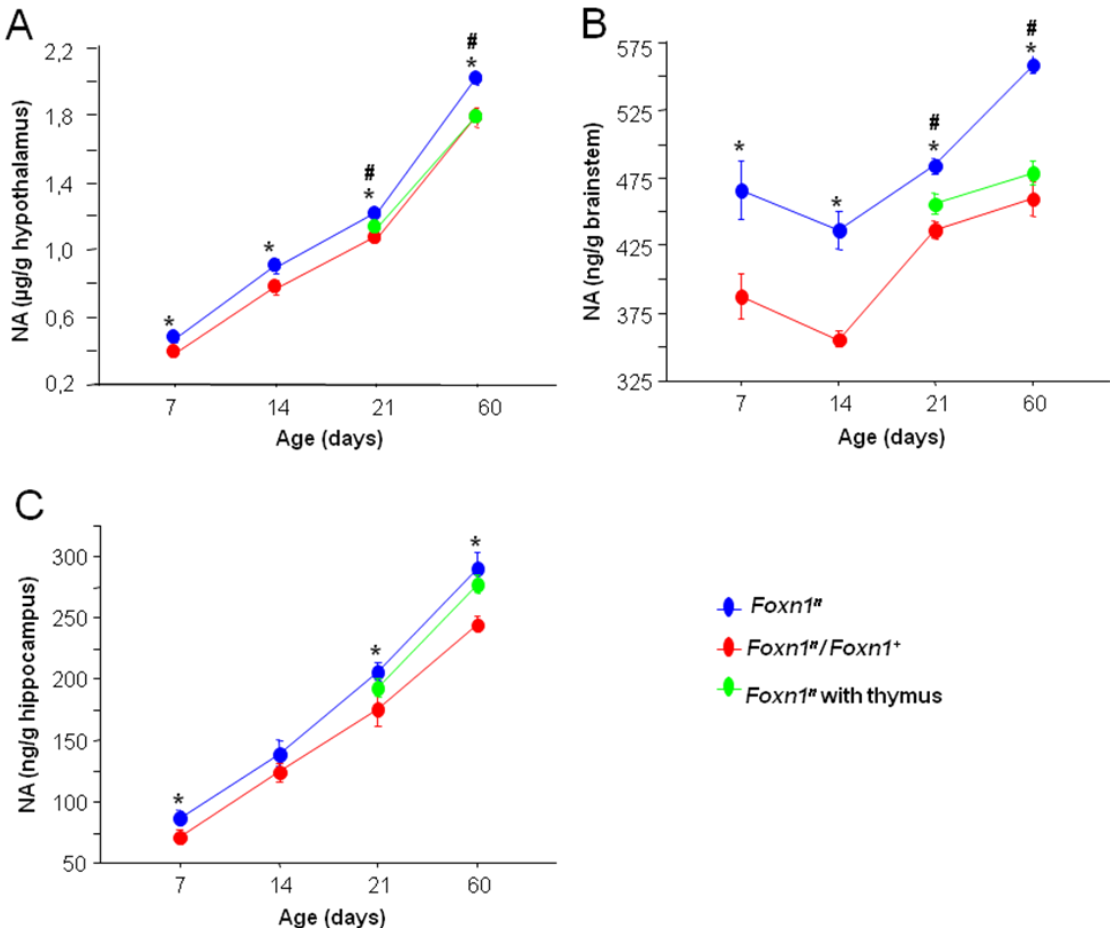


**Fig. 17. Increased NA concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth.** NA concentration in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were determined by HPLC in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice, and athymic mice implanted with a thymus at birth and sacrificed when they were 21 (n=9) or 60 (n=10) day-old (*Foxn1*$^{n}$ with thymus). Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and § *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$.

The concentration of DA, a catecholamine that gives rise to NA but also functions as a neurotransmitter itself in the brain, was also determined in the same brain regions of the same groups of mice and at the same time points.

While no differences between athymic mice and thymus-bearing heterozygous littermates were detected in the whole brain of newborn mice (Fig. 18), higher DA concentrations were detected in the hypothalamus, brainstem, and hippocampus of 7, 14, 21, and 60 day-old *Foxn1*$^{n}$ mice as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates. DA concentration was completely normalized in 21 and 60 day-old athymic mice that were implanted with a thymus at birth.

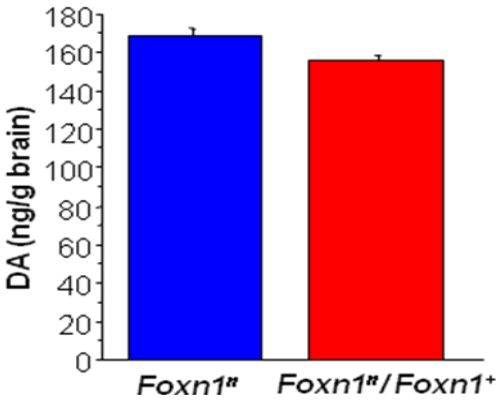


**Fig. 18.** No differences in DA concentration in the whole brain of athymic mice (*Foxn1*$^{n}$) mice and heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$); n= 7/group.

The differences in hypothalamic DA levels between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant (day 7: p<0,0003, day 14: p<0,03, day 21: p<0,004, day 60: p<0,0002), as well as between athymic and athymic mice implanted with thymus at birth (day 21 and day 60: p<0,003) (Fig 19 A).

Essentially the same differences were observed in the brainstem. DA levels in *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significantly different in 21 (p<0,001) and 60 (p<0,0001) day-old mice. The same tendency was observed in 7 and 14 day-old mice. The difference between athymic and athymic mice implanted with thymus at birth was statistically significant at both ages studied (21 day-old: p<0,004; 60 day-old: p<0,0002) (Fig 19 B).

The results obtained in the hippocampus are depicted in Figure 18 C, which shows that *Foxn1*$^{n}$ mice have more DA in this brain region than *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals when they were 7 (p<0,02) and 21 day-old (p<0,003). The same tendency was observed in 60 day-old mice. Thymus reconstitution at birth abrogated this difference, resulting in significant differences in DA concentration in the hippocampus of 21 day-old *Foxn1*$^{n}$ mice and *Foxn1*$^{n}$

implanted with thymus at birth (p<0,03). However, and contrary to what was observed in the other two brain regions studied, DA concentration in the hippocampus of 60 day-old *Foxn1*$^{n}$ mice implanted with thymus at birth was even higher than in *Foxn1*$^{n}$ (p<0,03), and *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,005) mice (Fig 19C).

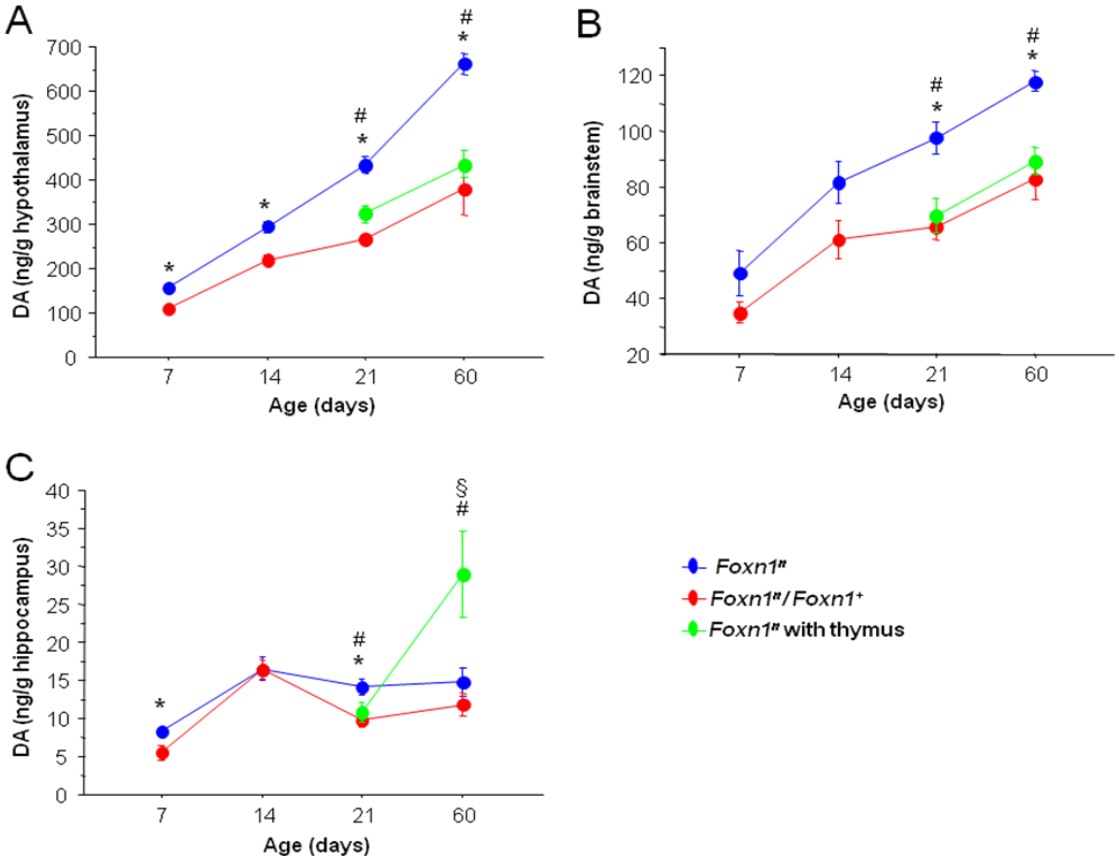


**Fig. 19. Increased DA concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth.** DA concentration in hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were determined by HPLC in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice, and athymic mice implanted with a thymus at birth and sacrificed when they were 21 (n=9) or 60 (n=10) day-old (*Foxn1*$^{n}$ with thymus). Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

Tyr, the amino acid precursor of NA, MHPG, a main metabolite of NA degradation in the brain, and DOPAC, an important metabolite of DA derived from the action of the enzyme monoaminooxidase (Eisenhofer et al., 2004), were evaluated in parallel to NA and DA in the same brain samples.

While no differences between athymic mice and thymus-bearing heterozygous littermates were detected in the whole brain of less than 24 hour-old mice (Fig. 20 A-C), higher Tyr, MHPG, and DOPAC concentrations were detected in the hypothalamus, brainstem, and hippocampus of *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates at later stages of development. Such differences were completely normalized in 21 and 60 day-old athymic mice implanted with thymus at birth.

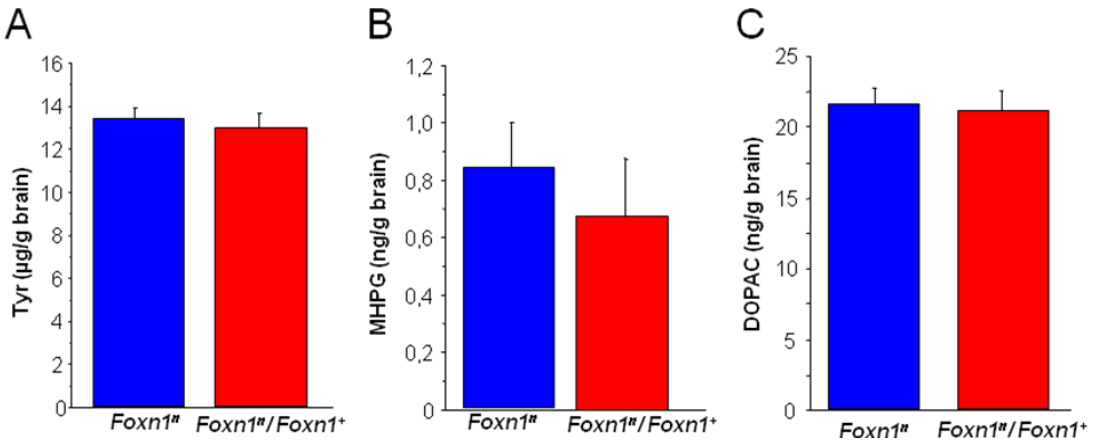


**Fig. 20.** No differences in Try, MHPG, and DOPAC concentrations in the whole brain of newborn athymic mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$). n= 7/group. **(A)** Tyr concentration, **(B)** MHPG concentration, and **(C)** DOPAC concentration.

The differences between hypothalamic Tyr levels in *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant at all ages studied (7day-old: p<0,04, 14 day-old: p<0,0001, 21 day-old: p<0,0001, 60 day-old: p<0,01). Such differences were abrogated in 21 (p<0,0001) and 60 (p<0,01) day-old athymic mice implanted with thymus at birth (Fig 21 A).

Comparable results were obtained in the brainstem at the same time-points. Try level in the brainstem of *Foxn1*$^{n}$ mice was statistically significantly higher than *Foxn1*$^{n}$/*Foxn1*$^{+}$ in 7 (p<0,002), 14 (p<0,002), 21 (p<0,0001), and 60 (p<0,0001) day-old mice. The difference between athymic and athymic mice implanted with thymus at birth was statistically significant in both ages studied (day 21: p<0,01, day 60: p<0,0001) mice. However, on day 21, Tyr concentration in the brainstem of athymic mice implanted with thymus at birth was not completely normalized yet, since it was significantly higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,003) (Fig 21B).

While no difference was found in 7 day-old mice, statistically significantly higher Tyr concentration in the hippocampus of 14 ($p<0,005$), 21 ($p<0,02$) and 60 ($p<0,005$) day-old *Foxn1*$^{n}$ mice than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals. Thymus implantation at birth abrogated these differences (21 day-old: $p<0,04$; 60 day-old: $p<0,003$) (Fig 21 C).

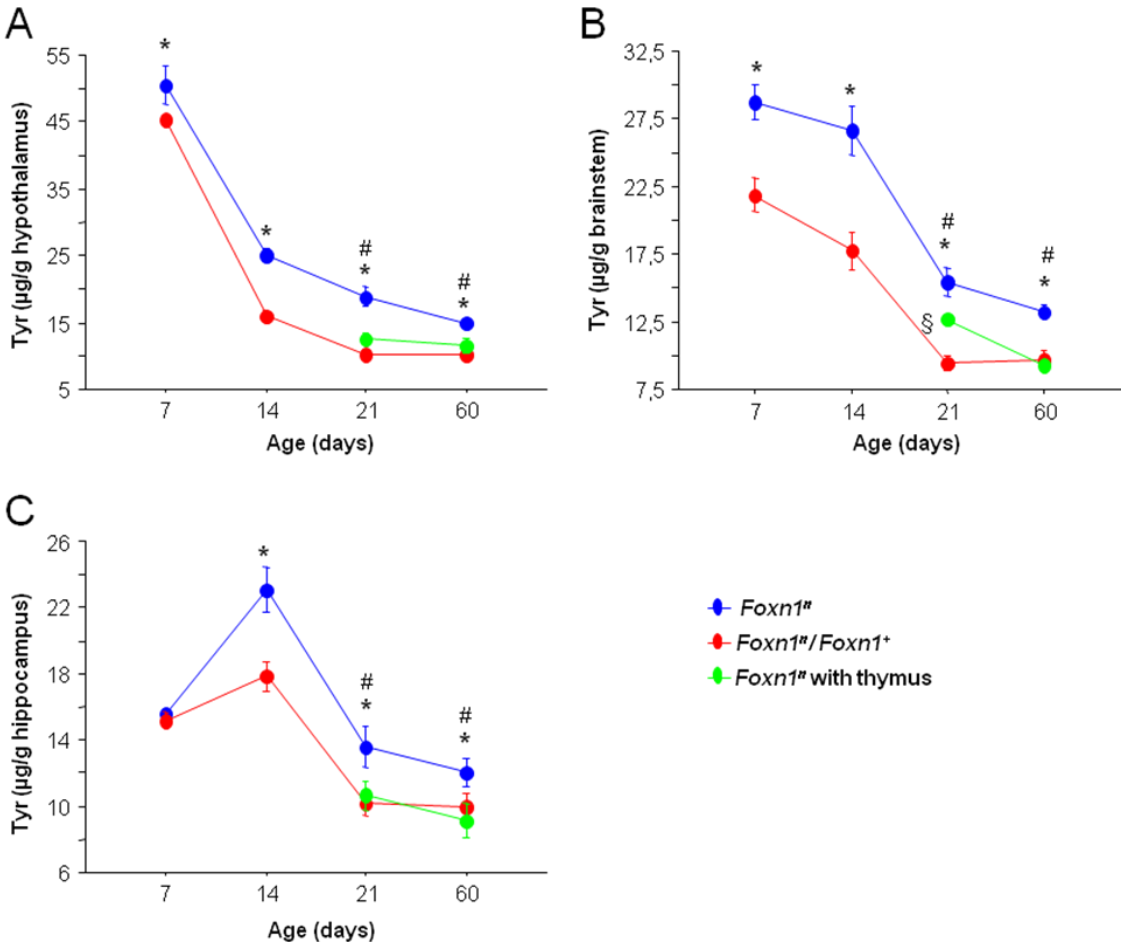


**Fig. 21. Increased Tyr concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth.** Tyr concentration in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were determined by HPLC in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old, and athymic mice implanted with a thymus at birth and sacrificed when they were 21 (n=9) or 60 (n=10) day-old (*Foxn1*$^{n}$ with thymus). Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and § *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The differences in MHPG levels in the hypothalamus of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant (day 7: $p<0,0001$; day 14: $p<0,0003$; day 21: $p<0,003$; day 60: $p<0,002$), as well as between athymic and

athymic mice implanted with thymus at birth (day 21: p<0,002, day 60: p<0,02). The concentration of MHPG in 60 day-old *Foxn1*$^{n}$ mice implanted with thymus at birth was even significantly lower than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,02) (Fig 22 A).

Essential the same differences were observed in the brainstem. MHPG levels in *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significantly different in 7 (p<0,02), 14 (p<0,0001), and 21 (p<0,0001) day-old mice. The same tendency was observed in 60 day-old mice. The difference between athymic and athymic mice implanted with thymus at birth was statistically significant in 21 day-old mice (p<0,003), and the same tendency was observed in 60 day-old mice (Fig 22 B).

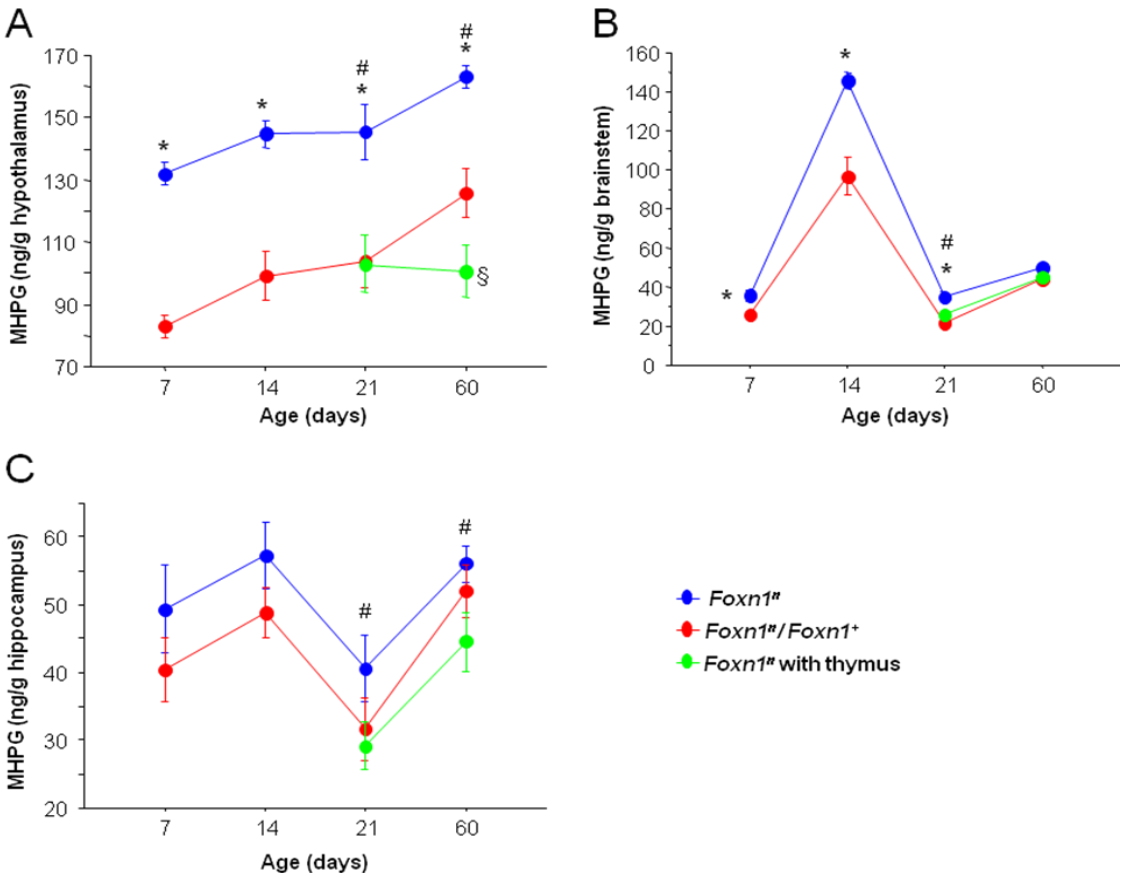


**Fig. 22. Increased MHPG concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth.** MHPG concentration in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were determined by HPLC in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old, and athymic mice implanted with a thymus at birth and sacrificed when they were 21 (n=9) or 60 (n=10) day-old (*Foxn1*$^{n}$ with thymus). Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and § *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

Although not reaching statistical significance, the same tendency was observed in MHPG concentration in the hippocampus of *Foxn1*$^{n}$ as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$. Thymus reconstitution at birth abrogated this difference, resulting in statistically significantly differences in MHPG concentration in the hippocampus of 21 ($p<0,04$), and 60 ($p<0,03$) day-old *Foxn1*$^{n}$ with thymus compared to *Foxn1*$^{n}$ mice (Fig 22 C).

Comparable results were obtained in DOPAC levels in the same brain regions at the same time-points. The differences between hypothalamic DOPAC levels of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant in 7 ($p<0,02$), 14 ($p<0,01$), 21 ($p<0,0001$), and 60 ($p<0,0001$) day-old mice. Such differences were abrogated in 21 ($p<0,0001$), and 60 ($p<0,0001$) day-old athymic mice implanted with thymus (Fig 23 A).

Essential the same differences were observed in the brainstem. DOPAC levels of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were also statistically significantly different in 7 ($p<0,0008$), 14 ($p<0,02$), 21 ($p<0,0001$), and 60 ($p<0,0001$) day-old mice. Thymus reconstitution at birth diminished this difference, resulting in significant differences in DOPAC concentration in the brainstem of *Foxn1*$^{n}$ mice and *Foxn1*$^{n}$ implanted with thymus at birth (21 day-old: $p<0,03$); 60 day-old: $p<0,0001$). However, DOPAC concentration in the brainstem of 60 day-old, thymus reconstituted *Foxn1*$^{n}$ was still higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals ($p<0,0004$) (Fig 23 B).

The results obtained in the hippocampus are depicted in Figure 22 C, which shows that 14 ($p<0,03$) and 21 ($p<0,0001$) day-old *Foxn1*$^{n}$ have more DOPAC in this brain region than *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals. The same tendency was observed in 7 day-old mice. However, thymus reconstitution at birth did not abrogate this difference, since the values were still significantly higher when compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ in 21 ($p<0,0003$), and 60 ($p<0,02$) day-old mice, and even higher than in *Foxn1*$^{n}$ in 60 day-old mice (p<0,003) (Fig 23 C).

The results were also expressed as ratio metabolite/catecholamine (MHPG/NA and DOPAC/DA) and precursor/catecholamine (Tyr/NA) in the same brain regions of the same groups of mice and at the same time point.

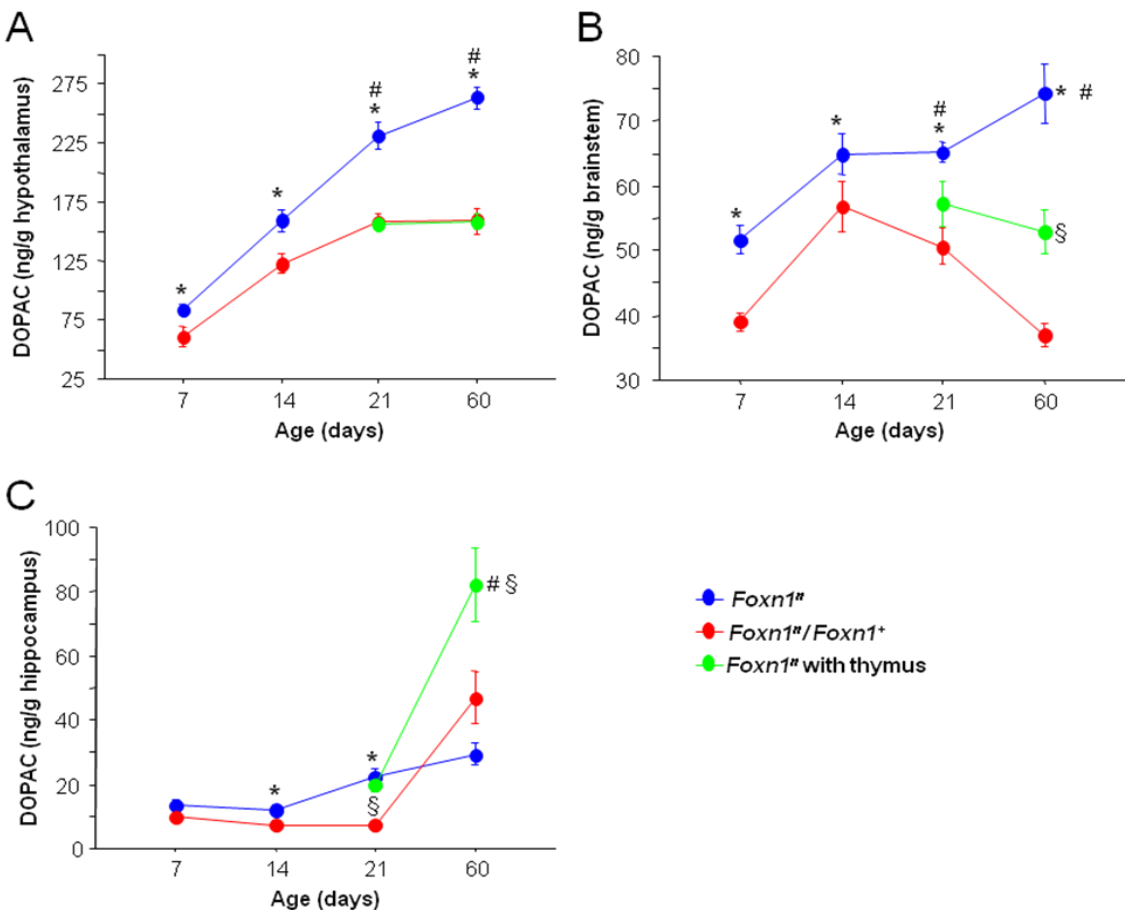


**Fig. 23. Increased DOPAC concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth**. DOPAC concentration in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were determined by HPLC in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old, and athymic mice implanted with a thymus at birth and sacrificed when they were 21 (n=9) or 60 (n=10) day-old (*Foxn1*$^{n}$ with thymus). Statistically significant difference: ∗ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and § *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

Statistically significant higher MHPG/NA ratios were detected in the hypothalamus of *Foxn1*$^{n}$ mice as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ in 7 ($p<0,0001$), 14 ($p<0,0003$), 21 ($p<0,0006$), and 60 ($p<0,003$) day-old mice. This ratio was normalized in athymic mice implanted with thymus at birth (21 day-old: $p<0,002$ and 60 day-old: $p<0,001$) (Fig. 24 A). However, no statistically differences were detected in MHPG/NA between these three groups in the brainstem and hippocampus (Fig. 24 B, C).

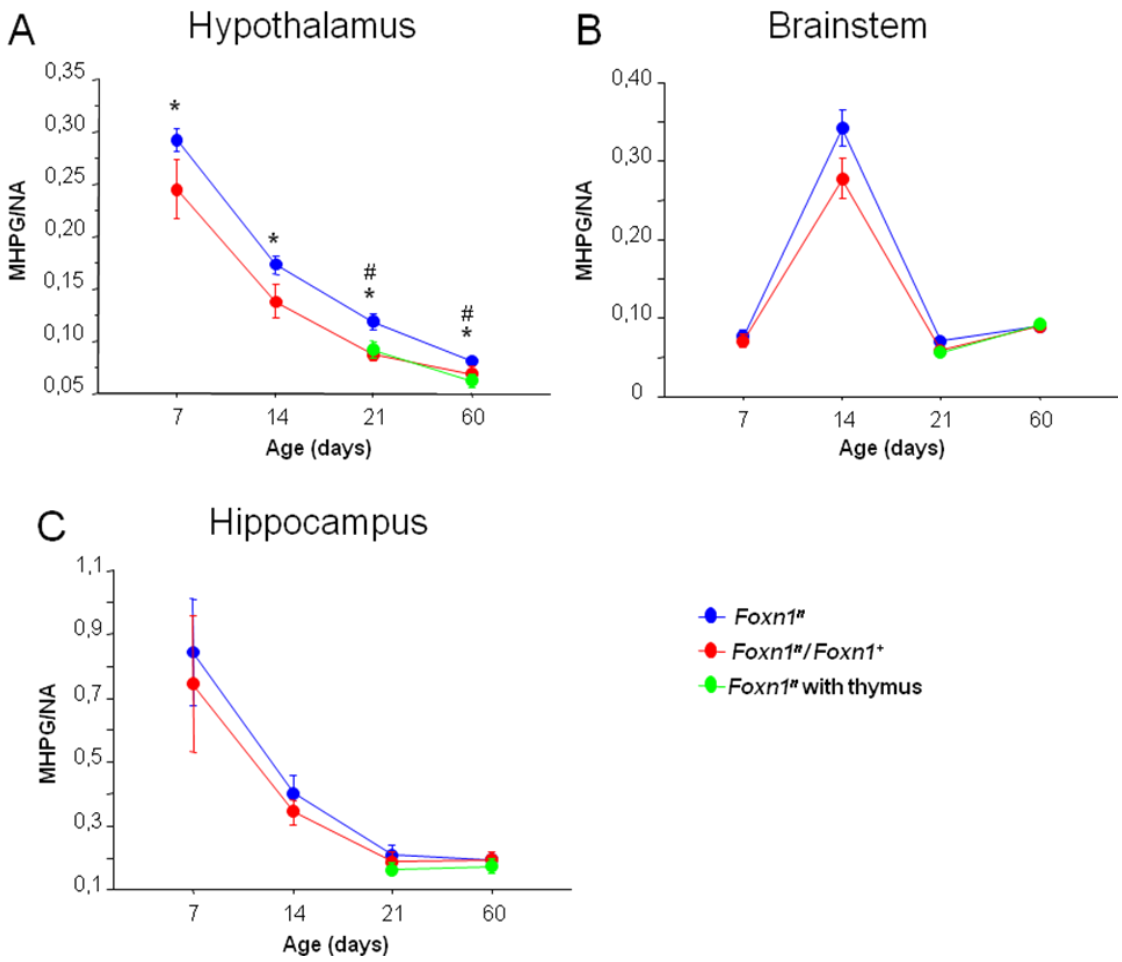


**Fig. 24. Increased MHPG/NA ratio in the hypothalamus of athymic mice during ontogeny and normalization following thymus implantation at birth.** MHPG/NA in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were calculated in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$), and athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) at the times indicated in the figure. Statistically significant difference: * *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; and # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus.

No statistically significant differences between *Foxn1*$^{n}$, *Foxn1*$^{n}$/*Foxn1*$^{+}$, and athymic mice implanted with thymus at birth were detected in the DOPAC/DA ratio in the hypothalamus, brainstem and hippocampus (Fig. 25 A-C). This ratio was statistically significantly higher only in the hippocampus of 60 day-old *Foxn1*$^{n}$/*Foxn1*$^{+}$ as compared to athymic mice ($p<0,006$) and athymic mice implanted with thymus at birth ($p<0,002$) (Fig. 25C).

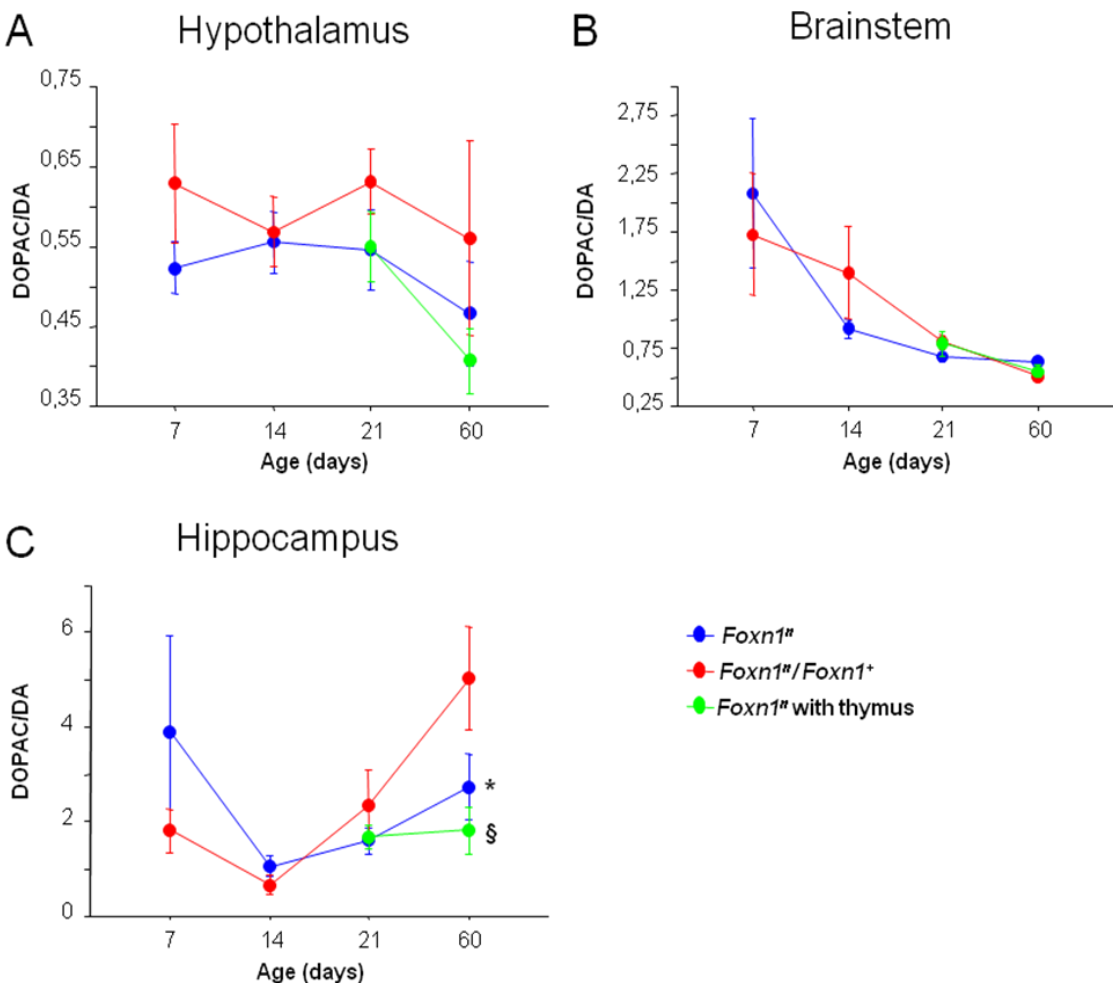


**Fig. 25. No difference in the DOPAC/DA ratio in hypothalamus, hippocampus and brainstem of athymic mice during the ontogeny**. DOPAC/DA ratio in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** was calculated in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$*/Foxn1*$^{+}$), and athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) at the time indicated in the figure. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$*/Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$*/Foxn1*$^{+}$**.**

While there were no differences in 7 day-old mice, statistically significantly higher Tyr/NA ratios in the hypothalamus of *Foxn1*$^{n}$ as compared to *Foxn1*$^{n}$*/Foxn1*$^{+}$ mice were detected at later stages of development (day 14: p<0,03, day 21: p<0,0001, day 60: p<0,03). Thymus reconstitution at birth abrogated the difference as compared to 21day-old athymic mice (p<0,002). The same tendency was observed in 60 day-old mice (Fig. 26 A).

Although not reaching statistical significance, the same tendency to increased Tyr/NA ratios was observed in the brainstem of *Foxn1*$^{n}$ as compared to *Foxn1*$^{n}$*/Foxn1*$^{+}$ mice. Such tendencies were abrogated by thymus implantation, and the ratio was significantly reduced in the brainstem of 60 day-

old, thymus-reconstituted mice when compared to *Foxn1*$^{n}$ mice (p<0,04). The same tendency was observed in 21 day-old mice (Fig. 26 B). However, no differences were detected between the three groups in the hippocampus at any age studied (Fig. 26 C).

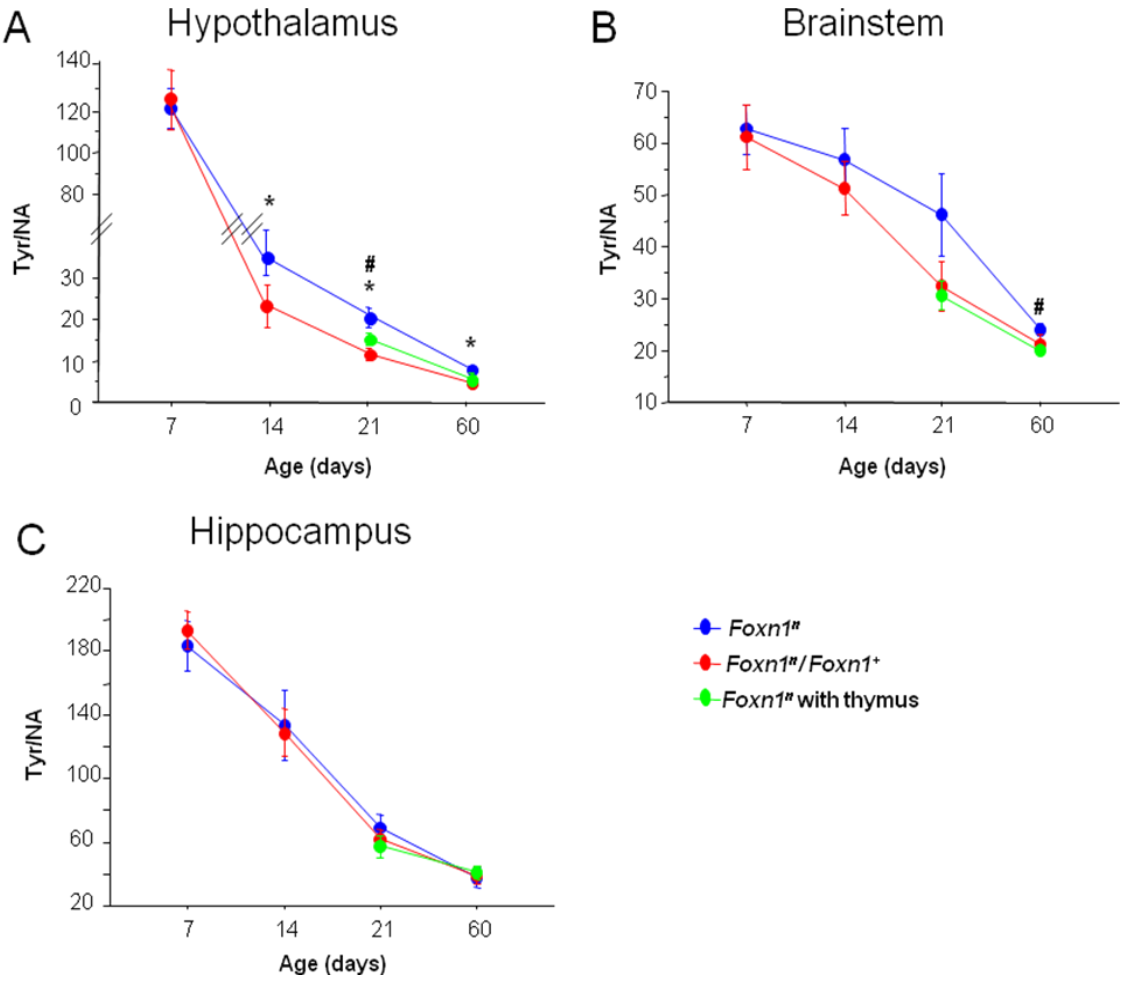


**Fig. 26. Increased Tyr/NA ratio in the hypothalamus and brainstem of athymic mice during ontogeny and normalization following thymus implantation at birth**. The Tyr/NA ratio in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** of athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$), and athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) at the time indicated in the figure was calculated. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; and # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus.

### 4.1.3. Alteration in tyrosine-hydroxylase-containing fibers in the spleen and brain of athymic mice

The results showing that there were clear differences between *Foxn1$^{n}$* and *Foxn1$^{n}$/Foxn1$^{+}$* mice in catecholamine concentration in the spleen and brain, and that these alterations were abrogated by thymus implantation at birth, led to perform immunohistochemical studies to detected fibers containing TH, the rate-limiting step in catecholamine synthesis (Elenkov et al., 2000; Purves D, 2008), in both organs and at different times during ontogeny.

No obvious differences between newborn *Foxn1$^{n}$* and *Foxn1$^{n}$/Foxn1$^{+}$* mice in the sympathetic innervation of the spleen were detected (Fig 27). However, there were clear immunohistochemical differences between the various groups latter in ontogeny. Enhanced immunofluorescence signals for TH were observed in the spleen of 7, 14, 21, and 60 day-old *Foxn1$^{n}$* mice as compared to *Foxn1$^{n}$/Foxn1$^{+}$* littermates. Representative examples are shown in Fig. 28.

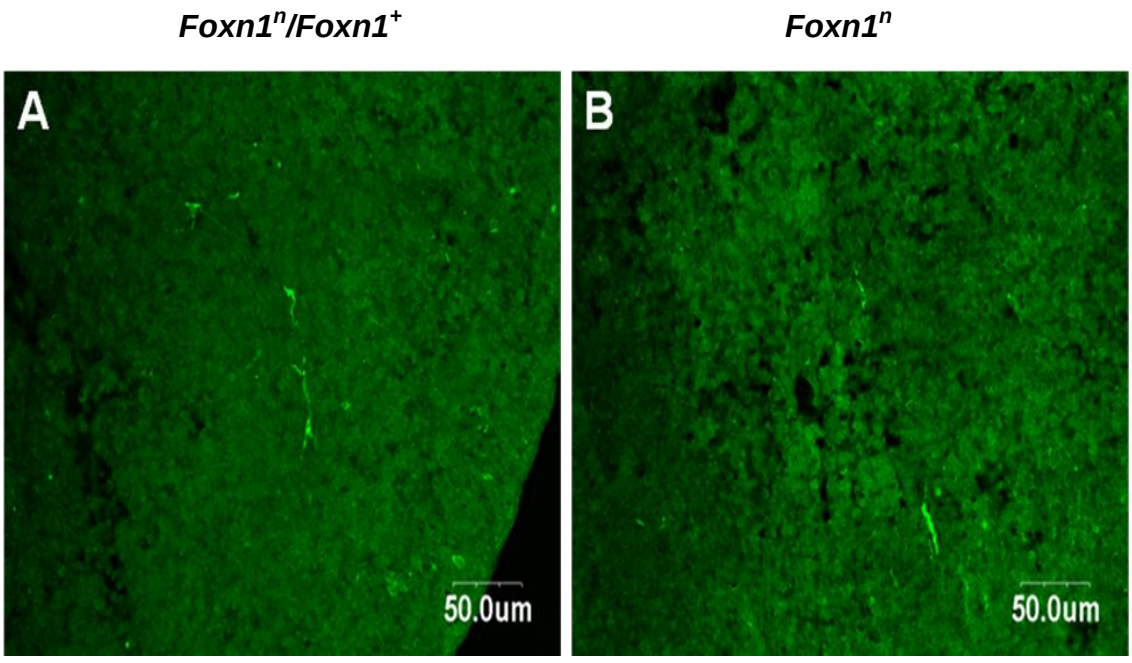


**Fig. 27. No marked difference in immunofluorescence signals for TH in the spleen of newborn *Foxn1$^{n}$* mice and *Foxn1$^{n}$/Foxn1$^{+}$* littermates.** The photos show a representative staining in *Foxn1$^{n}$/Foxn1$^{+}$* **(A)** and *Foxn1$^{n}$* **(B)**.

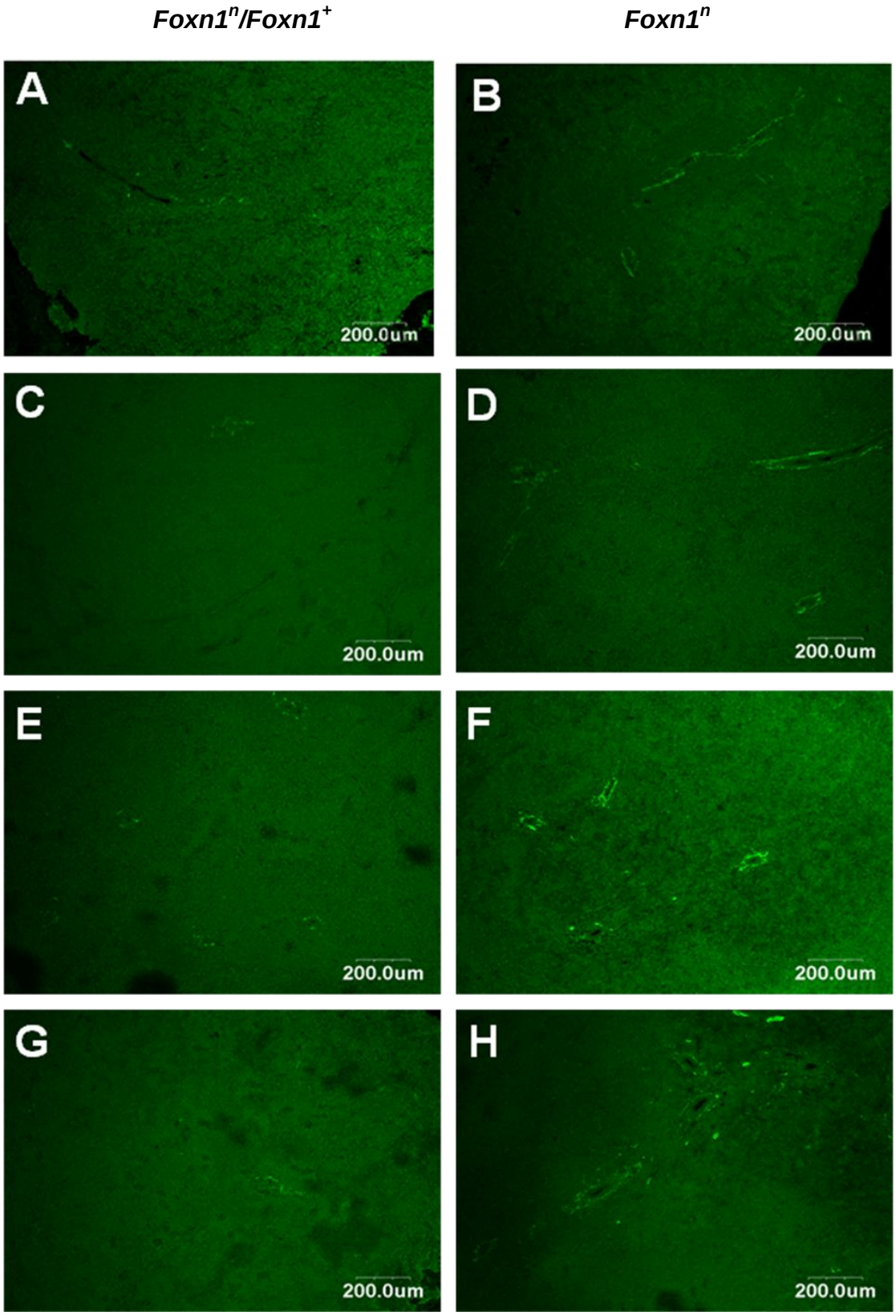


**Fig. 28. Increased intensity of immunofluorescent TH signals in the spleen of *Foxn1$^n$* mice**. Left panel: *Foxn1$^n$/Foxn1$^+$*; right panel: *Foxn1$^n$* mice. **A, B:** 7-day-old; **C, D:** 14-day-old; **E, F:** 21-day-old; **G, H:** 60-day-old.

As shown in a representative example in Fig. 29, these differences were abrogated by thymus implantation at birth.

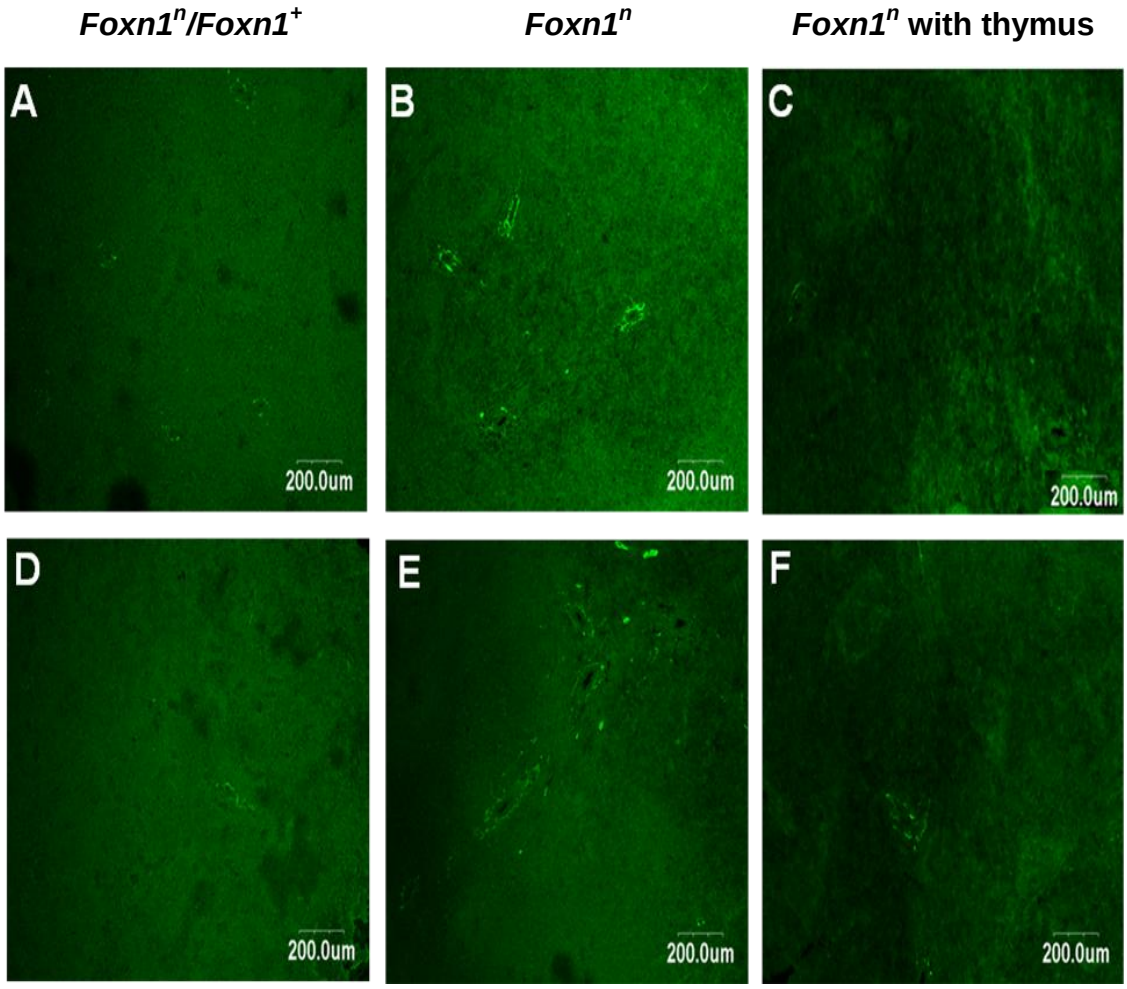


**Fig. 29. Implantation of a thymus into athymic newborn mice decreases immunofluorescent TH signals in the spleen**. The photos show representative results of TH-immunofluorescence in the spleen of *Foxn1*$^{n}$/*Foxn1*$^{+}$ (left), athymic *Foxn1*$^{n}$ mice (center), and *Foxn1*$^{n}$ mice implanted with thymus at birth (right). **A, B, C:** 21 day-old; **D, E, F:** 60 day-old.

The differences between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice in immunofluorescent signals for TH were even more marked in the hypothalamus at all ages studied (Fig.30). Enhanced TH-immunofluorescent signals were also observed in the hypothalamus of *Foxn1*$^{n}$ mice as compared to thymus-grafted *Foxn1*$^{n}$ mice (Fig. 31).

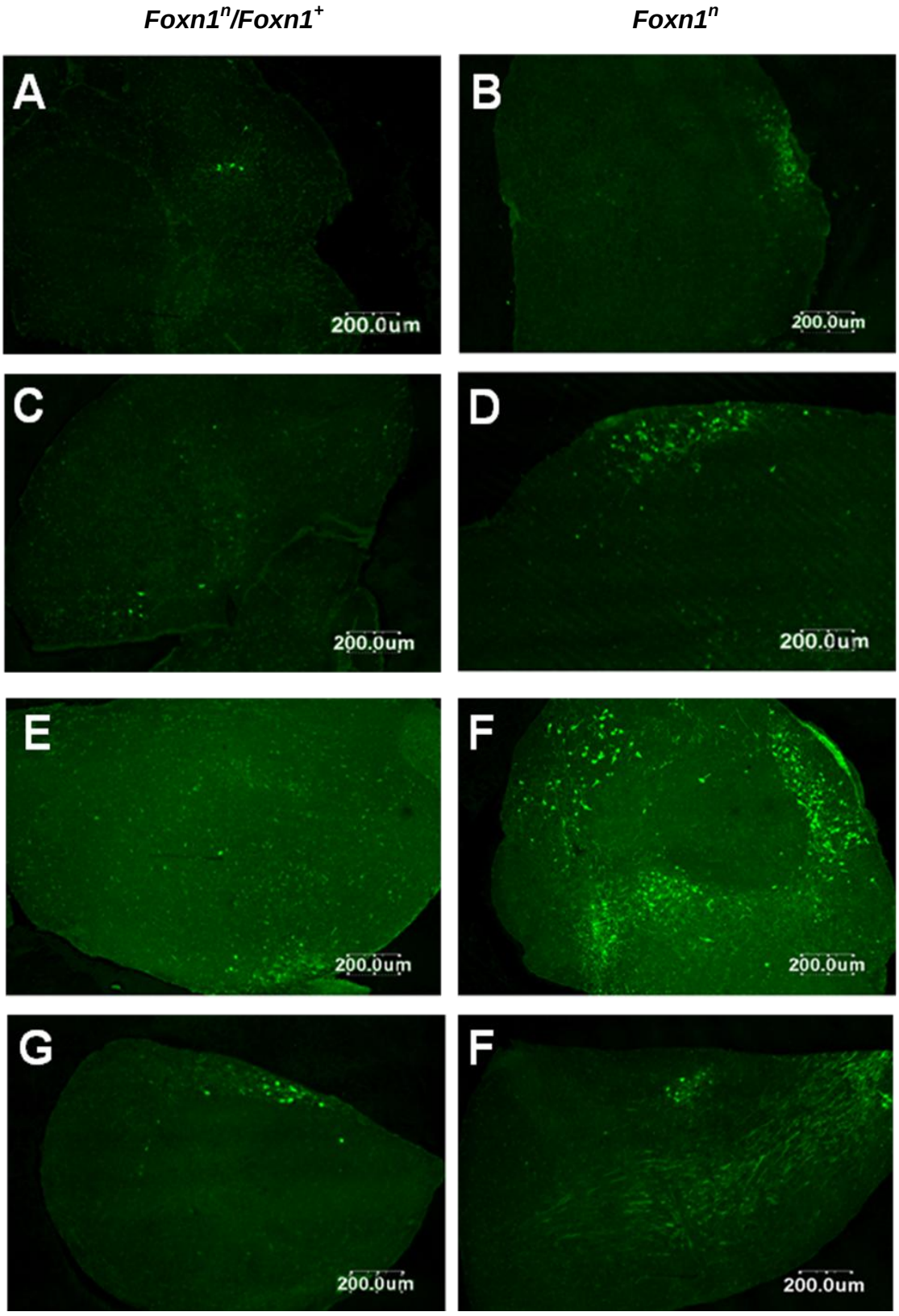


**Fig. 30. Increased intensity of TH-immunofluorescent signals in the hypothalamus of athymic mice.** Right panel: *Foxn1*$^{n}$ mice, left panel: *Foxn1*$^{n}$*/Foxn1*$^{+}$ littermates. **A, B:** 7-day-old, **C, D:** 14-day-old, **E, F:** 21-day-old, **G, H:** 60 day-old.

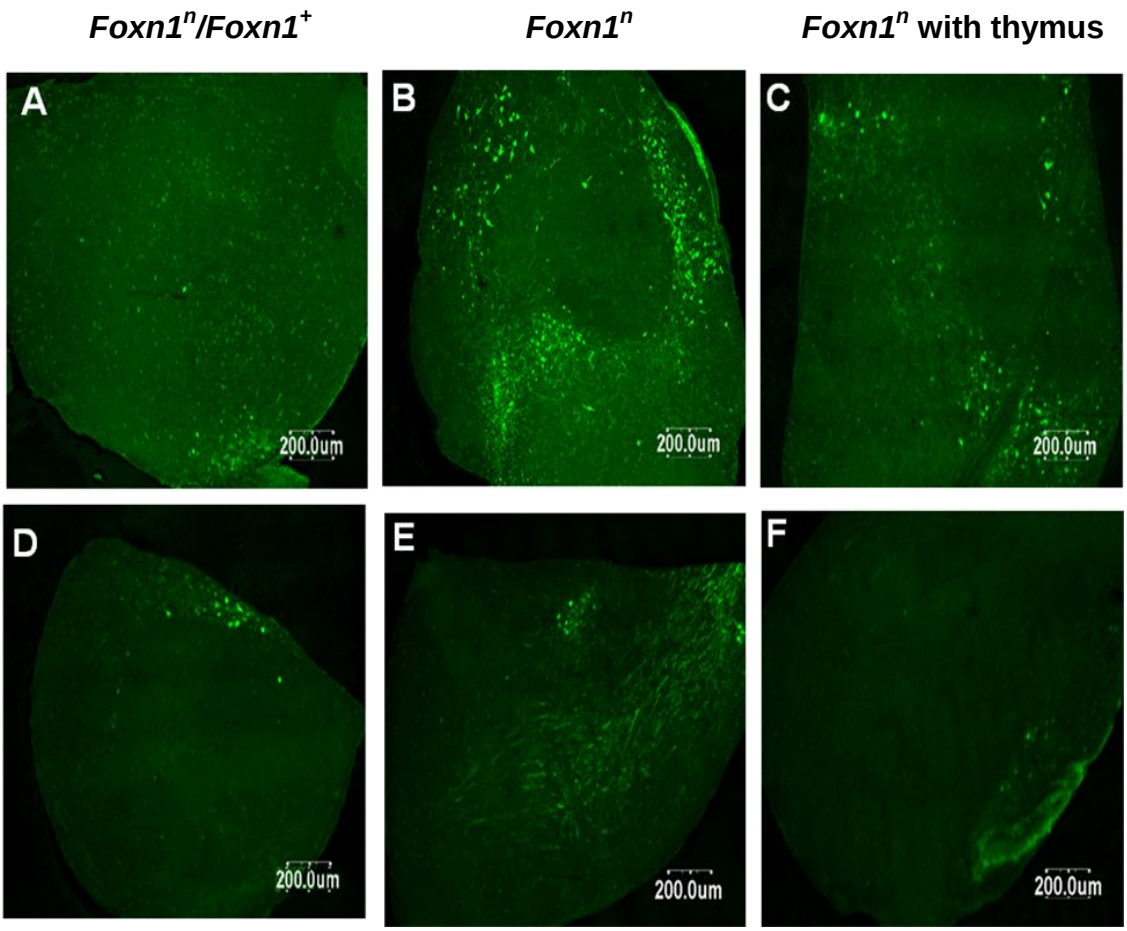


**Fig. 31. Implantation of a thymus into athymic newborn mice decreases TH-immunofluorescent signals in the hypothalamus**. The photos show representative results of TH-immunofluorescence in the hypothalamus of *Foxn1*$^{n}$/*Foxn1*$^{+}$ (left), *Foxn1*$^{n}$ mice (center), and *Foxn1*$^{n}$ mice implanted with thymus at birth (right). **A, B, C:** 21 day-old; **D, E, F:** 60 day-old.

## 4.2. The absence of the thymus affects indolamine, precursor, and metabolite concentrations in the spleen and brain

### 4.2.1. Effects on the spleen

In the periphery, 5-HT is mainly located in enterochromaffin cells in the gut. When it is secreted from these cells, it can reach other tissues via the blood, where it is taken up by platelets and stored. Some authors have reported that serotonin can be synthetized also by cells in the renal proximal tubules (Sole et al., 1986); and others that it can be synthetized in all tissues in which it is found, with the exception of platelets (Ghatak et al., 1998). Thus, 5-HT concentration was determined by HPLC in the spleen and kidney of the same mice used in the previous studies. The only small, but statistically significant difference in 5-

HT concentration in the spleen was observed in 21 day-old mice, in which 5-HT was higher in *Foxn1*$^{n}$ than *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,03), and the same tendency was observed in 60 day-old mice (Fig. 32 A). The same difference was observed when the results were expressed as total splenic 5-HT content, higher 5-HT levels were found only in 21 day-old *Foxn1*$^{n}$ than *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,02), and the same tendency was observed in 60 day-old (Fig. 31 C). No differences in 5-HT concentration or content were observed in the kidney at any time point (Fig.32 B, D).

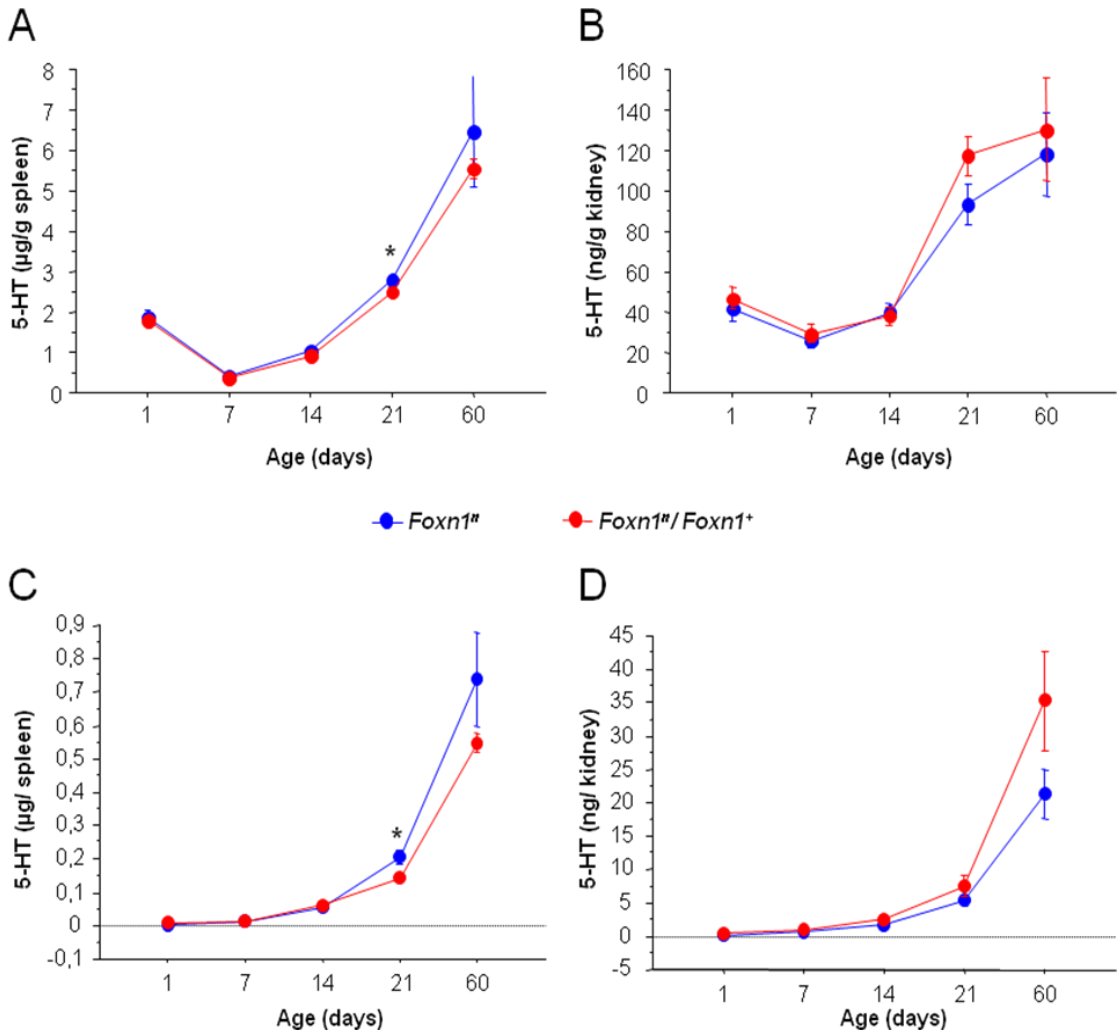


**Fig. 32. Increased 5-HT concentration and content in the spleen during ontogeny**. 5-HT concentration was determined in the spleen and left kidney of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/ *Foxn1*$^{+}$) littermates at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice. (**A, C)** spleen; **(B, D)** kidney. **A**, **B:** 5-HT concentration, **C**, **D:** total 5-HT in the organ. ***** Statistically significantly different from *Foxn1*$^{n}$/ *Foxn1*$^{+}$.

Thymus reconstitution at birth abrogated the differences in splenic 5-HT concentrations between athymic and normal littermates observed in 21 day-old mice, resulting now in significant differences between athymic and athymic mice implanted with thymus at birth (p<0,0001), and the concentration in thymus-reconstituted mice was even lower than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (p<0,004) (Fig.33A). The same tendency was observed in 60 day-old mice. No statistically significant differences between athymic mice implanted with thymus at birth and athymic mice were detected when 5-HT was expressed as total content in the spleen, and both groups significantly differed from *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,015) (Fig.33B).

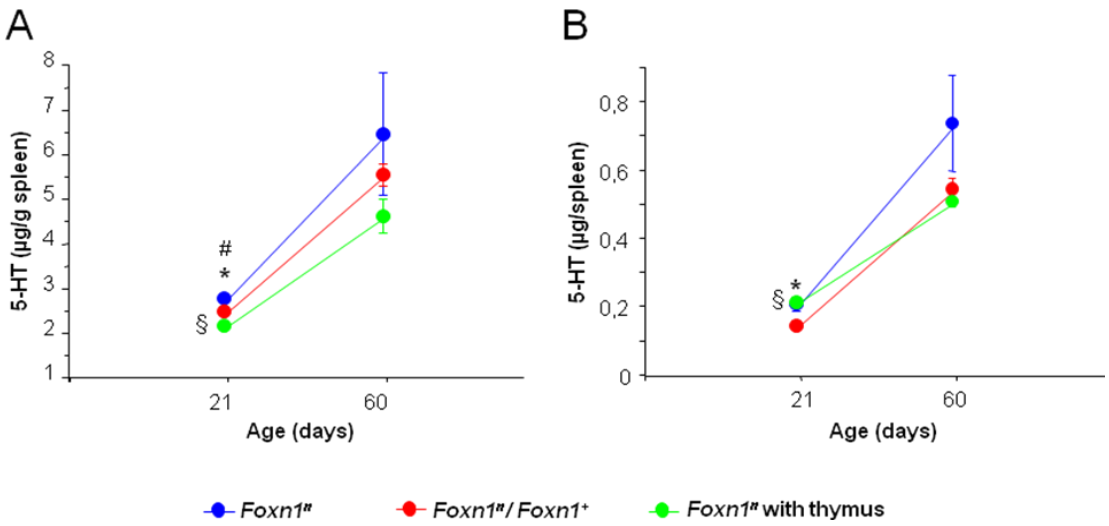


**Fig. 33. Thymus implantation into athymic newborn mice results in decreased 5-HT concentration in the spleen.** Two thymi from newborn *Foxn1*$^{n}$/*Foxn1*$^{+}$ donors were grafted into less than 24h-old athymic male nude mice (*Foxn1*$^{n}$ with thymus). 5-HT concentration in the spleen was determined when mice were 21 (n=9) or 60 (n=10) day-old mice. (**A)** 5-HT concentration. **(B)** Total 5-HT content. Statistically significant difference: ⋆ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The concentrations of Trp, the precursor of 5-HT, and 5-HIAA, its main metabolite, were determined in parallel.

Significantly higher Trp concentrations were found in the spleen of 1 (p<0,04), 14 (p<0,03), and 21(p<0,04) day-old *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates. The same tendency was observed in 7 and 60 day-old mice (Fig. 34 A). No significantly differences were detected at any time point in the kidney (Fig. 34 B).

Significantly higher 5-HIAA concentrations were found in the spleen of 1 (p<0,0002), 7 (p<0,02), and 14 (p<0,0003) day-old *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates, but not later in ontogeny (Fig. 34 C). Again, no differences were detected in the kidney (Fig. 34 D).

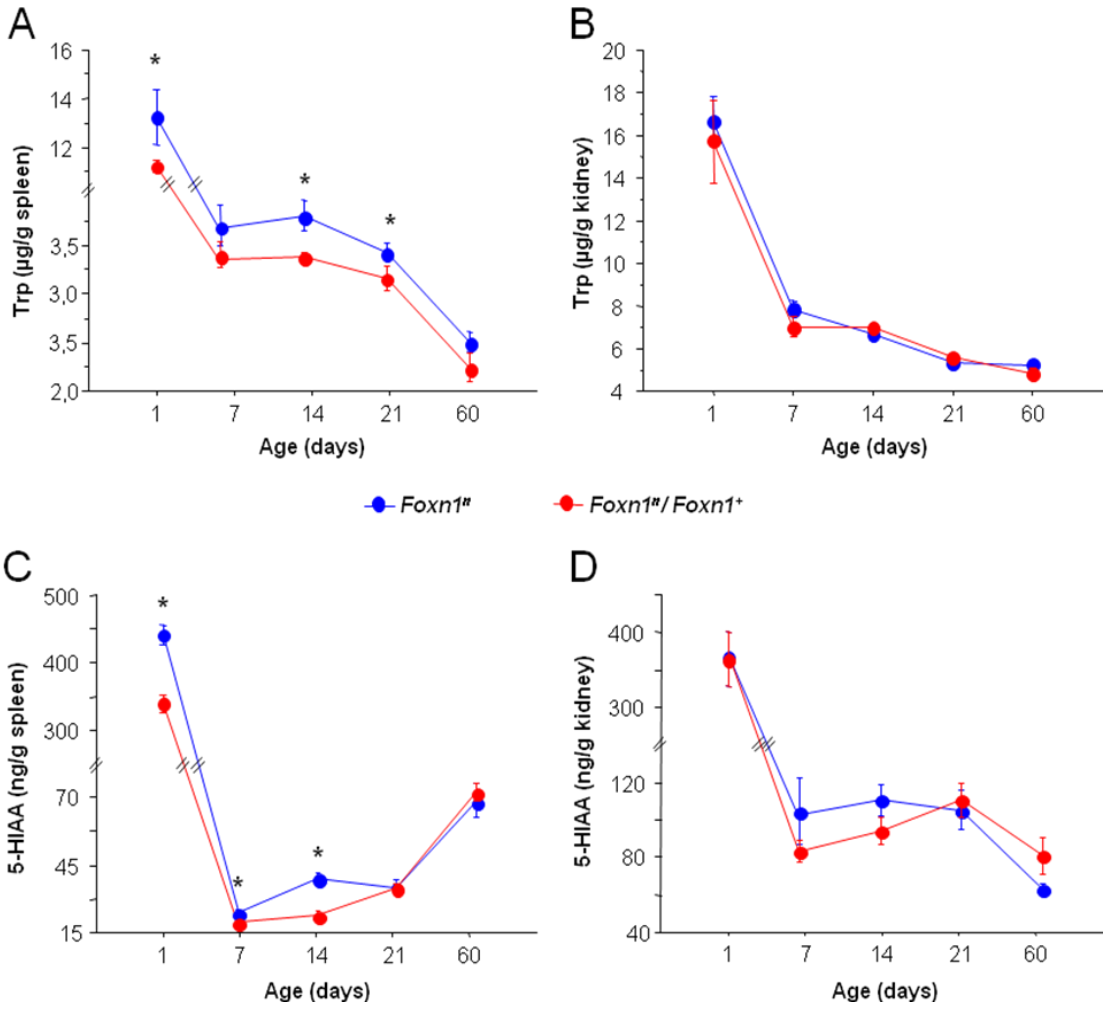


**Fig. 34. Increased splenic Trp and 5-HIAA concentrations in athymic mice during ontogeny.** Trp and 5-HIAA concentrations were determined in spleen and left kidney of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/*Foxn1*$^{+}$) littermates at different ages, 1 (n=7/group); 7 (n=7/group); 14 (n=13/group); 21 (n=10/group); and 60 (n=7/group) day-old mice. (**A, C)** spleen; **(B, D)** kidney. **A**, **B:** Trp concentration, **C**, **D:** 5-HIAA concentration. ***** Statistically significantly different from *Foxn1*$^{n}$/ *Foxn1*$^{+}$.

Thymus implantation at birth to athymic mice resulted in splenic Trp concentrations comparable to those of normal thymus-bearing littermates, and were significantly different from those of athymic *Foxn1*$^{n}$ mice when they were 21 day-old (p<0,045). The same tendency was observed in 60 day-old mice (Fig. 35 A). Thymus implantation did not induce any significant effect on splenic

5-HIAA concentrations, at a time in ontogeny in which its absence was not paralleled by changes in this metabolite (Fig. 35 B).

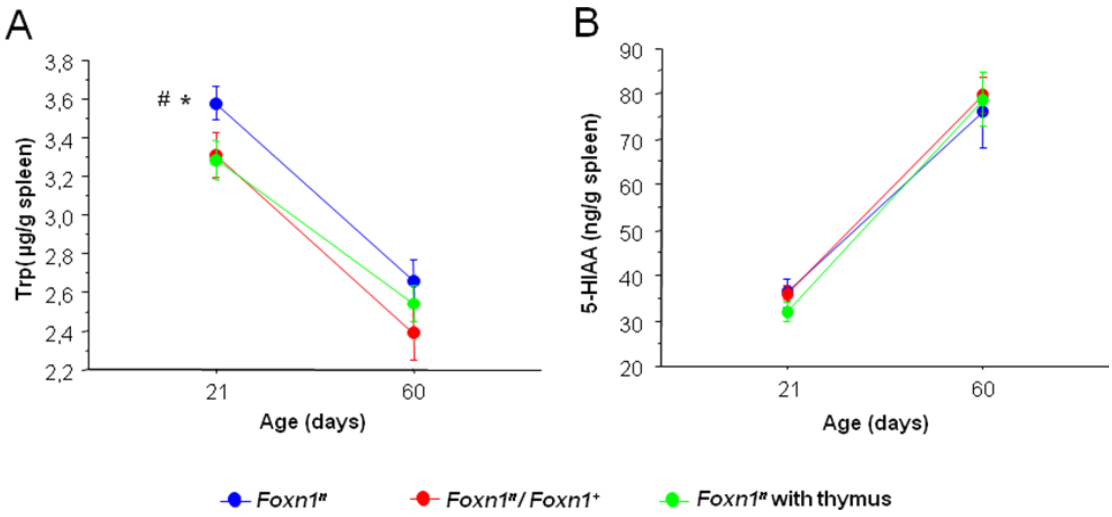


**Fig. 35. Thymus implantation into athymic newborn mice results in normalization of Trp concentrations in the spleen.** Two thymi from newborn Balb/c *Foxn1*$^{n}$/*Foxn1*$^{+}$ donors were grafted into less than 24h-old male athymic nude Balb/c mice (*Foxn1*$^{n}$ with thymus). Trp and 5-HIAA concentrations in the spleen were determined when mice were 21 (n=9) or 60 (n=10) day-old mice. **(A)** Trp concentration. **(B)** 5-HIAA concentration. Statistically significant difference: * *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; and # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus.

### 4.2.2. Effects on the brain

It is known that 5-HT can act as an immunomodulator in the periphery, but it functions as a neurotransmitter in the CNS (Ader et al., 1990; Neveu and Le Moal, 1990). This indolamine is found primarily in groups of neurons in the raphe region of the pons and upper brainstem, which have widespread projections to the forebrain (Purves D, 2008). Thus, 5-HT, Trp, and 5-HIAA concentrations were determined in several brain regions of the same groups of mice and at the same time points.

No differences in 5-HT, Trp, and 5-HIAA concentrations between athymic mice and thymus-bearing heterozygous littermates were detected in the whole brain of less than 24 hour-old mice (Fig. 36 A-C). However, higher 5-HT, Trp, and 5-HIAA concentrations were detected in the hypothalamus, brainstem, and hippocampus of *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates at later stages of development. Such differences were abrogated by thymus implantation at birth.

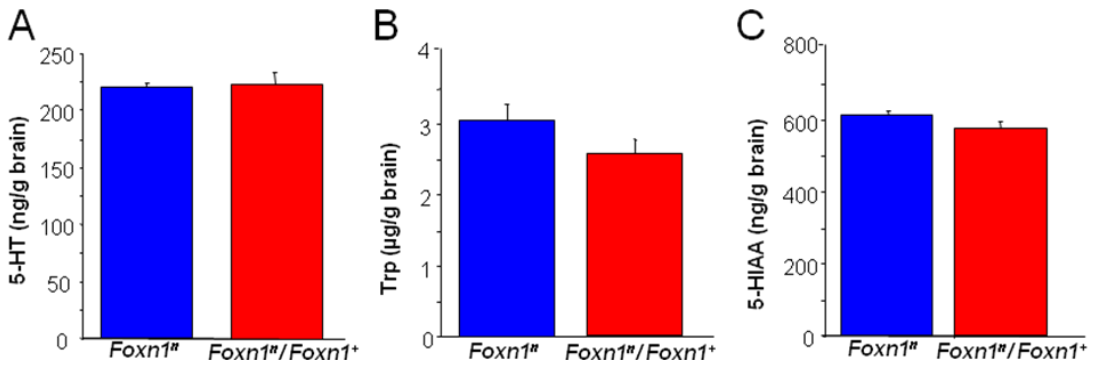


**Fig. 36.** No differences in 5-HT, Trp, and 5-HIAA concentrations in the whole brain of newborn athymic mice (*Foxn1*$^{n}$) and their heterozygous thymus- bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$). (n=7/group)

The differences between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice in hypothalamic 5-HT concentration were statistically significant at all ages studied (7day-old: p<0,002, 14 day-old: p<0,003, 21 day-old: p<0,0001, 60 day-old: p<0,0002). Thymus implantation at birth significantly decreased hypothalamic 5-HT concentration in 21 day-old athymic mice as compared to nude mice (p<0,0001). However, 5-HT concentration in the hypothalamus of 60 day-old *Foxn1*$^{n}$ implanted with thymus at birth was even higher than in *Foxn1*$^{n}$ (p<0,03) and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (p<0,001) (Fig. 37 A).

Comparable results were obtained in the brainstem and in the hippocampus at the same time-points, with the exception that in these regions, thymus implantation resulted in decreased 5-HT also in 60-day-old mice. The differences between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ in 5-HT concentration in the brainstem were statistically significant in 7 (p<0,0001), 14 (p<0,002), 21 (p<0,0001), and 60 (p<0,0001) day-old mice. The difference between athymic and athymic mice implanted with thymus at birth was statistically significant in 21 day-old (p<0,006) and 60 day-old (p<0,003) animals, but 5-HT concentration in the brainstem of 60 day-old athymic *Foxn1*$^{n}$ implanted with thymus at birth was still significantly higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,02) (Fig. 37 B).

5-HT in the hippocampus of 7 (p<0,002), 14 (p<0,003), 21 (p<0,0001) and 60 (p<0,0002) day-old *Foxn1*$^{n}$ mice was higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals, and than in 21 and 60 day-old athymic mice implanted with thymus at birth (p<0,01 and p<0,03, respectively). However, 5-HT concentration in the

hippocampus of 60 day-old athymic implanted with thymus at birth was still significant higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ (p<0,001) (Fig. 37 C).

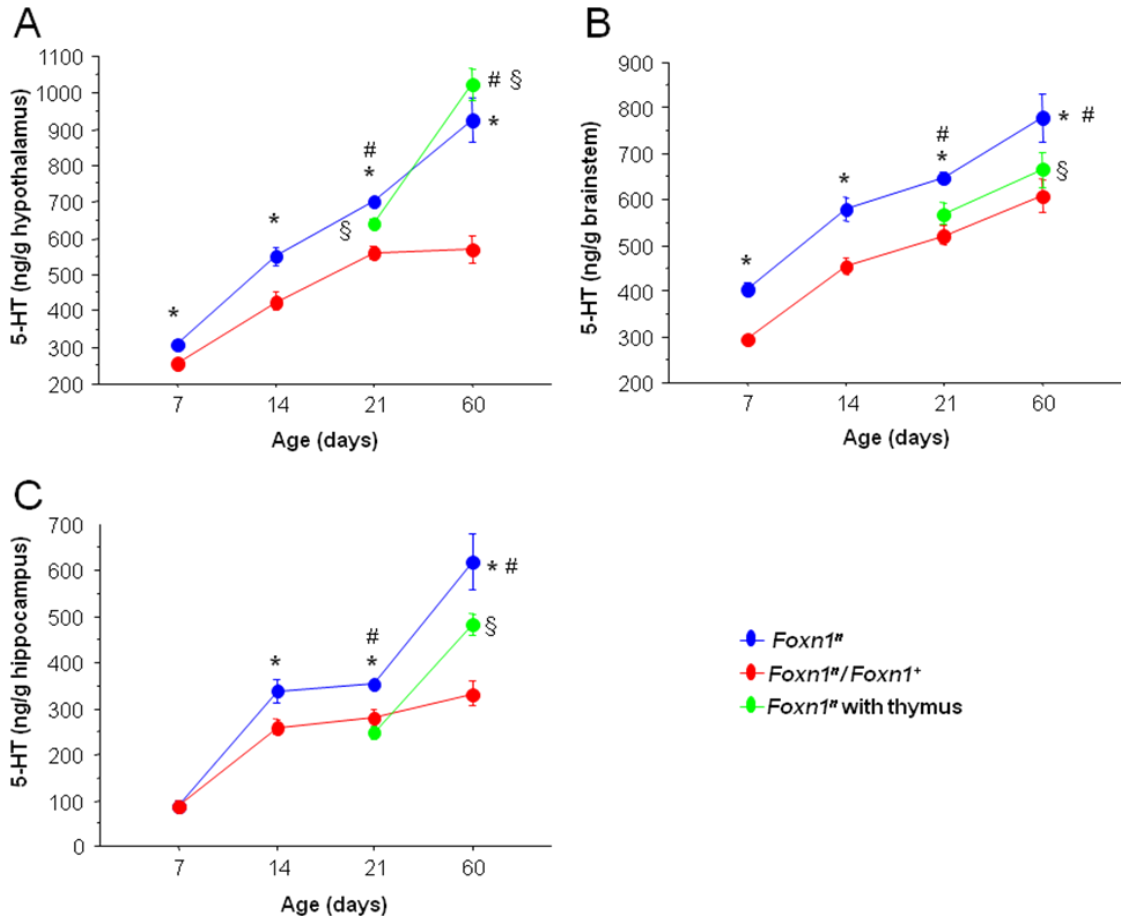


**Fig. 37. Increased 5-HT concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth.** 5-HT concentration was determined by HPLC in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** of athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages (days 1; n=7/group; 7: n=7/group); 14: n=13/group; 21: n=10/group; and 60: n=7/group), and of athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) and sacrificed when they were 21 (n=9) or 60 (n=10) day-old. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The differences in hypothalamic Trp levels between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant on days 14 (p<0,0001), 21 (p<0,02) and 60 (p<0,0007), and the same tendency was observed in 7 day-old mice. Thymus implantation at birth normalized the increased Trp levels observed in athymic mice (day 21: p<0,02, day 60: p<0,0002) (Fig 38 A).

Essentially the same differences were observed in the brainstem. Trp levels in *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significantly different at all ages studied (7 day-old: p<0,03, 14 day-old: p<0,01, 21 day-old: p<0,02, 60 day-old: p<0,0001). The difference between athymic and athymic mice implanted with thymus at birth was statistically significant on days 21 (p<0,005), and 60 (p<0,0001) (Fig 38 B).

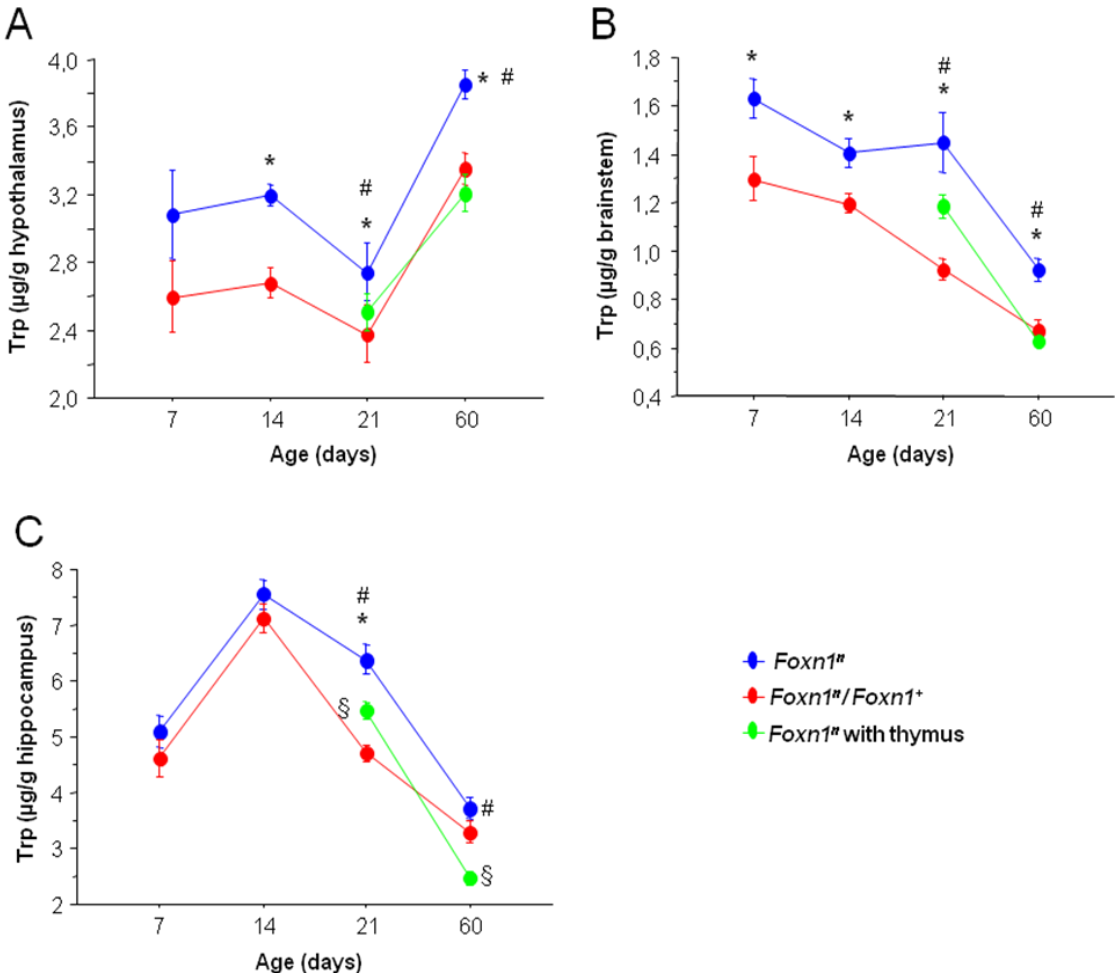


**Fig. 38. Increased Trp concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth.** Trp concentration was determined by HPLC in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** of athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages (days 1: n=7/group; 7: n=7/group; 14: n=13/group; 21: n=10/group; and 60: n=7/group), and of athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) and sacrificed when they were 21 (n=9) or 60 (n=10) day-old. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and § *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The results obtained in the hippocampus are depicted in Figure 38 C, which shows that *Foxn1*$^{n}$ mice have more Trp in this brain region than *Foxn1*$^{n}$/*Foxn1*$^{+}$

animals when they were 21 ($p<0,0001$) and 60 day-old ($p<0,004$). Thymus reconstitution at birth resulted in significant differences in Trp concentration in the hippocampus of *Foxn1*$^{n}$ and *Foxn1*$^{n}$ mice implanted with thymus at birth in 21 ($p<0,005$), and 60 ($p<0,004$) day-old mice. Trp concentration in the hippocampus of athymic mice implanted with thymus at birth was still higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ in 21 day-old animals ($p<0,01$) but even lower than in these mice when they were 60 day-old ($p<0,0001$) (Fig 38 C).

The differences between hypothalamic 5-HIAA levels of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant at all ages studied (7 day-old: $p<0,02$; 14 day-old: $p<0,001$; and $p<0,0001$ for 21 and 60 day-old). As mentioned, thymus implantation at birth normalized the hypothalamic levels of this metabolite in athymic mice. The difference between athymic and athymic mice implanted with thymus at birth was statistically significant in 21 ($p<0,0001$) and in 60 ($p<0,0001$) day-old mice (Fig 39 A).

Significant higher 5-HIAA levels were also detected in the brainstem of 7 ($p<0,003$), 14 ($p<0,0001$), 21 ($p<0,002$), and 60 ($p<0,0001$) day-old *Foxn1*$^{n}$ mice as compared to the *Foxn1*$^{n}$/*Foxn1*$^{+}$ littermates. Thymus implantation at birth to athymic mice resulted in 5-HIAA concentrations in the brainstem comparable to those of normal thymus-bearing littermates, and were significantly different from those of athymic mice at both ages studied (21 day-old: $p<0,0004$, and 60 day-old: $p<0,001$). However, 5-HIAA concentration in the brainstem of 60 day-old athymic *Foxn1*$^{n}$ mice implanted with thymus at birth was still higher than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ ($p<0,001$) (Fig 39 B).

Comparable results were obtained in the hippocampus at the same time-points. The differences between 5-HIAA concentration in the hippocampus of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ were statistically significant on days 21 ($p<0,04$) and day 60 ($p<0,0001$). The same tendency was observed in 7 and 14 day-old mice. Thymus implantation at birth resulted in completely normalized 5-HIAA concentrations in the hippocampus of 21 ($p<0,008$) and 60 day-old ($p<0,0001$) mice as compared to *Foxn1*$^{n}$ (Fig 39 C).

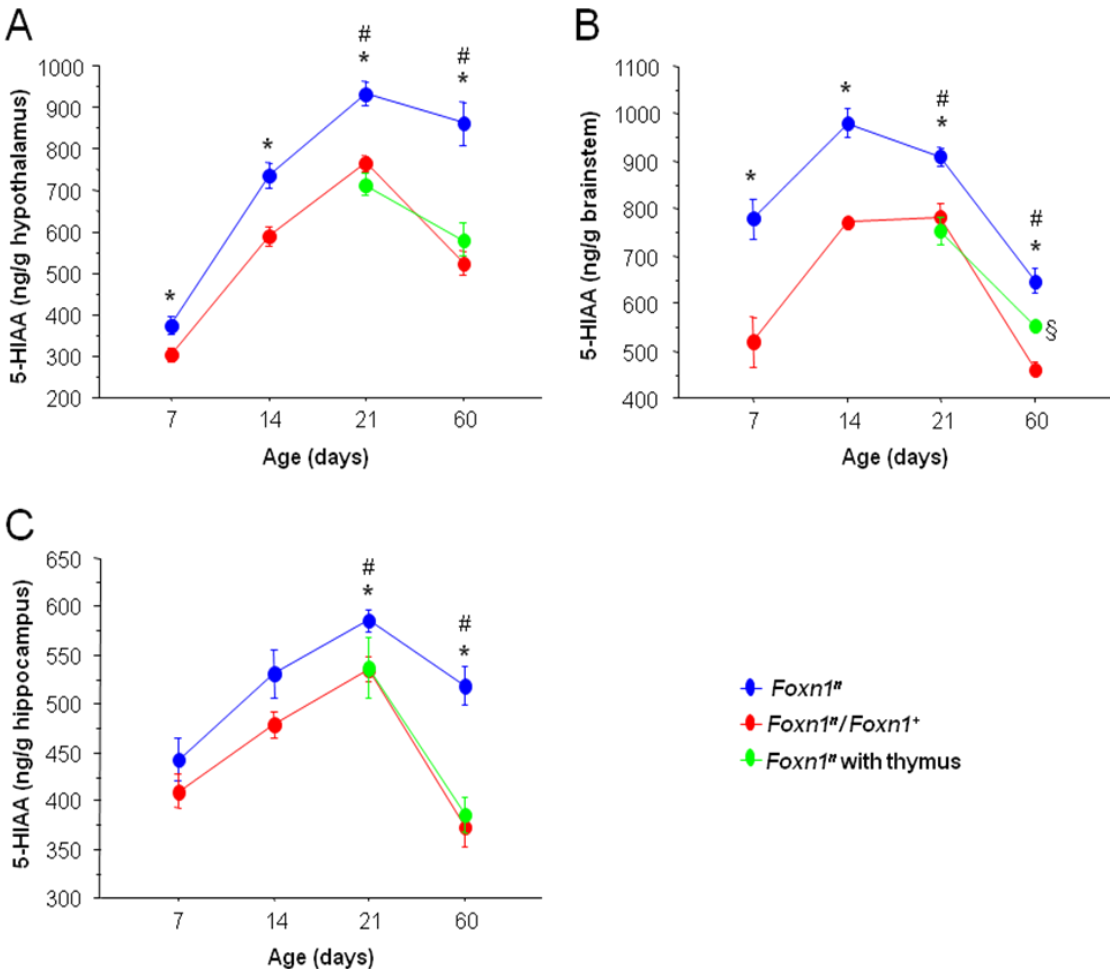


**Fig. 39. Increased 5-HIAA concentration in the hypothalamus, brainstem, and hippocampus of athymic mice during ontogeny and normalization following thymus implantation at birth**. 5-HIAA concentration was determined by HPLC in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** of athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages (days 1; n=7/group; 7: n=7/group; 14: n=13/group; 21: n=10/group; and 60: n=7/group), and of athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) and sacrificed when they were 21 (n=9) or 60 (n=10) day-old. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The ratio metabolite/indolamine (5-HIAA/5-HT) and precursor/indolamine (Trp/5-HT) were also calculated.

While no differences between athymic mice and thymus-bearing heterozygous littermates were detected in 5-HIAA/5-HT in the hypothalamus at any of the ages studied, statistically significantly lower 5-HIAA/5-HT ratios were observed in the hypothalamus of 21 day-old (p<0,002), and 60 day-old (p<0,004) athymic mice implanted with thymus at birth, as compared to *Foxn1*$^{n}$

and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (day 21: p<0,007: day 60: p<0,03) (Fig. 40 A). However, no differences were observed among these three groups in the 5-HIAA/5-HT ratio in the hippocampus and brainstem (Fig. 40 B,C).

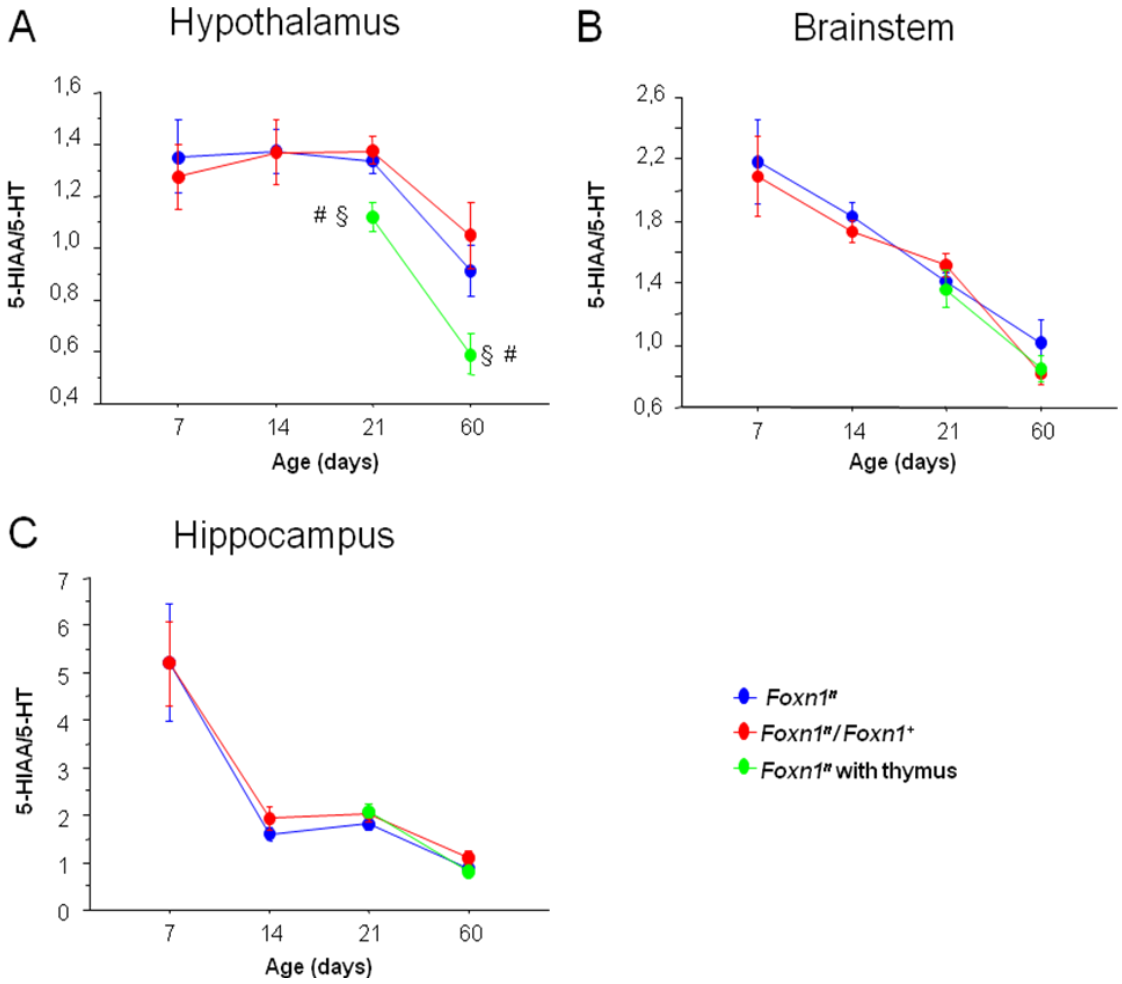


**Fig. 40. No difference in the ratio 5-HIAA/5-HT in the hypothalamus, brainstem, and hippocampus of athymic mice during the ontogeny**. 5-HIAA/5-HT ratio in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** of athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$), and athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) were calculated. Statistically significant difference: # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

The only statistically significantly difference in the Trp/5-HT ratio was observed in the hypothalamus of 60 day-old mice. This ratio was higher in *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice than in *Foxn1*$^{n}$ (p<0,0002), and *Foxn1*$^{n}$ mice implanted with thymus at birth (p<0,02) (Fig. 41 A). However, no differences were observed among these three groups neither at the other time-points, nor in the hippocampus and brainstem at any age studied (Fig. 41B,C).

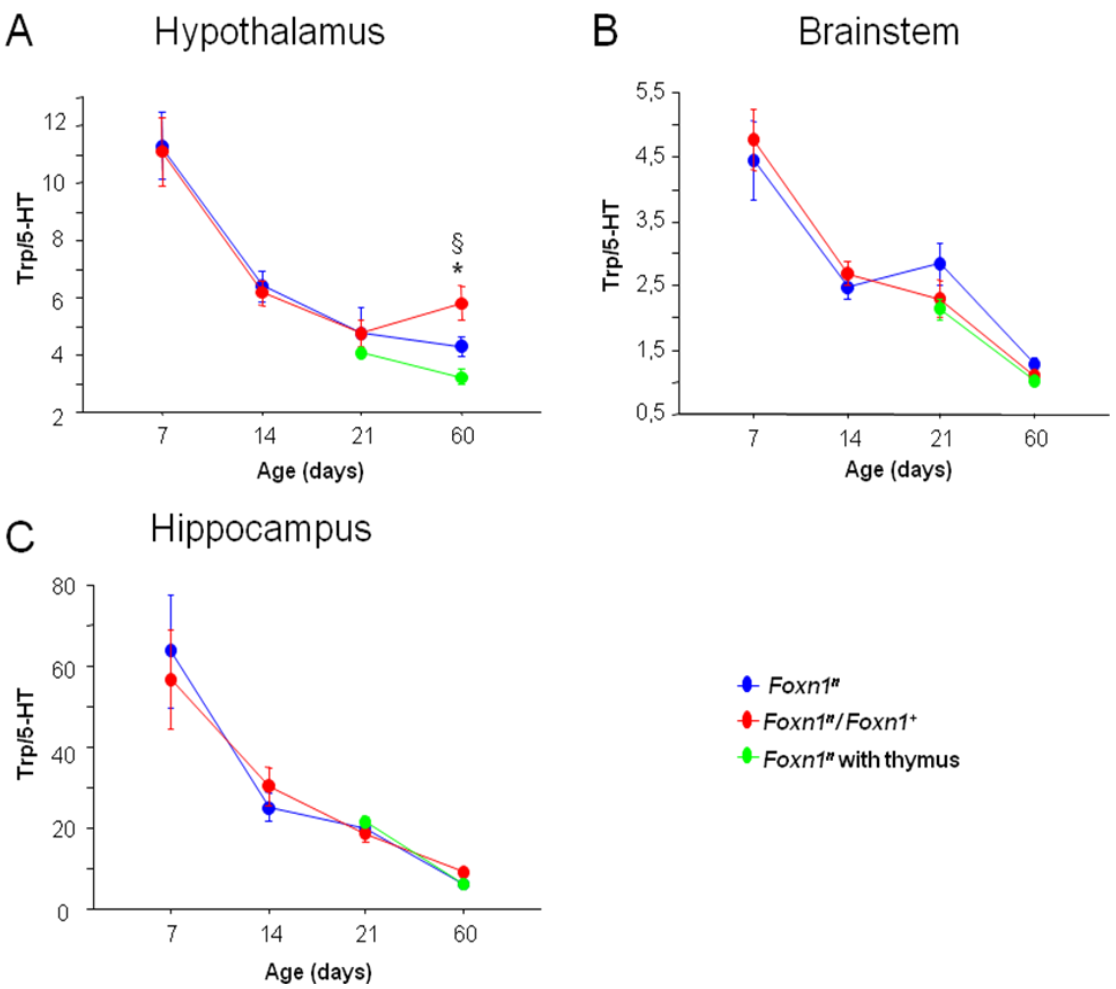


**Fig. 41. No difference in the ratio Trp/5-HT in the hypothalamus, brainstem, and hippocampus of athymic mice during the ontogeny**. Trp/5-HT ratios in the hypothalamus **(A)**, brainstem **(B)**, and hippocampus **(C)** were calculated. Statistically significant difference: * *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; and § *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$.

For a quick overview of the changes in neurotransmitters, precursors and metabolites in the spleen and in several brain regions described above, the results are schematically summarized in Table 4. The symbols indicate statistically significant differences between *Foxn1*$^{n}$ and *Foxn1*$^{n}$ mice implanted with thymus at birth as compared to normal, thymus-bearing *Foxn1*$^{n}$/*Foxn1*$^{+}$ animals.

**Table 4**. Differences in neurotransmitter, precursor and metabolite concentrations in the spleen and different brain regions of athymic *Foxn1*$^{n}$ and the effect of thymus reconstitution at birth

**A. Spleen**

| | Tyr | | NA | |
|---|---|---|---|---|
| **Age (days)** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** |
| **>1** | n.s. | n.d. | n.s. | n.d. |
| **7** | ⇑ | n.d. | ⇑ | n.d. |
| **14** | ⇗ | n.d. | ⇑ | n.d. |
| **21** | ⇗ | ⇔ | ⇑ | ⇔ |
| **60** | ⇑ | ⇔ | ⇑ | ⇔ |

| | DA | | DOPAC | |
|---|---|---|---|---|
| **Age (days)** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** |
| **>1** | n.s. | n.d. | n.s. | n.d. |
| **7** | n.s. | n.d. | n.s. | n.d. |
| **14** | n.s. | n.d. | n.s. | n.d. |
| **21** | ⇑ | ⇔ | n.s. | n.s. |
| **60** | ⇑ | ⇔ | ⇓ | ⇓ |

| | Trp | | 5-HT | | 5-HIAA | |
|---|---|---|---|---|---|---|
| **Age (days)** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** | ***Foxn1*$^{n}$ (athymic)** | ***Foxn1*$^{n}$ + thymus** |
| **>1** | ⇑ | n.d. | n.s. | n.d. | ⇑ | n.d. |
| **7** | ⇑ | n.d. | n.s. | n.d. | ⇑ | n.d. |
| **14** | ⇗ | n.d. | n.s. | n.d. | ⇑ | n.d. |
| **21** | ⇑ | ⇔ | ⇑ | ⇔ | n.s. | n.s. |
| **60** | ⇗ | ⇔ | ⇗ | ⇔ | n.s. | n.s. |

The symbols indicate statistically significant differences as compared to normal, thymus-bearing *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice. ⇑: increased; ⇗: increased but not significant; ⇓: decreased; ⇔: normalized; n.s. = not statistically significant; n.d. = not done. Less than 24 hour-old mice are not included since the brain was not dissected into different regions, but evaluated as a whole.

## B. Hypothalamus

| | Tyr | | NA | | MHPG | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 14 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |

| | MHPG/NA | | Tyr/NA | |
|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇑ | n.d. | n.s. | n.d. |
| 14 | ⇑ | n.d. | ⇑ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ |

| | DA | | DOPAC | | DOPAC/DA | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇑ | n.d. | ⇑ | n.d. | n.s. | n.d. |
| 14 | ⇑ | n.d. | ⇑ | n.d. | n.s. | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | n.s. | n.s. |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ | n.s. | n.s. |

| | Trp | | 5-HT | | 5-HIAA | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇗ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 14 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |
| 60 | ⇑ | ⇔ | ⇑ | ⇑ | ⇑ | ⇔ |

| | 5-HIAA/5-HT | | Trp/5-HT | |
|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | n.s. | n.d. | n.s. | n.d. |
| 14 | n.s. | n.d. | n.s. | n.d. |
| 21 | n.s. | ⇓ | n.s. | n.s. |
| 60 | n.s. | ⇓ | ⇓ | ⇓ |

The symbols indicate statistically significant differences as compared to normal, thymus-bearing *Foxn1*$^n$/*Foxn1*$^+$ mice. ⇑: increased; ⇗: increased but not significant; ⇓: decreased; ⇔: normalized; n.s. = not statistically significant; n.d. = not done. Less than 24 hour-old mice are not included since the brain was not dissected into different regions, but evaluated as a whole.

## C. Brainstem

| | Tyr | | NA | | MHPG | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 14 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ | ⇗ | ⇔ |

| | MHPG/NA | | Tyr/NA | |
|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | n.s. | n.d. | n.s. | n.d. |
| 14 | n.s. | n.d. | ⇗ | n.d. |
| 21 | n.s. | n.s. | ⇗ | ⇔ |
| 60 | n.s. | n.s. | ⇑ | ⇔ |

| | DA | | DOPAC | | DOPAC/ DA | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇗ | n.d. | ⇑ | n.d. | n.s. | n.d. |
| 14 | ⇗ | n.d. | ⇑ | n.d. | n.s. | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | n.s. | n.s. |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ | n.s. | n.s. |

| | Trp | | 5-HT | | 5-HIAA | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 14 | ⇑ | n.d. | ⇑ | n.d. | ⇑ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |

| | 5-HIAA/5-HT | | Trp/5-HT | |
|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | n.s. | n.d. | n.s. | n.d. |
| 14 | n.s. | n.d. | n.s. | n.d. |
| 21 | n.s. | n.s. | n.s. | n.s. |
| 60 | n.s. | n.s. | n.s. | n.s. |

The symbols indicate statistically significant differences as compared to normal, thymus-bearing *Foxn1*$^n$/*Foxn1*$^+$ mice. ⇑: increased; ⇗: increased but not significant; ⇓: decreased; ⇔: normalized; n.s. = not statistically significant; n.d. = not done. Less than 24 hour-old mice are not included since the brain was not dissected into different regions, but evaluated as a whole.

### D. Hippocampus

| | Tyr | | NA | | MHPG | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇗ | n.d. | ⇑ | n.d. | ⇗ | n.d. |
| 14 | ⇑ | n.d. | ⇗ | n.d. | ⇗ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | ⇗ | ⇔ |
| 60 | ⇑ | ⇔ | ⇑ | ⇔ | ⇗ | ⇔ |

| | MHPG/NA | | Tyr/NA | |
|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | n.s. | n.d. | n.s. | n.d. |
| 14 | n.s. | n.d. | n.s. | n.d. |
| 21 | n.s. | n.s. | n.s. | n.s. |
| 60 | n.s. | n.s. | n.s. | n.s. |

| | DA | | DOPAC | | DOPAC/ DA | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇑ | n.d. | ⇗ | n.d. | n.s. | n.d. |
| 14 | ⇗ | n.d. | ⇑ | n.d. | n.s. | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇑ | n.s. | n.s. |
| 60 | ⇗ | ⇔ | ⇓ | ⇑ | ⇓ | ⇓ |

| | Trp | | 5-HT | | 5-HIAA | |
|---|---|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | ⇗ | n.d. | n.s. | n.d. | ⇗ | n.d. |
| 14 | ⇗ | n.d. | ⇑ | n.d. | ⇗ | n.d. |
| 21 | ⇑ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |
| 60 | ⇗ | ⇔ | ⇑ | ⇔ | ⇑ | ⇔ |

| | 5-HIAA/5-HT | | Trp/5-HT | |
|---|---|---|---|---|
| Age (days) | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus | *Foxn1*$^n$ (athymic) | *Foxn1*$^n$ + thymus |
| 7 | n.s. | n.d. | n.s. | n.d. |
| 14 | n.s. | n.d. | n.s. | n.d. |
| 21 | n.s. | n.s. | n.s. | n.s. |
| 60 | n.s. | n.s. | n.s. | n.s. |

The symbols indicate statistically significant differences as compared to normal, thymus-bearing *Foxn1*$^n$/*Foxn1*$^+$ mice. ⇑: increased; ⇗: increased but not significant; ⇓: decreased; ⇔: normalized; n.s. = not statistically significant; n.d. = not done. Less than 24 hour-old mice are not included since the brain was not dissected into different regions, but evaluated as a whole.

## 4.3. The concentration of certain neurotrophins is affected by the absence of the thymus

These results presented above clearly show that there are differences between *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice in the concentration of neurotransmitters and their metabolites and precursors in the spleen and in defined brain regions. It has been well established that neurotrophins play an important role in the development, maintenance, and functioning of the nervous system, regulate axon growth, dendrite pruning, and the pattern of innervation, and are crucial for normal neuronal function (Huang and Reichardt, 2001; Snider, 1994). Neurotrophins also play a role in the immune system, and can be expressed by immune cells (Besser and Wank, 1999). It therefore appears likely that neurotrophins can contribute to a bi-directional cross-talk between the nervous and the immune systems (Kerschensteiner et al., 2003). Based on this evidence and on the results shown in the previous sections, the prediction was made that the presence of the thymus, and as consequence, of mature T cells or T cell-derived factors, can affect neurotrophin concentrations in lymphoid organs and in the brain.

The concentration of NGF, BDNF and NT-3 was determined in peripheral organs and brain regions of the same athymic, heterozygous thymus-bearing littermates and athymic mice implanted with thymus at birth used for the studies shown in the previous sections. The concentration of the neurotrophins was expressed per mg protein in the samples, as determined by the Bradford protein assay. However, it was first necessary to validate the ELISA kits to be used for neurotrophin determinations since no clear references were available for evaluations in tissues. The recovery of exogenous NGF, BDNF, and NT-3 was measured in mouse spleen and brain lysates by adding different known concentrations of neurotrophin standards. The recovery was calculated as the percentage of the ratio between the obtained and the expected values (Table 5).

As can be seen from the table, the recovery of the three neurotrophins was better in the middle concentration range for both organs. Thus, the dilutions for the determinations in the samples were done so that they were within this range.

**Table 5.** Neurotrophin recovery

**A. NGF recovery**

| Sample | Endogenous NGF (pg/ml) | Added (pg/ml) | Measured Conc. (pg/ml) | Expected Conc. (pg/ml) | Recovery (%) |
|---|---|---|---|---|---|
| Spleen | 890 | 1732 | 3500 | 2622 | 133 |
| | | 1090 | 2200 | 1890 | 116 |
| | | 623 | 1740 | 1513 | 115 |
| | | 290 | 1320 | 1180 | 111 |
| | | 130 | 1190 | 1020 | 116 |
| | | 63 | 1000 | 953 | 104 |
| Brain | 384 | 1732 | 2896 | 2116 | 137 |
| | | 1090 | 1652 | 1474 | 112 |
| | | 623 | 985 | 1007 | 98 |
| | | 290 | 691 | 674 | 103 |
| | | 130 | 582 | 514 | 113 |
| | | 63 | 486 | 447 | 109 |

**B. BDNF recovery**

| Sample | Endogenous BDNF (pg/ml) | Added (pg/ml) | Measured Conc. (pg/ml) | Expected Con. (pg/ml) | Recovery (%) |
|---|---|---|---|---|---|
| Spleen | 235 | 1026 | 990 | 1261 | 79 |
| | | 644 | 787 | 879 | 90 |
| | | 498 | 774 | 733 | 106 |
| | | 388 | 669 | 623 | 107 |
| | | 273 | 604 | 508 | 119 |
| | | 138 | 465 | 373 | 125 |
| Brain | 336 | 941 | 942 | 1070 | 88 |
| | | 734 | 893 | 807 | 111 |
| | | 471 | 770 | 693 | 111 |
| | | 357 | 618 | 594 | 104 |
| | | 258 | 585 | 492 | 119 |
| | | 156 | 588 | 412 | 143 |

**C. NT-3 recovery**

| Sample | Endogenous NT-3 (pg/ml) | Added (pg/ml) | Measured Con. (pg/ml) | Expected Con. (pg/ml) | Recovery (%) |
|---|---|---|---|---|---|
| Spleen | 391 | 964 | 1036 | 1261 | 82 |
| | | 703 | 938 | 879 | 107 |
| | | 446 | 792 | 733 | 108 |
| | | 318 | 765 | 623 | 123 |
| | | 201 | 664 | 508 | 131 |
| | | 102 | 570 | 373 | 153 |
| Brain | 405 | 964 | 1152 | 1370 | 84 |
| | | 703 | 912 | 1109 | 82 |
| | | 446 | 838 | 852 | 98 |
| | | 318 | 725 | 723 | 100 |
| | | 201 | 718 | 606 | 118 |
| | | 102 | 616 | 508 | 121 |

### 4.3.1. Effects on NGF concentration in the spleen and brain

As mentioned, the spleen and brain regions used in these studies derived from the same mice used for the neurotransmitter determinations. No significant differences between athymic and thymus-bearing mice in splenic NGF concentration were detected, although a marked tendency to increased concentration of this neurotrophin was observed on 1 day-old nude mice (Fig. 42 A).

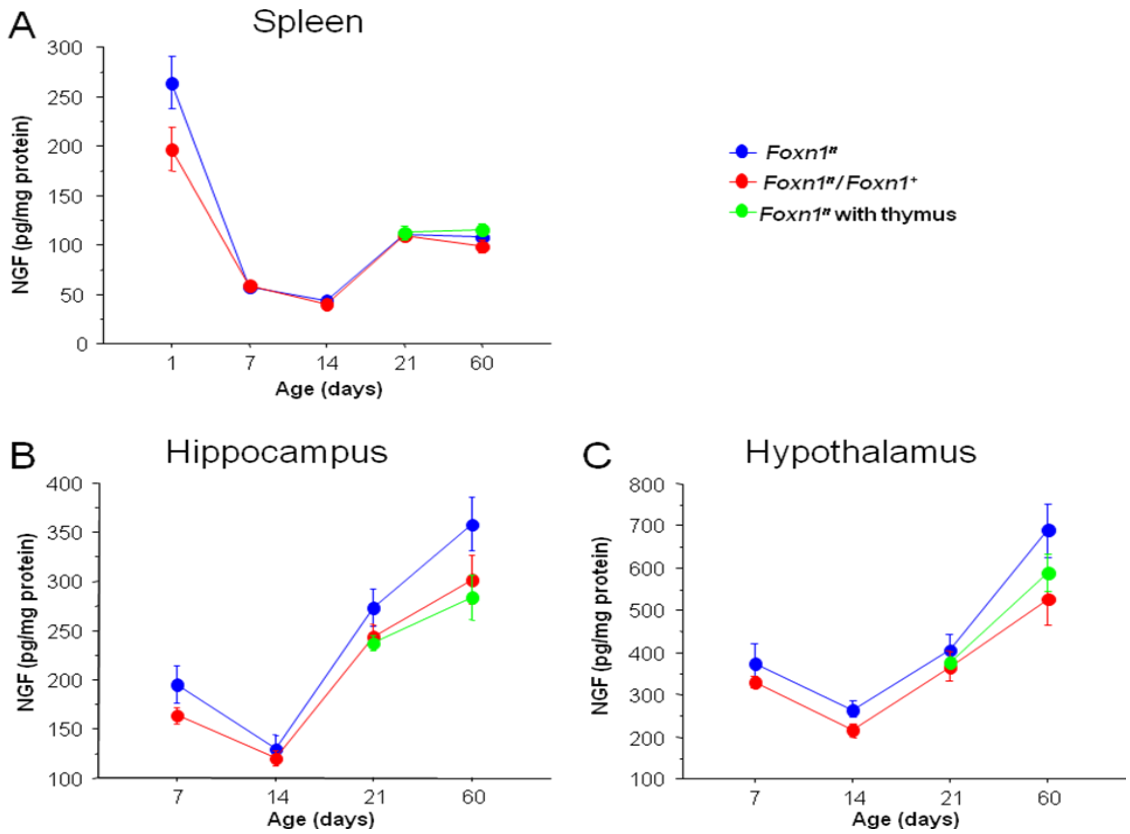


**Fig. 42. No significant differences between NGF concentrations in the spleen, hippocampus, and hypothalamus of athymic, thymus-bearing, and athymic mice implanted with thymus at birth.** NGF concentration in the spleen **(A)**, hippocampus **(B)**, and hypothalamus **(C)** were determined in athymic (*Foxn1*$^{n}$), heterozygous thymus-bearing littermates (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages (days 1; n=7/group; 7: n=7/group; 14: n=13/group; 21: n=10/group; and 60: n=7/group), and of athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) and sacrificed when they were 21 (n=9) or 60 (n=10) day-old.

Although the differences in the hypothalamus and in the hippocampus did not reach statistical significance either, higher NGF concentrations in *Foxn1*$^{n}$ mice than in *Foxn1*$^{n}$/*Foxn1*$^{+}$, were consistently detected at all ages studied. Furthermore, thymus implantation into newborn *Foxn1*$^{n}$ mice completely reverted this strong tendency (Fig. 42 B,C).

Interestingly, significantly correlations between NGF and NA concentrations were established in the spleen, hypothalamus, and hippocampus. The statistical significance of each of these correlations is indicated in Fig. 43. However, there were no statistically significant differences between the slopes of the regression lines of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice.

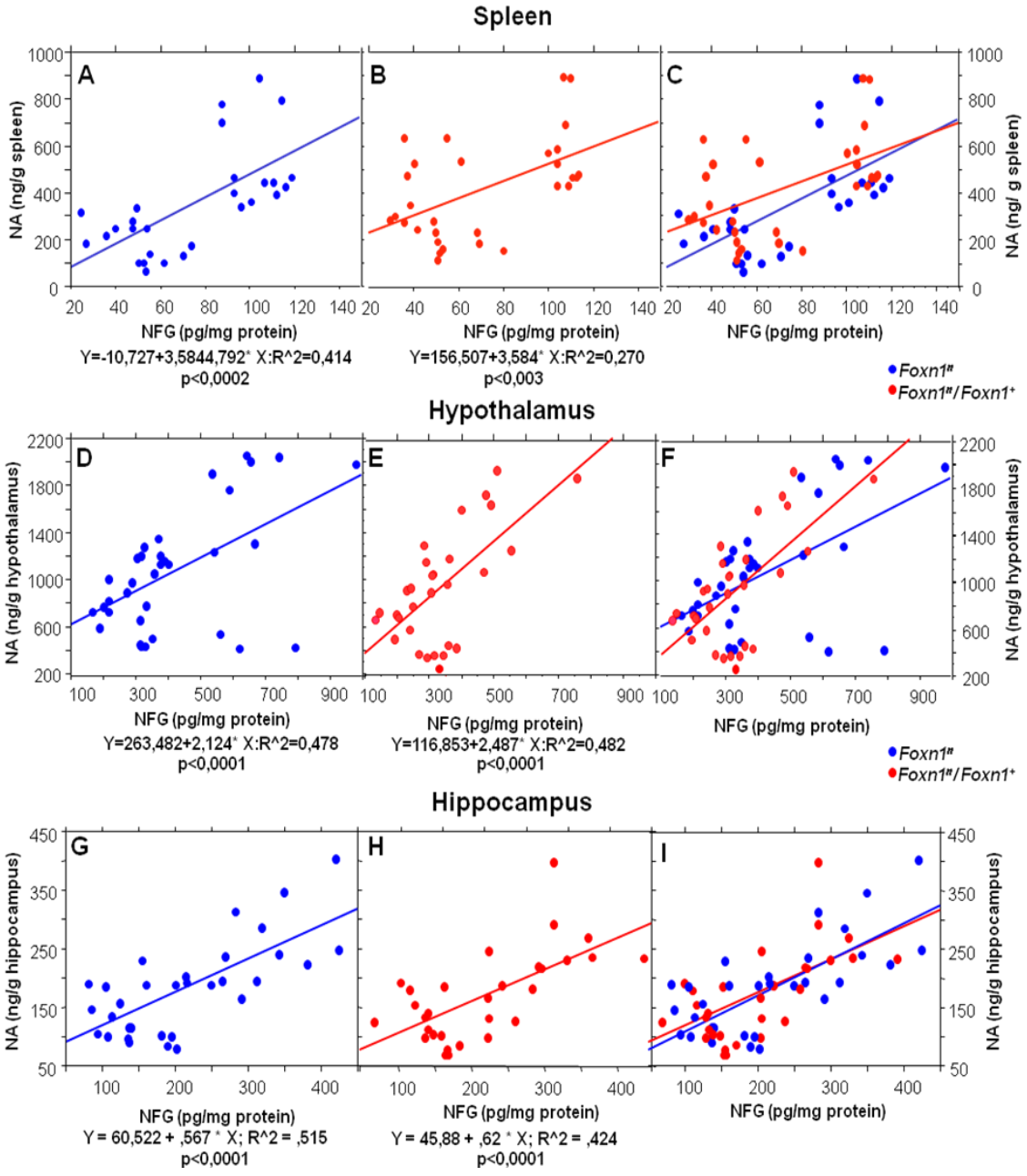


**Fig. 43. Significant correlations between NGF and NA concentrations in the spleen, hypothalamus, and hippocampus *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice.** There was no statistically significant difference between the slopes of the regressions lines of *Foxn1*$^{n}$ and *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice. The left panels **(A,D,G)** show the correlations observed in *Foxn1*$^{n}$; the panels in the center **(B,E,H)** those in *Foxn1*$^{n}$/*Foxn1*$^{+}$; the panels to the right show both correlations superimposed. **A,B,C:** spleen; **D,E,F:** hypothalamus; **G,H,I:** hippocampus.

### 4.3.2. Effects on BDNF concentration in the spleen and brain

The second neurotrophin evaluated in the spleen, kidney, and brain regions of athymic and thymus-bearing mice was BDNF, which was also determined by ELISA and the concentration expressed per mg protein.

While no differences were detected in 7 day-old mice, statistically significantly higher splenic BDNF concentrations were determined in 14 ($p<0,001$), 21 ($p<0,04$), and 60 ($p<0,03$) day-old *Foxn1*$^{n}$ as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice. BDNF concentrations were completely normalized in athymic mice implanted with thymus at birth, and were significantly lower than in 21 ($p<0,009$) and 60 ($p<0,0009$) day-old athymic mice ( Fig. 44 A). No differences in BDNF concentration were detected in the kidney at any time-point (Fig. 44 B).

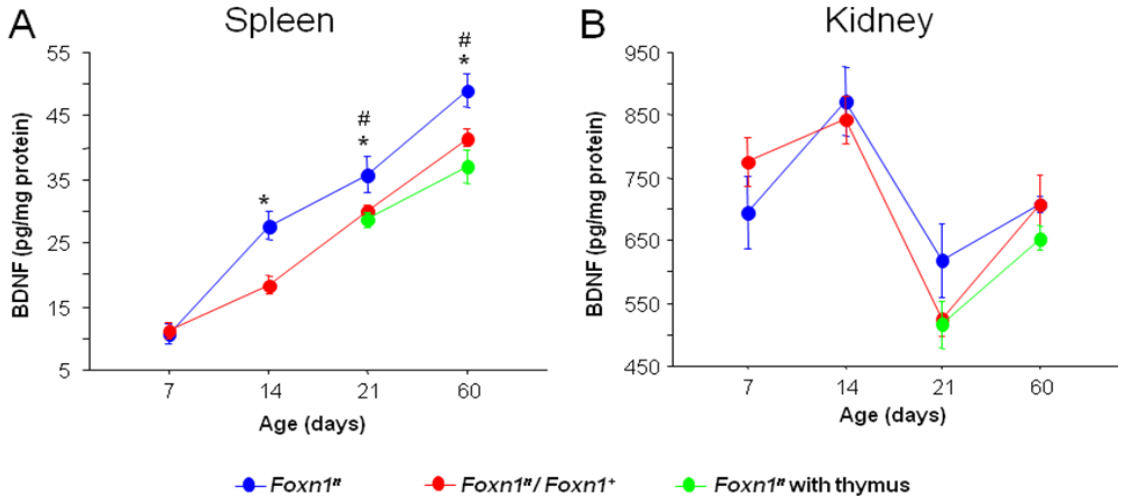


**Fig. 44. Increased BDNF concentration in the spleen of athymic mice during ontogeny and normalization by thymus implantation at birth.** BDNF determinations were performed in the spleen **(A)** and right kidney **(B)** of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages, and in 21 and 60 day-old athymic mice in which a thymus was implanted at birth (*Foxn1*$^{n}$ with thymus); n=8/group. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; and # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus.

The same determinations were performed in the hypothalamus and hippocampus of mice of the three experimental groups. Clearly higher BDNF concentrations were detected in both the hypothalamus and hippocampus of *Foxn1*$^{n}$ as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice. The differences were statistically significant in the hypothalamus of 7 ($p<0,002$) and 21 ($p<0,003$) day-old animals, and the same tendency was observed in 14 and 60 day-old mice.

BDNF concentrations were completely normalized in athymic mice implanted with thymus at birth, which had significantly lower concentrations of the neurotrophin than 21 day-old *Foxn1*$^{n}$ mice (p<0,0002). The same tendency was observed in 60 day-old mice (Fig. 45 A). Comparable results were obtained in the hippocampus: *Foxn1*$^{n}$ mice had more BDNF than *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (day 7: p<0,02; day 21: p<0,005; day 60: p<0,009), and the same tendency was observed in 14 day-old mice. Thymus implantation at birth also normalized the alterations observed in athymic mice. The differences in hippocampal BDNF concentration between *Foxn1*$^{n}$ and *Foxn1*$^{n}$ implanted with thymus at birth were statistically significant in 21 and 60 day-old mice (p<0,0002 and p<0,04, respectively) (Fig. 45 B).

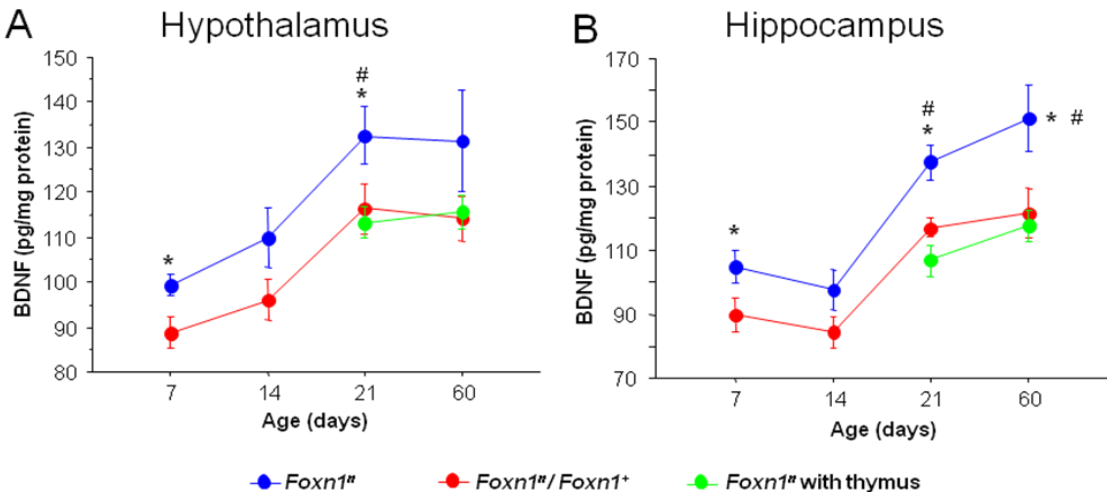


**Fig. 45. Increased BDNF concentration in the hypothalamus and hippocampus of athymic mice during ontogeny and normalization by thymus implantation at birth.** BDNF determinations were performed in the hypothalamus **(A)** and hippocampus **(B)** of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/ *Foxn1*$^{+}$) at different ages, and in 21 and 60 day-old athymic mice in which a thymus was implanted at birth (*Foxn1*$^{n}$ with thymus); n=8/group. Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; and # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus.

The results showing that there were clear differences between thymus-bearing *Foxn1*$^{n}$/*Foxn1*$^{+}$ and *Foxn1*$^{n}$ mice in BDNF concentration in the spleen and brain led to perform immunohistochemical studies. Although attempts were done to detect BDNF in the spleen immunohistochemically, this proved to be rather difficult due to the low concentration of this neurotrophin in this organ, and no results are shown. The hippocampus was chosen because BDNF concentration was here somewhat higher than in the hypothalamus.

There were clear immunohistochemical differences between the various groups in the hippocampus at all ages studied. Enhanced BDNF fluorescence intensity was observed in the hippocampus of *Foxn1*$^{n}$ mice as compared to normal *Foxn1*$^{n}$/*Foxn1*$^{+}$ (Fig. 46).

***Foxn1*$^{n}$/*Foxn1*$^{+}$** ***Foxn1*$^{n}$**

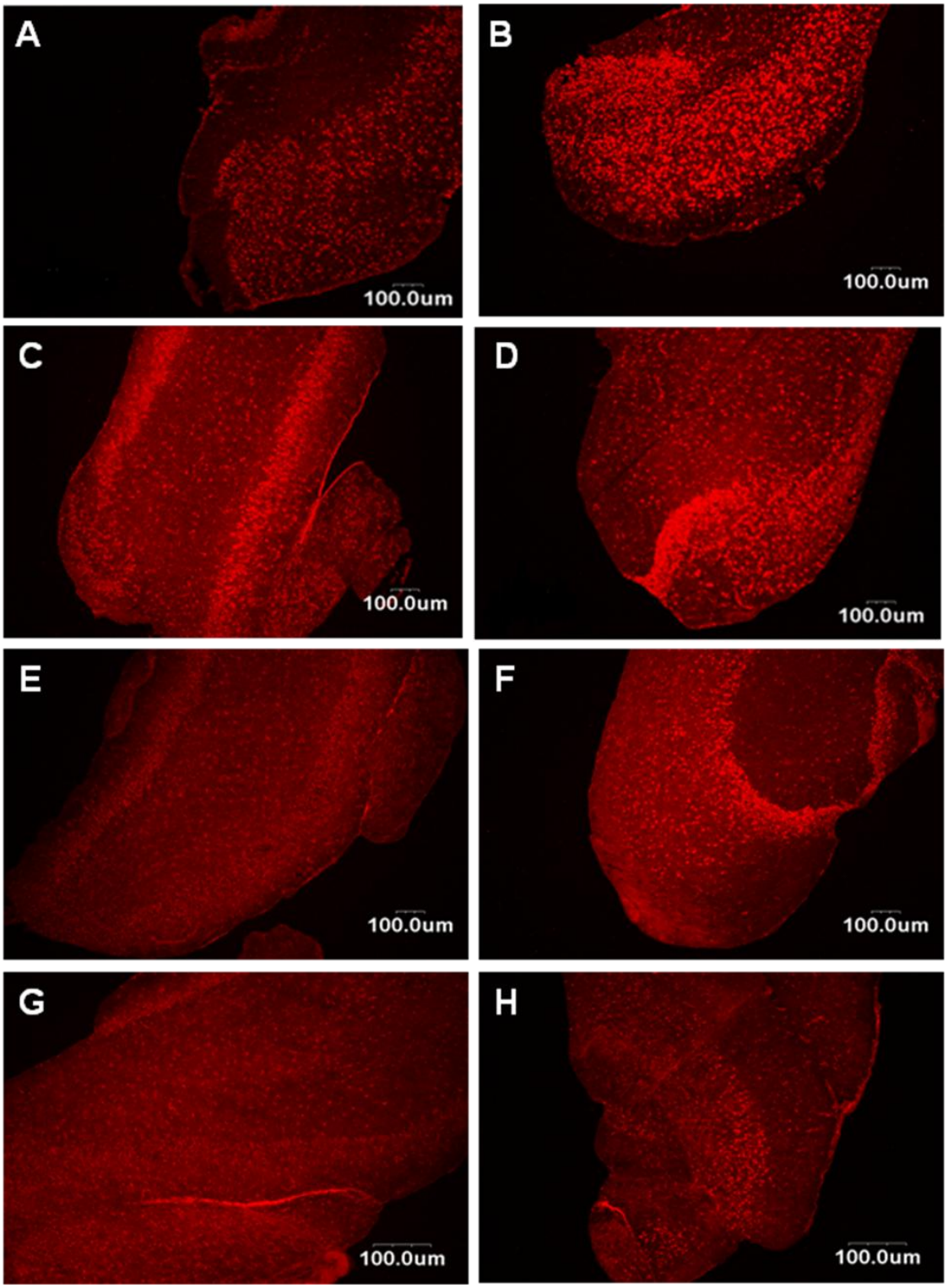


**Fig. 46. Increased intensity of BDNF positive signals in the hippocampus of athymic mice. (A,B)** 7-day-old; **(C,D)** 14-day-old; **(E,F)** 21-day-old; and **(G,H)** 60-day-old.

It could be appreciated that thymus implantation into newborn athymic mice also resulted in normalization of this parameter, namely BDNF fluorescent signals in the hippocampus decreased and were comparable to those of normal littermates in 21 and 60 day-old athymic mice that received a thymus transplant at birth (Fig. 47).

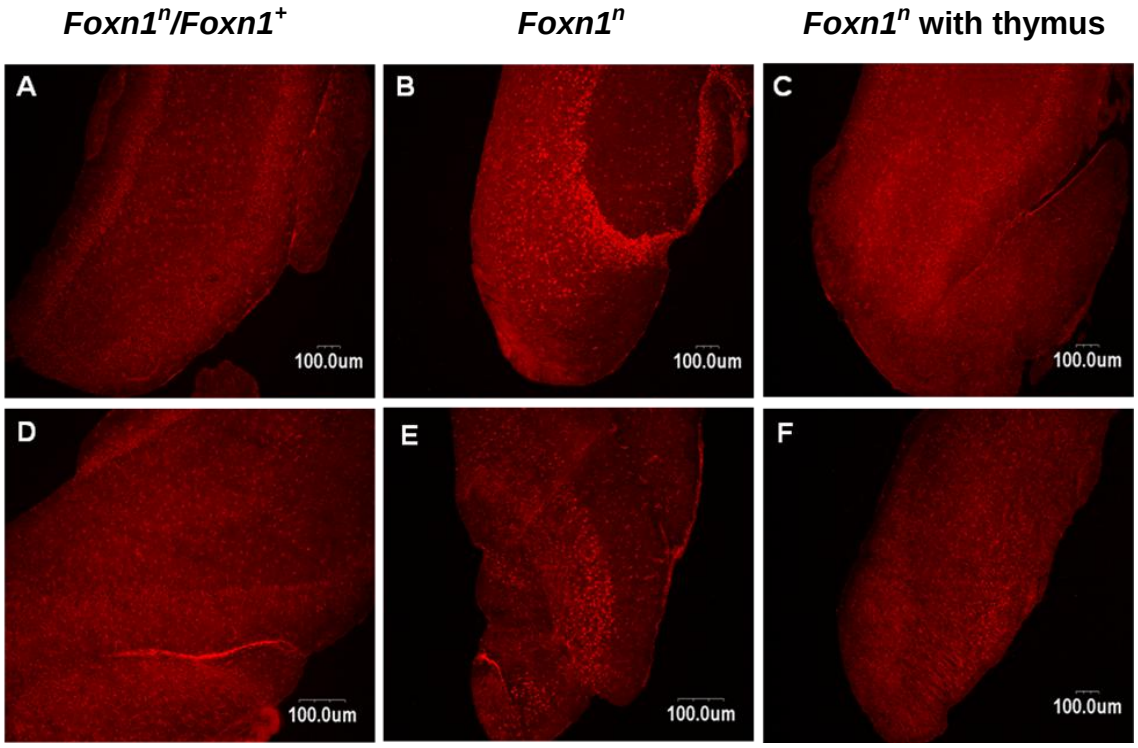


**Fig. 47. Thymus implantation into athymic newborn mice decreases the intensity of BDNF positive signals in the hippocampus.** The photos show representative examples of the hippocampus of *Foxn1$^{n}$/ Foxn1$^{+}$* (left), *Foxn1$^{n}$* (center), and *Foxn1$^{n}$* mice implanted with thymus at birth (right). Samples were collected when mice were 21 **(A, B, C)** or 60 **(D, E, F)** day-old, and stained for BDNF, as described in Methods.

It is well established that the highest BDNF concentration in the hippocampus is present in the CA1, CA2, and CA3 regions and in the DG (Conner et al., 1998; Thoenen et al.). These regions could be clearly identified in *Foxn1$^{n}$/Foxn1$^{+}$* hippocampal sections but not in those obtained from *Foxn1$^{n}$* mice, despite of the increased BDNF fluorescent intensity observed in these mice. This observation suggested that there might be also anatomical differences in the hippocampus of athymic mice. Thus, standard Nissl staining, commonly used for identifying basic neuronal structures, was performed in another brain sections. Longitudinal sections of the whole brain of 60 day-old *Foxn1$^{n}$* and *Foxn1$^{n}$/Foxn1$^{+}$* (n=3/group) were stained with Nissl staining, in

order to better compare the anatomical structure of the hippocampus of these mice. As illustrated in Fig. 48, regions such as CA1, CA2, CA3, and DG were not clearly defined in the hippocampus of *Foxn1*$^{n}$ mice. This interesting observation deserves a more detailed anatomical study.

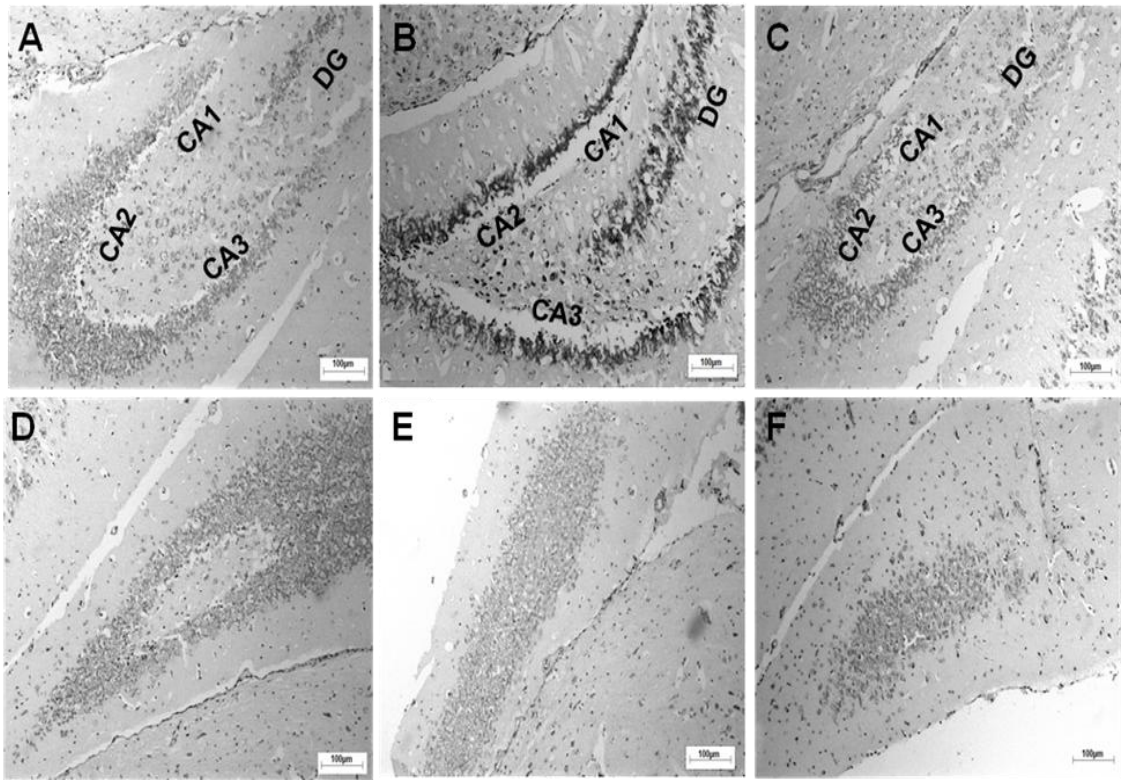


**Fig. 48. The anatomy of the hippocampus of 60 day-old athymic mice and thymus-bearing. (A,B,C)** thymus-bearing *Foxn1*$^{n}$/*Foxn1*$^{+}$, **(D,E,F)** athymic *Foxn1*$^{n}$ mice. There were differences between the anatomy of hippocampus of athymic mice and thymus bearing mice. The regions CA1, CA2, CA3, and DG are defined in the hippocampus of *Foxn1*$^{n}$/*Foxn1*$^{+}$ but undefined in the hippocampus of *Foxn1*$^{n}$ mice

### 4.3.3. Effects on NT-3 concentrations in the spleen and brain

The third neurotrophin that was evaluated in the spleen and hippocampus was NT-3. These determinations were performed in tissues obtained from 14, 21, and 60 day-old athymic mice and their heterozygous thymus-breading littermates, as well as in the group of athymic mice implanted with thymus at birth and sacrificed when they were 21 or 60 day-old. NT-3 concentrations were also determined by ELISA and the results expressed per mg protein. The kidney was used as an abdominal control organ also for this parameter. The

organs used to evaluate NT-3 were the same as those used for BDNF determinations, but not enough material was available from 7 day-old mice.

The results obtained for this neurotrophin were comparable to those of BDNF. Higher splenic NT-3 concentrations were detected in 14 ($p<0,005$) and 21 ($p<0,03$) day-old *Foxn1*$^{n}$ mice as compared to the age-matched *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice and when pair-wise comparisons between these groups were done, the difference was also statistically significant in 60 day-old mice ($p<0,04$). The concentrations were completely normalized in 21 day-old *Foxn1*$^{n}$ animals implanted with thymus at birth, and were significantly different from those in *Foxn1*$^{n}$ mice ($p<0,03$) (Fig 49 A). The same tendency was observed in 60 day-old mice. No significantly differences were detected in the kidney (Fig 49 B).

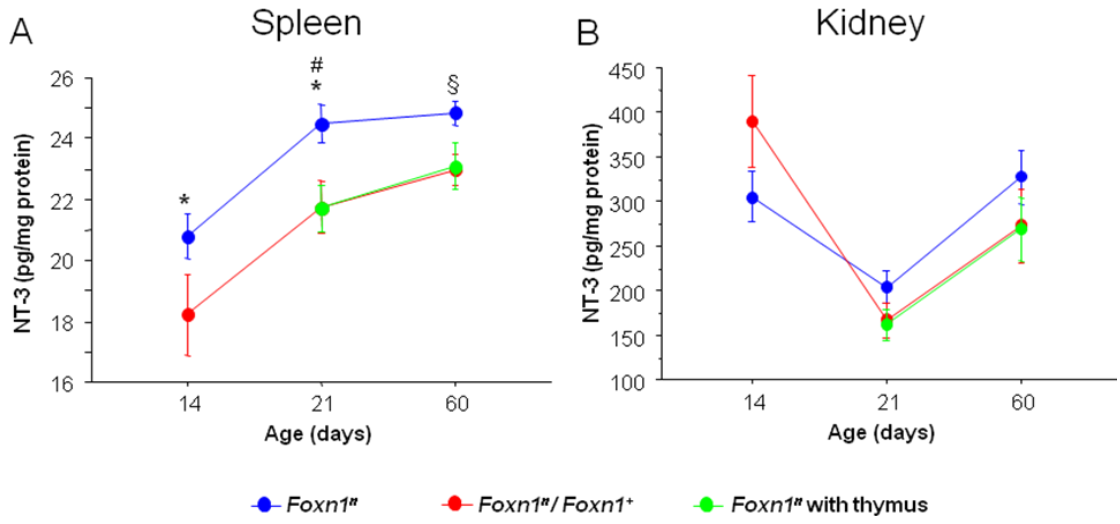


**Fig. 49. Increased NT-3 concentration in the spleen of athymic mice during ontogeny and normalization by thymus implantation at birth.** NT-3 determinations were performed in spleen **(A)** and right kidney **(B)** of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/ *Foxn1*$^{+}$) at different ages, and in 21 and 60 day-old athymic mice implanted with thymus at birth (*Foxn1*$^{n}$ with thymus). 14 day (n=5/group), 21 and 60 day (n=7/group). Statistically significant difference: ⁎ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$ when pair-wise comparisons between these group were done**.**

The differences in NT-3 concentrations in the hippocampus seemed to appear later in ontogeny than in BDNF, since no difference between athymic and normal mice was apparent in 14 day-old mice. However, higher NT-3 concentrations were detected in the hippocampus of *Foxn1*$^{n}$ mice as compared to *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice in older mice (day 21: $p<0.0001$; day 60: $p< 0,003$) NT-3 concentrations in athymic mice implanted with a thymus at birth reached

levels comparable to those of the normal littermates in 60 day-old mice. Interestingly, NT-3 levels in 21 day-old *Foxn1*$^{n}$ implanted with a thymus were even significantly lower than in *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice (p<0,007) (Fig. 50).

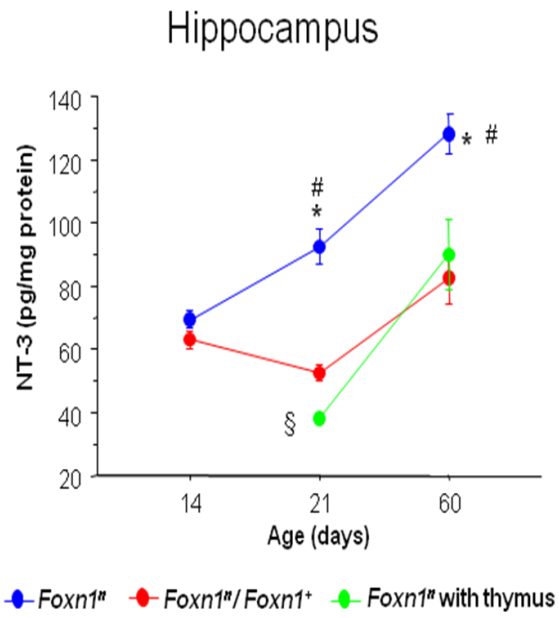


**Fig. 50. Increased NT-3 concentration in the hippocampus of athymic mice during ontogeny and normalization by thymus implantation at birth.** NT-3 determination in hippocampous of athymic male mice (*Foxn1*$^{n}$) and heterozygous thymus-bearing (*Foxn1*$^{n}$/ *Foxn1*$^{+}$) at different ages, and in 21, and 60 day-old athymic mice implanted with thymus at birth (*Foxn1*$^{n}$ with thymus); n=7/group. Statistically significant difference: * *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs.*Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

## 4.4. The absence of the thymus is paralleled by increased corticosterone blood levels

Normann et al. reported that corticosterone blood levels are higher in athymic mice than in euthymic mice (Normann et al., 1988). Others suggested that such levels are higher in athymic mice only under stress conditions (Spinedi et al., 1997). As shown here, NA concentration in the hypothalamus, is influenced by the presence of the thymus, and this effect might result in changes in the activity of the HPA axis (Besedovsky and del Rey, 1996). Thus, corticosterone concentrations in blood of the same animals used for neurotransmitter and NGF determinations were determined by ELISA.

While no difference was detected in 7 day-old mice, higher corticosterone concentrations were found in 1 (p<0,005), 14 (p<0,01), 21 (p<0,04), and 60 (p<0,0001) day-old *Foxn1*$^{n}$ mice as compared to age-matched *Foxn1*$^{n}$/*Foxn1*$^{+}$ mice. As with most of the parameters evaluated in this work, thymus transplantation to newborn nude mice resulted in normal corticosterone

concentrations when these mice were 21 day-old, and the values were significantly different from those of *Foxn1*$^{n}$ mice (p<0,03). The same tendency was observed in 60 day-old mice, although at this age, corticosterone levels of thymus-transplanted *Foxn1*$^{n}$ mice were still significantly higher than those of *Foxn1*$^{n}$/*Foxn1*$^{+}$-(p<0,002) (Fig. 51).

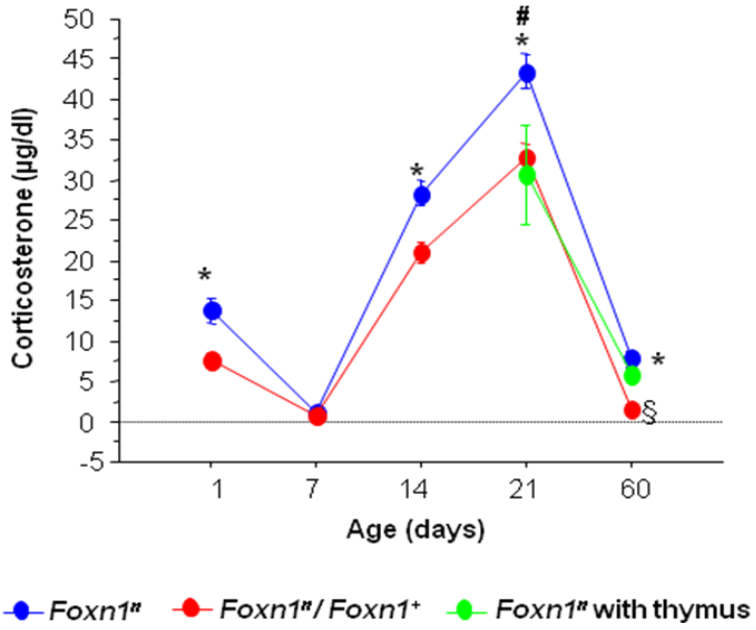


**Fig. 51. Increased corticosterone concentration in blood of athymic mice during ontogeny and normalization by thymus implantation at birth.** Corticosterone determinations were performed in blood of athymic male mice (*Foxn1*$^{n}$) and their heterozygous thymus-bearing (*Foxn1*$^{n}$/*Foxn1*$^{+}$) at different ages (day 1: n=7/group; day 7: n=7/group; day 14: n=13/group; day 21: n=10/group); day 60: n=7/group), and athymic mice implanted with a thymus at birth (*Foxn1*$^{n}$ with thymus) and sacrificed when they were 21 (n=9) or 60 (n=10) day-old. Statistically significant difference: ∗ *Foxn1*$^{n}$ vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$; # *Foxn1*$^{n}$ vs. *Fox1*$^{n}$ with thymus; and **§** *Fox1*$^{n}$ with thymus vs. *Foxn1*$^{n}$/*Foxn1*$^{+}$**.**

## 4.5. Sympathetic denervation results in changes in neurotrophin and corticosterone concentrations

Most of the alterations in neurotransmitter concentration and noradrenergic fibers, as well as in the concentration of certain neurotrophins in the spleen and in defined brain regions of athymic mice described in the previous sections could be reversed by thymus implantation at birth. These results show that the alterations detected in athymic mice are not directly linked to the *Foxn1*$^{n}$ mutation that nude mice carry, but rather phenotipical manifestations related to the absence of the thymus. Still, besides the defective development of the

thymus anlage, nude mice have other defects, such as abnormal hair growth and delayed sexual maturation. The question arises of whether the alterations in the catecholaminergic system and neurotrophin expression detected in nude mice reflect a more general condition that causally relates these parameters. As a first approach to answer this question, a model that could mimic a situation opposite to that observed in athymic mice regarding splenic NA concentrations was chosen. Thus, the sympathetic innervation in normal mice was decreased by chemical destruction of noradrenergic neurons and neurotrophin concentrations in the spleen and brain were determined in parallel. To clarify whether there is a critical time period in which catecholamine depletion would result in changes in neurotrophin and corticosterone concentrations, sympathectomy was performed at different ages.

For this purpose, the neurotoxin 6-OH-DA, which destroys noradrenergic fibers (Korsching and Thoenen, 1985), was injected into normal, male Balb/c mice at different ages, and groups of animals were sacrificed at different times after chemical denervation. At this stage of the studies, it was not feasible to include all possible combinations. Thus, based on previous reports and in preliminary results, the experiments were limited to four different groups of mice: 1) denervated at birth or 2) when they were 14 day-old, and sacrificed when they were 21 day-old; 3) denervated when they were 14 or 4) 60 day-old and killed when they were 67 day-old. Age- and sex-matched controls that received the vehicle alone (ascorbic acid) were included in each group. NA was determined in the spleen and brain to control for the degree of catecholamine depletion, and neurotrophin concentrations were evaluated in the same samples. Corticosterone blood levels were evaluated in parallel.

It is well established that 6-OH-DA injection at birth induces a permanent destruction of sympathetic nerves (Glatzel et al., 2001; Thoenen and Tranzer, 1968), while administration of the neurotoxin to adult mice results in a transient sympathectomy, i.e. sympathetic innervation is recovered after a few weeks (del Rey et al., 2002; Glatzel et al., 2001). However, the effect of denervating at around 14 days of life is not known. Also, the effect of peripheral administration of 6-OH-DA at different ages on central brain catecholamines is still poorly defined.

### 4.5.1 Sympathetic denervation decreases NA concentration

NA concentration in the spleen, hypothalamus, and hippocampus of mice that received 6-OH-DA or the vehicle alone was determined by HPLC. The results obtained are summarized in table 6.

NA concentration in the spleen of mice that received 6-OH-DA was significantly reduced as compared to the corresponding vehicle-injected controls, irrespectively of whether they received the neurotoxin when they were less than 1, 14, or 60 day-old (Table 6A).

NA concentration in the hypothalamus was also significantly reduced in 6-OH-DA-treated mice as compared to the corresponding control, with the exception of the group of mice that received the neurotoxin when they were 60 day-old and were sacrificed one week later (Table 6B).

**Table 6.** Decreased NA concentrations in the spleen, hypothalamus, and hippocampus of 6-OH-DA-treated mice

**A. Spleen**

| Age at injection (days) | Age at sacrifice (days) | Treatment | Splenic NA (ng/g) | p |
|---|---|---|---|---|
| **< 1 (birth)** | 21 | Vehicle | 445 ± 97 | |
| | | 6-OH-DA | 7 ± 1 | <0,0001 |
| **14** | 21 | Vehicle | 363 ± 71 | |
| | | 6-OH-DA | 4 ± 1 | <0,0001 |
| | 67 | Vehicle | 502 ± 102 | |
| | | 6-OH-DA | 88 ± 12 | <0,0001 |
| **60** | 67 | Vehicle | 790 ± 65 | |
| | | 6-OH-DA | 60 ± 27 | <0,0001 |

**B. Hypothalamus**

| Age at injection (days) | Age at sacrifice (days) | Treatment | hypothalamic NA (ng/g) | p |
|---|---|---|---|---|
| **< 1 (birth)** | 21 | Vehicle | 1031 ± 92 | |
| | | 6-OH-DA | 351 ± 82 | <0,0001 |
| **14** | 21 | Vehicle | 961 ± 36 | |
| | | 6-OH-DA | 516 ± 18 | <0,0001 |
| | 67 | Vehicle | 1691 ± 31 | |
| | | 6-OH-DA | 1488 ± 35 | <0,007 |
| **60** | 67 | Vehicle | 1471 ± 86 | |
| | | 6-OH-DA | 1316 ± 64 | n.s. |

**C. Hippocampus**

| Age at injection (days) | Age at sacrifice (days) | Treatment | Hippocampal NA (ng/g) | p |
|---|---|---|---|---|
| **< 1 (birth)** | 21 | Vehicle | 203 ± 42 | |
| | | 6-OH-DA | 27 ± 12 | <0,0001 |
| **14** | 21 | Vehicle | 252 ±15 | |
| | | 6-OH-DA | 62 ±20 | <0,0002 |
| | 67 | Vehicle | 355 ± 79 | |
| | | 6-OH-DA | 47 ± 19 | <0,0001 |
| **60** | 67 | Vehicle | 360 ± 14 | |
| | | 6-OH-DA | 308 ± 16 | <0,05 |

Mice received 6-OH-DA or the vehicle alone injected at birth, or when they were 14 or 60 day-old (n=6/group). NA concentration in the spleen **(A)**, hypothalamus **(B)**, and hippocampus **(C)** was determined at different times after injection, as indicated in the table.

Comparable results were obtained in the hippocampus, but statistically significant differences were also obtained in this brain region when 6-OH-DA was injected into adult (60 day-old) mice (Table 6C).

Although 6-OH-DA treatment resulted in NA depletion in the spleen and in the brain in all groups (with the only exception of the hypothalamus in adult mice mentioned above), the magnitude of the decrease was not the same. Thus, for better appreciation of the different effects, the results were also expressed as percentage of the simultaneous control group, and are summarized in Table 7. As a whole, 6-OH-DA administration during adulthood resulted in less NA depletion than when the neurotoxin was administered at birth or when mice were 14 day-old.

**Table 7.** Injection of 6-OH-DA at different ages results in different degrees of NA depletion

| Organs | Age at injection (days) | Age at sacrifice (days) | NA (% of control) | p |
|---|---|---|---|---|
| **Spleen** | < 1 (birth) | 21 | 2,0 ± 0,6 | vs. a: n.s.<br>vs. b: p<0,0001<br>vs. c: p<0,002 |
| | 14 | 21 [(a)] | 1,0 ± 0,0 | vs. b: p <0,0001<br>vs. c: p <0,004 |
| | | 67 [(b)] | 17,0 ± 1,0 | vs. c: p <0,0001 |
| | 60 | 67 [(c)] | 7,0 ± 1,8 | - |

| Organs | Age at injection (days) | Age at sacrifice (days) | NA (% of control) | p |
|---|---|---|---|---|
| Hypothalamus | < 1 (birth) | 21 | 34,0 ± 3,3 | vs. d: p<0,001<br>vs. e: p <0,002<br>vs. f: p <0,0001 |
| | 14 | 21 (d) | 53,5 ± 1,5 | vs. e: p <0,0001<br>vs. f: p <0,0001 |
| | | 67 (e) | 19,8 ± 1,2 | vs. f: p <0,0001 |
| | 60 | 67 (f) | 89,3 ± 2,6 | - |
| Hippocampus | < 1 (birth) | 21 | 13,3 ± 2,4 | vs. g: n.s.<br>vs. h: n.s.<br>vs. i: p <0,0007 |
| | 14 | 21 (g) | 35,5 ± 5,5 | vs. h: n.s.<br>vs. i: p<0,03 |
| | | 67 (h) | 23,6 ± 8,8 | vs. i: p <0,003 |
| | 60 | 67 (i) | 93,0 ± 5,7 | - |

Mice received 6-OH-DA injected i.p. at different ages and groups of animals were sacrificed at various times. NA concentration in the spleen, hypothalamus, and hippocampus of 6-OH-DA- and vehicle-treated mice was determined by HPLC. The results shown in the table were calculated taking as 100% the mean NA concentration of the corresponding age-matched control group. n= 6 per group. The statistical significances indicated correspond to the comparison of a given group vs those identified with a letter. n.s.: not statistically significantly different.

### 4.5.2. Sympathetic denervation affects NGF and BDNF concentration

NGF concentrations were determined by ELISA in the spleen, hippocampus, and hypothalamus of the same denervated and control mice whose NA concentration is shown in the table above. Protein concentrations in the samples were determined by the Bradford assay, and the results are expressed as pg of the neurotrophin per mg protein.

As a whole, the results indicate that the effects of noradrenergic denervation at birth or during adulthood on NGF concentration are different (Fig. 52).

NGF concentration was significantly lower in the spleen of mice treated with 6-OH-DA at birth as compared to corresponding control group ($p<0,03$). The same tendency was observed when 6-OH-DA was injected into 14 day-old mice and killed when animals were 21 or 60 day-old. However, exactly the opposite results were obtained when chemical sympathectomy was performed

in adult mice that were killed one week later: NGF concentration was significantly higher in the spleen of denervated mice as compared to the control group (p<0,0007) (Fig. 52A).

Essentially the same effects were observed in the hippocampus: NGF concentration was significantly lower in the hippocampus of mice treated with 6-OH-DA at birth as compared to the corresponding control group (p<0,03), and there was the same tendency in 21 or 60 day-old mice that received 6-OH-DA when they were 14 day-old. As in the spleen, denervation during adulthood resulted in the opposite effect also in the hippocampus: NGF concentrations were significantly higher in 6-OH-DA-treated mice than in the control group (p<0,01) (Fig. 52B).

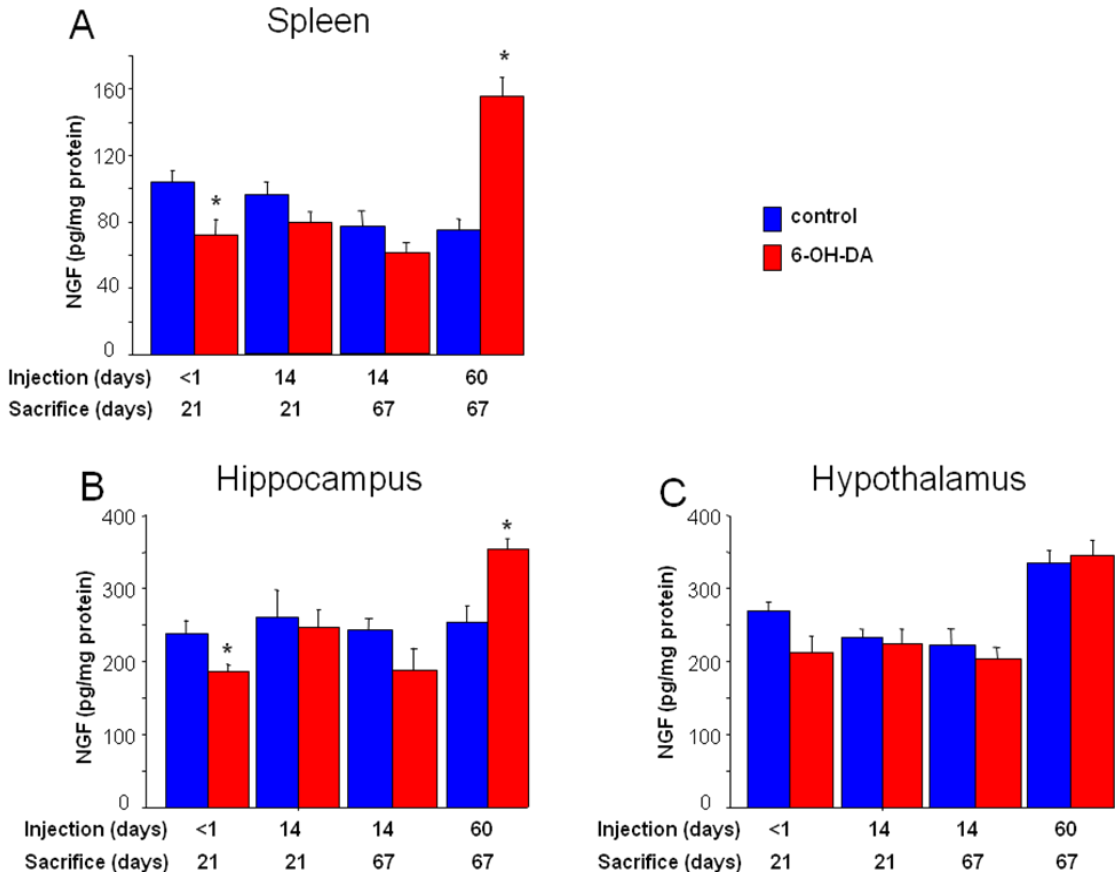


**Fig. 52. Effect of noradrenergic denervation at different ages on NGF concentration.** NGF concentrations were determined in the spleen **(A)**, hippocampus **(B)**, and hypothalamus **(C)** of 6-OH-DA- and vehicle-treated mice (n=6/group). The corresponding NA concentrations of these organs are given in table 4 A-C. ***** Statistically significantly different from the age-matched, vehicle-treated controls.

In the hypothalamus, the differences between 6-OH-DA- and vehicle-treated mice did not reached statistical significance, but showed the same tendency (Fig. 52C).

The remaining aliquots of the spleen and hippocampus obtained from the 6-OH-DA- and vehicle-treated mice shown above were used to determine BDNF concentration by ELISA. The results obtained for this neurotrophin are also expressed as pg BDNF per mg protein. Unfortunately, not enough material from the hypothalamus was available to determine BDNF concentrations. However, the results of the spleen and hippocampus are included here to illustrate the effect of denervation on other neurotrophin, which was essentially the same as for NGF.

BDNF concentration was significantly lower in the spleen of mice that received 6-OH-DA at birth or when they were 14 day-old as compared to the control group ($p<0,006$ and $p< 0,03$, respectively) (Fig 53A). The same tendency was observed in the hippocampus (Fig 53B). Conversely, significantly higher BDNF concentrations were found in both, spleen and hippocampus when mice were denervated during adulthood ($p<0,002$, and $p<0,03$) (Fig. 53 A,B).

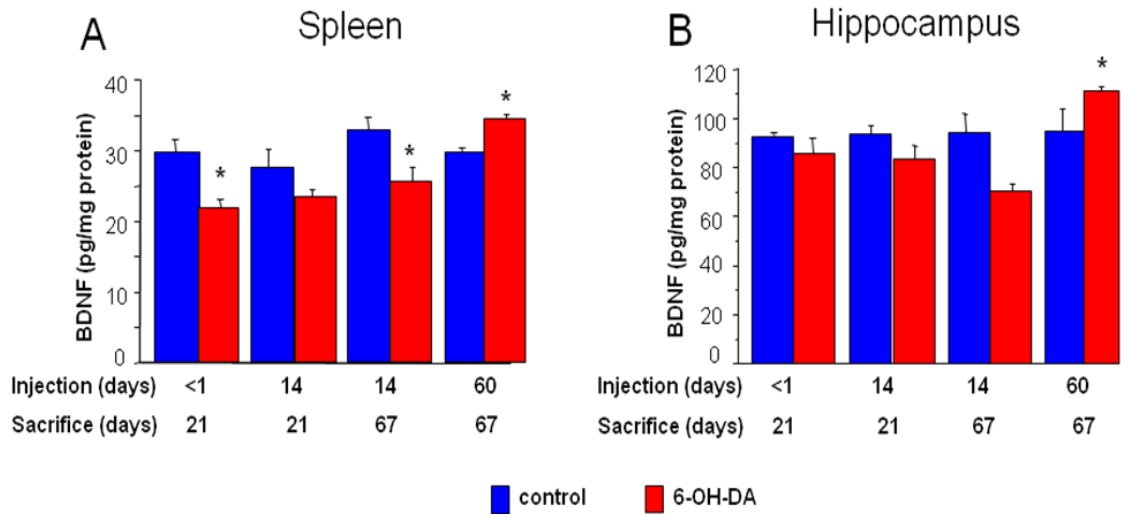


**Fig. 53. Effect of noradrenergic denervation at different ages on BDNF concentration.** BDNF concentrations were determined in the spleen **(A)**, and hippocampus **(B)** of 6-OH-DA- and vehicle-treated mice (n=6/group). The corresponding NA concentrations in these organs are given in table 4 A-C. * Statistically significantly different from the age-matched, vehicle-treated control.

### 4.5.3. Corticosterone concentration

Since permanent and transient sympathectomy affected NA content in the hypothalamus, which may lead to changes in the activity of the HPA axis, corticosterone concentration in blood of the denervated and control mice used above was evaluated using a commercially available ELISA kit.

Corticosterone concentrations were statistically significantly lower in blood of mice treated with 6-OH-DA at birth or when they were 14 day-old, and sacrificed on day 21, as compared to the corresponding controls ($p<0,007$ and $p<0,02$, respectively). However, the concentration of the hormone was significantly higher in blood of denervated adult mice than in the vehicle-injected controls, independently of whether they received 6-OH-DA when they were 14 ($p<0,0001$) or 60 ($p<0,002$) day-old (Fig. 54).

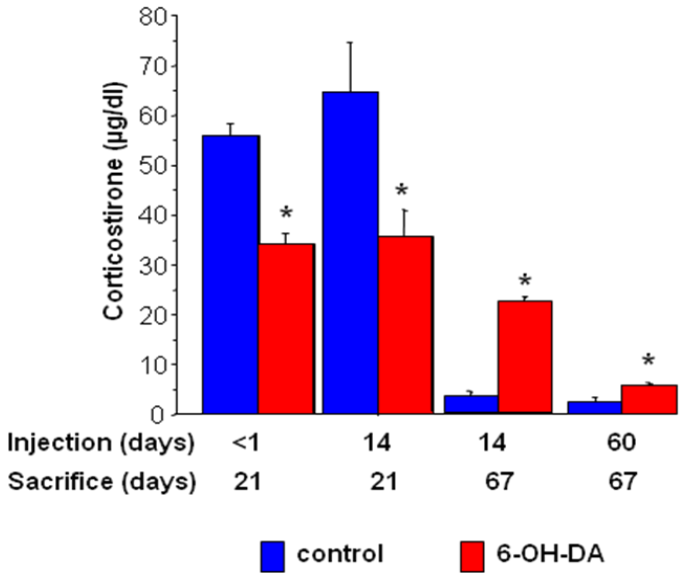


**Fig. 54. Effect of noradrenergic denervation at different ages on corticosterone concentration.** Corticosterone concentrations were determined in blood of 6-OH-DA- and vehicle-treated mice (n=6/group). The corresponding NA concentrations in these organs are given in table 4 A-C. * Statistically significantly different from the age-matched, vehicle-treated control.

# 5. Discussion

There is good evidence demonstrating the interplay between the nervous, endocrine and the immune systems. This work addressed a particular aspect of these interactions, namely whether the absence of a functional thymus, and therefore the resulting missing population of the host with mature T cells, can affect the development of noradrenergic innervation in lymphoid organs and in defined brain regions. For this purpose, homozygous *Foxn1*$^{nu}$ mice, which congenitally lack a thymus, have been used as a model. These mice offer the advantage that immunocompetence can be easily reversed by thymus implantation (Loor and Kindred, 1974). Therefore, three groups of mice were used in the first part of this study: athymic mice, their heterozygous thymus-bearing littermates, and athymic mice implanted with a thymus at birth.

For reasons of simplicity, in some parts in this work, it is referred to the effect of T cells on the variables studied, although in a strict sense, the experiments reported here do not allow to distinguish between effects of T cells or their products and potential effects of the thymus itself, for example some of the hormones derived from its epithelium (Podosinnikov et al., 1986; Reggiani et al., 2009). However, based on previous reports, this last possibility seems unlikely and this aspect will be discussed below.

The spleen was chosen as a secondary lymphoid organ, and the kidney was used as an abdominal, control non-lymphoid organ. The hypothalamus was chosen because it controls certain activities of the ANS and neuro-endocrine responses. Its function is regulated by the classical monoamine neurotransmitters NA, DA and 5-HT (for review see Qiu et al., 1996). The brainstem was used as a second brain region because it is involved in the regulation of neural pathways between the CNS and the endocrine system, and also because most of the noradrenergic innervation of the hypothalamus arises from cell bodies in brainstem nuclei, (Livnat et al., 1985). The hippocampus was also included because it has been recently reported that T lymphocytes can affect hippocampal neurogenesis and spatial learning abilities (Ziv et al., 2006).

## 5.1. The lack of a functional thymus affects catecholamine and indolamine concentration in the spleen and brain

It is well established that the sympathetic innervation of several peripheral organs in rodents is not completely developed until relatively late in ontogeny (De Champlain and Smith, 1974). This situation is also observed in the spleen, where the T cell compartment also develops late in postnatal life (Bach et al., 1975). In athymic mice, the thymus-dependent areas in the spleen are not populated by mature T cells because T cell lymphopoiesis is interfered with early in embryogenesis (Pritchard and Micklem, 1974). The results reported in this work show that athymic *Foxn1*$^{n}$ mice have higher splenic NA concentration, the main sympathetic neurotransmitter, and TH-fluorescent positive signals than their normal, thymus-bearing littermates *Foxn1*$^{n}$/*Foxn1*$^{+}$. This situation does not reflect a general sympathetic hyperactivity in nude mice since no such differences are detected in the kidney. Furthermore, thymus grafting into newborn nude mice normalizes splenic NA concentration and the number of TH-fluorescent positive signals. The results reported here are in agreement with previous works showing that: 1) NA concentration is increased in the spleen of young athymic mice (Besedovsky et al., 1987); 2) reduced immunological activity in germ-free animals results in increased NA levels in lymphoid organs (del Rey et al., 1981); and 3) NA levels decrease in the spleen of rats during the course of an immune response to innocuous antigens (Besedovsky et al., 1979; del Rey et al., 1982), and at late phases of the response to superantigens in mice (del Rey et al., 2002); 4) NA concentration and TH-containing fibers are markedly decreased during the development of an autoimmune lymphoproliferative disease (del Rey et al., 2006). Most importantly, it has been shown that these changes in NA concentration have an impact on the immune response. Furthermore, there is a negative correlation between NA levels in the spleen and lymphocyte proliferation (Bellinger et al., 2008; Leposavic et al., 2010). From this evidence, it can be concluded that the degree of immune activity plays an important role in regulating the sympathetic innervation of the spleen.

The results also show that, as compared to normal thymus-bearing littermates, athymic mice have higher splenic concentrations of DA and Tyr (the precursor of NA and DA synthesis), particularly more marked at later stages of

development, and that thymus grafting into newborn nude mice normalizes these concentrations. No differences were detected in the kidney.

While the NA present in the spleen is most likely derived from the sympathetic nerves, the source of DA is not so clear. It has been reported that DA is preferentially metabolized to the acid metabolite DOPAC (Eisenhofer et al., 2004), and that the conversion of DA to NA in sympathetic nerves is about 90%. Since there is more DA and its metabolites than NA excreted in the urine, it was originally thought that this reflects inefficient conversion of DA to NA in sympathetic nerves (Kopin, 1985). However, it is at present believed that a substantial amount of the DA produced in the body is derived from sources independent of the sympathetic nerves, the adrenal medulla, or the brain; and that about a half is produced in mesenteric organs (Eisenhofer et al., 2004). Since DA is also found in the circulation, it is likely that the DA detected in the spleen derives partially from incomplete conversion to NA in sympathetic nerves and partially from the blood. The tissue concentrations of DOPAC significantly differed in the spleen of 60 day-old mice, showing lower values in athymic mice. A possibility would be that a local reduction in the activity of the metabolic enzymes monoamine oxidase (MAO) and alcohol dehydrogenase (ADH) together with an increased supply of Tyr might result in elevated NA and DA concentrations in the spleen of nude mice. The results show that thymus implantation abrogates the differences in NA and DA, but not DOPAC, concentration in adult mice. It is therefore possible that other mechanisms are affected by the absence of mature T cells since several other enzymes are involved in the metabolism of catecholamines (Eisenhofer et al., 2004). To add more complexity to this system, it has been also reported that picomol levels of DA are found in lymphocytes, neutrophils and macrophages. For example, it has been shown that normal mouse spleen cells contain more DA than macrophages, and that T cells contain more DA than B cells (Bergquist et al., 1998). Furthremore, the presences of an active DA uptake system in murine and human lymphocytes and bone marrow cells was also suggested (Basu et al., 1993).

As already mentioned, a large body of *in vivo* and *in vitro* experimental evidence has shown that sympathetic nerves, and NA itself, can have marked effects on the functioning of the immune system (for review see Elenkov et al.,

2000). However, much less is known about the immunoregulatory role that peripheral DA may play, and only speculations can be done. The presence of intracellular DA in lymphocytes (Bergquist et al., 1998) and of DA receptors on murine and human T and B cells (Ricci and Amenta, 1994) suggest that this catecholamine may affect lymphoid cell activity in an autocrine regulatory way. *In vitro* studies demonstrated that DA can induce thymic cell apoptosis in mice (Offen et al., 1995), and inhibit lymphocyte proliferation and T cell-mediated cytotoxicity, acting through D1/D5, and D3 dopamine receptors (Cosentino et al., 2007; Ghosh et al., 2003). Increased plasma catecholamine concentrations, including DA, and decreased catecholamine biosynthesis were found in the spleen of rats exposed to a stressful situation (Gavrilovic et al., 2012).

The studies reported here show that athymic *Foxn1*$^{n}$ mice have also higher NA and DA concentrations in the hypothalamus, brainstem, and hippocampus. Increased positive signals in TH-containing fibers have also been detected in the hypothalamus, and thymus grafting into newborn athymic mice normalizes these alterations. It has been previously reported that NA turnover rate in the hypothalamus is reduced by immunological processes (Besedovsky et al., 1983), and that the decrease of central NA concentration following immune stimulation is localized to specific regions (Carlson et al., 1987). Furthermore, it is known that T cells produce soluble mediators that have the capacity to evoke both central autonomic and endocrine changes (Besedovsky et al., 1983; Besedovsky et al., 1981). It is therefore likely that the absence of mature T cells in nude mice results in increased NA concentration in certain brain regions, as shown in this work. In addition, as already mentioned in the Introduction, there are different views on whether lymphoid cells can enter into a healthy brain (Engelhardt and Coisne, 2011; Kleine and Benes, 2006). In any case, taking this evidence as a whole, it can be concluded that T cells can affect noradrenergic innervation in the CNS, either directly, by T cells trafficking into the brain, or indirectly, by releasing specific molecular messengers.

This study showed that athymic mice have also higher concentrations of Tyr (the catecholamine precursor), and MHPG (NA metabolite), and DOPAC (DA metabolite), in the hypothalamus, brain stem, and hippocampus as compared to normal thymus-bearing littermates, and that thymus grafting into newborn athymic mice results in normalization of their levels in the brain. It has been

reported that brain Tyr concentration affects the synthesis of brain NA (Gibson and Wurtman, 1978). Furthermore, the simultaneous evaluation of precursor amino acid, monoamine, and metabolites is usually taken as an index of neuronal activity in the monoaminergic system (Maas et al., 1987; Murai et al., 1988; Muskiet et al., 1978). Increased Tyr/NA and MHPG/NA ratios were found in the hypothalamus of athymic mice, probably reflecting an accelerated NA synthesis and metabolism. These ratios were normalized by thymus implantation. The ratio DOPAC/DA did not significantly differ between athymic and thymus-bearing littermates, since metabolite and precursor increased both in the same proportion in athymic mice. The ratios MHPG/NA and DOPAC/DA were not significantly different in the brain stem and in the hippocampus of nude mice as compared to thymus-bearing littermates, also most likely due to the fact that metabolite and neurotransmitter were increased in the same proportion in *Foxn1*$^{n}$ mice. The exception was a decreased DOPAC/DA ratio in the hippocampus on day 60, which might be interpreted as a decreased DA metabolism in this brain region in athymic mice. This was the only time-point, the only monoaminergic metabolite, and the only brain region in which a decrease in DOPAC concentration was observed in nude mice, and this was not normalized, but even increased after thymus grafting. More experiments are needed to clarify if this finding represents a specific regional effect of the lack of mature T cells. It has been reported that NA concentration is selectively decreased in the PVN of the hypothalamus at the peak of an immune response. Decreases in NA concentration were not detected in other hypothalamic sites or in the A1 cell group of the medulla, which sends noradrenergic projections to the hypothalamus, suggesting that the effect is selective and regional. Morphometric evaluation of varicosities revealed no alterations in the density of catecholamine-containing varicosities in the PVN, further suggesting that the decrease in NA concentration is a metabolic effect and not a loss or redistribution of fibers. NA concentration also was decreased in the hippocampus at the rising phase of the immune response (Carlson et al., 1987).

Regarding indolamines, most of the 5-HT in the periphery is produced and stored in enterochromaffin cells in the gut. It is also partially stored in platelets, which can release 5-HT in multiple peripheral organs. Besides its functions in the CNS, 5-HT is involved in the regulation of vasoconstriction and vasodilation

(Frohlich and Meston, 2000) and has also been implicated in several physiological and pathological functions in peripheral organs and tissues, including the immune system (Abdouh et al., 2004; Idzko et al., 2004; Iken et al., 1995; Lesurtel et al., 2006; O'Connell et al., 2006; Walther et al., 2003).It has also been reported that platelets and 5-HT are destroyed in the spleen (Mellinkoff et al., 1962). Interesting in the context of this work is the proposal that NA alters 5-HT concentration in splenic platelets (Walker and Codd, 1985), and that 5-HT can be accumulated by noradrenergic nerve terminals (for review see Mossner and Lesch, 1998).

The only statistically significant changes in splenic 5-HT concentration and content in nude mice was an increase observed on day 21, although the same tendency was observed in 60 day-old mice. However, both the concentration of the amino acid precursor Trp and of its main metabolite 5-HIAA were significantly increased in the spleen of nude mice as compared to normal mice at most time points studied. This might indicate the reaching of a steady state after a challenge of the serotonergic system. Thymus transplantation normalized splenic Trp concentration in nude mice, while in terms of 5-HIAA, the changes induced by thymic reconstitution did not reach statistical significance.

For 20 years, 5-HT has been proposed as a modulator of T cell functions. Early studies, mainly pharmacological, already showed that 5-HT can stimulate T cell proliferation and implicated the $5\text{-HT}_{1A}$ receptor in this effect. More progress has been done recently. For example, Leon-Ponte et al. showed that the selective stimulation of $5\text{-HT}_{7}$ receptors plays an important role in the activation of naïve T cells (Leon-Ponte et al., 2007). Other work also showed that 5-HT can be transported and even synthetized by immune cells. 5-HT uptake is optimized during late stages of dendritic cell functions, such as interactions with T cells in lymphoid tissues. Similarly, B lymphocytes exhibit a marked increase in the expression of 5-HT transporters upon activation (Meredith et al., 2005), a relevant finding given the recent report that T lymphocytes can synthesize 5-HT (Leon-Ponte et al., 2007), and that T cells can also uptake 5-HT. These results are well summarized in a recent review (Ahern, 2011). As a whole, this evidence may indicate that the changes in Trp

and 5-HIAA concentration observed in the spleen of nude mice may be a consequence of the lack of mature T cells.

About 10% of the total 5-HT found in the body is synthesized in serotonergic neurons in the CNS, where it exerts several functions, including regulation of mood, appetite, sleep, and some cognitive functions, such as memory and learning, and is the target of several classes of pharmacological antidepressants (for review see Young et al., 2011). It has been reported that IL-2 injection reduces extracellular 5-HT levels in several brain regions, and that it has dose-dependent effects on depressive-related behavior after delayed, but not acute, testing, but no effects on anxiety-like behavior (Karrenbauer et al., 2011). It has also been proposed that antidepressants that target the uptake of serotonin into neurons also affect its uptake by immune cells (Ahern, 2011).

In this work, it is shown that athymic mice exhibited significantly increased concentrations of 5-HT, its precursor Trp, and its main metabolite 5-HIAA in the hypothalamus, hippocampus, and brainstem, at all ages studied. These alterations characterize an increased serotonergic activity in these brain regions, and they were all normalized to control values by thymus transplantation at birth. The ratios Trp/5-HT and 5-HIAA/5-HT were comparable in nude and normal, thymus-bearing mice. Again, also in these ratios, this is due to the fact that both substances were proportionally increased in athymic mice. The only significant effect of thymus graft on these ratios was a decrease in the hypothalamus even below the values of normal mice.

The evidence so far available regarding effects of a peripheral immune response on 5-HT levels in the brain is scarce and somewhat contradictory (for review see Mossner and Lesch, 1998). Even using the same antigen (sheep red blood cells, SRBC) to trigger the immune response, some authors reported an early increase in 5-HT concentration in the hippocampus and in the nucleus tractus solitarius, and decrease in the PVN (Carlson et al., 1987), while others found that its levels are decreased in the hypothalamus and cortex late during the immune response (Gardier et al., 1994). A third group reported increased 5-HT and HIAA in the hypothalamus, followed by a marked decrease in 5-HT level at later stages of the immune response, with similar observations in the brainstem and hippocampus (Qiu et al., 1996).

It is at present very difficult to attempt at integrating the changes in

catecholamines and indolamines observed in the spleen and in the brain of athymic mice. Some brain regions involved in the control of splenic immune function have been identified in brain lesion and stimulation studies (Irwin, 1994). However, it is unclear how these regions are interconnected and whether other brain areas are also involved (Tyagi and Divya, 2012). Stimulation or ablation of central hypothalamic nuclei is correlated with changes in electrical activity of the splenic nerve (Tyagi and Divya, 2012). For example, lesions in the preoptic nucleus of the hypothalamus raise splenic nerve activity and suppress NK cell cytotoxicity. It has also been shown that central catecholamine depletion inhibits the primary antibody response to SRBC (Cross et al., 1986; Cross and Roszman, 1988) and mitogen-stimulated splenic lymphocyte proliferation and cytokine production (Pacheco-Lopez et al., 2003). Other studies reported impairment or enhancement of splenic T-lymphocyte proliferation after injection of 6-OH-DA into the striatum and nucleus accumbens (Deleplanque et al., 1992; Deleplanque et al., 1994; Neveu and Le Moal, 1990). Whether central dopaminergic activity can contribute to regulate splenic functions needs still to be determined (Mok et al., 1990; Sim and Hsu, 1990). However, there is at present enough evidence available showing that brain NA, DA and 5-HT can contribute to the regulation of splenic functions by the CNS (Idova et al., 2012; Tyagi and Divya, 2012).

The studies reported in this work add evidence for the converse situation, namely that the homozygous presence of the *Foxn1* mutation, with the consequent lack of functional T lymphocytes, affects central and peripheral noradrenergic and serotonergic mechanisms, and that these alterations can be reversed by thymus implantation at birth.

## 5.2. The absence of a functional thymus affects corticosterone blood levels

The present results show that athymic mice have higher corticosterone blood levels than normal thymus-bearing littermates, and that, thymus grafting into newborn athymic mice normalizes the concentration of this hormone. Corticosterone blood levels in less than 1-day-old athymic mice were significantly higher than in the thymus-bearing littermates. Since it has been reported that there is a lack of response of the HPA axis to stress in early postnatal life due to the immaturity of this axis (Sapolsky and Meaney, 1986), It

is possible that such elevated levels of corticosterone derive from the blood of the mother. However, this would not explain why corticosterone blood levels in newborn mice are higher in athymic than in thymus-bearing littermates. Whether athymic mice have a different corticosterone metabolism than thymus-bearing littermates is not known, and further experiments are needed to clarify this difference. With the only exception of 7 day-old mice, the levels of corticosterone were also significantly higher in athymic than in thymus-bearing mice at all other ages studied. Independently of the presence of a thymus, corticosterone levels were higher in 14 and 21 day-old mice. Since these mice were still with the mother at the moment of killing, such increased glucocorticoid levels could have been the result of an acute stress situation at removal from the cage. Sixty-day-old mice were individually caged one week before sample collection, and this resulted in lower basal corticosterone levels. Also in this age group, athymic mice had higher glucocorticoid levels than the heterozygous control mice, and this difference was abrogated by thymus implantation at birth.

It has been shown that supernatants of activated T cells can increase glucocorticoid levels upon injection into normal mice (Besedovsky et al., 1985). It has been also demonstrated that NA levels are selectively decreased in PVN during the immune response (Carlson et al., 1987) . Since cell bodies producing CRH are localized in the PVN, NA changes in this nucleus during the immune response may be important for activation of the neuroendocrine axis, resulting in increased corticosterone production (Besedovsky et al., 1981). Interestingly, corticosterone blood levels and inflammatory responses are higher in athymic mice than in euthymic mice (Normann et al., 1988). One possibility is that these effects are mediated by IL-1, since macrophages from athymic mice produce more IL-1 than macrophages from euthymic mice when stimulated by lipopolysaccharide *in vitro* (Normann et al., 1988). Other authors demonstrated that, under basal conditions, Swiss nude mice have increased ACTH and VAP levels than normal mice, but similar corticosterone blood levels. However, when subject to certain stressful conditions, higher glucocorticoid levels were found in athymic mice as compared to the controls (Spinedi et al., 1997). On the other hand, after insulin-induce hypoglycemia and

CRH stimulation, Swiss nude mice showed significantly lower ACTH and corticosterone levels than the controls-(Gaillard et al., 1998).

The studies reported in this work showed that nude mice had increased plasma corticosterone concentrations, probably under stressful conditions, as observed in 14- and 21-day-old mice, and also under basal conditions, as in 60-day-old mice, and that the levels can be normalized by thymus implantation at birth.

## 5.3. The concentration of certain neurotrophins is affected by the absence of the thymus

The data above collectively show that the absence of a thymus is paralleled by increased NA concentration and nerve density in a lymphoid organ and in certain brain regions. One possibility to explain these results would be that athymic mice have increased levels of neurotrophins. Previous work in this Research Group has shown that the sympathetic innervation in the spleen of Fas-deficient lpr/lpr mice, which develop an autoimmune lymphoproliferative disease, is lost as the disease progresses, and that this alteration contributes to aggravate the disease (del Rey et al., 2006). As continuation of this work, it was later found that supernatants from spleen cells obtained from lpr/lpr mice inhibit dendrite growth (Diplomarbeit J.S. Grigoleit, manuscript in preparation), and significantly decrease dopamine metabolism (Diplomarbeit A. Wagner, manuscript in preparation) by PC12 cells, which are commonly used as model of sympathetic neurons. As a whole, these results indicate that immune cells produce factors that can affect noradrenergic cell growth and activity. Another interesting result derived from this work was that, in parallel to decreased NA concentration, NGF concentration is significantly lower in the spleen of lpr/lpr mice than in normal littermates.

Neurotrophins are essential for neuronal growth and differentiation during development, and important regulators of survival and maintenance of nerve cells during adulthood. They are also target-derived modulators of neuronal function in postnatal viscera (Lommatzsch et al., 2005). It is established that neurotrophin expression is altered in pathological conditions of internal organs, such as urinary bladder (Vizzard et al., 2000) and salivary gland (Lipps, 2002).

The thymic stroma generates many signals that regulate T cell development (Boyd et al., 1993). A number of thymic stroma-derived cytokines and growth factors also play an important role in driving T cell development (Lommatzsch et al., 2005), and the neurotrophic microenvironment within the thymic stroma can contribute to this process (Maroder et al., 1996). Indeed, thymic stromal cells are able to produce neurotrophic factors, such as NGF, BDNF, NT-4, and ciliary neurotrophic factor (CNTF) (Maroder et al., 1996; Screpanti et al., 1992; Screpanti et al., 1993; Screpanti et al., 1995). These factors may sustain the development and modulation of an appropriate intrathymic neural cell population able to produce selected neuropeptides and/or neurotransmitters that may, in turn, influence thymocyte development. Indeed, the expression of several neuropeptides and neurotransmitters such as catecholamines, (Leposavic et al., 2007), which are traditionally regarded as being synthesized and secreted only by neurons, has been described in thymic stroma (Ericsson et al., 1990; Geenen et al., 1986). Kurz et al. (Kurz et al., 1997) suggested that NA can influence T cell maturation by acting, not only directly on developing T cells, but also indirectly, on thymic non-lymphoid cells. In addition, Pilipović et al. (Leposavic et al., 2010) reported that thymic lymphoid and non-lymphoid cells can produced NA.

It is well established that BDNF is also a sympathetic neuron-derived factor. Developing and mature sympathetic neurons synthesize BDNF, and preganglionic neurons express the full-length BDNF/TrkB receptor. When the concentration of sympathetic neuron-derived BDNF is increased, preganglionic cell bodies and axons hypertrophy, and the preganglionic synaptic innervation to sympathetic neurons is increased. Conversely, when BDNF synthesis is blocked, the preganglionic synaptic innervation to sympathetic neurons is decreased (Causing et al., 1997). It has also been reported that NT-3 knockout mice develop severe deficits in the peripheral sensory and sympathetic nervous systems (Ernfors et al., 1995).

In this study, it is shown that athymic mice have higher BDNF and NT-3 concentrations in the spleen than their normal thymus-bearing littermates, and that this situation does not reflect a general increase in neurotrophin concentrations, since no significant differences were found in the kidney. Furthermore, the results also indicate that such alterations are not genetically

programmed, since thymus grafting into newborn athymic mice normalizes BDNF and NT-3 concentrations.

It has been reported that NGF levels correlate with the density of sympathetic innervation, and that sympathetic ganglia contain the highest NGF levels of all tissues studied (Korsching and Thoenen, 1983). This observation may explain the positive correlation between NA and NGF concentrations reported here.

Neurotrophins also play an important role in the CNS. Neurotrophins have emerged as major modulators of synaptic plasticity (Lu, 2003; McAllister et al., 1999), the dominant underlying mechanism for brain function (for review see Je et al., 2011). For example, it is well established that NGF is essential for the formation of central pain circuitry. Exogenous administration of this neurotrophin to rodents results in the rapid onset of hyperalgesia (Taiwo et al., 1991). Together with its receptors (p75 and TrkA), it plays a critical trophic role on forebrain cholinergic neurons that degenerate during brain aging and neurodegenerative disorders (Fischer et al., 1987; Sofroniew et al., 2001). During development, both NGF and BDNF regulate naturally occurring cell death, synaptic connectivity, fiber guidance, dendritic morphology, and neuroplasticity (for review see Berry et al., 2012). It has also been established that BDNF affects hippocampal development and neuroplasticity. Increased BDNF signaling enhances neurogenesis, neurite sprouting, electrophysiological activity, and other processes reflective of a general enhancement of hippocampal function. Increased hippocampal activity may mediate beneficial effects, such as enhanced learning and memory (for review see Murray and Holmes, 2011). Interestingly, it was also found that T cells can affect spatial learning and memory, and the expression of BDNF in the DG (Ziv et al., 2006). It has also been reported that microglia are a source of trophic factors known to support the development and normal function of CNS cells. On the other hand, BDNF and NT-3 induce microglia proliferation and phagocytic activity *in vitro* (Elkabes et al., 1996). The loss of BDNF during earlier stages of development causes hyperactivity and more pronounced hippocampal-dependent learning deficits. The absence of BDNF in the forebrain attenuates the actions of the anti-depressant desipramine, in the forced swim test, suggesting the involvement of BDNF in anti-depressant efficacy (Monteggia et al., 2004).

Reduced NGF and BDNF signaling in the adult brain may be involved in the pathophysiology of psychiatric disorders, such as depression (for review see Berry et al., 2012). Interestingly, depressed patients have decreased serum levels of BDNF, and the treatment with anti-depressants promotes an increase in these levels. Anti-depressants drugs, which mainly act by increasing the levels of the monoamines serotonin and noradrenaline in the synaptic cleft, are associate with several structural and neurochemical changes in which the levels of neurotrophins, particularly of BDNF, are altered (for review see Neto et al., 2011). It is also interesting to note that BDNF, but not NGF, decreases 5-HT uptake in some B lymphoblast lines, which would functionally lead to increased extracellular 5-HT levels (Mossner et al., 2000). Indeed, in studies in the rat, it has been found that BDNF increases 5-HT levels in the brain and spinal cord (Siuciak et al., 1994). In a subsequent study, this group showed that BDNF increases tryptophan hydroxylase mRNA levels in the rat brain (Siuciak et al., 1998).

In this study, it is shown that athymic mice have higher BDNF and NT-3 concentrations in defined brain regions than their normal thymus-bearing littermates. The results also indicate that such alterations are not genetically programmed since thymus grafting into newborn athymic mice normalizes BDNF and NT-3 concentrations.

In addition, although there are some tendencies, the results showing that there are no statistically significant differences between athymic mice and their normal thymus-bearing littermates in NGF concentration in the spleen and brain regions, indicate that there is some selectivity in the neurotrophins affected by the lack of a functional thymus. On the other hand, significant correlations between NGF and NA concentrations were detected in both athymic mice and their normal thymus-bearing littermates. It seems that this correlation was independent of the presence of T cells, because there were no differences in the slope of the regression curves between athymic and thymus-bearing littermates. At the same time, they also indicate that there is a relation between the concentrations of NGF and NA present in an organ.

Immunohistochemical studies also showed higher BDNF fluorescent signals in the hippocampus of athymic mice as compared to normal thymus-bearing littermates, and that thymus grafting into newborn athymic mice abrogated this

difference. These studies also showed a different distribution of these signals in the hippocampus of athymic mice compared with their normal thymus-bearing littermates. Again, thymus grafting into newborn athymic mice abrogated this difference. While performing these studies, it became apparent that the histological pattern of the different regions in the hippocampus of athymic mice differed from that of the normal mice. In fact, the standard histological studies that were performed as consequence of these observations showed that regions such as CA1, CA2, and CA3, and the DG are ill defined in the hippocampus of athymic mice. More detailed histological studies are needed to clarify this aspect. In this context, it is interesting to note that the groups of J. Kipnis (USA) and M. Schwartz (Israel) have recently shown that T cells can affect adult neurogenesis by affecting progenitor-cell proliferation and neuronal differentiation, as well as particular learning abilities. Ziv et al. (Ziv et al., 2006) also found that there is less dendritic arborization in the DG of nude as compared to wild-type mice, and less percentage of cells expressing early neuronal differentiation markers.

## 5.4. Sympathetic denervation at different stages of life affects NA, neurotrophin, and corticosterone concentrations

The previous parts of this work showed that the increase in noradrenergic innervation in the spleen and brain of athymic mice is paralleled by increased concentration of the neurotrophins BDNF and NT-3. The fact that both changes could be reverted by thymus implantation indicated that it is unlikely that these alterations are fortuitously and independently associated to the *Foxn1* genetic mutation, or to other alterations observed in athymic mice, such as lack of hairs. The positive correlations established between NA and NGF concentrations also suggested that these parameters are related. Although no causal effects can be concluded from these correlations alone, it is well established that neurotrophins are essential for neuronal development and functioning. Different approaches can be followed to study whether manipulation of one of these parameter results in changes in the other. For example, one possibility would be to study the effect of increasing NA concentration on the amount of neurotrophins that are produced *in vivo*, or vice versa. However, this type of experiments is difficult to interpret, since it is

almost impossible to mimic local increases on neurotransmitter or neurotrophins in tissues by administration of noradrenergic agonists or neurotrophic factors. The disadvantage is also that systemic administration of noradrenergic agonists or blockers results in many other changes in the host, and are, in general, of acute nature. Another alternative is to determine the impact that selective depletion of noradrenergic fibers has on neurotrophin concentration. This was the approach chosen here, since it is well known that chemical sympathectomy results in prolonged changes in the sympathetic tonus, and that sympathectomized animals can well survive this treatment as long as they do not have to cope with very stressful situations or marked changes in ambient temperature. Although this approach has also drawbacks, it was chosen as a first attempt to study whether decreasing noradrenergic innervation at a given stage of development affects neurotrophin concentration in the spleen and brain. For this purpose, 6-OH-DA was injected into normal mice at different ages and neurotrophin concentration was determined in the spleen and some brain regions.

It is well established that the neurotoxin 6-OH-DA destroys sympathetic nerve terminals. Injection of this neurotoxin at birth results in permanent destruction of sympathetic nerve terminals (Glatzel et al., 2001; Thoenen and Tranzer, 1968), while administration of 6-OH-DA during adult life results in a more transient sympathectomy (del Rey et al., 2002; Glatzel et al., 2001). A large percentage of the cell bodies in sympathetic ganglia are completely and permanently destroyed by 6-OH-DA administered at birth due to the sensitivity of the perikaryon to the neurotoxin (Angeletti, 1971; Angeletti and Levi-Montalcini, 1970a, b). It has been proposed that the sensitivity to 6-OH-DA is decreased in adult animals due to less accumulation of the neurotoxin in the perikaryon than in the terminal nerve endings (Tranzer, 1971). Others authors argued that the sensitivity of the perikarya to 6-OH-DA cannot account for the different effects because there are no big differences between young and adult animals in the size of the neuronal cell body (Furness, 1971). Some authors suggested that the fact that cell bodies are incompletely covered by a layer of satellite cells in the neonate could explain the different susceptibility to 6-OH-DA (Bloom, 1971). The size of the dendritic tree may be also important in sequestering significant amounts of 6-OH-DA. In the neonate, more 6-OH-DA

is available for uptake by perikarya due to less arborization of terminal plexuses (Cheah et al., 1971).

Four groups were used in the studies reported here, in which 6-OH-DA was injected to newborn, 2 week-old, and adult mice, and animals were sacrificed at different ages. The aim was to study the effect of destroying sympathetic nerve fibers at different stages of development on NA and neurotrophin concentrations in the spleen and brain.

A substantial and significant decrease in splenic NA concentration was observed in all groups. It has been previously shown that 6-OH-DA administration at birth results in a permanent reduction of noradrenergic fibers in peripheral organs. The results reported here show that a permanent splenic denervation is also observed when the neurotoxin is administered into 14 day-old mice. A nearly complete (approximately 90%) decrease in splenic NA concentration is as well observed shortly (7 days) after adult sympathectomy, and previous findings in this Research Group have shown that the levels of the neurotransmitter in the spleen are practically normalized 2 weeks later (unpublished results).

The effect of 6-OH-DA on NA concentration in the brain observed in the experiments reported here differed from that in the spleen, since the reduction in the concentration of the neurotransmitter was less pronounced, particularly in the hippocampus, when the neurotoxin was administered to adult mice. It has been reported that 6-OH-DA administered peripherally can cross the still not completely developed BBB in newborn animals, thus reducing NA content in the CNS much more significantly than in adult animals (Lytle et al., 1972). The results obtained here showing that the degree of NA depletion in the hypothalamus and hippocampus was more marked when mice were denervated at birth than when the neurotoxin was injected into adult mice, agree with this and several other reports. Interestingly, the depletion of NA was remarkably more pronounced in the hippocampus than hypothalamus. It has been reported that all brain regions can be affected by 6-OH-DA administration, albeit not all to the same degree (for review see Kostrzewa and Jacobowitz, 1974).

As mentioned above, the main scope of these experiments was to study whether alterations in the SNS at different ages result in changes in neurotrophin concentration. It is shown here that NGF and BDNF

concentrations were decreased in the spleen and in defined brain regions of mice denervated at birth or when they were 14 day-old, conditions under which sympathetic nerve terminals are nearly completely and permanently destroyed, as compared to the corresponding control groups. These results are in agreement with the results reported above, showing that BDNF concentrations are higher in the spleen and brain of athymic mice, which have more noradrenergic innervation than normal thymus-bearing mice. The results are also in accordance with the positive correlation between NA and NGF concentrations detected in the spleen and brain regions, as reported in this work, and with previous findings showing that a decrease in the density of sympathetic innervation ia paralleled by decreased NGF concentration (Korsching and Thoenen, 1983). It has also been reported that BDNF transgenic and knockout mice have increased, respectively decreased, sympathetic innervation (Causing et al., 1997)

However, administration of 6-OH-DA into 60 day-old mice, in which also NA concentrations were decreased, resulted in increased NGF and BDNF concentrations in the spleen and hippocampus. Several factors may explain the opposite effects of denervation on neurotrophin concentrations. Among them, the age at which 6-OH-DA was administered, resulting either in permanent or transient denervation might be particularly relevant. It has been proposed that the accumulation of NGF in target tissues after 6-OH-DA injection may be due not only to the lack of NGF removal by retrograde transport, but also reflect an increased rate of synthesis influenced by the presence or activity of the sympathetic nerve terminals (Barth et al., 1984). It has also been reported that the damage of dopaminergic neurons by intra nigral or intra striatal 6-OH-DA injection may result in increased NGF (Nitta et al., 1992) and BDNF (Branchi et al., 2010) synthesis in brain. This evidence and the differences in experimental design mentioned above might contribute to explain the results showing that neurotrophin concentrations were increased in the spleen and brain following administration of 6-OH-DA to adult animals. It is clear that additional experiments are needed to further clarify this problem. However, as a whole, the results presented in this work show that: 1) the effect of NA depletion on neurotrophin levels depend on the age at which the neurotoxin is administered,

and 2) NA depletion for a relatively short time has different effects than a more prolonged absence of noradrenergic fibers.

Previous work in this Research Group has shown that local surgical denervation of the spleen or general chemical sympathectomy by 6-OH-DA results in increased immune responses (del Rey et al., 1981), indicating that the immune cells involved are released from the suppressive action of NA (Besedovsky et al., 1979). As continuation of this work, this group also found that sympathetic denervation results in decreased SEB-induced cell proliferation and IL-2 production, and impedes the specific clonal deletion induced by the superantigen in normal mice, without affecting anergy (del Rey et al., 2002). A number of other studies have also used 6-OH-DA to investigate the effects of sympathetic denervation on various aspects of the immune response, including *in vivo* (Madden et al., 1994) and *in vitro* (Kruszewska et al., 1995) proliferation of lymphocytes, mitogen-induced T cell proliferation (Lyte et al., 1991), and cytokine production (Lyte et al., 1991). Also, the destruction of noradrenergic fibers by 6-OH-DA leads to the recruitment of phagocytic cells (Perry et al., 1987). In turn, these cells can release cytokines such as IL-1β and IL-6, both of which have been implicated in the activation of the HPA axis (Besedovsky et al., 1986; Kim et al., 2009; Watkins et al., 1995). In addition, destruction of peripheral noradrenergic fibers in adult mice may stimulate feedback mechanisms that ultimately signal activation of the HPA axis (Leo et al., 1998). This mode of activation may be mediated by the connections between catecholaminergic nuclei in the brainstem and the PVN of the hypothalamus (Leibowitz et al., 1989; Leo et al., 1998; Swanson and Kuypers, 1980).

As it happened with the concentration of the neurotrophins, also corticosterone blood levels were differentially affected, depending on the age at which 6-OH-DA was injected. Administration of the neurotoxin to newborn or 14 day-old mice resulted in decreased corticosterone blood levels when mice were 21 day-old. As in the experiments performed in athymic mice, the litters were still with the mothers at the moment of sacrifice, and not individually caged, as it was done with adult mice. This procedure could have resulted in a considerable acute stress, which may explain the high levels of corticosterone determined at this age in the control mice. Albeit this observation, the results show that, under the same conditions, corticosterone levels in denervated mice

were significantly lower than in vehicle-injected mice. This finding is in agreement with the report by Jiang et al (Jiang et al., 2004) showing that i.p. injection of 6-OH-DA blocks cold stress-induced suppression of NK cytotoxicity. In this publication, it was also shown that cold stress-induced suppression of splenic NK activity, Fos expression in the PVN, and the elevation of the plasma corticosterone concentration were abrogated by central administration of the neurotoxin (Jiang et al., 2004).

In this work, it is reported that peripheral injection of 6-OH-DA also results in decreased NA concentration in the hypothalamus, although to different degrees depending on the age at which the neurotoxin is administered. Although NA has been long considered as an inhibitor of the HPA axis, further studies have demonstrated that it can also have stimulatory influences (for review see Locatelli et al., 2010). These apparent discrepancies are most likely due to the different experimental approaches used to address this matter, raging from situations of stress of different magnitude, or infusion of adrenergic agonists or antagonists to *in vitro* experiments. Furthermore, it seems at present that whether the HPA axis is inhibited or stimulated by NA depends also on the type of adrenergic receptors that are involved, $\alpha$ receptors being stimulatory and $\beta$ inhibitory. Both types of effects have been found in the experiments reported in this work. The relatively modest decrease in NA concentration in the hypothalamus of denervated, adult mice was paralleled by increased corticosterone blood levels, which would agree with an inhibitory effect of the neurotransmitter on the HPA axis. Conversely, decreased corticosterone blood levels were detected in young mice denervated when they were 1- or 14 day-old, and in which NA concentration in the hypothalamus was reduced by more than 50% by peripheral injection of 6-OH-DA. These results would agree with those obtained in athymic mice, in which higher catecholamine concentrations in the hypothalamus were paralleled by increased corticosterone blood levels.

It should be considered, however, that several other factors could contribute to changes in the activity of the HPA axis following denervation, for example, an increase in the release of pro-inflammatory cytokines, as mentioned above. Increased levels of certain cytokines could also explain the results observed in athymic mice. For example, it has been reported that, as compared to euthymic mice, macrophages from athymic mice stimulated *in vitro* with

lipopolysaccharide produce more IL-1 β (Normann et al., 1988), a cytokine that can strongly stimulate the HPA axis (Besedovsky et al., 1986),.

## 5.5. Conclusions and perspectives

The evidence available indicates that the effect of sympathetic neurotransmitters and neuro-endocrine mechanisms under the control of central noradrenergic neurons on immune cell activity does not represent a unidirectional process, but that such influences are bi-directional. Indeed, the presence of a functional thymus, and the consequent reconstitution of the host by T cells, can also influence the development of neural structures. The results in nude mice reported here are in agreement with the view that, in general, immune cells play an inhibitory role on the development of the sympathetic innervation of lymphoid organs and/or in the activity of the SNS and of central noradrenergic neurons (Besedovsky et al., 1979; del Rey et al., 1981). This mutual immune-neural effects re-inforce the concept that a neuro-endocrine-immune network of interactions operates during development and adult life.

In brief, the results described in this work show that: 1) the absence of a functional thymus results in increased neurotransmitter and neurotrophin concentrations in the spleen and in defined brain regions, and in corticosterone concentrations in blood; 2) these alterations are completely normalized when athymic mice are implanted with a thymus at birth; 3) the levels of neurotrophins in the spleen and brain are affected by the degree of noradrenergic innervation, as shown by a pharmacological manipulation that destroys of sympathetic nerve terminals. These results indicate that the alterations observed in athymic mice are not just a genetic epiphenomenon fortuitously associated with the absence of a functional thymus, but rather that mature T cells, by acting either directly or indirectly, exert an inhibitory influence on the development of splenic sympathetic innervation and noradrenergic innervation of the CNS. At the same time, these findings open further questions and offer many perspectives for future work. Some of them are briefly commented below.

It would be certainly necessary to formally prove that the alterations detected in athymic mice, in particular those at central levels, are caused by

the lack of mature T cells, by directly reconstituting the animals with this cell type. Also, it would be interesting to study the contribution of the innate immune system to the changes detected, since the animals used for the studies reported here were not bred under germ-free or specific-pathogen free conditions and nude mice have a rather active innate immune system.

This work concentrated on the study of neurotrophins as possible contributors to the altered innervation observed in athymic mice. However, it is conceivable that other mediators, such as semaphorins, could be involved as well. Semaphorins are a class of secreted and membrane-bound proteins that, by acting as repellent or attractant factors, affect axon and dendrite growth, and control synapse formation and function (for review see Yoshida, 2012), and also play a relevant role in immune function (for review see Takamatsu and Kumanogoh, 2012).

As mentioned, while performing the studies reported here, it was found that there are marked alterations in the anatomy of the hippocampus. These studies should be also extended to other brain regions, since changes in BDNF and NT-3 concentrations can affect neurogenesis in the whole brain.

Besides affecting many physiological functions, most of the neurotransmitters and neurotrophins, whose levels are altered in the brain of nude mice, are also involved in the control of behavior. In fact, as already mentioned, it has been shown that T lymphocytes are important to maintain hippocampal neurogenesis and spatial learning abilities (Ziv et al., 2006). Coupled to this recent evidence, the results reported in this work offer several perspectives for future research in other related disciplines. For example, it is interesting to mention here that, while breeding athymic mice, it was observed that heterozygous *Foxn*1$^{n}$/*Foxn1*$^{+}$ mothers seem to have a preference in the handling of normal thymus-bearing pups as compared to the athymic littermates. A very interesting, although complex, aspect to investigate would be to study if athymic pups, already at birth, would show a different behavior that could justify this observation.

The human equivalent of the murine nude phenotype was first reported in 1996 (Pignata et al., 1996). As in mice, also in humans this form of immunodeficiency is characterized by an intrinsic defect of the thymus, together with congenital alopecia and nails dystrophy, and is due to mutations in the *Foxn1* gene. This mutation in human results in prenatal blockage of CD4+ T cell maturation and

severe impairment of CD8+ cells (Vigliano et al., 2011). It would be of basic and clinical relevance to ascertain whether alterations in neurotransmitters and neurotrophins similar to those reported here in mice carrying the mutated *Foxn1* gene, are also present in these patients. The discovery of the mutation in humans is rather recent, but it is interesting to mention in this context that brain alterations have been reported in two human fetuses carriers of this mutation (Amorosi et al., 2010).

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# 7. Appendix

## 7.1. Abbreviations

| | |
|---|---|
| 5-HIAA | 5-hydroxy-indol-acetic acid |
| 5-HT | Serotonin |
| 6-OH-DA | 6-hydroxydopamine |
| A | Adrenaline |
| Ab | antibody |
| ACTH | adrenocorticotrophic hormone |
| ANS | autonomic nervous system |
| APTES | amino propyl triethoxy saline |
| AVP | arginine vasopressin |
| BBB | blood-brain barrier |
| BCA | bicinchoninic acid |
| BDNF | brain-derived neurotrophic factor |
| BSA | bovines serum albumin |
| CNS | central nervous system |
| CRH | corticotrophin releasing hormone |
| CSF | cerebrospinal fluid |
| DA | Dopamine |
| DBH | Dopamine-ß-hydroxylase |
| dist.w. | distilled water |
| DG | dentate gyrus |
| DOPAC | dihydroxylphenylacetic acid |
| ELISA | enzyme linked immunosorbent assay |
| Fig | figure |
| *Foxn1* | forkhead box |
| HPA axis | hypothalamus- pituitary- adrenal axis |
| HPLC | high performance liquid chromatography |
| HRP | horseradish peroxidare |
| i.p | intraperitoneal |
| IL-1 | Interleukin-1 |
| IL-2 | Interleukin-2 |
| IL-6 | Interleukin-6 |

| | |
|---|---|
| MHPG | 4-hydroxy-3-methoxyphenylglycol-hemipiperinium salt |
| min | minute |
| NA | Noradrenaline |
| NGF | Nerve growth factor |
| NK | Natural killer |
| NT-3 | Neurotophins-3 |
| NT-4 | Neurotrophins-4 |
| OD | optical density |
| PALS | periarterial lymphatic sheath |
| PBS | phosphate buffer saline |
| PNS | peripheral nervous system |
| PVN | paravertricular nucleus |
| RT | room temperature |
| SCID | severe combined immunodeficiexy |
| SNS | sympathetic nervous system |
| TH | Tyrosine hydroxylase |
| TMB | tetramethylbenzidine |
| TNFα | tumor necrosis factor-α |
| Trk | Tyrosine kinase |
| Trp | Tryptophan |
| Tyr | Tyrosine |

## 7.2. Cirrculum vitae

### Personal Details

| | |
|---|---|
| Name: | Jamela Aesa Kadhem Jouda |
| Place and date of birth: | Maysan (Iraq), 05.06.1975 |
| Address | Am Richtsberg 88, Appartment: 909, 3539 Marburg |

### Education

| | |
|---|---|
| 1993: | Al-Najat Gymnasium, Baghdad, Iraq. Qualification level: Abitu/very good |
| 1994-19997: | B.Sc. in Microbiology, College of Science, University of Al-Mustansiriyah, Baghdad, Iraq. Degree: very good |
| 2000-2003: | M.Sc. in Animal Science (Histology and Physiology), College of Science, University of Al- Mustansiriyah, Baghdad, Iraq. Degree: very good.<br>Diploma thesis: Sclerotherapy evaluation of simple renal cyst. |
| 2009-2012: | Ph.D. study under the supervision of Prof. Dr. A. del Rey, Dept. Immunphysiology, Institute of Physiology and Pathophysiology, Medical Faculty, Philipps University, Marburg, Germany. Project: Interaction between the endocrine, the nervous, and the immune system. |

### Qualifying Courses

| | |
|---|---|
| 1999: | Third qualifying courses for Computer, The Center of Electronic Computer, Al-Mustansiriyah University, Iraq. |
| 2003: | Education qualifying courses in teaching methods; Education College, Al-Mustansiriyah University, Iraq |
| 2007: | Language Courses (Germany), speak and write, Marburg, Germany. |
| 2009: | 4$^{th}$ German-Endocrine-Brain-Immune-Network (GEBIN) Educational Course on Immune-Neuro-Endocrine Interactions; Frankfurt, Germany |
| 2011: | ISNIM/German-Endocrine-Brain-Immune-Network (GEBIN) Educational Course on Immune-Neuro-Endocrine Interaction; Dresden, Germany. |
| 2012: | Animal experimental training; Gießen, Germany.<br>Application and withdrawal of blood techniques in mause and rat; Frankfort, Germany. |

### Fellowship

- Ministery of Education of Iraq to perform the Doctoral Thesis in Germany
- Volkswagen Stiftung (to attend the 18$^{th}$ International Congress of the International Society of NeuroImmunoModulation, Dresden, Germany, 20-22 October 2011).

- Ministery of Education of Iraq to attend the Training Corse on Animal Expermentation, Gießen, Germany.

**Professional experience**

1997-2003: Research Assistant, College of Science, University of Al-Mustansiriyah, Iraq

1999-2001: Research Assistant, Al-Ataa Laboratory, Medical Al-Ataa Society, Baghdad, Medical Cooperative Society Iraq. Experience in laboratory diagnosis (Hematology, Microbiology, Biochemistry, and Serology).

2003-2008: Assistant Teacher, Department of Biology/Zoology, College of Science, University of Al-Mustansiriyah, Iraq
Teaching courses: Animal Histology and Physiology, Medical Parasitology.
Supervision of research of under graduate studies.

**Oral presentation in Congresses**

2011: 8th congress of the international Society for Neuro-Immuno-Medulation (ISNIM) orgaized together with the German Endocrine-Brain-Immune Network (GEBIN); Dresden, Germany.
Title: T cells affect central and peripheral noradrenergic mechanisms and neurotrophin concentration in the spleen and hypothalamus.

**Publications**

J. Jouda, J. Wildmann, M.Schäfer, H.O. Besedovsky, and A. del Rey. T cells affect central and peripheral noradrenergic mechanisms and neurotrophin concentration in the spleen and hypothalamus, NeuroImmunoModulation, 18:380, 2011 (abstract)

J. Jouda, J. Wildmann, M. Schäfer, E. Roggero, H. O.Besedovsky, and A. del Rey. T cells affect central and peripheral noradrenergic mechanisms and neurotrophin concentration in the spleen and hypothalamus, Ann. N.Y. Acad. Sci., 1261: 18–25, 2012

**Language skills**

Arabic: Native language
English: Speak and write well
German: Speak and write acceptable

### 7.3. Academic teacher

My academic teachers in Irak were:

Prof. Dr. Aesa
Prof. Dr. Al-Ahny
Dr. Al-Azawi
Dr. Al-rekabi
Dr. Al-Saech
Dr. Authman
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Prof. Dr. Ehssan
Prof. Dr. Hamudi
Dr. Khamas
Prof. Dr. Khattab
Dr. Manklo
Dr. Manssor
Dr. Mohamad Ali
Dr. Nachelan
And other professors and doctors of Biology department of Science college of Al-Mustansiriya university, Iraq.

In Marburg:
Prof. Dr. Phil. A. del Rey
Prof. Dr. H.O. Besedovsky
Dr. J. Wildman
Dr. M. Schäfer
Dr. M. Wöher

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