Neuroserpin and brain-derived neurotrophic factor in neuroendocrine and neuronal plasticity

Functional studies in (transgenic) *Xenopus* intermediate pituitary cells

Neuroserpin and brain-derived neurotrophic factor in neuroendocrine and neuronal plasticity- Functional studies in (transgenic) *Xenopus* intermediate pituitary cells/ Dorien M. de Groot/ PhD thesis Radboud University Nijmegen, The Netherlands

ISBN-10: 90-9021251-5
ISBN-13: 978-90-9021251-7

Cover illustration: Drawing of a neuronal network presented as a jigsaw puzzle
Cover design: Dorien de Groot

Printed by: PrintPartners Ipskamp, Enschede

Neuroserpin and brain-derived neurotrophic factor in neuroendocrine and neuronal plasticity

Functional studies in (transgenic) *Xenopus* intermediate pituitary cells

Een wetenschappelijke proeve op het gebied van de Natuurwetenschappen, Wiskunde en Informatica

Proefschrift

ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van de rector magnificus prof. dr. C.W.P.M. Blom, volgens besluit van het College van Decanen in het openbaar te verdedigen op vrijdag 19 januari 2007 om 10:30 precies door

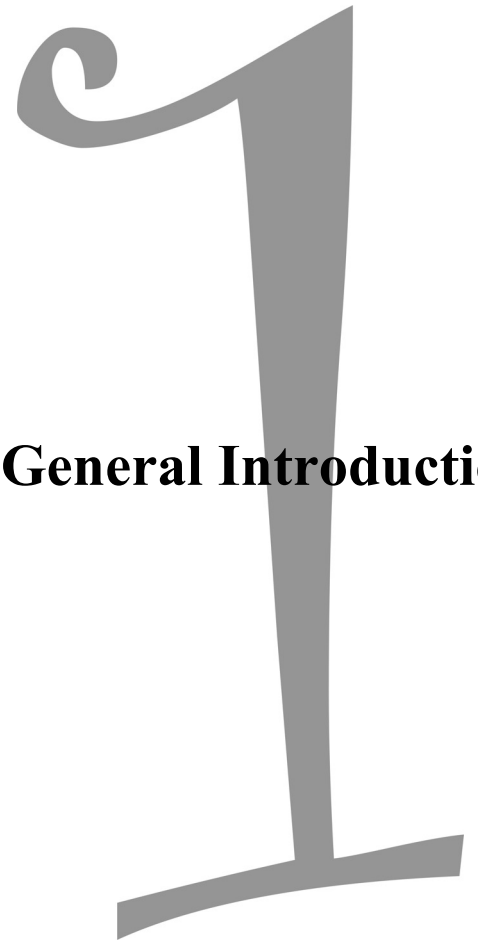
Dorien Martine de Groot

geboren op 24 september 1978 te Buchten

Promotor:
Prof. dr. G.J.M. Martens

Manuscriptcommissie:
Prof. dr. E.J.J. van Zoelen
Prof. dr. H. Vaudry (l'Université de Rouen, France)
Dr. P. Wesseling

Contents



General Introduction

In the brain, a high degree of neuronal flexibility is needed for proper development of its neuronal network and because the connections between neurons continuously change, *e.g.* during learning and memory formation. To allow flexibility, the brain is able to reorganize itself by forming new neuronal connections throughout life and neurons alter the strength of their synaptic connections with other neurons in response to activity and experience (a process called synaptic plasticity), resulting in an overall neuronal plasticity. Thus, neuronal plasticity plays an essential role in information processing in the nervous system. The molecular mechanisms underlying this process are, however, still far from being completely understood. In this thesis, two proteins that are involved in neuronal plasticity are examined, namely the neuroendocrine-specific serine protease inhibitor neuroserpin and the neurotrophin brain-derived neurotrophic factor (BDNF).

The serine protease inhibitors (serpins) and the group of proteins they inhibit, the serine proteases, play a role in the mechanisms responsible for the interaction of a (nerve) cell with its environment. The environment in which cells reside and which provides the structural integrity of tissues is formed by the extracellular matrix (ECM). The integrity of the ECM is tightly controlled by a balance between matrix-degrading proteases and protease inhibitors. The ECM consists of a complex of collagen, glycoproteins and proteoglycans, and an increasing amount of data supports the involvement of several ECM molecules in neurite outgrowth and synaptic plasticity (1-4). The serine proteases and serpins, and in particular neuroserpin, will be discussed in more detail below.

The second protein dealt with in this thesis is BDNF, a member of the neurotrophin (NT) family and implicated in inter-cellular signaling in the nervous system. Neurotrophic factors are traditionally viewed as secretory proteins that regulate long-term survival and differentiation of neurons. In addition, it has now been well accepted that NTs play an important role in synapse development and plasticity (reviewed in 5-10). The family of NTs and especially BDNF will also be described in more detail below.

Serine proteases and serine protease inhibitors

In addition to their role in maintaining ECM integrity, proteases are involved in many other biological processes, such as the digestion of proteins to supply amino acids, proenzyme activation, clot formation and lysis, angiogenesis and complement activation. To meet these needs, a series of families of proteases have evolved, namely the serine proteases, threonine proteases, cysteine proteases, aspartyl proteases and metalloproteases. The serine proteases form an important group of proteases and their name is derived from the active serine residue in their catalytic site. Serine proteases can be divided into two main evolutionary groups, namely the chymotrypsin-like serine proteases and the subtilisin-like proprotein convertases, and both groups can be further classified into families. The family of trypsin-like enzymes belongs to the chymotrypsin-like group, includes trypsin, tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), kallikreins and

plasmin, and has been most extensively characterized (11, 12). For example, t-PA is known to cleave plasminogen into plasmin (13) and, in turn, plasmin can cleave ECM components involved in neuronal plasticity, such as laminin (14) or the cell-adhesion molecule L1 (15). Examples of the group of subtilisin-like proprotein convertases are furin and the prohormone convertases 1 and 2.

The evolution of serine proteases is paralleled by the development of serine protease inhibitors, termed serpins, which constitute a superfamily that currently consists of ~500 members. Examples of serpin family members are α1-antitrypsin, antithrombin III, plasminogen activator inhibitor-1 and -2, C1 inhibitor, thyroxine-binding globulin, maspin and neuroserpin, one of the two proteins dealt with in this thesis. Serpins differ from other enzyme-inhibiting families by their capacity to undergo a marked conformational change and to act as suicide substrate inhibitors; serpins can form an unusually tight and SDS-stable interaction with their substrate protease after which drastic conformational changes take place, rendering both the serine protease and its serpin inactive (reviewed in 16, 17) (Figure 1). However, this inherent molecular flexibility also makes serpins susceptible to point mutations that result in intermolecular linkage and polymer formation. The effect of such protein aggregation is cumulative, with a progressive loss of cellular function, eventually resulting in a disease, collectively named serpinopathies (18-21).

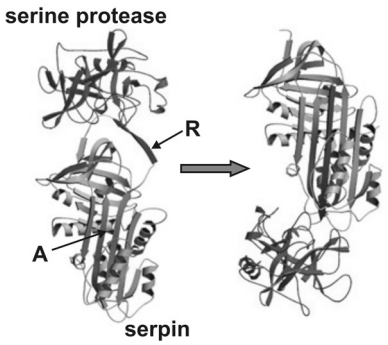


Figure 1: **Conformational transition of serpin family members**. Members of the serpin superfamily, such as the depicted α1-antitrypsin, undergo a striking conformational transition to inhibit their target protease. After docking, the target serine protease is inactivated by movement from the upper to the lower pole of the protein. This is associated with the insertion of the reactive centre loop of the serpin (R) as an extra strand into β-sheet A of the serpin. Adapted from Lomas and Carrell (20).

Neuroserpin

Neuroserpin was discovered more than 16 years ago as a secreted protein from chicken dorsal root ganglia cells (22) and received its name because of its expression in the developing and adult brain (23, 24). Neuroserpin has also been identified in neuroendocrine cells, such as in rat pituitary and adrenal glands (25, 26). Like other serpin family members, neuroserpin undergoes a marked conformational transition in order to be able to function as an inhibitor. Consequently, a number of mutations in the neuroserpin gene lead to a serpinopathy. Thus far, five mutations have been described which lead to polymerization

and accumulation of the protein, resulting in neuronal inclusion bodies and subsequent neuronal degeneration. These mutations result in the recently described autosomal-dominant dementia 'familial encephalopathy with neuroserpin inclusion bodies' (FENIB) (20, 27, 28).

In vitro studies have assigned t-PA as a target protease for neuroserpin (24, 29). This was supported by the *in vivo* finding that injection of neuroserpin markedly delays the progression of seizure activity in wild-type, but not in t-PA-deficient, mice (30-32). Nevertheless, gene-targeting of neuroserpin in mice has implicated the inhibitor to play a role in the regulation of emotional behaviour through a mechanism that is at least in part independent of t-PA activity (33). Thus, the cognate target of neuroserpin may be another, as yet unidentified, serine protease.

The exact physiological function of neuroserpin in the nervous system remains to be elucidated. The temporo-spatial pattern of expression and the fact that it is secreted from axons implicate neuroserpin as a regulator of proteolytic events associated with neuronal plasticity (23, 24, 29, 34, 35). Since small changes in neuroserpin expression result in changes in the number of neuronal cells extending neurits and total neurite length following nerve growth factor treatment, neuroserpin may as well play a role in cell communication, cell adhesion and/or cell migration (25, 26). Thus, further studies are necessary to unravel the exact physiological role of neuroserpin. Part of the research described in this thesis involved our attempts to increase the understanding of this serpin.

(Pro-)neurotrophins

The second subject of this thesis, the NT family member BDNF, has also been implicated in the mechanism of neuronal plasticity, as will be described in more detail in the next section. The NT family consists of structurally related neurotrophic factors that exert a wide array of effects in the central and peripheral nervous systems. Currently, the family consists of nerve growth-factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), and two members that have been identified only in fish and not in other vertebrates, namely neurotrophin-6 (NT-6) and -7 (NT-7) (reviewed in 36).

NTs exert their actions via two classes of transmembrane receptors, the receptor tyrosine kinases (Trk)-A, -B, and –C, and the p75 NT receptor ($p75^{NTR}$), a member of the tumor necrosis factor (TNF) receptor superfamily (reviewed in 37). Whereas $p75^{NTR}$ binds all NTs equally well, TrkA preferentially interacts with NGF, TrkB with BDNF and NT-4/5, and TrkC with NT-3. Interaction of NTs with $p75^{NTR}$ causes the activation of various signaling pathways, such as increased *c-jun* N-terminal kinase or NF- κB activities, resulting in effects on cellular apoptosis, survival, neurite elongation and growth arrest. Binding of a NT dimer to a specific Trk receptor induces the receptors tyrosine kinase activity, leading to the activation of phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and phospholipase C-γ (PLC-γ) pathways, resulting in

cell survival and differentiation (reviewed in 37-40). In addition to the intact Trks, carboxy-terminal truncated forms of TrkB and TrkC that lack a tyrosine kinase domain have also been described (41-44). The roles of the truncated receptors remain enigmatic, but various functions have been suggested, including growth and development (45-47), negative modulation of TrkB receptor expression and function (48-50), and calcium signaling in glial cells (51).

Like many other growth factors, the NTs are synthesized as 30-35-kDa immature precursors that are proteolytically cleaved intracellularly, by furin and other proconvertases, to release mature ligands of 12-14 kDa (52). In addition, it was recently found that also uncleaved proNTs can be released by cells (53-56). NTs are secreted constitutively from nonneuronal cells (52), but secretion can be mediated by both constitutive and regulated secretory pathways in neurons and neuroendocrine cells (55). Remarkably, the pro-domain sequences of individual NTs are highly conserved among vertebrate species, suggesting that the prodomains may have an important function. Several regions in the pro-domains have been proposed to assist in proper folding and secretion of NTs (54, 57-59). Furthermore, it was recently found that proNTs are high-affinity ligands for the $p75^{NTR}$ and can induce $p75^{NTR}$-dependent apoptosis (60). Thus, pro- and mature NTs can elicite opposite functional effects by differential interactions with p75 and Trk receptors.

BDNF and neuronal plasticity

In addition to their classic effects on neuronal cell survival and differentiation, NTs, and in particular BDNF, have emerged as major regulators of neuronal plasticity. BDNF and its receptors are widely expressed in the nervous system, with particularly high expression in areas of both developmental and adult synaptic plasticity (reviewed in 61). Furthermore, the expression and secretion of both proBDNF and mature BDNF are tightly regulated during development and by neuronal activity (6, 62-66). The observations that BDNF modulates the efficacy of synaptic transmission in certain forms of short-term synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) (reviewed in 7, 67-69), or the growth of dendrites and axons (reviewed in 70-73) led to the hypothesis that BDNF serves as a molecular mediator of synaptic plasticity (reviewed in 74). However, a comprehensive understanding of the molecular and cellular mechanisms of BDNF function in neuronal plasticity is still lacking. Many synaptic functions of BDNF seem to be mediated by the TrkB receptor, which triggers intracellular signaling cascades that can alter gene expression of for example synapse-associated proteins (75). BDNF appears to exert both pre- and postsynaptic roles, since it has been shown to modify presynaptic transmitter release (5, 76-79), and to have an effect on postsynaptic ion channels (reviewed in 78, 80-82). Currently, several hypotheses have been proposed to clarify how the diffusible molecule BDNF can in fact achieve local and synapse-specifc modulation (reviewed in 6, 83). In addition, BDNF not only acts as a modulator of ion channels that are otherwise gated by membrane voltage

or ligands, but also can rapidly gate a Na+ channel, giving BDNF the properties of a classical excitatory transmitter (84-86).

Besides their roles in neuronal cell survival, differentiation and plasticity, there is growing evidence that NTs play an even broader role in nervous system functionality. For example, NGF and BDNF may be key regulators of the myelination process in the CNS and PNS (87-90), enhance neurogenesis (91-93) and stimulate the proliferation of brain astrocytes, cardiac myocytes, and microglia (94-99). Other responses to NTs include changes in morphology and differentiation (100, 101). Finally, a number of studies have reported that BDNF and NGF are involved in malignancies (102-106). Thus, although NTs, and more specifically BDNF, have already been characterized extensively, new data continuously extend our knowledge about their physiological function.

BDNF in the intermediate pituitary of the amphibian *Xenopus laevis*

To gain more insight into the roles of pro- and mature BDNF, we use the South-African claw-toad frog *Xenopus laevis* as a model system, and more specifically the melanotrope cells of the intermediate pituitary (described in more detail below). In the *Xenopus* melanotrope cells, both proBDNF and mature BDNF proteins have been found (107, 108). BDNF secreted by the melanotrope cells can stimulate the release of α-melanophore stimulating hormone (α-MSH) and the biosynthesis of the prohormone proopiomelanocortin (POMC) in an autocrine manner (107) (Figure 2).

Xenopus laevis intermediate pituitary melanotrope cells as a model system to study neuronal plasticity in a neuroendocrine interface

The melanotrope cells of the intermediate pituitary of *Xenopus laevis* constitute a valuable model system to examine the secretory pathway as well as aspects of neuroendocrine and neuronal plasticity. The cells play a central role in the process of background adaptation of the amphibian. By placing the frog on either a black or a white background, the activity of the melanotrope cells and their regulatory neuronal input can be manipulated *in vivo* in a physiological way. In animals that are adapted to a black background, the melanotrope cells are biosynthetically very active and produce vast amounts of POMC, which is processed to a number of bioactive peptides, including α-MSH. This hormone causes pigment dispersion in skin melanophores, giving the animal a black appearance. On a white background, the melanotrope cells are inhibited and thus inactive; consequently, the release of α-MSH is inhibited and animals appear white. The activity of the melanotrope cells is controlled by several brain centres of which the hypothalamic suprachiasmatic nucleus inhibits and the

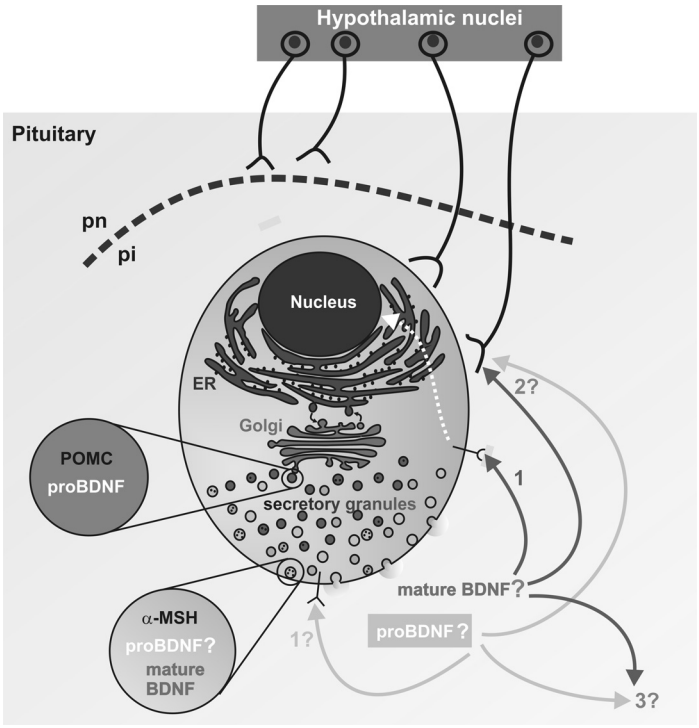


Figure 2: **BDNF in the neurointermediate pituitary of *Xenopus laevis*.**
Schematic representation of a *Xenopus* intermediate pituitary melanotrope cell, whose activity is controlled through direct synaptic innervation from hypothalamic nuclei. In the melanotrope cell, immature and mature secretory granules are indicated in dark grey and light grey, respectively. Some of the characteristics of pro- and mature BDNF are hypothesized and therefore indicated by question marks. Pro- and mature BDNF may have an effect on the melanotrope cell itself (1), on the innervating hypothalamic neurons (2) or on other cells (3). Abbreviations: pn, pars nervosa; pi, pars intermedia; ER, endoplasmic reticulum; POMC, proopiomelanocortin; pro- and mature BNDF, pro- and mature brain-derived neurotrophic factor; α-MSH, α-melanophore stimulating hormone.

magnocellular nucleus stimulates the biosynthesis and release of α-MSH (reviewed in 109-111). Neurons from the suprachiasmatic nucleus (suprachiasmatic melanotrope-inhibiting neurons; SMINs) make direct synaptic contacts with the melanotrope cells and the structure of these so-called varicosities depends on the adaptation state of the animal: more than double the number of active synaptic zones are present on the melanotropes of white- relative to black frogs (112) (Figure 3).

During white-background adaptation, the physiological response (drop of blood plasma α-MSH levels) takes place within 30 minutes after the animal is placed on a white background (113). This is thought to be regulated by the initial release of the neurotransmitter GABA from hypothalamic SMINs (114). However, immediate synaptic

changes are likely also necessary to ensure that already during the first few days of adaptation the melanotrope cells will stay inactive. During a more prolonged adaptation to a white background (at least a week), the melanotrope cells themselves undergo drastic morphological changes and become biosynthetically inactive (115). Conversely, when a white animal has to adapt to a black background, the immediate response involves the release of α-MSH that is stored in storage granules in the inactive melanotrope cells of the white animal (115, 116). Again, for a more prolonged adaptation, in this case to a black background, gradual morphological changes will lead to larger and biosynthetically active melanotrope cells and less elaborate inhibitory varicosities. Thus, manipulation of melanotrope cell activity is accompanied by differences in neuronal plasticity.

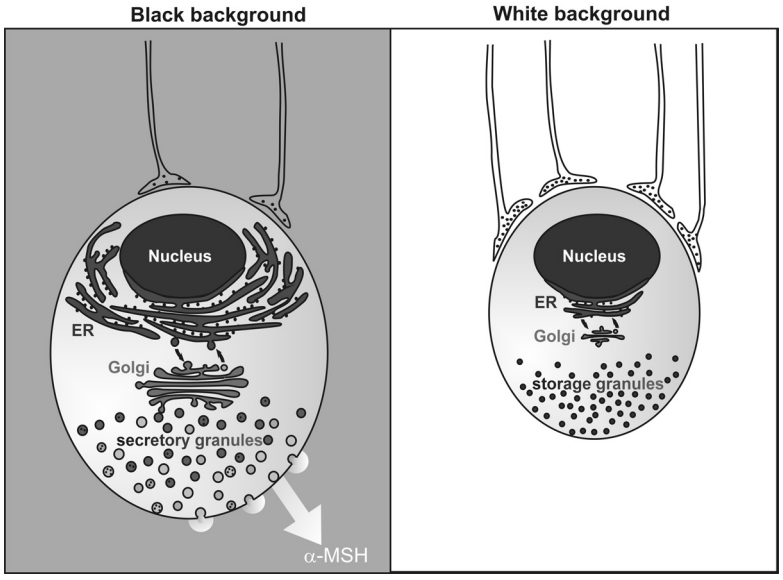


Figure 3: **Neuroendocrine plasticity of *Xenopus* intermediate pituitary melanotrope cells**.
Schematic representation of synaptic and endocrine plasticity at the level of the intermediate pituitary melanotrope cell during the process of background adaptation of the amphibian *Xenopus laevis*. See text for details. Abbreviations: ER, endoplasmic reticulum; α-MSH, α-melanophore stimulating hormone.

Xenopus laevis transgenesis for functional studies

To perform functional studies on neuroserpin and BDNF, we used the technique of stable *Xenopus laevis* transgenesis (117, 118). With this technique we can direct the expression of the protein of interest to various tissues by using cell type-specific promoter sequences in front of the protein-encoding cDNA. For example, using the POMC gene promoter (119)

transgenic animals can be generated that overexpress the transgene (in this thesis neuroserpin or BDNF) specifically in the melanotrope cells of the intermediate pituitary. An important advantage of the use of this promoter is that the level of transgene expression can be regulated by placing the frog on a white or black background, resulting in low or high transgene promoter activity, respectively.

Aim and outline of thesis

Research on the functioning of the brain fascinates and intrigues many people. Up to date, still many aspects of brain functioning remain elusive and need further investigation, one aspect being the molecular mechanism of neuronal plasticity. As mentioned above, neuroserpin and BDNF may play a role in neuronal plasticity. However, the exact physiological function of neuroserpin remains unclear and although BDNF has been quite extensively characterized, new data continue to broaden the functional spectrum of this protein in nervous system functionality. In this thesis, aspects of the physiological roles of neuroserpin and BDNF are therefore explored. In **chapter 2,** the identification and expression profile of neuroserpin in *Xenopus laevis* is presented. We found that *Xenopus* neuroserpin shows a high degree of amino acid sequence identity with neuroserpin proteins from other species, and that it was expressed mainly in neuronal and neuroendocrine tissues. In the neuroendocrine intermediate pituitary, neuroserpin formed a stable complex with a putative substrate, which was dependent on the degree of melanotrope cell activation (**chapter 3**). In an attempt to learn more about the physiological role of neuroserpin, we used the technique of stable *Xenopus* transgenesis in combination with the POMC gene promoter to overexpress neuroserpin and a mutant form of the protein specifically in the intermediate pituitary melanotrope cells. The generation and analyses of these animals is described in **chapter 4**. To explore the physiological role of BDNF in more detail, transgenic frogs were generated and analysed with overexpression of pro- and mature BDNF specifically in the melanotrope cells. We found that unprocessed proBDNF may result in endoplasmic reticulum stress in the melanotrope cells and a block of transport of a number of secretory pathway proteins (**chapter 5**). In addition, we observed that a relatively high level of transgene expression of mature BDNF in the melanotrope cells resulted in glial cell proliferation and axonal outgrowth and myelination (**chapter 6**). Finally, **in chapter 7** the results described in this thesis are summarized, discussed and placed into a broader context.

References

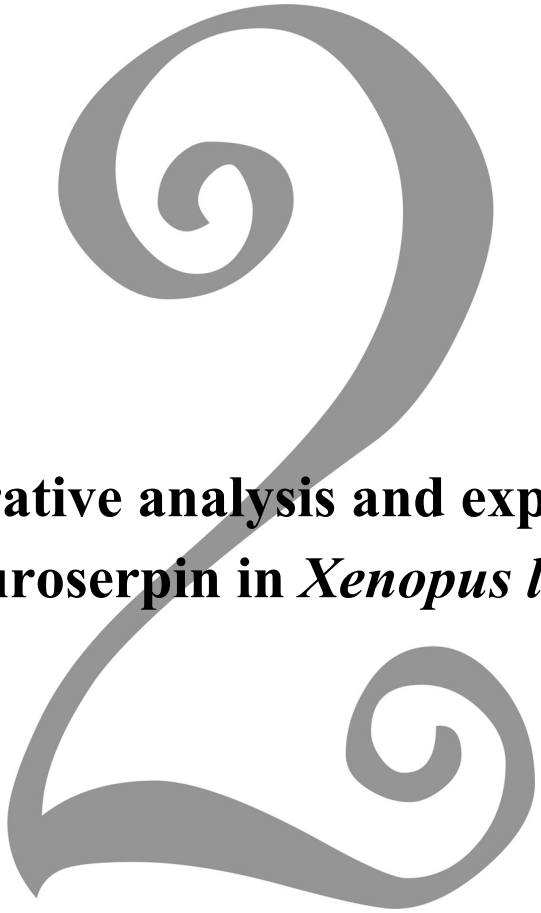
1. Dityatev A, Schachner M. Extracellular matrix molecules and synaptic plasticity. Nat Rev Neurosci 2003;4:456-68.
2. Pavlov I, Lauri S, Taira T, Rauvala H. The role of ECM molecules in activity-dependent synaptic development and plasticity. Birth Defects Res C Embryo Today 2004;72:12-24.
3. Theodosis DT, Piet R, Poulain DA, Oliet SH. Neuronal, glial and synaptic remodeling in the adult hypothalamus: functional consequences and role of cell surface and extracellular matrix adhesion molecules. Neurochem Int 2004;45:491-501.
4. Rauch U. Extracellular matrix components associated with remodeling processes in brain. Cell Mol Life Sci 2004;61:2031-45.
5. Schinder AF, Poo M. The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci 2000;23:639-45.
6. Lu B. BDNF and activity-dependent synaptic modulation. Learn Mem 2003;10:86-98.
7. Lu B. Acute and long-term synaptic modulation by neurotrophins. Prog Brain Res 2004;146:137-50.
8. Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat Rev Neurosci 2005;6:603-14.
9. Caleo M, Maffei L. Neurotrophins and plasticity in the visual cortex. Neuroscientist 2002;8:52-61.
10. Blum R, Konnerth A. Neurotrophin-mediated rapid signaling in the central nervous system: mechanisms and functions. Physiology (Bethesda) 2005;20:70-8.
11. Krem MM, Rose T, Di Cera E. Sequence determinants of function and evolution in serine proteases. Trends Cardiovasc Med 2000;10:171-6.
12. Yousef GM, Elliott MB, Kopolovic AD, Serry E, Diamandis EP. Sequence and evolutionary analysis of the human trypsin subfamily of serine peptidases. Biochim Biophys Acta 2004;1698:77-86.
13. Yepes M, Lawrence DA. New functions for an old enzyme: nonhemostatic roles for tissue-type plasminogen activator in the central nervous system. Exp Biol Med (Maywood) 2004;229:1097-104.
14. Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci 2000;20:2003-10.
15. Nayeem N, Silletti S, Yang X, et al. A potential role for the plasmin(ogen) system in the posttranslational cleavage of the neural cell adhesion molecule L1. J Cell Sci 1999;112 (Pt 24):4739-49.
16. Huntington JA, Carrell RW. The serpins: nature's molecular mousetraps. Sci Prog 2001;84:125-36.
17. Ye S, Goldsmith EJ. Serpins and other covalent protease inhibitors. Curr Opin Struct Biol 2001;11:740-5.
18. Lomas DA, Belorgey D, Mallya M, et al. Polymerisation underlies alpha1-antitrypsin deficiency, dementia and other serpinopathies. Front Biosci 2004;9:2873-91.
19. Lomas DA, Belorgey D, Mallya M, et al. Molecular mousetraps and the serpinopathies. Biochem Soc Trans 2005;33:321-30.
20. Lomas DA, Carrell RW. Serpinopathies and the conformational dementias. Nat Rev Genet 2002;3:759-68.
21. Lomas DA, Mahadeva R. Alpha1-antitrypsin polymerization and the serpinopathies: pathobiology and prospects for therapy. J Clin Invest 2002;110:1585-90.
22. Stoeckli ET, Lemkin PF, Kuhn TB, Ruegg MA, Heller M, Sonderegger P. Identification of proteins secreted from axons of embryonic dorsal-root-ganglia neurons. Eur J Biochem 1989;180:249-58.
23. Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. Embo J 1996;15:2944-53.
24. Krueger SR, Ghisu GP, Cinelli P, et al. Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J Neurosci 1997;17:8984-96.
25. Hill RM, Parmar PK, Coates LC, Mezey E, Pearson JF, Birch NP. Neuroserpin is expressed in the pituitary and adrenal glands and induces the extension of neurite-like processes in AtT-20 cells. Biochem J 2000;345 Pt 3:595-601.
26. Hill RM, Coates LC, Parmar PK, Mezey E, Pearson JF, Birch NP. Expression and functional characterization of the serine protease inhibitor neuroserpin in endocrine cells. Ann N Y Acad Sci 2002;971:406-15.
27. Davis RL, Shrimpton AE, Holohan PD, et al. Familial dementia caused by polymerization of mutant neuroserpin. Nature 1999;401:376-9.
28. Davis RL, Shrimpton AE, Carrell RW, et al. Association between conformational mutations in neuroserpin and onset and severity of dementia. Lancet 2002;359:2242-7.

29. Osterwalder T, Cinelli P, Baici A, et al. The axonally secreted serine proteinase inhibitor, neuroserpin, inhibits plasminogen activators and plasmin but not thrombin. J Biol Chem 1998;273:2312-21.
30. Yepes M, Lawrence DA. Neuroserpin: a selective inhibitor of tissue-type plasminogen activator in the central nervous system. Thromb Haemost 2004;91:457-64.
31. Yepes M, Sandkvist M, Coleman TA, et al. Regulation of seizure spreading by neuroserpin and tissue-type plasminogen activator is plasminogen-independent. J Clin Invest 2002;109:1571-8.
32. Yepes M, Lawrence DA. Tissue-type plasminogen activator and neuroserpin: a well-balanced act in the nervous system? Trends Cardiovasc Med 2004;14:173-80.
33. Madani R, Kozlov S, Akhmedov A, et al. Impaired explorative behavior and neophobia in genetically modified mice lacking or overexpressing the extracellular serine protease inhibitor neuroserpin. Mol Cell Neurosci 2003;23:473-94.
34. Hastings GA, Coleman TA, Haudenschild CC, et al. Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. J Biol Chem 1997;272:33062-7.
35. Berger P, Kozlov SV, Cinelli P, Kruger SR, Vogt L, Sonderegger P. Neuronal depolarization enhances the transcription of the neuronal serine protease inhibitor neuroserpin. Mol Cell Neurosci 1999;14:455-67.
36. Hallbook F. Evolution of the vertebrate neurotrophin and Trk receptor gene families. Curr Opin Neurobiol 1999;9:616-21.
37. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;4:299-309.
38. Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 2001;11:272-80.
39. Barker PA. p75NTR is positively promiscuous: novel partners and new insights. Neuron 2004;42:529-33.
40. Nykjaer A, Willnow TE, Petersen CM. p75NTR--live or let die. Curr Opin Neurobiol 2005;15:49-57.
41. Barbacid M. The Trk family of neurotrophin receptors. J Neurobiol 1994;25:1386-403.
42. Barbacid M. Structural and functional properties of the TRK family of neurotrophin receptors. Ann N Y Acad Sci 1995;766:442-58.
43. Klein R, Conway D, Parada LF, Barbacid M. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 1990;61:647-56.
44. Middlemas DS, Lindberg RA, Hunter T. trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 1991;11:143-53.
45. Luikart BW, Nef S, Shipman T, Parada LF. In vivo role of truncated trkb receptors during sensory ganglion neurogenesis. Neuroscience 2003;117:847-58.
46. Yacoubian TA, Lo DC. Truncated and full-length TrkB receptors regulate distinct modes of dendritic growth. Nat Neurosci 2000;3:342-9.
47. Fryer RH, Kaplan DR, Kromer LF. Truncated trkB receptors on nonneuronal cells inhibit BDNF-induced neurite outgrowth in vitro. Exp Neurol 1997;148:616-27.
48. Haapasalo A, Sipola I, Larsson K, et al. Regulation of TRKB surface expression by brain-derived neurotrophic factor and truncated TRKB isoforms. J Biol Chem 2002;277:43160-7.
49. Haapasalo A, Koponen E, Hoppe E, Wong G, Castren E. Truncated trkB.T1 is dominant negative inhibitor of trkB.TK+-mediated cell survival. Biochem Biophys Res Commun 2001;280:1352-8.
50. Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF. Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 1996;16:3123-9.
51. Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A. Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature 2003;426:74-8.
52. Seidah NG, Benjannet S, Pareek S, Chretien M, Murphy RA. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett 1996;379:247-50.
53. Heymach JV, Jr., Kruttgen A, Suter U, Shooter EM. The regulated secretion and vectorial targeting of neurotrophins in neuroendocrine and epithelial cells. J Biol Chem 1996;271:25430-7.
54. Farhadi HF, Mowla SJ, Petrecca K, Morris SJ, Seidah NG, Murphy RA. Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J Neurosci 2000;20:4059-68.
55. Mowla SJ, Pareek S, Farhadi HF, et al. Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 1999;19:2069-80.
56. Mowla SJ, Farhadi HF, Pareek S, et al. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J Biol Chem 2001;276:12660-6.

57. Egan MF, Kojima M, Callicott JH, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 2003;112:257-69.
58. Suter U, Heymach JV, Jr., Shooter EM. Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. Embo J 1991;10:2395-400.
59. Rattenholl A, Ruoppolo M, Flagiello A, et al. Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. J Mol Biol 2001;305:523-33.
60. Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science 2001;294:1945-8.
61. McAllister AK, Katz LC, Lo DC. Neurotrophins and synaptic plasticity. Annu Rev Neurosci 1999;22:295-318.
62. Goodman LJ, Valverde J, Lim F, et al. Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol Cell Neurosci 1996;7:222-38.
63. Kohara K, Kitamura A, Morishima M, Tsumoto T. Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. Science 2001;291:2419-23.
64. Gartner A, Staiger V. Neurotrophin secretion from hippocampal neurons evoked by long-term-potentiation-inducing electrical stimulation patterns. Proc Natl Acad Sci U S A 2002;99:6386-91.
65. Thoenen H. Neurotrophins and activity-dependent plasticity. Prog Brain Res 2000;128:183-91.
66. Lessmann V, Gottmann K, Malcangio M. Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 2003;69:341-74.
67. Pang PT, Lu B. Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Res Rev 2004;3:407-30.
68. Binder DK, Scharfman HE. Brain-derived neurotrophic factor. Growth Factors 2004;22:123-31.
69. Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol 2005;76:99-125.
70. Horch HW. Local effects of BDNF on dendritic growth. Rev Neurosci 2004;15:117-29.
71. Cohen-Cory S, Lom B. Neurotrophic regulation of retinal ganglion cell synaptic connectivity: from axons and dendrites to synapses. Int J Dev Biol 2004;48:947-56.
72. Tucker KL. Neurotrophins and the control of axonal outgrowth. Panminerva Med 2002;44:325-33.
73. Ide C. Peripheral nerve regeneration. Neurosci Res 1996;25:101-21.
74. Poo MM. Neurotrophins as synaptic modulators. Nat Rev Neurosci 2001;2:24-32.
75. Ring RH, Alder J, Fennell M, Kouranova E, Black IB, Thakker-Varia S. Transcriptional profiling of brain-derived-neurotrophic factor-induced neuronal plasticity: a novel role for nociceptin in hippocampal neurite outgrowth. J Neurobiol 2006;66:361-77.
76. Lessmann V, Gottmann K, Heumann R. BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. Neuroreport 1994;6:21-5.
77. Lessmann V. Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. Gen Pharmacol 1998;31:667-74.
78. Berninger B, Poo M. Fast actions of neurotrophic factors. Curr Opin Neurobiol 1996;6:324-30.
79. Tyler WJ, Perrett SP, Pozzo-Miller LD. The role of neurotrophins in neurotransmitter release. Neuroscientist 2002;8:524-31.
80. Manabe T. Does BDNF have pre- or postsynaptic targets? Science 2002;295:1651-3.
81. Schuman EM. Neurotrophin regulation of synaptic transmission. Curr Opin Neurobiol 1999;9:105-9.
82. Berninger B, Poo M. Exciting neurotrophins. Nature 1999;401:862-3.
83. Nagappan G, Lu B. Activity-dependent modulation of the BDNF receptor TrkB: mechanisms and implications. Trends Neurosci 2005;28:464-71.
84. Blum R, Kafitz KW, Konnerth A. Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature 2002;419:687-93.
85. Kafitz KW, Rose CR, Thoenen H, Konnerth A. Neurotrophin-evoked rapid excitation through TrkB receptors. Nature 1999;401:918-21.
86. Rose CR, Blum R, Kafitz KW, Kovalchuk Y, Konnerth A. From modulator to mediator: rapid effects of BDNF on ion channels. Bioessays 2004;26:1185-94.
87. McTigue DM, Horner PJ, Stokes BT, Gage FH. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci 1998;18:5354-65.
88. Chan JR, Cosgaya JM, Wu YJ, Shooter EM. Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci U S A 2001;98:14661-8.
89. Cosgaya JM, Chan JR, Shooter EM. The neurotrophin receptor p75NTR as a positive modulator of myelination. Science 2002;298:1245-8.

90. Tolwani RJ, Cosgaya JM, Varma S, Jacob R, Kuo LE, Shooter EM. BDNF overexpression produces a long-term increase in myelin formation in the peripheral nervous system. J Neurosci Res 2004;77:662-9.
91. Zigova T, Pencea V, Wiegand SJ, Luskin MB. Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Mol Cell Neurosci 1998;11:234-45.
92. Benraiss A, Chmielnicki E, Lerner K, Roh D, Goldman SA. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. J Neurosci 2001;21:6718-31.
93. Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 2001;21:6706-17.
94. Yokoyama M, Black IB, Dreyfus CF. NGF increases brain astrocyte number in culture. Exp Neurol 1993;124:377-80.
95. Ikeda T, Puro DG. Nerve growth factor: a mitogenic signal for retinal Muller glial cells. Brain Res 1994;649:260-4.
96. Lin MI, Das I, Schwartz GM, Tsoulfas P, Mikawa T, Hempstead BL. Trk C receptor signaling regulates cardiac myocyte proliferation during early heart development in vivo. Dev Biol 2000;226:180-91.
97. Elkabes S, DiCicco-Bloom EM, Black IB. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 1996;16:2508-21.
98. Zhang J, Geula C, Lu C, Koziel H, Hatcher LM, Roisen FJ. Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. Exp Neurol 2003;183:469-81.
99. Lambert WS, Clark AF, Wordinger RJ. Effect of exogenous neurotrophins on Trk receptor phosphorylation, cell proliferation, and neurotrophin secretion by cells isolated from the human lamina cribrosa. Mol Vis 2004;10:289-96.
100. Hutton LA, Perez-Polo JR. In vitro glial responses to nerve growth factor. J Neurosci Res 1995;41:185-96.
101. Liu ZZ, Zhu LQ, Eide FF. Critical role of TrkB and brain-derived neurotrophic factor in the differentiation and survival of retinal pigment epithelium. J Neurosci 1997;17:8749-55.
102. Chou TT, Trojanowski JQ, Lee VM. Neurotrophin signal transduction in medulloblastoma. J Neurosci Res 1997;49:522-7.
103. Pearse RN, Swendeman SL, Li Y, Rafii D, Hempstead BL. A neurotrophin axis in myeloma: TrkB and BDNF promote tumor-cell survival. Blood 2005;105:4429-36.
104. Yang ZF, Ho DW, Lam CT, et al. Identification of brain-derived neurotrophic factor as a novel functional protein in hepatocellular carcinoma. Cancer Res 2005;65:219-25.
105. Dolle L, Adriaenssens E, El Yazidi-Belkoura I, Le Bourhis X, Nurcombe V, Hondermarck H. Nerve growth factor receptors and signaling in breast cancer. Curr Cancer Drug Targets 2004;4:463-70.
106. Montano X, Djamgoz MB. Epidermal growth factor, neurotrophins and the metastatic cascade in prostate cancer. FEBS Lett 2004;571:1-8.
107. Kramer BM, Cruijsen PM, Ouwens DT, et al. Evidence that brain-derived neurotrophic factor acts as an autocrine factor on pituitary melanotrope cells of Xenopus laevis. Endocrinology 2002;143:1337-45.
108. Wang LC, Meijer HK, Humbel BM, Jenks BG, Roubos EW. Activity-dependent dynamics of coexisting brain-derived neurotrophic factor, pro-opiomelanocortin and alpha-melanophore-stimulating hormone in melanotrope cells of Xenopus laevis. J Neuroendocrinol 2004;16:19-25.
109. Roubos EW, Scheenen WJ, Jenks BG. Neuronal, neurohormonal, and autocrine control of Xenopus melanotrope cell activity. Ann N Y Acad Sci 2005;1040:172-83.
110. Roubos EW. Background adaptation by Xenopus laevis: a model for studying neuronal information processing in the pituitary pars intermedia. Comp Biochem Physiol A Physiol 1997;118:533-50.
111. Kramer BM, Kolk SM, Berghs CA, et al. Dynamics and plasticity of peptidergic control centres in the retino-brain-pituitary system of Xenopus laevis. Microsc Res Tech 2001;54:188-99.
112. Berghs CA, Roubos EW. Background adaptation and synapse plasticity in the pars intermedia of Xenopus laevis. Neuroscience 1996;70:833-41.
113. van Zoest ID, Heijmen PS, Cruijsen PM, Jenks BG. Dynamics of background adaptation in Xenopus laevis: role of catecholamines and melanophore-stimulating hormone. Gen Comp Endocrinol 1989;76:19-28.
114. Jenks B, Buzzi M, Dotman C, et al. The significance of multiple inhibitory mechanisms converging on the melanotrope cell of Xenopus laevis. Ann N Y Acad Sci 1998;839:229-34.
115. de Rijk EP, Jenks BG, Wendelaar Bonga SE. Morphology of the pars intermedia and the melanophore-stimulating cells in Xenopus laevis in relation to background adaptation. Gen Comp Endocrinol 1990;79:74-82.

116. Martens GJ, Weterings KA, van Zoest ID, Jenks BG. Physiologically-induced changes in proopiomelanocortin mRNA levels in the pituitary gland of the amphibian Xenopus laevis. Biochem Biophys Res Commun 1987;143:678-84.
117. Kroll KL, Amaya E. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 1996;122:3173-83.
118. Sparrow DB, Latinkic B, Mohun TJ. A simplified method of generating transgenic Xenopus. Nucleic Acids Res 2000;28:E12.
119. Jansen EJ, Holling TM, van Herp F, Martens GJ. Transgene-driven protein expression specific to the intermediate pituitary melanotrope cells of Xenopus laevis. FEBS Lett 2002;516:201-7.



Comparative analysis and expression of neuroserpin in *Xenopus laevis*

Dorien M. de Groot, Christine Pol and Gerard J.M. Martens

Department of Molecular Animal Physiology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Institute for Neuroscience, Faculty of Science, Radboud University, Nijmegen, the Netherlands

Neuroendocrinology (2005) 82: 11-20

Abstract

Serine protease inhibitors form a diverse family of proteins of which most members inhibit target serine proteases. Neuroserpin is a member of this family. Here, we have characterized neuroserpin in the non-mammalian species *Xenopus laevis* and found a high degree of amino acid sequence conservation, especially of the reactive center loop, of the *Xenopus* protein compared to mammalian and chicken neuroserpin sequences, suggesting a conserved target specificity. Neuroserpin mRNA and protein were expressed throughout *Xenopus* development, while in the adult frog high mRNA expression was found in neuronal and neuroendocrine tissues, and the reproductive organs, and the neuroserpin protein was detected mainly in brain and pituitary. More specifically, in *Xenopus* pituitary neuroserpin mRNA was expressed higher in the neurointermediate lobe than in the pars distalis. At the protein level, we detected a 55-kDa neuroserpin protein in the pars nervosa, two neuroserpin proteins of 44- and 50-kDa in the melanotrope cells of the pars intermedia, and a 46-kDa product in the pars distalis. On the basis of its relatively high degree of sequence conservation and its expression pattern, we conclude that *Xenopus* neuroserpin may play an important physiological role, *e.g.* as a serine protease inhibitor during development, and for proper neuronal and neuroendocrine cell functioning.

Introduction

The proteolytic cleavage of peptide bonds in proteins is a critical step in many biological processes, such as digestion of proteins to supply amino acids, proenzyme activation, clot formation and lysis, tissue remodelling, angiogenesis, complement activation and the maintenance of the extracellular matrix. An important class of proteolytic enzymes are the serine proteases, named after the active serine in their catalytic site. To maintain homeostasis, the proteolytic activity of proteases needs to be tightly regulated. Inhibition of serine proteases is provided by a superfamily of serine protease inhibitors (serpins) which currently consists of ~500 known members. Serpins differ from other enzyme inhibitory families in that they are able to undergo a marked conformational change and act as suicide target inhibitors; they enter into a covalent interaction with their target protease, after which drastic conformational changes take place, rendering both the serine protease and its serpin inactive (reviewed in 1-3).

Neuroserpin is a serpin that was first identified as an axonally secreted protein from chicken dorsal root ganglion cultures (4). The protein is primarily expressed in the nervous system throughout embryonic development and adulthood (5-7), and in neuroendocrine cells such as rat pituitary and adrenal glands (8, 9). Thus far, the protein has been characterized in only a number of mammalian species (*e.g.* human, rat, mouse) and chicken. The primary structure of neuroserpin suggested that this serpin inhibits trypsin-like serine proteases, like tissue-type plasminogen activator (t-PA), urokinase-type plasminogen

activator (u-PA) and trypsin, and interactions of neuroserpin with these target proteases have indeed been confirmed experimentally *in vitro* (6, 10-12). Based on kinetic analyses and expression patterns, t-PA appears to be the most likely target for neuroserpin in the nervous system (10, 13), although the *in vitro* interaction of t-PA with neuroserpin is short-lived, suggesting that the physiological effect of a t-PA-neuroserpin interaction may be more complex than previously thought (14).

In this study, we have isolated the full-length cDNA for *Xenopus laevis* neuroserpin, have identified from the database a second *Xenopus* neuroserpin sequence, and performed a comparative analysis of the now known *Xenopus* sequences with other known neuroserpin amino acid sequences. We also studied the expression of *Xenopus* neuroserpin mRNA and protein during development of the frog and in the adult animal, in particular in the neuroendocrine pituitary gland.

Materials and methods

Animals

South African claw-toed frogs, *Xenopus laevis*, were reared in the Central Animal Facility of the Radboud University of Nijmegen, The Netherlands. The animals were adapted to a black background by keeping them in black buckets for at least three weeks at 22°C, with a light/dark cycle of 12 h. Experimental procedures were performed under the guidelines of the Dutch law concerning animal welfare.

Antibodies

Two anti-neuroserpin antibodies were used: rabbit polyclonal IgG against human recombinant neuroserpin (α-NS-L; generous gift of Dr. D.A. Lawrence, American Red Cross Holland Laboratory, Rockville, Maryland, USA; 10) and rabbit polyclonal IgG against human recombinant neuroserpin (α-NS-M; generous gift of Dr. Lomas, University of Cambridge, Cambridge, UK; 15). The dilutions used for Western blotting were 1:5,000 and 1:10,000 for α-NS-L and α-NS-M, respectively, and 1:300 for immunocytochemistry (α-NS-L). Tubulin was detected using the monoclonal E7 anti-tubulin antibody (1:1,500; Developmental Studies Hybridoma Bank, Rockland, Gilbertsville, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a rabbit polyclonal antibody (1:10,000; Trevigen, Gaithersburg, MD, USA).

RNA isolation

For RNA isolation, 10-20 embryos of a number of developmental stages (generated by *in vitro* fertilization) and various dissected *Xenopus* tissues were frozen in liquid nitrogen. Next, total RNA was isolated using Trizol reagent, according to the manufacturer's

instructions (Life Technologies, Inc.). The RNA was dissolved in 20 µl RNase-free H_2O and the concentration was measured with a GeneQuant RNA/DNA calculator (Pharmacia).

Reverse transcription PCR (RT-PCR)

For RT-PCR, two µg total RNA was reverse transcribed into cDNA in a 20 µl reaction mix containing first strand buffer (Invitrogen), 10 mM DTT, 0.5 mM dNTPs, 20 U RNasin (Promega) and 100 U Superscript II (Invitrogen) for 1.5 hrs at 37°C. The full-length cDNA clone of *Xenopus* neuroserpin was obtained by performing a PCR using *Xenopus* brain cDNA as a template. The following primer set was developed on the basis of database *Xenopus* neuroserpin-related ESTs (accession numbers NCBI nucleotide database: BG264770, BG407876, BI312920, BG233110, BF428341 and BI315484): NS-fw(*BamHI)*: 5'-GGCGGATCCATGCATCACTTCATATTACTC-3', starting at the putative start codon (underlined), and NS-rev(*Xba*I): 5'-GCGTCTAGATTAGAGCTCTTCAAAATCATGG-3', ending at the putative stop codon (underlined). The PCR amplification was carried out in a reaction mixture containing Pfu buffer (Fermentas), 0.05 units/µl Pfu Turbo DNA polymerase (Fermentas), 200 µM dNTPs, 0.5 µM of each primer and 1 µl cDNA (35 amplification cycles: 30 sec at 95°C, 2 min at 49°C and 1 min 30 sec at 72°C). To examine *Xenopus* neuroserpin mRNA expression in a number of developmental stages and tissues from adult frogs, the following forward and reverse primers were used: 5'-ATTGAAAGCCCATTTGATTGAAG-3' and 5'- GGATGCATGACCCTTCCCAT-3', respectively. As controls, *Xenopus* GAPDH cDNA (forward primer 5'-GCCGTGTATGTGGTGGAATCT-3' and reverse primer 5'-AAGTTGTCGTTGATGACCTTTGC-3') and ornithine decarboxylase (ODC) cDNA (forward primer 5'- GTCAATGATGGAGTGTATGGATC-3'and reverse primer 5'-TCCATTCCGCTCTCCTGAGCAC- 3') were amplified. Of each primer 0.5 µM was used in a 25-µl reaction volume containing PCR-buffer (Fermentas), 200 µM dNTPs, 3 mM $MgCl_2$, 1 unit Taq polymerase (Fermentas) and 2 µl cDNA. The neurointermediate lobe (NIL) and pars distalis (PD) cDNAs were diluted 5 times before use. Thirty amplification cycles were performed: 1 min at 94°C, 1 min at 45°C (for neuroserpin and ODC) or 53°C (for GAPDH), and 1 min at 72°C. PCR-products were analyzed on a 2% agarose gel. To quantify the relative amounts of neuroserpin mRNA in the NIL and PD of the pituitary, detection of the PCR products was performed using a BioChemi imaging system and signals were analyzed using LabWorks 4.0 software (UVP BioImaging systems, Cambridge, UK). Total raw densities of 390-bp neuroserpin were normalized to the corresponding 230-bp GAPDH samples (n = 4). A paired t test was used for statistical analysis.

Cloning and sequence analysis of Xenopus neuroserpin

For cloning, the *Xenopus* neuroserpin cDNA generated by RT-PCR was isolated from an agarose gel using the QIAEX II Agarose gel extraction kit (Qiagen). After digestion with

*BamH*I and *Xba*I, the neuroserpin 1245-bp cDNA insert was ligated into the pCS2+ vector (16), resulting in the pCS2+-NS construct. Finally, full-length *Xenopus* neuroserpin cDNA was cycle sequenced using the ABI 310 Prism. For this, three different primers were used: NS-fw(*BamH*I), NS-rev(*Xba*I) and NS-fw366 (5'-CCATATCAGCGACAAGTTCATCCAG-3') (accession number *Xenopus* neuroserpin: AM050698). Next, the deduced *Xenopus* neuroserpin amino acid sequence was aligned with other species neuroserpin amino acid sequences using the Vector NTI program (InforMax, Inc.). Amino acid sequences were obtained from the NCBI protein database and the amino acid sequence of pig neuroserpin was deduced from its mRNA sequence found in the NCBI nucleotide database. Accession numbers were: *Xenopus laevis* MGC84260 protein, AAH74366; human neuroserpin, CAB03626; mouse neuroserpin, CAA04939; pig neuroserpin, AY609517; chicken neuroserpin, CAA96493; *Drosophila* Spn4.1, CAD21892; human protease nexin-1, P07093; human antithrombin III, NP_000479; human angiotensinogen, CAI14864; and human pigment epithelium-derived factor, NP_002606.

Western blotting

For Western blot analyses, homogenates of the pituitary pars intermedia (PI), pars nervosa (PN), NIL (the PI and PN together), and PD, embryos of various developmental stages, neuroserpin mRNA-injected embryos, and tissues from adult *Xenopus*, were made in lysis buffer (250 mM sucrose, 1% Triton X-100, 10 mM Tris (pH 8.0), 1 mM EDTA, 1μM PMSF, 0.1 mg/ml soybean trypsin inhibitor) using a glass homogenizer. After 20 min incubation on ice, the samples were centrifuged for 20 min (18,000g). The supernatant was separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (0.45 μM Hybond-P, Amersham Pharmacia Biotech) or nitrocellulose (Protran; used for the analysis of adult tissue samples) using a BioRad mini-protean II cell system (Bio-Rad). For immunodetection, blots were washed one time in PBS and incubated for at least one hour in blocking buffer (5% skimmed milk and 1% Tween in PBS). Subsequently, blots were incubated with primary antiserum in blocking buffer for 16 hours (4°C). After three rinses in wash buffer (1% skimmed milk and 1% Tween in PBS), blots were incubated in horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (1:5,000) or HRP-conjugated goat-anti-mouse IgG (1:5,000) for 45 min. Following three rinses in wash buffer and one rinse in PBS, the proteins were visualized by incubating blots in Lumi-Light^{PLUS} Western blotting substrate (Roche) and subsequent exposure to X-ray film (Eastman Kodak). For quantification of neuroserpin protein levels in brain tissue, remaining tissue of the head and the tail of stage 49/50 tadpoles, detection was performed using a BioChemi imaging system and signals were analyzed using LabWorks 4.0 software (UVP BioImaging systems, Cambridge, UK). Total densities of 55-kDa neuroserpin were normalized to the corresponding tubulin samples (n = 4). An unpaired t test was used for statistical analysis.

Immunocytochemistry

For microscopic analysis of neuroserpin protein expression, *Xenopus* brain with the pituitary attached was dissected and fixed in 4% paraformaldehyde in PBS. After cryoprotection in 10% sucrose-PBS, sagittal 20-μm cryosections were mounted on poly-L-lysine coated slides, dried for 2 h at 45°C and immunohistochemistry was performed as described previously (17), using α-NS-L as a primary antibody at a dilution of 1:300 and goat-anti-rabbit FITC (Nordic) as a second antibody (dilution 1:50). To examine FITC fluorescence, cryosections were viewed under a Leica DM RA fluorescenct microscope and photographs were taken with a Cohu High-Performance CCD Camera using the Leica Q Fluoro software. Slides were also analyzed with an MRC 1024 CLSM.

RNA injections

For oocyte injection, neuroserpin RNA was produced according to the High Yield Capped RNA Transcription Kit (Ambion), using the pCS2+-NS vector. Next, 1-cell *Xenopus* embryos (generated by *in vitro* fertilization) were injected with 1 ng RNA or 0.1x MR solution (10 mM NaCl, 0.18 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM Hepes-NaOH, pH 7.6). After 2 h, 4-cell embryos were selected and subsequently monitored in time. A number of embryos were frozen 24, 48 and 72 h post injection, and used for analysis on Western blot.

Results

Cloning of *Xenopus* neuroserpin and comparative analysis of *Xenopus*, mammalian, chicken and *Drosophila* neuroserpin sequences

Since neuroserpin is a recently identified new member of the serpin superfamily and has only been described in a limited number of species, we were interested in identifying neuroserpin in our model system, the amphibian *Xenopus laevis*. To this end, we performed RT-PCR and amplified, cloned and sequenced a specific product of 1248 bp. Alignment of the deduced amino acid sequence with the mammalian and chicken neuroserpin sequences, and with the *Drosophila* Spn4.1 amino acid sequence (the closest *Drosophila* homologue to neuroserpin; 18), revealed an overall identity of 64, 73 and 32%, respectively (Fig. 1A). From the comparative analysis, we conclude that we have identified neuroserpin in *Xenopus laevis*. In addition, during the preparation of this article a database search yielded *Xenopus laevis* unidentified protein MGC84260, which showed 95% amino acid sequence identity with the *Xenopus* neuroserpin protein we had cloned and represents a second *Xenopus* neuroserpin (Fig. 1A). A number of other *Xenopus* protein pairs have been shown to occur, including for POMC (19), prohormone convertase PC2 (20), amyloid precursor protein APP (21), D2 dopamine receptor (22) and serum albumins (23), and these pairs are present because a whole-genome duplication event has occurred in the species *Xenopus*

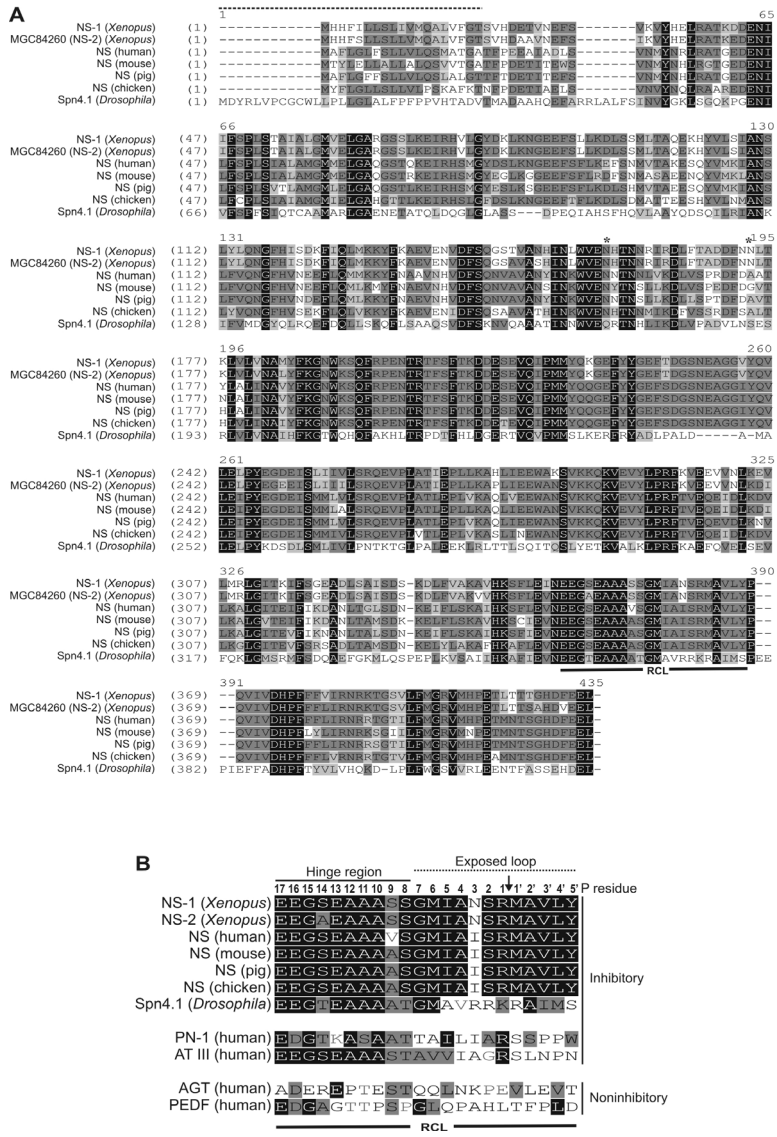


Figure 1: Amino acid sequence comparison between *Xenopus* neuroserpin and mammalian, chicken and insect neuroserpin proteins.

(A) Alignment of the amino acid sequences of *Xenopus laevis* neuroserpin (NS-1), *Xenopus laevis* MGC84260 protein (NS-2), and human, mouse, pig and chicken neuroserpin (NS) proteins and the *Drosophila* Spn4.1 protein. The one-letter amino acid notation is used. Residues identical among all four species are white on a black background, while residues conserved in three or more species are black on a dark grey background. Conservative amino acid changes are depicted black on a light grey background. The reactive center loop (RCL) is underlined. The two putative N-linked glycosylation sites are indicated with an asterisk, and the predicted signal peptide sequences are overlined with a dashed line. (B) Alignment of the RCLs of various neuroserpin sequences, and the human inhibitory serpins protease nexin-1 (PN-1) and antithrombin III (AT III), and noninhibitory serpins

angiotensinogen (AGT) and pigment epithelium-derived factor (PEDF). Residues conserved in three or more species are white on a black background and conservative amino acid changes are depicted black on a dark grey background. The amino acids are numbered P17–P5', which corresponds to the nomenclature of Schechter and Berger (25). The cleavage site between the P1 and P1' residues is indicated with an arrow.

laevis ~35 million years ago (24). Since the magnitude of sequence identity observed between the two *Xenopus* neuroserpin proteins (95%) is similar to those found for the other *Xenopus* protein pairs (92-95%), it is likely that the two *Xenopus* neuroserpins do not correspond to allelic variants but rather that they are also the result of the genome duplication. On the basis of its homology with other serpins, we have annotated the reactive center loop (RCL) of *Xenopus* neuroserpin as the segment ranging from Glu 346 to Tyr 367 (underlined in Fig. 1A). The amino acids flanking the scissile bond of an RCL are generally denoted P1 and P1', and adjacent amino acid residues in the amino-terminal and carboxy-terminal directions are numbered P2, P3, P4, etc. and P2', P3', P4', etc., respectively (25). The RCL of *Xenopus* neuroserpin is highly similar to that of the mammalian and chicken neuroserpin sequences and differed only by two P residues (a serine at position P9 and an asparagine at position P3; Fig. 1B). This is consistent with a function for *Xenopus* neuroserpin as an inhibitory serpin, since noninhibitory serpins show sequence divergence from inhibitory serpins, especially in the P12-P9 residues of the hinge region. More specifically, inhibitory serpins, such as neuroserpin, protease-nexin 1 (PN-1) and antithrombin III (AT III), contain alanines at positions P12-P9 in >50% of the cases, whereas noninhibitory serpins, such as angiotensinogen (AGT) and pigment epithelium-derived factor (PEDF), lack this consensus sequence (2) (Fig. 1B). The reactive site P1 residue in the neuroserpin proteins in all species examined is an arginine, suggesting tha neuroserpin inhibits trypsin-like serine proteases, similar to PN-1 and AT III that also have an arginine at the P1 position. In contrast, the region around the P1 site of the *Drosophila* Spn4.1 protein consists of the basic amino acids arginine (P3)-arginine (P2)-lysine (P1)-arginine (P1'), which suggests that Spn4.1 targets serine proteases with a basic recognition site. This prediction was confirmed by several groups that have found *in vitro* complex formation of Spn4.1 with furin, a member of the family of subtilisin-like proprotein convertases, with a consensus arginine (P4) - X (P3) - arginine/lysine (P2) - arginine (P1) cleavage site (18, 26, 27).

Neuroserpin mRNA and protein expression during *Xenopus* development

We then investigated the expression of neuroserpin mRNA and protein during the development of *Xenopus laevis* up to stage 47 (staging of embryos according to 28). Using semi quantitative RT-PCR to analyse the two *Xenopus* neuroserpin gene transcripts, we found expression of neuroserpin mRNA throughout development (Fig. 2A). More specifically, neuroserpin mRNA was already expressed at relatively high levels during all stages of oocyte development (stages I-VI), indicating that it was present maternally.

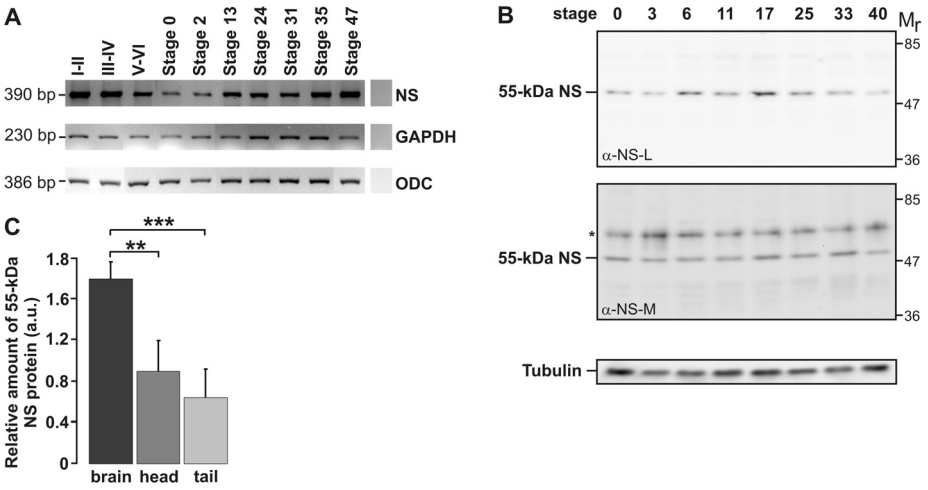


Figure 2: Neuroserpin mRNA and protein expression during *Xenopus* development
(A) Neuroserpin (NS) mRNA levels were measured with RT-PCR in three stages of oocyte development (stages I-II, III-IV and V-VI) and several stages of tadpole development. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and ornithine decarboxylase (ODC) mRNA expression were used as internal controls for RNA isolation and cDNA synthesis (32, 33). The results shown are a representative of two independent experiments. (B) NS protein expression in various developmental stages was examined by Western blotting using the anti-neuroserpin antibodies α-NS-L and α-NS-M. Analysis of tubulin protein expression served as a control for the loaded amount of protein. The asterisk indicates a non-specifically bound immunoreactive product. (C) Relative expression of 55-kDa neuroserpin in brain, remaining tissue of the head and tail of stage 49/50 embryos. Values of 55-kDa neuroserpin expression were normalized to tubulin protein levels and are expressed in arbitrary units (a.u.) ± SEM. Significant differences are indicated by asterisks (** for $p<0.01$ and *** for $p<0.001$; n=4).

In addition, expression of the neuroserpin protein was examined in various developmental stages. After western blotting using two different anti-neuroserpin antisera (α-NS-L and α-NS-M), we detected with both antibodies a specific neuroserpin product of ~55-kDa in all stages examined (Fig. 2B). At developmental stage 49/50, 55-kDa neuroserpin was more abundantly expressed in brain tissue than in the remaining tissue of the head (two-fold lower) or tail (three-fold lower) of the tadpole (Fig. 2C), suggesting that during development expression of neuroserpin is restricted to neuronal tissue. In an attempt to clarify the function of neuroserpin during *Xenopus* development, we injected *Xenopus* fertilized eggs in the one-cell stage with *Xenopus* neuroserpin RNA. This resulted in an overexpression of the *Xenopus* neuroserpin protein, but no abnormal phenotype of the injected embryos was observed (data not shown).

Tissue distribution of neuroserpin mRNA and protein in adult *Xenopus laevis*

Next, we performed RT-PCR analysis to examine neuroserpin mRNA expression in various tissues of adult *Xenopus laevis*. Relatively high expression of neuroserpin mRNA was found in brain, testis, oviduct and oocytes. Neuroserpin mRNA was moderately expressed in liver, spleen, kidney, lung and heart, whereas weak to no expression was found in stomach, intestine, gall bladder, bladder and skeletal muscle (Fig. 3A). Neuroserpin protein expression was examined by Western blotting using the two anti-neuroserpin antisera α-NS-L and α-NS-M, and a specific product with a molecular weight of ~55 kDa was detected only in brain (Fig. 3B and data not shown).

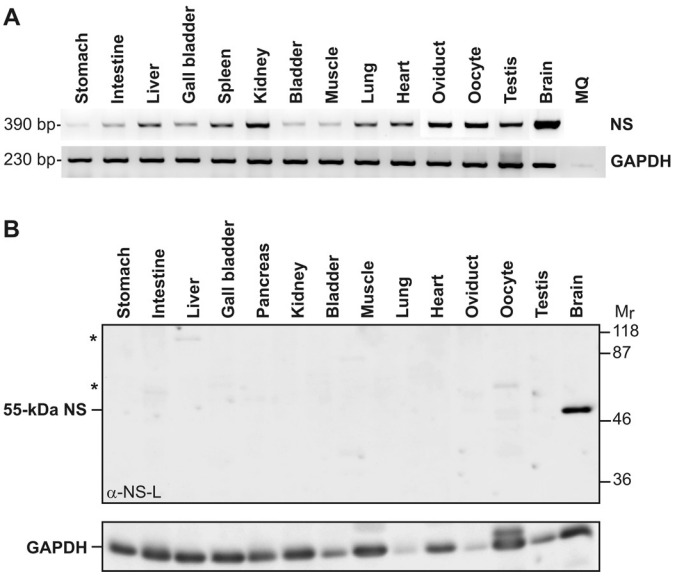


Figure 3: Neuroserpin mRNA and protein expression in various *Xenopus* tissues

(A) Neuroserpin (NS) mRNA levels were examined in various *Xenopus* tissues by RT-PCR. Analysis of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA expression served as a control. The results shown are a representative of two independent experiments. (B) NS protein expression in various adult *Xenopus* tissues was examined by Western blotting using the anti-neuroserpin antibody α-NS-L. Analysis of GAPDH protein expression was used as a control for the loaded amount of protein. The asterisks indicate non-specifically bound products.

Neuroserpin mRNA and protein expression in *Xenopus* pituitary

Finally, we examined in more detail the expression of neuroserpin mRNA and protein in a neuroendocrine tissue, namely in *Xenopus* pituitary. Using RT-PCR we found a three-fold higher expression of neuroserpin mRNA in the NIL compared with the PD (Fig. 4A, B). Neuroserpin protein expression in the two parts of the pituitary (NIL and PD) and in brain

was analyzed by Western blotting using the anti-neuroserpin antibodies α-NS-L and α-NS-M. In the NIL, we found a major product of ~55 kDa that was expressed in lower amounts in the PD. This product corresponded to the main neuroserpin protein detected in brain (Figs. 3B and 4C). In addition, neuroserpin products of ~44 and ~50 kDa were found in the NIL, and a ~46-kDa product was detected in the PD (Fig. 4C).

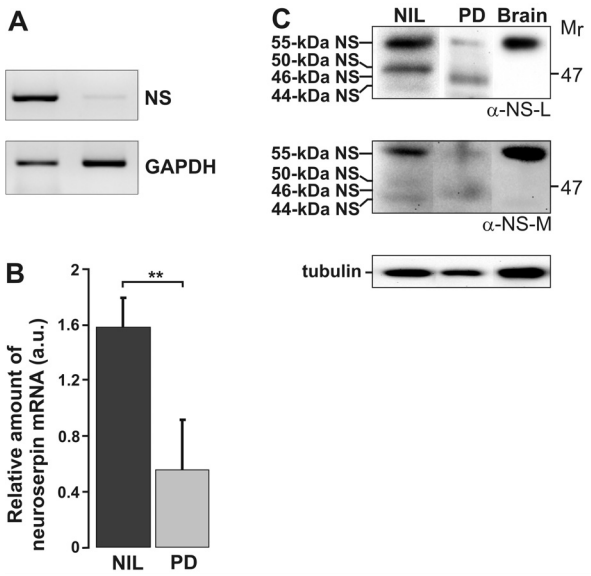


Figure 4: Neuroserpin mRNA and protein expression in *Xenopus* pituitary
(A) Neuroserpin (NS) mRNA levels were examined in the pituitary neurointermediate lobe (NIL) and pars distalis (PD) by RT-PCR. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA expression was measured as a control. The results are a representative of four independent experiments. (B) Quantification of relative amounts of NS mRNA in the NIL and PD. Values were normalized to GAPDH mRNA levels and expressed as means in arbitrary units (a.u.) ± SEM. A significant difference is indicated with two asterisks ($p<0.01$; $n=4$). (C) NS protein expression in the NIL and PD of the pituitary, and in brain tissue was detected by Western blotting using two different polyclonal anti-neuroserpin antisera (anti-NS-L and anti-NS-M). Analysis of tubulin protein expression was used as a control for the loaded amount of protein.

To study the localization of neuroserpin protein in the pituitary, cryosections were made and neuroserpin was detected using anti-neuroserpin antibody α-NS-L. We found expression of neuroserpin in the PN, PI and in parts of the PD (Fig. 5A). More specifically, in the PN neuroserpin protein was found in fibre-like structures, whereas in the PI the protein was localized in vesicle-like structures throughout the melanotrope cells (Fig. 5B).

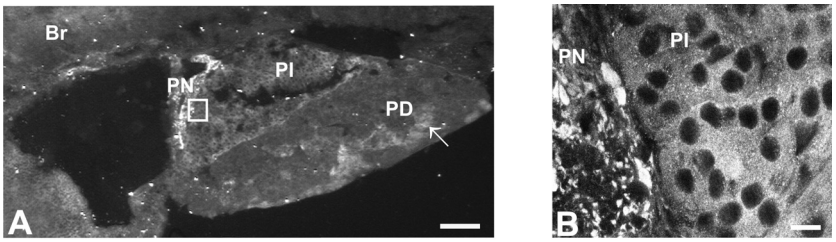


Figure 5: Localization of neuroserpin protein in *Xenopus* pituitary cells
(A) Cryosections were made of part of *Xenopus* brain (Br), and the pars nervosa (PN), pars intermedia (PI) and pars distalis (PD) of the pituitary. Neuroserpin protein was detected using anti-neuroserpin antibody α-NS-L. The white arrows indicate neuroserpin-positive regions in the PD and the area enclosed by the white box is enlarged in B. Scale bar = 200 μm. (B) Closer inspection of neuroserpin protein expression in the PN and PI of the pituitary, using confocal scanning laser microscopy. Scale bar = 10 μm.

Discussion

Serpins are important regulators of intra- and extracellular proteolysis by serine proteases. Neuroserpin represents a recently identified serpin family member, which is expressed primarily in neuronal and neuroendocrine tissues. Thus far, several roles for neuroserpin have been proposed, among which an involvement in neuronal plasticity and learning (10), neuronal cell survival (12), neurite outgrowth, and intercellular communication in mature neuroendocrine tissues by the regulation of cell adhesion (29).

In this study, we identified two *Xenopus laevis* neuroserpin cDNAs. It is likely that the two sequences represent products derived from two *Xenopus* neuroserpin-A and -B genes, since *Xenopus* is a tetraploid species as a result of a whole-genome duplication that occurred ~35 million years ago (24). Comparative analysis of the deduced *Xenopus* neuroserpin amino acid sequences with known mammalian and chicken neuroserpin and *Drosophila* Spn4.1 sequences revealed that the neuroserpin RCL comprises one of the most conserved regions. Since the RCL is an important domain of serpins responsible for target specificity, this high degree of sequence identity implies a possible conservation of the target of neuroserpin in *Xenopus*. Thus far, the target for neuroserpin is elusive, but several (trypsin-like) proteases have been suggested, of which t-PA is considered a likely target (6, 11, 12). In contrast, the *Drosophila* Spn4.1 sequence has a stretch of basic amino acids in its RCL which constitutes a recognition sequence for subtilisin-like proprotein convertases, and binding of the proprotein convertase furin to *Drosophila* Spn4.1 has indeed recently been described (18, 26, 27).

Neuroserpin is expressed during development, and in mouse embryos and human foetal tissues mRNA expression was detected mainly in the nervous system (6, 10). We found that neuroserpin mRNA and protein were expressed throughout *Xenopus*

development, and that neuroserpin protein expression became restricted to brain tissue. Interestingly, we detected neuroserpin mRNA already in all stages of oocyte development, indicating that this mRNA is maternally derived. We could not attribute a clear function to neuroserpin during early *Xenopus* development, since neuroserpin RNA micro-injections did not result in a change of phenotype of the developing embryos.

Neuroserpin mRNA expression was also detected in almost all *Xenopus* tissues examined, with relatively high expression in brain, pituitary and the reproductive organs, moderate expression in liver, kidney, lung, and heart, and weak to no expression in stomach, intestine, gall bladder, spleen, bladder and skeletal muscle. In mouse and human, neuroserpin mRNA was predominantly expressed in neuronal tissues and weak expression has been found in heart, kidney, testis and pancreas (6, 7, 10). In these studies with mammalian tissues, Northern blot analysis was used to detect neuroserpin mRNA, whereas we measured mRNA levels with the more sensitive technique of RT-PCR. This could explain why in *Xenopus* we found a broader tissue distribution of neuroserpin mRNA than has been reported for mouse and human. At the protein level, neuroserpin was detected predominantly in *Xenopus* brain and pituitary tissue, in line with findings in mouse and rat (6, 8).

We examined the expression of neuroserpin protein in the *Xenopus* pituitary in more detail. A main neuroserpin product of 55 kDa was detected in nerve fibers of the PN and in brain, indicating that it likely represents a neuronal form of neuroserpin. Furthermore, we found differently sized neuroserpin products in the NIL (44 and 50 kDa) compared with the PD (46 kDa). These data suggest that various forms of neuroserpin protein are expressed in different pituitary cell types. In rat pituitary, a number of forms of the protein have also been found in the PI and PD, with molecular weights of 55 and 53 kDa, respectively (8). The multiple forms we detected in the *Xenopus* pituitary cell types probably represent post-translational modifications of the neuroserpin protein. Previously, we found that 44-kDa neuroserpin and the neuronal 55-kDa protein are non-N-linked-glycosylated neuroserpins, whereas the 50-kDa protein has sugar groups attached at two asparagines (30). In addition, the 46-kDa form of neuroserpin, detected in the PD was also N-linked glycosylated (data not shown).

In the melanotrope cells of the pituitary PI, neuroserpin was present in vesicles, in line with findings of Hill *et al*. (8) who detected the neuroserpin protein in rat neurendocrine dense-cored secretory vesicles. Also in PC12 and AtT20 cell lines transfected with neuroserpin, the protein was localized in secretory granules (31). Proposed functions of neuroserpin in the pituitary include the regulation of intragranular proteases and, following regulated secretion, inhibition of enzymes regulating cell-extracellular matrix interactions (29). On the basis of results from studies on *Drosophila* Spn4.1, Osterwalder *et al*. (18) also suggested that neuroserpin, the closest vertebrate Spn4.1 orthologue, might function intracellularly as a regulator of prohormone processing. However, unlike Spn4.1, neuroserpin lacks the di-basic amino acid recognition sequence

for proprotein convertases in its RCL and therefore it is unlikely that neuroserpin will inhibit prohormone convertases, such as furin.

In conclusion, we identified neuroserpin in the amphibian *Xenopus laevis* and compared the deduced amino acid sequence with known mammalian and chicken neuroserpin and *Drosophila* Spn4.1 sequences. The high degree of sequence identity, especially in the RCL, suggests a conserved target specificity of the protein. Neuroserpin mRNA was expressed throughout *Xenopus* development and in a number of tissues in the adult animal, with high expression levels in neuronal and neuroendocrine tissues. Neuroserpin protein was also detected throughout development and in the adult animal mainly in brain and pituitary. Furthermore, we found expression of various forms of the neuroserpin protein in different pituitary cell types, namely a neuronal neuroserpin protein of 55 kDa in the nerve fibers of the PN, products of 44 and 50 kDa in vesicle-like structures in the neuroendocrine melanotrope cells of the PI, and a 46-kDa protein in distinct cell populations of the PD. Together, our results support an important role for neuroserpin during development and in neuronal and neuroendocrine cell functioning.

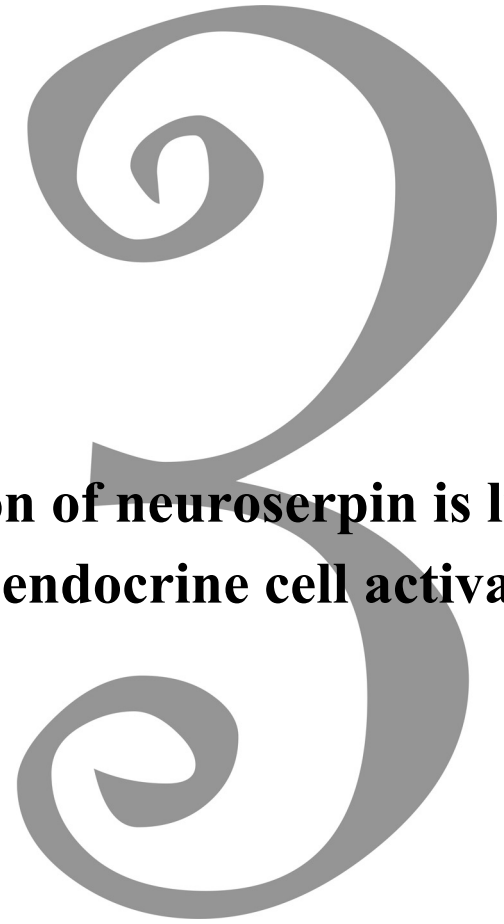
Acknowledgements

We gratefully thank Drs. D.A. Lawrence and D.A. Lomas for providing antibodies, Tony Coenen for performing the cryosectioning and immunocytochemistry, and Ron Engels for animal care.

References

1. Silverman GA, Bird PI, Carrell RW, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 2001;276:33293-6.
2. Gettins PG. Serpin structure, mechanism, and function. Chem Rev 2002;102:4751-804.
3. Huntington JA, Carrell RW. The serpins: nature's molecular mousetraps. Sci Prog 2001;84:125-36.
4. Stoeckli ET, Lemkin PF, Kuhn TB, Ruegg MA, Heller M, Sonderegger P. Identification of proteins secreted from axons of embryonic dorsal-root-ganglia neurons. Eur J Biochem 1989;180:249-58.
5. Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. Embo J 1996;15:2944-53.
6. Krueger SR, Ghisu GP, Cinelli P, et al. Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J Neurosci 1997;17:8984-96.
7. Schrimpf SP, Bleiker AJ, Brecevic L, et al. Human neuroserpin (PI12): cDNA cloning and chromosomal localization to 3q26. Genomics 1997;40:55-62.
8. Hill RM, Parmar PK, Coates LC, Mezey E, Pearson JF, Birch NP. Neuroserpin is expressed in the pituitary and adrenal glands and induces the extension of neurite-like processes in AtT-20 cells. Biochem J 2000;345 Pt 3:595-601.
9. Hill RM, Brennan SO, Birch NP. Expression, purification, and functional characterization of the serine protease inhibitor neuroserpin expressed in Drosophila S2 cells. Protein Expr Purif 2001;22:406-13.
10. Hastings GA, Coleman TA, Haudenschild CC, et al. Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. J Biol Chem 1997;272:33062-7.
11. Osterwalder T, Cinelli P, Baici A, et al. The axonally secreted serine proteinase inhibitor, neuroserpin, inhibits plasminogen activators and plasmin but not thrombin. J Biol Chem 1998;273:2312-21.
12. Yepes M, Sandkvist M, Wong MK, et al. Neuroserpin reduces cerebral infarct volume and protects neurons from ischemia-induced apoptosis. Blood 2000;96:569-76.
13. Yepes M, Lawrence DA. Neuroserpin: a selective inhibitor of tissue-type plasminogen activator in the central nervous system. Thromb Haemost 2004;91:457-64.
14. Barker-Carlson K, Lawrence DA, Schwartz BS. Acyl-enzyme complexes between tissue-type plasminogen activator and neuroserpin are short-lived in vitro. J Biol Chem 2002;277:46852-7.
15. Miranda E, Romisch K, Lomas DA. Mutants of neuroserpin that cause dementia accumulate as polymers within the endoplasmic reticulum. J Biol Chem 2004;279:28283-91.
16. Turner DL, Weintraub H. Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev 1994;8:1434-47.
17. Jansen EJ, Holling TM, van Herp F, Martens GJ. Transgene-driven protein expression specific to the intermediate pituitary melanotrope cells of Xenopus laevis. FEBS Lett 2002;516:201-7.
18. Osterwalder T, Kuhnen A, Leiserson WM, Kim YS, Keshishian H. Drosophila serpin 4 functions as a neuroserpin-like inhibitor of subtilisin-like proprotein convertases. J Neurosci 2004;24:5482-91.
19. Martens GJ. Expression of two proopiomelanocortin genes in the pituitary gland of Xenopus laevis: complete structures of the two preprohormones. Nucleic Acids Res 1986;14:3791-8.
20. Braks JA, Guldemond KC, van Riel MC, Coenen AJ, Martens GJ. Structure and expression of Xenopus prohormone convertase PC2. FEBS Lett 1992;305:45-50.
21. van den Hurk WH, Bloemen M, Martens GJ. Expression of the gene encoding the beta-amyloid precursor protein APP in Xenopus laevis. Brain Res Mol Brain Res 2001;97:13-20.
22. Martens GJ, Groenen PM, Groneveld D, Van Riel MC. Expression of the Xenopus D2 dopamine receptor. Tissue-specific regulation and two transcriptionally active genes but no evidence for alternative splicing. Eur J Biochem 1993;213:1349-54.
23. Westley B, Wyler T, Ryffel G, Weber R. Xenopus laevis serum albumins are encoded in two closely related genes. Nucleic Acids Res 1981;9:3557-74.
24. Bisbee CA, Baker MA, Wilson AC, Haji-Azimi I, Fischberg M. Albumin phylogeny for clawed frogs (Xenopus). Science 1977;195:785-7.
25. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun 1967;27:157-62.
26. Richer MJ, Keays CA, Waterhouse J, Minhas J, Hashimoto C, Jean F. The Spn4 gene of Drosophila encodes a potent furin-directed secretory pathway serpin. Proc Natl Acad Sci U S A 2004;101:10560-5.
27. Oley M, Letzel MC, Ragg H. Inhibition of furin by serpin Spn4A from Drosophila melanogaster. FEBS Lett 2004;577:165-9.

28. Nieuwkoop PD, Faber J, Hubrecht-Laboratorium (Embryologisch Instituut). Normal table of Xenopus laevis (Daudin); a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. 2d ed. Amsterdam,: North-Holland Pub. Co., 1967.
29. Hill RM, Coates LC, Parmar PK, Mezey E, Pearson JF, Birch NP. Expression and functional characterization of the serine protease inhibitor neuroserpin in endocrine cells. Ann N Y Acad Sci 2002;971:406-15.
30. de Groot DM, Martens GJ. Expression of Neuroserpin Is Linked to Neuroendocrine Cell Activation. Endocrinology 2005.
31. Parmar PK, Coates LC, Pearson JF, Hill RM, Birch NP. Neuroserpin regulates neurite outgrowth in nerve growth factor-treated PC12 cells. J Neurochem 2002;82:1406-15.
32. Bassez T, Paris J, Omilli F, Dorel C, Osborne HB. Post-transcriptional regulation of ornithine decarboxylase in Xenopus laevis oocytes. Development 1990;110:955-62.
33. Holling TM, van Herp F, Durston AJ, Martens GJ. Differential onset of expression of mRNAs encoding proopiomelanocortin, prohormone convertases 1 and 2, and granin family members during Xenopus laevis development. Brain Res Mol Brain Res 2000;75:70-5.



Expression of neuroserpin is linked to neuroendocrine cell activation

Dorien M. de Groot and Gerard J.M. Martens

Department of Molecular Animal Physiology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Institute for Neuroscience, Faculty of Science, Radboud University, Nijmegen, the Netherlands

Endocrinology (2005) 146: 3791-3799

Abstract

Inhibitors of serine proteases (serpins) are important regulators of intra- and extracellular proteolytic pathways, and they function by forming an irreversible complex with their substrate. Neuroserpin represents a neuroendocrine-specific serpin family member that is expressed in brain regions displaying synaptic plasticity. In this study, we explored the biosynthesis of endogenous neuroserpin in a neuroendocrine model system, namely the melanotrope cells of *Xenopus* intermediate pituitary. The biosynthetic activity of these cells can be physiologically manipulated (high and low production of the prohormone proopiomelanocortin [POMC] in black and white animals, respectively), resulting from a synaptic plasticity in innervating hypothalamic neurons. We found that neuroserpin was also differentially expressed in the *Xenopus* intermediate, but not anterior, pituitary with a three-fold higher mRNA- and >30-fold higher protein expression in the active versus the inactive melanotrope cells. Two newly synthesized glycosylated forms of the neuroserpin protein (47 and 50 kDa) were produced and secreted by the active cells. Intriguingly, neuroserpin was found in an ~130-kDa sodium dodecyl sulphate-stable complex in the active, but not in the inactive, melanotrope cells, which correlated with the high and low POMC expression levels, respectively. In conclusion, we report on the biosynthesis of neuroserpin in a physiological context, and we find that the induction of neuroserpin expression and the formation of the 130-kDa neuroserpin-containing complex are linked to neuroendocrine cell activation.

Introduction

Serine proteases, such as plasminogen activators, plasmin and thrombin, are known primarily for their role in blood coagulation and fibrinolysis. In addition, the proteolytic activity of these proteases is important for maintaining the integrity of the extracellular matrix (ECM). In the nervous system, serine proteases regulate plasticity in the developing as well as the mature brain (reviewed in 1, 2). For example, tissue-type plasminogen activator (t-PA) is expressed in the nervous system and has been implicated to play a role in synaptic plasticity and memory development (3, 4). Serine protease inhibitors (serpins) form a large family that can be found both intracellularly and extracellularly and regulates a number of physiological processes, such as coagulation, fibrinolysis, complement activation, angiogenesis and apoptosis. The primary function of most members is to neutralize intracellular and extracellular proteolytic pathways. One characteristic of the inhibitory serpin family members is the way in which they inhibit their substrate. Also known as suicide inhibitors, these serpins form sodium dodecyl sulphate (SDS)-stable complexes with their target protease, and complex formation is considered to be biologically irreversible (reviewed in 5). The rapid clearance of the serpin-protease

complex takes place by binding of the complex to its receptor and subsequent internalization (6, 7).

Neuroserpin is a newly identified member of the serpin family primarily expressed in brain during late stages of development and during adulthood in regions that exhibit synaptic plasticity (8, 9). Furthermore, neuroserpin has been identified in neuroendocrine cells, *e.g.* in rat pituitary and adrenal glands (10). t-PA has been considered to be a target protease for neuroserpin (9, 11, 12), although gene-targeting of neuroserpin in mice has implicated the inhibitor as playing a role in the regulation of emotional behavior through a mechanism that is, at least in part, independent of t-PA activity (13). Like other serpin family members, neuroserpin undergoes a marked conformational transition to be able to function as an inhibitor. Inevitably, this delicate process also renders the molecule susceptible to point mutations. Five mutations in the neuroserpin gene have been described, and they indeed lead to polymerization and accumulation of the protein, resulting in neuronal inclusion bodies and subsequent neuronal degeneration. This process underlies the recently described autosomal-dominant dementia known as familial encephalopathy with neuroserpin inclusion bodies (FENIB) (14, 15; reviewed in 16).

Thus far, the biosynthesis of only transfected recombinant neuroserpin has been studied (17). In this study, we examined the expression and biosynthesis of endogenous neuroserpin in the neuroendocrine melanotrope cells of the intermediate pituitary of *Xenopus laevis*. The process of background adaptation of this amphibian provides the opportunity to manipulate in a physiological way, *in vivo,* the activity of the melanotrope cells as well as their regulatory neuronal input. In animals adapted to a black background, the melanotrope cells are very active and produce vast amounts of the prohormone proopiomelanocortin (POMC), which is processed to a number of bioactive peptides, including α-melanophore-stimulating hormone (α-MSH). This hormone causes pigment dispersion in skin melanophores, giving the animal a black appearance. In white-background-adapted animals, the activity of the melanotrope cells is inhibited by neurons originating from the hypothalamic suprachiasmatic nucleus, which make direct synaptic contacts with the melanotrope cells. Thus, manipulation of melanotrope cell activity is accompanied by differences in synaptic plasticity (reviewed in 18). Here we find that neuroserpin mRNA and protein expression are also induced in the biosynthetically active melanotrope cells from black-adapted animals compared with the inactive cells of white animals. Neuroserpin was synthesized as a 47-/50-kDa protein and formed, presumably with its substrate, a stable ~130-kDa complex, the formation of which was dependent on the color of the background of the animal and thus the melanotrope cell activity.

Materials and Methods

Animals

South African claw-toed frogs, *Xenopus laevis*, were reared in the Central Animal Facility of the Radboud University of Nijmegen, The Netherlands. The animals were adapted to their background by keeping them in either white or black buckets for at least three weeks at 22°C, with a light/dark cycle of 12 h. Experimental procedures were performed under the guidelines of the Dutch law concerning animal welfare.

Antibodies

Four anti-neuroserpin antibodies were used: mouse monoclonal IgG and rabbit polyclonal IgG against human recombinant neuroserpin (mα-NS and α-NS-L, respectively; generous gifts of D.A. Lawrence, American Red Cross Holland Laboratory, Rockville, Maryland, USA; 19), rabbit polyclonal IgG against human recombinant neuroserpin (α-NS-M, generous gift of D. Lomas, University of Cambridge, Cambridge, UK; 17), and rabbit polyclonal IgG directed against the C-terminal region of rat neuroserpin (CGRVMHPETMNTSGHDFEEL; α-NS-PEP) (generous gift of N. Birch, University of Auckland, New Zealand; 10). The dilutions used were 1:400 (α-NS-L) and 1:300 (mα-NS and α-NS-PEP) for immunoprecipitation and 1:5,000 (α-NS-L), 1:2,500 (mα-NS) and 1:10,000 (α-NS-M) for Western blotting. Tubulin, POMC and p24δ_2 were identified on Western blots using a monoclonal anti-tubulin antibody (1:1,500) (generous gift of Dr. J. Fransen, Radboud University, Nijmegen, The Netherlands; 20), a polyclonal anti-POMC antibody (1:20,000) (ST62; generous gift of Dr. S. Tanaka, Shizuoka University, Shizuoka, Japan; 21) and the polyclonal anti-p24$\delta_{1/2}$ antibody 1262CH (1:5,000) (as described previously in 22), respectively.

Separation of Xenopus pars nervosa and intermediate pituitary

To compare neuroserpin protein expression in the pars nervosa with that in the intermediate pituitary, the two lobes were separated. To isolate the melanotrope cells from the neurointermediate lobe (NIL), NILs were dissected, washed several times in sterile *Xenopus* XL15 (10 mM glucose, 2 mM $CaCl_2$, 1% kanamycin (Life Technologies, Inc.), 1% antibiotic/antimycotic (Life Technologies, Inc.) in 67% Leibovitz's-15 medium (Life Technologies, Inc.) and transferred to XL15 containing 0.25% (wt/vol) trypsin. After incubating for 45 min at 20°C, XL15 was added and the lobes were suspended by seven passages through a siliconized Pasteur's pipet. The cell suspension was transferred to a syringe and filtered through a nylon filter (pore size 60 μm). Finally, the melanotrope cells were collected by centrifugation (10 min at 6000 rpm) and the remaining pars nervosa was collected from the filter. Alternatively, the pars nervosa and intermediate pituitary were separated by dissection under a microscope. After separation, homogenates of cells and

lobes were made in lysis buffer (250 mM sucrose, 1% Triton X-100, 10 mM TrisCl, 1 mM EDTA (pH7.4), 1μM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml soybean trypsin inhibitor) using a glass homogenizer. After 20 min incubation on ice, the samples were centrifuged for 20 min (153,000 rpm, 4°C). Sample buffer (50 mM TrisCl (pH6.8), 100 mM dithiothreitol (DTT), 2% SDS, 0.1% bromephenol blue, 10% glycerol) was added to the supernatant, and the samples were heated for 30 min at 37°C.

Western blotting

Homogenates of the NIL, the pars nervosa, intermediate pituitary, and isolated melanotrope cells were prepared as described above. Samples (corresponding to 25-33% of one NIL) were separated on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (PVDF) membrane (0.45 μM Hybond-P, Amersham Pharmacia Biotech) using a BioRad (Hercules, CA) mini-protean II cell system. Molecular weight markers were run alongside the samples to be analyzed. For immunodetection, blots were washed one time in PBS and incubated for at least 1h in blocking buffer (5% skimmed milk and 1% Tween 20 in PBS). Subsequently, blots were incubated with primary antiserum in blocking buffer for 16 h (4°C). After three rinses in wash buffer (1% skimmed milk and 1% Tween 20 in PBS), blots were incubated in horseradish peroxidase (HRP)-conjugated goat-anti-rabbit or mouse IgG (1:5,000) for 1 h. After three rinses in wash buffer and one rinse in PBS, the proteins were visualized by incubating blots in Lumi-Light^{PLUS} Western blotting substrate (Roche Diagnostics, Manheim, Germany) and by subsequent exposure to X-ray film (Eastman Kodak, Rochester, NY). For quantification of relative neuroserpin protein levels in black- compared with white-adapted animals, detection was performed using a BioChemi imaging system (UVP, Inc., Upland, CA) and signals were analyzed using LabWorks 4.0 software (UVP BioImaging systems, UVP, Inc., Cambridge, UK). Total densities of 44-, 47-/50- and 55-kDa neuroserpin products were normalized to the corresponding tubulin samples (n = 3). Subsequently, the values found for 44-kDa neuroserpin in white-adapted animals were set at 1, and relative protein levels of 44-, 47-/50- and 55-kDa neurserpin were calculated. The mean differences of 44- and 47-/50-kDa neuroserpin were plotted in a graph and a paired t test was used for statistical analysis.

Metabolic cell labeling and immunoprecipitation

For metabolic cell labeling, NILs of black-adapted *Xenopus* were rapidly dissected and preincubated in Ringer's medium (112 mM NaCl, 15 mM Hepes pH 7.4, 2 mM KCl, 2 mM $CaCl_2$, 2 mg/ml glucose and 0.3 mg/ml BSA) for 15 min at 22°C. Radioactive labeling of newly synthesized proteins was performed by incubating the NILs in Ringer's medium containing 5 mCi/ml Tran^{35}S label (ICN Radiochemicals, Irvine, CA) for 30 or 90 min at 22°C. After the pulse labeling, NILs were rinsed in Ringer's medium and, in case a chase was performed, incubated in Ringer's medium supplemented with 0.5 mM L-methionine for 150 min at 22°C. Subsequently, lysates were prepared as described above.

Immunoprecipitation was performed in TTD buffer (50 mM HEPES, 140 mM NaCl, 0.1% Triton-X100, 1% Tween-20, 1 mM EDTA, 1 mg/ml deoxycholate (DOC), 1 µM PMSF, 0.1 mg/ml soybean trypsin inhibitor) supplemented with SDS (final concentration of 0.075%) and antiserum. After overnight rotation at 4°C, immune complexes were precipitated with protein A-sepharose (Amersham-Pharmacia Biotech), washed four times with TTD buffer and analyzed on a 10% SDS-polyacrylamide gel.

N-glycosidase F treatment

For protein deglycosylation, N-glycosidase F (which cleaves N-linked sugar chains from proteins) was used. The NIL lysates were boiled for 10 min in 6 mM HEPES (pH7.4) containing 0.06% SDS and subsequently supplemented with 0.5% Nonidet P-40, 10 µg/ml soybean trypsin inhibitor, and 0.1 µM PMSF, and incubated with or without 40 mU/ml N-glycosidase F (Roche Diagnostics) for 1.5 h at 37°C.

Two-dimensional gel electrophoresis and protein analysis

For two-dimensional gel electrophoretic analysis, NILs were dissected and the tissues were homogenized using a glass potter in 40 mM Tris base (pH9.5) and 10 mM Pefablock (Roche). Proteins were trichloroacetic acid precipitated, air-dried, and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% DTT and 0.8% IPGphor buffer (pH3-10; Amersham-Bioscience, Little Chalfont, Buckinghamshire, UK). The samples were separated by isoelectric point (pI) (pH3-10 gradient in the first dimension) and subsequently by molecular weight on 10% SDS-PAGE. Neuroserpin products were then identified by Western blotting using the α-NS-L antibody. Protein spots were analyzed using matrix-assisted laser desorption ionization-time-of-flight-mass spectrometry (MALDI-TOF MS) and liquid chromatography-electrospray ionization mass spectrometry (LC/ESI-MS) as described previously (23).

Real-time quantitative RT-PCR analysis

The NIL and anterior lobe (AL) of the pituitary of black- and white-adapted *Xenopus* were dissected collected in Trizol (Life Technologies, Inc.) and total RNA was isolated according to the manufacturer's instructions. The RNA was then dissolved in 20 µl RNase-free H_2O and the concentration was measured with a GeneQuant RNA/DNA calculator (Biochem Ltd., Cambridge, UK). One µg total RNA was reverse transcribed; first-strand cDNA synthesis was performed using 11 µl RNA and 1 µl $pd(N)_6$ (random primers, 5 mU/µl, Roche) at 70°C for 10 min, followed by double-strand synthesis in 20 µl strand buffer (Life Technologies, Inc.) with 10 mM DTT, 20 U RNAsin (Promega, Madison, WI), 0.5 mM dNTPs (Roche), and 100 U reverse transcriptase (SuperScript II, Life Technologies, Inc.) at 37°C for 90 min. Samples were diluted five times in RNase-free H_2O

and subjected to real-time quantitative RT-PCR on a 5700 GeneAmp PCR system (PE Applied Biosystems, Wellesley, MA) as follows: 5 µl template cDNA was added to 20 µl SYBR green buffer (PE Applied Biosystems) with 3 mM $MgCl_2$, 0.2 m dNTPs (PE Applied Biosystems), 0.6 µM of each primer, and 0.625 U AmpliTaq gold (PE Applied Biosystems). To amplify the neuroserpin nucleotide sequence, the forward primer 5'-ATTGAAAGCCCATTTGATTGAAG -3' and reverse primer 5'-TCAAGACCTCCTTTAGGTTCACTAC-TT -3' were used and for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) the primers 5'-GCCGTGTATGTGGTGGAATCT-3' and 5'-AAGTTGTCGTTGATGACCTTTGC-3', respectively. PCR conditions were 95°C for 10 min followed by 40 reaction cycles of 95°C for 15 sec, and 60°C for 1 min each. For each reaction, the cycle threshold (Ct) was determined, i.e. the cycle number at which fluorescence was detected above an arbitrary threshold (0.5). At this threshold, Ct values were within the exponential phase of the amplification. Ct values for neuroserpin mRNAs in lobes from black- vs. white-adapted animals were normalized to that of the internal standard (GADPH) mRNA. To examine the length of the amplified DNA, the reaction products were run on a 2.5% agarose gel and visualized with ethidium bromide.

Results

Neuroserpin mRNA and protein expression in the pituitary of black- and white-adapted *Xenopus*

We used quantitative RT-PCR to examine neuroserpin mRNA expression in the NIL and the AL of the pituitary of black- and white-adapted *Xenopus*. Neuroserpin mRNA was detected in both lobes of the pituitary. A three-fold higher level of neuroserpin mRNA was found in the NIL of black-adapted animals than in that of white-adapted animals (3.2 ± 1.1-fold induction, n=6). Neuroserpin mRNA levels were not significantly different in the ALs of black and white animals (Fig. 1). Thus, neuroserpin mRNA expression was up-regulated specifically in the biosynthetically active *Xenopus* melanotrope cells.

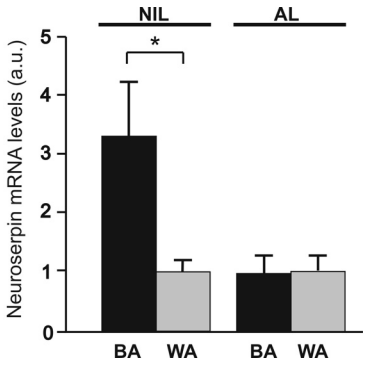


Figure 1: Neuroserpin mRNA levels in the neurointermediate and anterior pituitary of black- and white-adapted *Xenopus*.
Neuroserpin mRNA levels in the neurointermediate lobe (NIL) and the anterior lobe (AL) of the pituitary of black-adapted (BA; black bars) compared to those in white-adapted (WA; grey bars) animals were measured by quantitative RT-PCR. All values were normalized to GAPDH mRNA levels and are expressed as means ± SEM. A significant difference is indicated by an asterisk ($p<0.05$).

Western blot analysis with a number of anti-neuroserpin antibodies (α-NS-L, α-NS-M, α-NS-PEP and mα-NS) was performed to study the steady-state neuroserpin protein levels in the NILs of black- and white-adapted *Xenopus*. In NIL lysates of black-adapted animals, we detected with α-NS-L, α-NS-M and mα-NS expression of ~44-, ~47-/50- and ~55-kDa neuroserpin proteins. Furthermore, using the two polyclonal anti-neuroserpin antisera α-NS-L and α-NS-M, an immunoreactive product of ~130 kDa was found (Fig 2A); the three additional products of ~57-, ~60- and ~70 kDa are considered to be nonspecifically interacting products, as in each case they were recognized by only one of the two antibodies (Fig 2A, asterisks). The NS-PEP antibody was not appropriate for Western blot analysis, and the fact that the 130-kDa product was not detected with the monoclonal antibody mα-NS was due presumably to epitope masking. Using α-NS-L, α-NS-M and mα-NS for analysis of NILs of white-adapted animals, only the 44-, 47-/50- and 55-kDa proteins, but not the 130-kDa product, were found (Fig. 2A). At least a 30-fold higher level of expression of the 130-kDa neuroserpin-containing product was detected in the active melanotrope cells of black-adapted animals, compared with the inactive cells of white animals. The 44-kDa neuroserpin protein was up-regulated three-fold in the NIL of black- compared with white-adapted animals (2.8 ± 0.3-fold induction, n=3), whereas 47-/50- and 55-kDa neuroserpin expression did not differ significantly (Fig. 2B and data not shown).

The 130-kDa neuroserpin-containing complex is expressed in *Xenopus* intermediate pituitary cells

Because the NIL consists of the intermediate pituitary (the neuroendocrine melanotrope cells) and the pars nervosa (endings of neurons originating from the hypothalamus), we decided to study the site of neuroserpin protein expression in the *Xenopus* NIL in more detail. For this purpose, we separated the pars nervosa from the intermediate pituitary cells using two different strategies. First, the pars nervosa and intermediate pituitary were physically separated by dissection under a microscope. In a second approach, the melanotrope cells of the intermediate pituitary were isolated from the NIL by trypsin treatment; the remaining tissue after this treatment consisted mainly of the pars nervosa. Remarkably, we found that 55-kDa neuroserpin was expressed exclusively in the pars nervosa from black- and white-adapted *Xenopus* (Fig. 2C), whereas 47-/50-kDa neuroserpin and the 130-kDa complex were present in the melanotrope cells of the intermediate pituitary in black-adapted animals (Fig. 2C, upper panel). In the NIL of white-adapted animals, 47-/50-kDa neuroserpin expression levels were below the detection limit. The low amount of the 130-kDa product found in the pars nervosa following dissection or trypsin treatment probably originated from residual intermediate pituitary melanotrope cells, caused by incomplete separation of the two parts of the pituitary. The observed differences in the ratios between the 130-kDa complex and 55-kDa neuroserpin in the

NIL(*e.g*. Figs. 2A and 2D) were therefore probably caused by variations in tissue dissection (especially concerning the amount of pars nervosa tissue).

We next wanted to investigate whether neuroserpin is glycosylated in the *Xenopus* NIL. After N-glycosidase-F treatment of a NIL lysate, the migrations of the 130-kDa product and 47-/50-kDa neuroserpin shifted to products of ~115 kDa and ~44 kDa, respectively, whereas the migration of 55-kDa neuroserpin was not affected (Fig. 2D). This finding indicates that only the 130-kDa product and 47-/50-kDa neuroserpin are N-linked glycosylated.

Biosynthesis and post-translational modification of neuroserpin in the *Xenopus* neurointermediate pituitary

Pulse metabolic cell labeling of the *Xenopus* NIL for 30 or 90 min and subsequent immunoprecipitation analysis of the newly synthesized proteins with three anti-neuroserpin antisera (α-NS-L, NS-PEP, and mα-NS) revealed the production of two newly synthesized neuroserpin proteins with molecular weights of ~47- and ~50 kDa (Fig. 3A and data not shown). Following a 2.5-h chase period, the newly synthesized neuroserpins were secreted into the incubation medium as products with slightly higher molecular weights (~48- and ~51 kDa) than the two neuroserpin products in the cells (Fig. 3B). Treatment of the immunoprecipitated newly synthesized neuroserpins with N-Glycosidase-F gave an ~44-kDa product in both the cells and the medium, indicating that the 47- and 50-kDa neuroserpins and the two secreted forms are N-linked glycosylated (presumably on one residue in the 47-/48-kDa and two in the 50-/51-kDa forms) (Fig. 3C). The observed differences in molecular weights between the neuroserpins produced in the cells and secreted into the medium may thus be due to a modification of the N-linked sugar groups. After a 2.5-h chase period, but not after a 1-h pulse labeling, a product of ~130 kDa was immunoprecipitated from the cell lysates, suggesting that this product may represent a complex of newly synthesized neuroserpin with another protein and that this complex is not formed immediately after neuroserpin synthesis; we did not detect this complex in the media (Fig. 3C). Because treatment of the immunoprecipitate with N-glycosidase-F resulted in a shift of the newly-synthesized 130-kDa to an ~115-kDa product, the complex was N-linked glycosylated (Fig. 3C), in line with the western blot results (Fig. 2D).

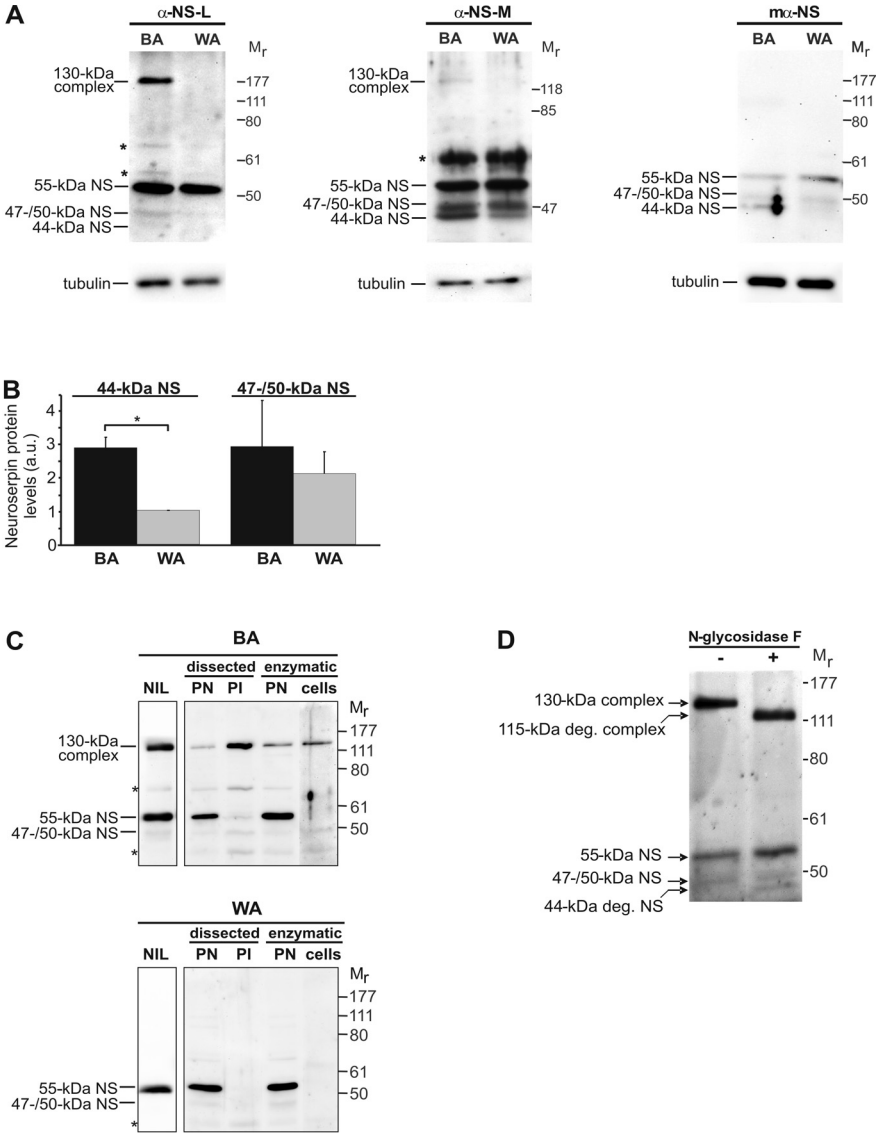


Figure 2: Neuroserpin protein levels in the neurointermediate pituitary of black- and white-adapted *Xenopus*.

(A) Lysates of neurointermediate lobes (NILs) of black- and white-adapted frogs (BA and WA, respectively) were separated by SDS-PAGE. After Western blotting, neuroserpin (NS) was identified using the polyclonal anti-neuroserpin antisera α-NS-L and α-NS-M and the monoclonal anti-neuroserpin antibody mα-NS. Tubulin was used as a control for loading equal amounts of protein. Nonspecifically bound proteins are indicated with asterisks. (B) Quantification of 44- and 47-/50-kDa neuroserpin (NS) protein expression in NILs of BA and WA animals relative to 44-kDa neuroserpin expression in WA animals, which was set at 1. Values were normalized to tubulin protein levels and expressed as mean differences ± SEM. A significant difference is indicated by an asterisk ($p<0.05$). (C) The 130-kDa complex is expressed in the melanotrope cells of the intermediate pituitary of black-

adapted *Xenopus*. Melanotrope cells of the intermediate pituitary were isolated from the neurointermediate lobe (NIL) of BA and WA animals by either dissection of the pars intermedia (PI) tissue from the pars nervosa (PN) (dissected) or trypsin treatment of the NIL tissue (enzymatic). Extracts of PI- and PN-tissues were separated on 10% SDS-PAGE and subjected to Western blot analysis with anti-neuroserpin antibody α-NS-L. Nonspecifically interacting proteins are indicated with asterisks. (D) NIL lysate of BA animals was treated with N-glycosidase F and subsequently subjected to SDS-PAGE and immunoblotting with α-NS-L.

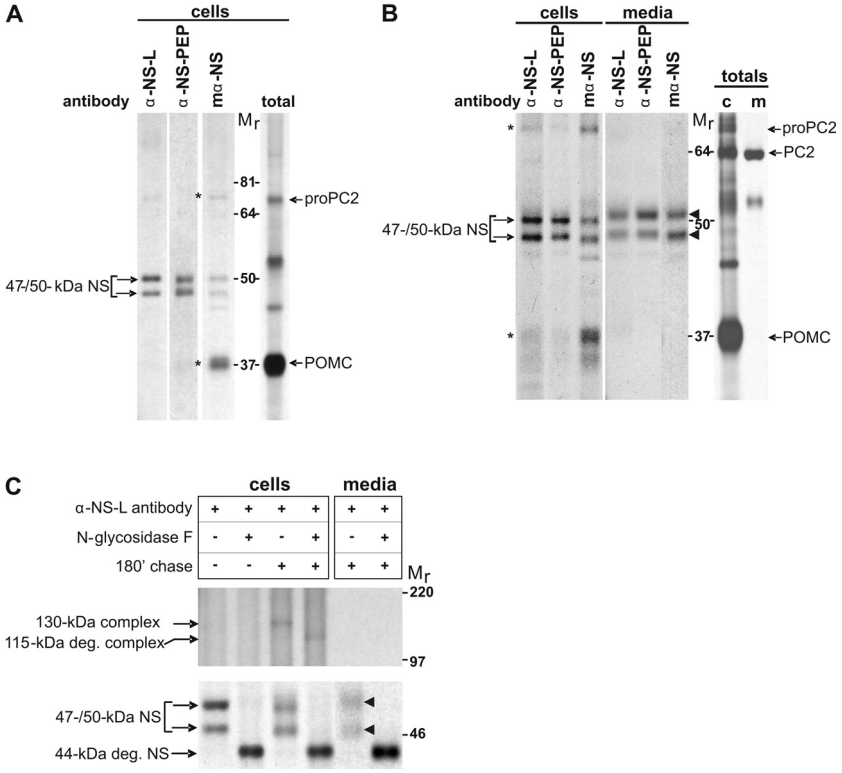


Figure 3: Biosynthesis and release of neuroserpin by the intermediate pituitary cells of *Xenopus*.
(A) Neurointermediate lobes (NILs) were pulse labeled for 90 min. Cell lysates were directly analysed on SDS-PAGE (total, representing 5% of the total cell lysate) or immunoprecipitated using the anti-neuroserpin antibodies α-NS-L, α-NS-PEP, or mα-NS, followed by resolving the immunoprecipitates with SDS-PAGE. When using mα-NS in the immunoprecipitation, a number of newly synthesized proteins, such as the proform of the prohormone convertase PC2 (proPC2) and POMC bound nonspecifically to the antibody (asterisks). (B) NILs were pulse labeled for 90 min and chased for 2.5 h, and the cell lysate (c) and incubation medium (m) were directly analyzed on SDS-PAGE (totals, representing 5% of the total cell lysate and 20% of the incubation medium, respectively) or immunoprecipitated using α-NS-L, α-NS-PEP, or mα-NS, followed by SDS-PAGE. The secreted neuroserpin (NS) proteins and nonspecifically bound proteins are indicated with arrow heads and asterisks, respectively. (C) NILs were pulse labeled for 60 min or pulse labeled for 60 min and chased for 2.5 h. Cell lysates and media were treated with N-glycosidase F before immunoprecipitation and subsequent SDS-PAGE. The abbreviations used are: NS, neuroserpin; proPC2, pro-prohormone convertase 2; POMC, proopiomelanocortin; deg., deglycosylated.

The 130-kDa neuroserpin-containing complex is acidic

Because of the striking characteristics of the 130-kDa neuroserpin-containing product (difference in expression levels in the NILs of black- and white-adapted *Xenopus*, and the newly synthesized 130-kDa product is not formed in the first hour of chase) we were interested in the nature of this high-molecular weight product. In view of its size, it is not likely that the 130-kDa product would simply represent a multimerization of the 47-/50-kDa neuroserpin. One feature of serpins is their unusually strong binding to their substrate (reviewed in 24). We hypothesized that the 130-kDa product might be an SDS-stable complex of neuroserpin with a serine protease such as t-PA, urokinase-type plasminogen activator (u-PA), plasmin, or thrombin, since these four proteins have been found to be *in vitro* substrates for neuroserpin (19, 9, 11). These potential substrates are basic proteins with a calculated pI of ~8.2, ~8.8, ~7.2, and ~7.3, respectively. To estimate the pI of the 130-kDa neuroserpin-containing complex in the NIL, two-dimensional gel electrophoresis of NIL protein extracts combined with immuodetection of neuroserpin was performed. We found that, in line with the calculated pI of *Xenopus* neuroserpin (pI 5.4), 47-/50-kDa neuroserpin, 55-kDa neuroserpin, and the 130-kDa complex are acidic products with a pI of ~5.6, ~5.2, and ~5.3, respectively (Fig. 4), indicating that the 130-kDa neuroserpin-containing complex displays a pI similar to that of neuroserpin itself. This observation implies that the binding partner of neuroserpin in the 130-kDa complex is also an acidic protein and makes it unlikely that, in the intermediate pituitary of black-adapted *Xenopus,* this complex is formed by binding of neuroserpin to the basic proteins t-PA, u-PA, plasmin, or thrombin.

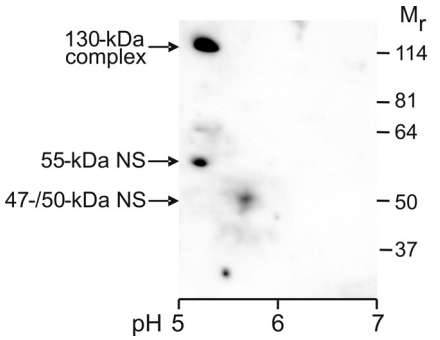


Figure 4: Neuroserpin and the neuroserpin-containing complex are acidic in the neurointermediate pituitary of *Xenopus*.
Two-dimensional gel electrophoresis (pH gradient in the first dimension and SDS-PAGE in the second dimension) of a lysate of the neurointermediate lobe of black-adapted *Xenopus* followed by Western blot analysis with anti-neuroserpin antibody α-NS-L.

In an attempt to identify the substrate of neuroserpin in the 130-kDa complex, we separated NIL proteins by two-dimensional gel electrophoresis, recovered a number of protein spots from the region in which the 130-kDa complex migrated, and analyzed the spots by MALDI-TOF-MS or by directly combined LC/ESI-MS. Unfortunately, the protein amounts were below the detection limit (data not shown), presumably due to the low expression level of the complex.

Dynamics in the expression of the 130-kDa complex in the intermediate pituitary during background adaptation of *Xenopus*

An interesting characteristic of *Xenopus laevis* is its ability to adapt its skin color to its background. The process of background adaptation is mediated by α-MSH, a cleavage product of POMC that is produced at high levels in the intermediate pituitary melanotrope cells of a black-adapted animal. Because of the clear differential expression of the 130-kDa neuroserpin-containing complex in the inactive and active melanotrope cells of white and black animals, respectively, we were interested in the time course of the formation of this complex during background adaptation. For this analysis, the expression of the 130-kDa complex in the NIL was examined in white animals adapting to a black background and vice versa for various time periods. In white animals adapting to a black background, we first detected the complex after five days of adaptation; its level gradually increased until maximum expression levels were reached after 21 days, similar to the level found in fully black-adapted animals (*i.e.* adapted to a black background for more than three weeks). The time course of expression of the 130-kDa complex was similar to that of POMC and the p24δ_2 protein (a putative receptor for endoplasmic reticulum to Golgi cargo transport) with in each case a change of >30-fold (Fig. 5A). In black animals adapting to a white background, the expression levels of the 130-kDa complex dropped considerably already after one day of adaptation, followed by a slow decrease such that, after 16 days, the expression was not detectable anymore. In contrast, during this adaptation the levels of POMC and p24δ_2 only decreased gradually (Fig. 5B). Thus, during adaptation to a white background, the time course of expression of the 130-kDa complex differed from those of POMC and p24δ_2.

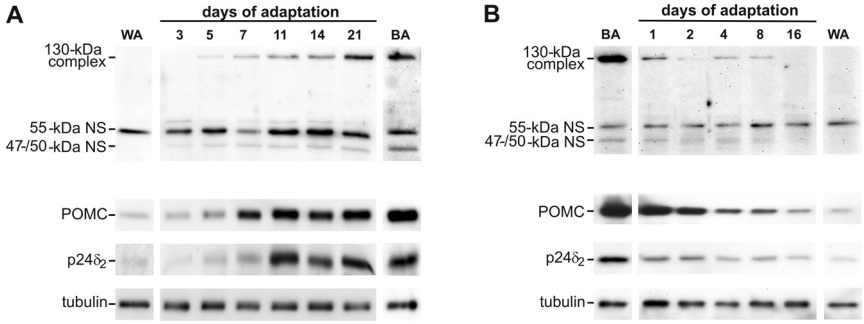


Figure 5: Dynamics in the expression of the neuroserpin-containing complex in the intermediate pituitary during background adaptation of *Xenopus*.

(A) Fully white-adapted (>21 days; WA) animals were adapted to a black background for 3, 5, 7, 11, 14 or >21 days (fully black-adapted; BA). The neurointermediate lobes were isolated and analyzed on Western blot using anti-neuroserpin antibody α-NS-L. For reference, protein expression of POMC, p24δ_2, and tubulin was analyzed. (B) Fully BA animals were adapted to a white background for 1, 2, 4, 8, 16 or >21 days (WA). Experimental conditions as under A. In both A and B, representatives of three independent experiments are shown.

Discussion

Serine proteases form a large family of intra- and extracellular proteases, and have been implicated in a wide variety of biological processes. For example, in the nervous system, proteolysis of the ECM by serine proteases is an important step in the process of synaptic plasticity (25). Obviously, these proteolytic events ask for a delicate control that is provided by regulatory antiproteases called serpins. Neuroserpin is a recently described serpin that is mainly expressed in neuronal and neuroendocrine cells. In this study, the expression and biosynthesis of neuroserpin in the neuroendocrine melanotrope cells of the intermediate pituitary of the amphibian *Xenopus laevis* were examined. The *Xenopus* neurointermediate pituitary forms a highly plastic system that can be physiologically manipulated. The plasticity is exhibited by both the innervating hypothalamic neurons and the melanotrope cells, at the levels of proteins, subcellular structures, and synapses (reviewed in 18).

We found that, in the *Xenopus* melanotrope cells, neuroserpin was initially synthesized as a 44-kDa protein that was subsequently once or twice N-linked glycosylated to products of 47- and 50 kDa, respectively. Glycosylation occurred rapidly after synthesis, as mainly the glycosylated forms were detected during the metabolic cell labeling studies. Both 47- and 50-kDa neuroserpin were secreted into the incubation medium in a slightly modified form, probably as a result of a modification of the N-linked sugar chains, the nature of which remains to be determined. These results constitute the first report on the biosynthesis of neuroserpin under physiological conditions. In the only other biosynthetic study reported thus far, transfected cells were used, and four forms of newly synthesized neuroserpin (45, 47/48, 50 and 55 kDa) were found of which the 47-/48-, 50- and 55-kDa forms were glycosylated (17). Previous Western blot analyses of mammalian tissues and transfected cells have revealed that the molecular weights for steady-state neuroserpin range from 40 to 55 kDa (19, 9, 26, 10). The sizes we detected for immunoreactive *Xenopus* neuroserpin (44-, 47-/50- and 55-kDa forms) lie within the same range. Because 55-kDa neuroserpin was expressed specifically in the *Xenopus* pars nervosa, which consists of biosynthetically inactive nerve endings, it was not surprising that we did not detect newly synthesized 55-kDa neuroserpin when the NIL was metabolically labeled. Furthermore, we found the 55-kDa neuroserpin protein in *Xenopus* brain extracts (data not shown) suggesting that this neuroserpin product represents a neuronal form, whereas 44- and 47-/50-kDa neuroserpin produced in the melanotrope cells of the intermediate pituitary are neuroendocrine forms of the protein. Remarkably, 55-kDa neuroserpin was not N-linked glycosylated and is thus probably otherwise post-translationally modified. Some serpins are regulated in their antiproteolytic activity by binding of a ligand such as heparin (27). However, heparin-binding seems unlikely for neuroserpin as previous studies have suggested that the antiproteolytic activity of neuroserpin is heparin independent (26).

In addition to 44-, 47-/50- and 55-kDa neuroserpin, a further immunoreactive product with a molecular weight of 130 kDa was detected. In principle, a neuroserpin

multimer may explain the presence of the high-molecular weight product. Although polymerization of certain serpins is generally caused by mutant forms of the protein (reviewed in 16), serpins may indeed show spontaneous polymerization under physiological conditions (28, 29). However, *Xenopus* neuroserpin dimer or trimer formation would have resulted in a complex of ~100- and ~150 kDa, respectively. Since the newly synthesized 130-kDa product was immunoprecipitated only after the 2.5-h chase and not after the 1-h pulse incubation, the occurrence of this product likely reflects the coimmunoprecipitation of neuroserpin with a tightly bound substrate. In general, serpins form a tight complex with their substrate (reviewed in 5). We therefore hypothesize that the 130-kDa neuroserpin-containing product represents an SDS-stable complex of 47-/50-kDa neuroserpin with a substrate. Characterization of the 130-kDa neuroserpin-containing complex using two-dimensional gel electrophoresis revealed that both neuroserpin and the 130-kDa complex are acidic (pI ~5.4). Because the currently known (*in vitro*) serine protease substrates for neuroserpin (t-PA, u-PA, plasmin, and thrombin) are basic proteins, the observed pI of the complex indicates that these enzymes do not constitute the substrate for *Xenopus* neuroserpin. Unfortunately, our attempts to further characterize the 130-kDa complex using MALDI-MS and LC/ESI-MS were not successful because of the low endogenous expression levels of the complex. This is not a unique situation. For instance, Misra *et al*. (30) observed a high-molecular mass complex containing a serine protease with presumably a serpin, but they could also not identify the associated protein. Recently, *Drosophila* serine protease inhibitor 4 (SP-4), the closest invertebrate ortholog of neuroserpin, has been found to inhibit *in vitro* the subtisilin-like proprotein convertase furin (31). However, the amino acid sequence of the reactive site of *Xenopus* neuroserpin (with an arginine and a methionine at positions P1 and P1', respectively) suggests that this serpin will preferably bind a trypsin-like substrate rather than a proprotein convertase such as furin, as the type of target protease is thought to be specified by the positions P1 and P1' of the reactive site (32, 33); an arginine and methionine at P1 and P1', respectively, are also found in chicken neuroserpin which most likely targets trypsin-like proteases (26). Moreover, in view of the molecular weight of furin (~57 kDa) a neuroserpin-furin complex of 130 kDa would be unlikely. In mouse cortical cultures and embryonic fibroblasts, the very low density lipoprotein receptor (VLDL receptor) and the low density lipoprotein receptor-related protein (LRP) have been shown to bind and internalize neuroserpin (34). Again, because of their sizes it is not to be expected that the *Xenopus* VLDL receptor or LRP (~100- and ~101 kDa, respectively) will form a 130-kDa complex with *Xenopus* neuroserpin. Thus, at present the identity of the substrate interacting with *Xenopus* neuroserpin remains unclear.

Of special interest was the observation that the 130-kDa complex was expressed specifically in the melanotrope cells of the intermediate pituitary and not in the pars nervosa, and that in our biosynthetic studies the complex was not detected in the medium. The complex may thus be formed intracellularly during the transport of neuroserpin through the secretory pathway or in the melanotrope ECM. Furthermore, the complex was

found in the melanotropes of only black- and not white-adapted animals, correlating with high and low POMC expression levels, respectively. These findings suggest that the formation of the complex is linked to melanotrope cell activation. The melanotrope cells play a key role in the physiological process of background adaptation of *Xenopus*. When the animal is on a black background, the melanotrope cells are biosynthetically highly active, producing large amounts of POMC and releasing α-MSH. On a white background, the cells are inactive, α-MSH release is inhibited and the animal turns white. Inhibition of the melanotrope cells is effected by contacting synapses that appear at the ultrastructural level as axon varicosities of neurons originating from the suprachiasmatic nucleus of the hypothalamus (suprachiasmatic melanotrope-inhibiting neurons, SMINs) (35-38). The terminal varicosities of these inhibitory neurons are much larger and more numerous in white- than in black-adapted animals (more than double the number of active synaptic zones are present on the melanotropes of white- relative to black frogs) (35). When a black animal is placed on a white background, a fast inhibition of melanotrope cell activity is necessary for the animal to shut off α-MSH secretion instantly. The immediate physiological response (drop of plasma α-MSH levels) takes place within 30 min after the animal is placed on a white background (39) and may be regulated by the initial release of the neurotransmitter GABA from the SMINs (40). However, to ensure that the melanotrope cells stay inactive during the first few days of adaptation, immediate synaptic changes are also necessary. Obviously, the morphological changes associated with the conversion of the highly active melanotrope cell into a biosynthetically inactive cell take more time (at least one week), which is reflected by only a gradual decrease of POMC expression during such a time period. In black animals adapting to a white background we found a relatively fast decrease in the amount of the 130-kDa neuroserpin-protease complex, possibly through internalization of the complex by the melanotrope cells, implying that the protease and thus proteolytic activity in the melanotrope ECM would be readily available. In melanotropes of white animals adapting to a black background, the 130-kDa complex was detected only after five days of adaptation, allowing protease activity in the cell matrix to be still present during the first days of black-background adaptation. Thus, in the first days of adaptation to either a black- or a white background, protease activity is available in the melanotrope ECM and may regulate the formation of the pertinent matrices; for maintenance of the ECM, a proper balance between matrix proteases and their inhibitors is essential (reviewed in 1). Because the ECM is thought to play an important role in synaptic plasticity (reviewed in 25, 41-43), changes in the melanotrope ECM will allow the synaptic plasticity of the hypothalamic neurons and thus enable the dramatic morphological changes that occur in the melanotrope cell during background adaptation of *Xenopus*.

In conclusion, we report for the first time on the biosynthesis of neuroserpin under physiological conditions, and find that neuroserpin and a 130-kDa neuroserpin-containing complex are up-regulated in the biosynthetically active neuroendocrine melanotrope cells

of the intermediate pituitary of black-adapted *Xenopus laevis*. Because the formation of the 130-kDa complex is linked to melanotrope cell activation, neuroserpin may be somehow involved in the shaping of the melanotrope ECM and thus in the regulation of the synaptic plasticity of hypothalamic neurons that directly innervate this cell.

Acknowledgements

We would like to thank Ron Engels for animal care and Karel Janssen (in memoriam) for technical assistance. We also thank D.A. Lawrence, N. Birch, D.A. Lomas, J. Fransen and S. Tanaka for providing antibodies.

References

1. Romanic AM, Madri JA. Extracellular matrix-degrading proteinases in the nervous system. Brain Pathol 1994;4:145-56.
2. Shiosaka S. Serine proteases regulating synaptic plasticity. Anat Sci Int 2004;79:137-44.
3. Qian Z, Gilbert ME, Colicos MA, Kandel ER, Kuhl D. Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature 1993;361:453-7.
4. Calabresi P, Napolitano M, Centonze D, et al. Tissue plasminogen activator controls multiple forms of synaptic plasticity and memory. Eur J Neurosci 2000;12:1002-12.
5. Silverman GA, Bird PI, Carrell RW, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 2001;276:33293-6.
6. Pizzo SV. Serpin receptor 1: a hepatic receptor that mediates the clearance of antithrombin III-proteinase complexes. Am J Med 1989;87:10S-14S.
7. Andreasen PA, Sottrup-Jensen L, Kjoller L, et al. Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. FEBS Lett 1994;338:239-45.
8. Stoeckli ET, Lemkin PF, Kuhn TB, Ruegg MA, Heller M, Sonderegger P. Identification of proteins secreted from axons of embryonic dorsal-root- ganglia neurons. Eur J Biochem 1989;180:249-58.
9. Krueger SR, Ghisu GP, Cinelli P, et al. Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J Neurosci 1997;17:8984-96.
10. Hill RM, Parmar PK, Coates LC, Mezey E, Pearson JF, Birch NP. Neuroserpin is expressed in the pituitary and adrenal glands and induces the extension of neurite-like processes in AtT-20 cells. Biochem J 2000;345 Pt 3:595-601.
11. Osterwalder T, Cinelli P, Baici A, et al. The axonally secreted serine proteinase inhibitor, neuroserpin, inhibits plasminogen activators and plasmin but not thrombin. J Biol Chem 1998;273:2312-21.
12. Yepes M, Sandkvist M, Wong MK, et al. Neuroserpin reduces cerebral infarct volume and protects neurons from ischemia-induced apoptosis. Blood 2000;96:569-76.
13. Madani R, Kozlov S, Akhmedov A, et al. Impaired explorative behavior and neophobia in genetically modified mice lacking or overexpressing the extracellular serine protease inhibitor neuroserpin. Mol Cell Neurosci 2003;23:473-94.
14. Davis RL, Shrimpton AE, Carrell RW, et al. Association between conformational mutations in neuroserpin and onset and severity of dementia. Lancet 2002;359:2242-7.
15. Davis RL, Shrimpton AE, Holohan PD, et al. Familial dementia caused by polymerization of mutant neuroserpin. Nature 1999;401:376-9.
16. Lomas DA, Carrell RW. Serpinopathies and the conformational dementias. Nat Rev Genet 2002;3:759-68.
17. Miranda E, Romisch K, Lomas DA. Mutants of neuroserpin that cause dementia accumulate as polymers within the endoplasmic reticulum. J Biol Chem 2004.
18. Kramer BM, Kolk SM, Berghs CA, et al. Dynamics and plasticity of peptidergic control centres in the retino-brain-pituitary system of Xenopus laevis. Microsc Res Tech 2001;54:188-99.
19. Hastings GA, Coleman TA, Haudenschild CC, et al. Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. J Biol Chem 1997;272:33062-7.
20. Cuppen E, Wijers M, Schepens J, Fransen J, Wieringa B, Hendriks W. A FERM domain governs apical confinement of PTP-BL in epithelial cells. J Cell Sci 1999;112 (Pt 19):3299-308.
21. Berghs CA, Tanaka S, Van Strien FJ, Kurabuchi S, Roubos EW. The secretory granule and pro-opiomelanocortin processing in Xenopus melanotrope cells during background adaptation. J Histochem Cytochem 1997;45:1673-82.
22. Kuiper RP, Waterham HR, Rotter J, Bouw G, Martens GJ. Differential induction of two p24delta putative cargo receptors upon activation of a prohormone-producing cell. Mol Biol Cell 2000;11:131-40.
23. Devreese B, Janssen KP, Vanrobaeys F, Van Herp F, Martens GJ, Van Beeumen J. Automated nanoflow liquid chromatography-tandem mass spectrometry for a differential display proteomic study on Xenopus laevis neuroendocrine cells. J Chromatogr A 2002;976:113-21.
24. Carrell RW, Huntington JA. How serpins change their fold for better and for worse. Biochem Soc Symp 2003:163-78.
25. Shiosaka S, Yoshida S. Synaptic microenvironments--structural plasticity, adhesion molecules, proteases and their inhibitors. Neurosci Res 2000;37:85-9.

26. Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. Embo J 1996;15:2944-53.
27. Patston PA, Church FC, Olson ST. Serpin-ligand interactions. Methods 2004;32:93-109.
28. Nawata S, Suminami Y, Hirakawa H, et al. Nondenaturing two-dimensional electrophoretic analysis of loop-sheet polymerization of serpin, squamous cell carcinoma antigen-2. Electrophoresis 2001;22:161-4.
29. Benning LN, Whisstock JC, Sun J, Bird PI, Bottomley SP. The human serpin proteinase inhibitor-9 self-associates at physiological temperatures. Protein Sci 2004;13:1859-64.
30. Misra S, Hecht P, Maeda R, Anderson KV. Positive and negative regulation of Easter, a member of the serine protease family that controls dorsal-ventral patterning in the Drosophila embryo. Development 1998;125:1261-7.
31. Osterwalder T, Kuhnen A, Leiserson WM, Kim YS, Keshishian H. Drosophila serpin 4 functions as a neuroserpin-like inhibitor of subtilisin-like proprotein convertases. J Neurosci 2004;24:5482-91.
32. Lawrence DA, Strandberg L, Ericson J, Ny T. Structure-function studies of the SERPIN plasminogen activator inhibitor type 1. Analysis of chimeric strained loop mutants. J Biol Chem 1990;265:20293-301.
33. Sherman PM, Lawrence DA, Yang AY, et al. Saturation mutagenesis of the plasminogen activator inhibitor-1 reactive center. J Biol Chem 1992;267:7588-95.
34. Makarova A, Mikhailenko I, Bugge TH, List K, Lawrence DA, Strickland DK. The low density lipoprotein receptor-related protein modulates protease activity in the brain by mediating the cellular internalization of both neuroserpin and neuroserpin-tissue-type plasminogen activator complexes. J Biol Chem 2003;278:50250-8.
35. Berghs CA, Roubos EW. Background adaptation and synapse plasticity in the pars intermedia of Xenopus laevis. Neuroscience 1996;70:833-41.
36. Tuinhof R, Artero C, Fasolo A, et al. Involvement of retinohypothalamic input, suprachiasmatic nucleus, magnocellular nucleus and locus coeruleus in control of melanotrope cells of Xenopus laevis: a retrograde and anterograde tracing study. Neuroscience 1994;61:411-20.
37. Tuinhof R, Gonzalez A, Smeets WJ, Roubos EW. Neuropeptide Y in the developing and adult brain of the South African clawed toad Xenopus laevis. J Chem Neuroanat 1994;7:271-83.
38. Tuinhof R, Gonzalez A, Smeets WJ, Scheenen WJ, Roubos EW. Central control of melanotrope cells of Xenopus laevis. Eur J Morphol 1994;32:307-10.
39. van Zoest ID, Heijmen PS, Cruijsen PM, Jenks BG. Dynamics of background adaptation in Xenopus laevis: role of catecholamines and melanophore-stimulating hormone. Gen Comp Endocrinol 1989;76:19-28.
40. Jenks B, Buzzi M, Dotman C, et al. The significance of multiple inhibitory mechanisms converging on the melanotrope cell of Xenopus laevis. Ann N Y Acad Sci 1998;839:229-34.
41. Pavlov I, Lauri S, Taira T, Rauvala H. The role of ECM molecules in activity-dependent synaptic development and plasticity. Birth Defects Res Part C Embryo Today 2004;72:12-24.
42. Dityatev A, Schachner M. Extracellular matrix molecules and synaptic plasticity. Nat Rev Neurosci 2003;4:456-68.
43. Wright JW, Kramar EA, Meighan SE, Harding JW. Extracellular matrix molecules, long-term potentiation, memory consolidation and the brain angiotensin system. Peptides 2002;23:221-46.

2

Transgene expression of neuroserpin in *Xenopus* intermediate pituitary cells affects the structure of the extracellular matrix

Dorien M. de Groot and Gerard J.M. Martens

Department of Molecular Animal Physiology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Institute for Neuroscience, Faculty of Science, Radboud University, Nijmegen, The Netherlands

In preparation for submission

Abstract

Neuroserpin is a member of the serine protease inhibitor (serpin) superfamily that is predominantly expressed in neuronal and neuroendocrine tissues. The exact physiological role of neuroserpin remains unclear. Mutations in the neuroserpin gene (*e.g.* G392E) are linked to the autosomal dominant dementia Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB). To study functional aspects of neuroserpin, we used the *Xenopus* intermediate pituitary neuroendocrine melanotrope cell as a model system. This cell regulates the process of background adaptation of the frog, and exhibits plasticity at both the synaptic and endocrine level. Via stable *Xenopus* transgenesis green fluorescent protein (GFP)-tagged neuroserpin or mutant neuroserpin(G392E) fusion products were expressed specifically in the melanotrope cells. Newly synthesized and steady-state levels of the transgene fusion products varied from relatively low (mutant neuroserpin) to high (neuroserpin), and in the transgenic melanotrope cells the fusion proteins were transported through the secretory pathway and secreted. Transgene expression of the fusion proteins did not affect endogenous neuroserpin protein levels and melanotrope cell functioning was not detectably altered, as determined by metabolic cell labeling and Western blot analysis of various secretory pathway proteins. Intriguingly, at the ultrastructural level we observed intercellular gaps between the transgenic melanotrope cells that were not detected between wild-type cells, implying an effect of the exogenous (mutant) neuroserpin on the extracellular matrix of the melanotrope cell. Thus, the neuroendocrine serine protease inhibitor neuroserpin may affect the extracellular matrix of neuroendcrine cells.

Introduction

The superfamily of serine protease inhibitors (serpins) is involved in a number of fundamental biological processes and is associated with numerous familial disorders (1-4). One characteristic of serpins is their ability to undergo drastic conformational changes when inhibiting their substrates. After binding of the substrate protease to the reactive center loop (RCL) of the serpin, the P1-P1' bond of the RCL will be cleaved, and a drastic rearrangement will incorporate the N-terminal residues of the loop into the middle of the A-β-sheet as strand 4A. This conformational change inactivates the protease by translocating it over 70 Å to the opposite pole of the serpin and disrupting the catalytic site (reviewed in 5, 6). As a consequence of this molecular flexibility of serpins, mutations affecting the structural stability can result in aberrant intermolecular linkage and the formation of polymers (reviewed in 7).

Neuroserpin is a member of the serpin superfamily that is primarily expressed in neuronal- and neuroendocrine tissues (8-13). Specific mutations in the neuroserpin gene lead to a disease called 'familial encephalopathy with neuroserpin inclusion bodies'

(FENIB). This disorder is characterized clinically as an autosomal dominantly inherited dementia, histologically by unique neuronal inclusion bodies and biochemically by polymers of neuroserpin (14). Currently, five different point mutations in the neuroserpin gene have been described that result in FENIB, which provided evidence that the number of inclusions is directly related to the molecular instability caused by the mutation and inversely proportional to the age of onset of dementia. For example, the least disruptive S49P mutation caused dementia after age 45 and the presence of neuroserpin inclusion bodies in only a few neurons. By contrast, the more severely disruptive G392E mutation resulted at age 13 in progressive myoclonus epilepsy, with many inclusions present in almost all neurons (15). Although it is now mechanistically understood how polymerization of mutated neuroserpin takes place, little information is available concerning the intracellular trafficking and processing of the protein.

The physiological role of neuroserpin in the nervous system and neuroendocrine tissues remains elusive. A number of functions for the protein have been proposed, such as a regulatory role in synaptic growth and in the development of synaptic plasticity in the nervous system (9, 10, 12, 16-22), as well as in secretory vesicle function or intercellular communication between endocrine cells (13). Furthermore, there is a growing body of evidence demonstrating the participation of tissue-type plasminogen activator (t-PA) in a number of physiological and pathological events in the central nervous system, and the role of neuroserpin in these processes as the natural regulator of t-PA activity (22-26).

To gain more insight into the physiological role of neuroserpin and the pathophysiological role of mutated neuroserpin, we used the intermediate pituitary melanotrope cells of the South-African claw-toed frog *Xenopus laevis* as a model system. These neuroendocrine cells are responsible for the process of background adaptation of this amphibian by coordinating the release of the proopiomelanocortin (POMC) cleavage product α-melanophore stimulating hormone, which causes pigment dispersion in skin melanophores. On a black background, the melanotrope cells are very active, producing vast amounts of POMC, whereas on a white background the activity of the cells is inhibited by hypothalamic neurons, which make direct synaptic contacts with the melanotrope cells. Thus, the biosynthetic activity of these neuroendocrine cells and the degree of their innervation by regulatory hypothalamic neurons can be manipulated in a physiological way by placing the animal on a black or a white background (reviewed in 27-29).

In this study, we generated and analyzed transgenic *Xenopus* with overexpression of green fluorescent protein (GFP)-tagged neuroserpin or mutant neuroserpin(G392E) specifically in the intermediate pituitary melanotrope cells.

Materials and methods

Animals

South African claw-toed frogs, *Xenopus laevis*, were reared in the Central Animal Facility of the Radboud University of Nijmegen (Nijmegen, The Netherlands). The animals were adapted to their background by keeping them in either white or black buckets, with the lights turned on in cycles of 12 h. Experimental procedures were performed under the guidelines of the Dutch law concerning animal welfare, and permit RBD0166 (H10) to generate and house transgenic *Xenopus.*

Antibodies

For Western blotting, immunoprecipitation analysis and immunocytochemistry, the following antibodies were used. To detect neuroserpin, we used a rabbit polyclonal IgG against human recombinant neuroserpin (α-NS-L; generous gift of Dr. D.A. Lawrence, American Red Cross Holland Laboratory, Rockville, Maryland, USA; 9). GFP was detected with an anti-GFP antibody (generous gift of Dr. B. Wieringa, Radboud University, Nijmegen, The Netherlands; 30) and tubulin was detected with the monoclonal anti-tubulin antibody E7 (Developmental Studies Hybridoma Bank, Rockland, Gilbertsville). *Xenopus* calnexin was detected with an anti-calnexin antibody (31), BiP with an anti-BiP antibody (32) (generous gifts of Dr. K. Geering, University of Lausanne, Lausanne, Switzerland), POMC with an anti-POMC antibody (ST62; generous gift of Dr. S. Tanaka, Shizuoka University, Japan; 33), POMC and a POMC-derived product with an anti-adrenocorticotropic hormone (ACTH) antibody (34), prohormone convertase PC2 with an anti-PC2 antibody (generous gift of Dr. W. Van de Ven, University of Leuven, Belgium; 35) and p24$\delta_{1/2}$ with anti-p24-antibody 1262N (36).

Generation of constructs used for Xenopus laevis transgenesis

For transgenesis, two different constructs encoding the fusion proteins GFP-neuroserpin and GFP-neuroserpin(G392E) were generated. For the construct encoding GFP-neuroserpin, neuroserpin-encoding DNA was amplified by PCR using Pfu enzyme (Fermentas), forward primer NS-*Xba*I sense 2 (5'-GGGGTCTAGATTAGAGCTCTTCAAAATCA-TGG-3'), reverse primer NS-*Xho*I-sense (5'-GGGGCTCGAGCGTTTGGTACTAGTGTCCATG-3') and 1 ng pCS2+-NS vector (described in 8) as template DNA, according to the manufacturer's instructions. The amplified neuroserpin PCR-fragment was then cloned in frame behind the GFP sequence of pPOMC(A)2+-SP-GFP (described in reference 37), resulting in the pPOMC-SP-GFP-NS construct. To generate the construct encoding the mutant GFP-neuroserpin(G392E) fusion protein, we used the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with the pPOMC-SP-GFP-NS construct as input DNA and the following primer: 5'-GGATCAGTCTTGTTTATGGAAAGGGTCATGCATCCAG-3' (mutation

leading to amino acid change is underlined), resulting in the pPOMC-SP-GFP-NS(G392E) construct.

Xenopus laevis transgenesis

To create transgenic frogs, linear 2829-kb *Sal*I/*Not*I DNA fragments were generated from the pPOMC-SP-GFP-NS and pPOMC-SP-NS(G392E) constructs, respectively. These linear fragments were used for stable *Xenopus* transgenesis (38, 39) (Fig. 1). A number of injection rounds resulted in animals transgenic for the fusion protein GFP-NS and the mutant fusion protein GFP-NS(G392E). To generate F1 offspring, the testes of male transgenic *Xenopus* laevis frogs were isolated and used for *in vitro* fertilization of eggs harvested from wild-type *Xenopus laevis* females, resulting in transgenic line 55 (pPOMC-SP-GFP-NS) and line 84 (pPOMC-SP-GFP-NS(G392E)).

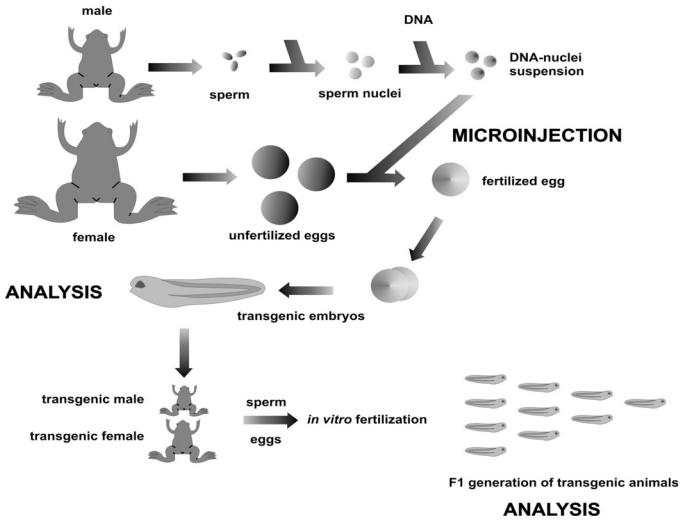


Figure 1: **Flow diagram of the stable *Xenopus* transgenesis procedure and the generation of F1 transgenic animals**.
This scheme represents the modified transgenesis technique described by the group of Mohun (38). For the generation of F1 transgenic animals, we used either transgenic sperm and wild-type eggs or transgenic eggs in combination with wild-type sperm.

Isolation of Xenopus melanotrope cells

Intermediate pituitary melanotrope cells from wild-type and transgenic *Xenopus* were isolated as described previously (40). Briefly, neurointermediate lobes (NILs) were dissected, washed several times in sterile *Xenopus* XL15 (10 mM glucose, 2 mM CaCl2, 1% kanamycin (Life Technologies, Inc.), 1% antibiotic/antimycotic (Life Technologies, Inc.) in 67% Leibovitz's-15 medium (Life Technologies, Inc.)) and transferred to XL15 containing 0.25% trypsin. After 45 min incubation, XL15 was added and the melanotrope cells were mechanically dissociated from the NIL by 7 passages through a siliconized Pasteur's pipet. Next, the cell suspension was filtered and the melanotrope cells were

collected by centrifugation. For primary culturing, cells were resuspended in a small volume of serum-free XL15 medium, and seeded on poly-L lysine-coated coverslips. After 1 hour, XL15/10% FCS was added and the cells were cultured for two days at room temperature (RT) in a humified chamber before using them for immunocytochemistry.

Immunocytochemistry

For immunocytochemistry, primary cultures of melanotrope cells were washed three times in *Xenopus* PBS (XPBS; 69% PBS), fixed in 2% paraformaldehyde/PBS for 1.5 hr at RT, and subsequently washed three times in 50 mM NH_4Cl/PBS and three times in 0.1% Triton/XPBS (XPBS-T). Antibody incubations were performed sequentially for 1 hr in 2% bovine serum albumine (BSA)/XPBS-T at RT. Anti-ACTH (1:2000) was used as a primary antibody and Alexa 568-conjugated goat anti-rabbit antibody (1:500) was used to visualize the first antibody-antigen complex. Finally, cells were washed three times in XPBS-T, two times in XPBS and once in MQ, and cover slips were mounted in Mowiol (10% [wt/vol]; CalBiochem, La Jolla, CA; 15% glycerol; 100 mM Tris-HCl, pH 8.5; 2.5% NaN_3). Fluorescence was analyzed with an MRC 1024 confocal laser scanning microscope (BioRad).

Metabolic cell labeling and immunoprecipitation

Metabolic cell labeling was performed as described previously (40). Briefly, *Xenopus* NILs were rapidly dissected, pre-incubated for 15 min in Ringer's medium (112 mM NaCl, 15 mM Hepes (pH7.4), 2 mM KCl, 2 mM $CaCl_2$, 2 mg/ml glucose, and 0.3 mg/ml BSA), and newly synthesized proteins were radioactively labelled with 5 mCi/ml Tran^{35}S label for 30 min. Following a 180-min chase period in Ringer's medium supplemented with 0.5 mM L-methionine and with or without $1*10^{-7}$ M or $2*10^{-8}$ M apomorphine, lysates were made in 100 µl lysis buffer (50 mM Hepes, 140 mM NaCl, 0.1% Triton-X100, 1% Tween 20, 1 mM EDTA, 1 mg/ml deoxycholate, 1 µM phenylmethylsulfonyl fluoride, 0.1 mg/ml soybean trypsin inhibitor). The lysates and newly synthesized proteins secreted into the incubation media were resolved by SDS-PAGE and visualized by fluorography. The amounts of newly synthesized 37-kDa POMC and of the 18-kDa POMC cleavage product (both the cellular and secreted protein) were quantified by densitometry using a phosphoimager (BioRad). The relative processing of 37-kDa POMC was estimated by the ratio of 37-kDa to 18-kDa POMC (total of cellular and secreted protein), and the relative secretion by the ratio of secreted 18-kDa to cellular 18-kDa POMC (n=3). An unpaired t test was used for statistical analysis. Immunoprecipitation was performed in lysis buffer with 0.075% SDS in the presence of the anti-GFP antiserum (1:1000). After overnight rotation at 4°C, immune complexes were precipitated with protein-A-sepharose (Amersham Pharmacia Biotech), washed four times with lysis buffer and analysed via SDS-polyacrylamide gel electrophoresis.

Western blot analysis

To examine steady-state protein expression levels by Western blot analysis, NIL lysates (described above) were separated on a polyacrylamide gel with or without SDS (denaturing and native conditions, respectively) and proteins were transferred to nitrocellulose membranes (protran, Schleicher & Schuell, Dassel, Germany) by electroblotting. Blots were blocked for 1 hour in blocking buffer (5% non-fat dried milk in PBS containing 1% Tween 20) and incubated overnight with primary antiserum (1:5000 anti-NS-L; 1:5000 anti-GFP; 1:500 anti-tubulin; 1:10000 anti-Cal; 1:10000 anti-BiP; 1:20000 anti-POMC; 1:5000 anti-PC2 or 1:5000 anti-p24) in blocking buffer. Bound antibodies were detected with peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antiserum followed by chemiluminescence (Lumilight plus, Roche Diagnostics, Manheim, Germany). Signals were visualized with a BioChemi Imaging System, and relative quantification by densitometry was performed with Labworks 4.0 software (UVP BioImaging systems, Cambridge, UK). The densities of the various signals were normalized to the corresponding tubulin signals (n = 3). An unpaired t test was used for statistical analysis.

Electron microscopy

For ultrastructural studies, freshly dissected NILs of wild-type *Xenopus* and *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E) were used. Whole lobes were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.3). Following rinsing in the same buffer, fixed tissues were osmicated for one hour in 1% osmium tetroxide in 0.1 M PB, rinsed in PB, dehydrated through graded series of ethanol and embedded in Epon 812. One-micron thick sections were cut and stained with toludine blue. Subsequently, the sections were examined in a phase-contrast microscope (Dialux 20, Leitz). Ultrathin sections were cut, double contrasted with uranyl-acetate/lead-citrate and photographed in a transmission electron microscope (JEOL 1010).

Results

Generation of *Xenopus* with transgene expression of neuroserpin or mutant neuroserpin(G392E) specifically in intermediate pituitary melanotrope cells

To study the role of neuroserpin in a physiological context, we generated via stable *Xenopus laevis* transgenesis F0 and F1 lines with cell-specific transgene expression of GFP-neuroserpin (line 55) or GFP-neuroserpin(G392E) (line 84) (Fig. 2A). Melanotrope cell-specific transgene expression was accomplished by cloning the GFP-neuroserpin DNA fragments behind a *Xenopus* POMC gene promoter fragment (41). The transgenic tadpoles were identified by direct screening for GFP fluorescence in the pituitary (Fig. 2B).

We next examined the biosynthesis of the newly synthesized transgene products in the melanotrope cells of transgenic lines 55 and 84. For this purpose, NILs of wild-type and

transgenic animals were metabolically labeled for 30 min and chased for 180 min. Following immunoprecipitation analysis of the lysates using an anti-GFP antibody, we detected newly-synthesized GFP-neuroserpin and GFP-neuroserpin(G392E) fusion proteins of ~80 kDa in the transgenic NILs (Fig. 3A, left panel). The levels of newly synthesized GFP-neurserpin (line 55) were ~5-fold higher than newly synthesized mutant GFP-neuroserpin(G392E) (line 84), which was detected only after prolonged exposure of the film.

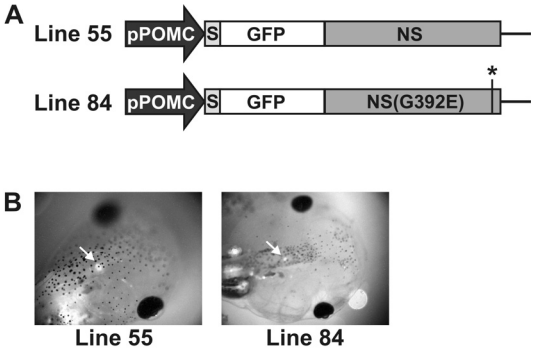


Figure 2: Generation of transgenic *Xenopus laevis* with GFP-tagged neuroserpin or mutated neuroserpin(G392E) expression specifically in the intermediate pituitary melanotrope cells.
(A) Schematic representation of the constructs that were used to generate transgenic line 55 (neuroserpin) and line 84 (neuroserpin(G392E)) *Xenopus*. Abbreviations: pPOMC, promoter fragment of the *Xenopus* proopiomelanocortin gene; S, signal peptide; GFP, green fluorescent protein; NS, neuroserpin; NS(G392E), neuroserpin with a G392E point mutation, which is indicated with an asterisk. (B) GFP fluorescence in intermediate pituitaries (indicated with a white arrow) of transgenic tadpoles of lines 55 and 84.

To examine the steady-state expression levels of the transgene products in line-55 and -84 animals, we performed Western blot analysis of NIL lysates with an anti-neuroserpin and anti-GFP antibody. With both antibodies, we detected an ~6-fold higher expression level of ~80-kDa GFP-neuroserpin in lobes of line-55 animals than the expression level of ~80-kDa mutant GFP-neuroserpin(G392E) in lobes of transgenic line-84 animals (Fig. 3B; left panels), in line with the results of the biosynthetic studies.

We then wondered whether the transgene products were also secreted by the transgenic melanotrope cells. Following metabolic cell labeling, we therefore performed immunoprecipitation analysis of the incubation media using an anti-GFP antibody, and detected ~80-kDa GFP-neuroserpin and GFP-neuroserpin(G392E) products in the media of the transgenic NILs (Fig. 3A, right panel). Additionally, the media were analyzed by Western blotting using an anti-neuroserpin antibody, which revealed an ~80-kDa GFP-neuroserpin product in the incubation medium of transgenic line 55 (Fig. 3B, right panel), whereas the expression levels of GFP-neuroserpin(G392E) were probably too low to detect the secreted transgene product in the medium (Fig. 3B, right panel).

Using the anti-neuroserpin antibody, we also detected endogenous neuroserpin products of ~47-, 50-, 55- and 130-kDa in wild-type and transgenic animals (Fig. 3B, upper left panel; 8, 40). The expression levels of the endogenous neuroserpin proteins and the 130-kDa complex appeared not to be affected by GFP-neuroserpin or GFP-neuroserpin(G392E) transgene expression, since we did not detect significant differences in endogenous neuroserpin expression levels between the NILs of wild-type and transgenic line 55 or 84 animals (Fig. 3B, upper left panel).

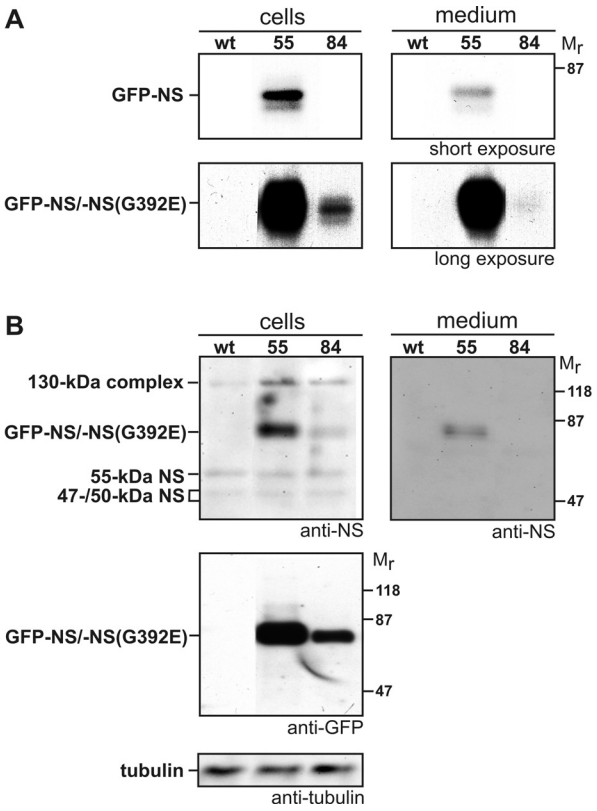


Figure 3: Newly-synthesized and steady-state levels of GFP-neuroserpin and GFP-neuroserpin(G392E) in transgenic *Xenopus* intermediate pituitary melanotrope cells and secreted into the incubation medium.
(A) Newly synthesized green fluorescent protein (GFP)-neuroserpin or GFP-neuroserpin(G392E) fusion proteins produced in the neurointermediate lobes (cells) and secreted into the incubation media (medium) after metabolic labeling of wild-type (wt) and transgenic lines-55 and -84 lobes. Immunoprecipitation was performed using an anti-GFP antibody. Both a short and long exposure of the gel are shown (upper and lower panel, respectively). (B) Western blot analysis of neurointermediate lobe lysates (cells) and incubation media (medium) of adult wt and transgenic line-55 and line-84 animals using an anti-neuroserpin (anti-NS) or anti-GFP antibody. Analysis of tubulin protein expression served as a control for protein loading. Abbreviations: NS, neuroserpin; GFP-NS, GFP-tagged neuroserpin fusion protein; GFP-NS(G392E), GFP-tagged neuroserpin protein with a G392E mutation.

Thus, transgenic *Xenopus* were generated with relatively high transgene expression of GFP-neuroserpin (line 55) and relatively low transgene expression of mutant GFP-neuroserpin(G392E) (line 84) in the melanotrope cells of the intermediate pituitary. The transgene products were secreted by the melanotrope cells and furthermore did not have a detectable effect on the steady-state expression levels of the endogenous neuroserpin proteins.

Intracellular localization of the neuroserpin fusion proteins in the transgenic *Xenopus* melanotrope cells

We next wondered about the intracellular localization of the transgene GFP-neuroserpin and GFP-neuroserpin(G392E) fusion products, and we therefore isolated melanotrope cells from wild-type and transgenic line-55 and -84 animals to examine direct GFP fluorescence using confocal microscopy. In the melanotropes of transgenic lines 55 (GFP-neuroserpin) and 84 (GFP-neuroserpin(G392E)), the fusion proteins were localized in vesicle-like structures (Fig. 4; left panels). To find out whether these vesicle-like structures were part of the secretory pathway of the transgenic melanotrope cells, immunocytochemistry was performed on the cells using an anti-ACTH antibody, recognizing POMC and a POMC-derived cleavage product. As expected, in the melanotrope cells fluorescence was detected in the ER and in vesicles of the secretory pathway (Fig. 4; right panels). The observed fluorescence of GFP-neuroserpin (line 55) and GFP-neuroserpin(G392E) (line 84) partially colocalized with the fluorescence of POMC and the POMC-derived cleavage product in the melanotropes, indicating that a portion of the transgene GFP-neuroserpin and GFP-

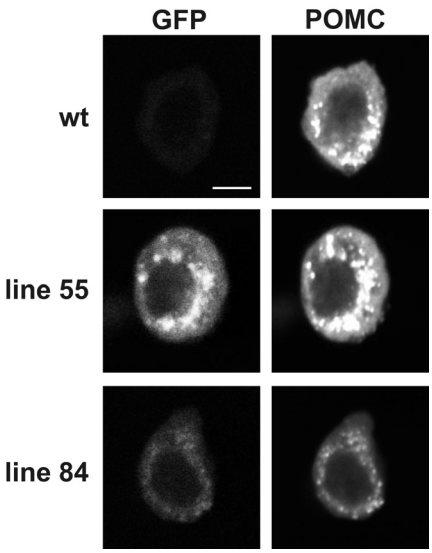


Figure 4: Localization of GFP-neuroserpin and GFP-neuroserpin(G392E) in the transgenic *Xenopus* melanotrope cells.
Intracellular localization of GFP-neuroserpin or GFP-neuroserpin(G392E) fusion proteins (left panels) and of proopiomelanocortin (POMC and a POMC-derived product in primary-cultured melanotrope cells of black-adapted wild-type (wt), and transgenic line-55 and line-84 *Xenopus*. Shown are confocal microscopy pictures of the direct fluorescence of the transgene GFP-neuroserpin products (GFP) or immunofluorescence of the POMC/POMC-derived product (POMC). Scale bar: 5 μm.

neuroserpin(G392E) fusion products were localized in the secretory pathway. The other part may well be localized in the endosomal and lysosomal compartment of the melanotrope cell. Localization in the secretory pathway is in line with immunoprecipitation and Western blot analyses in which the transgene fusion products were detected in the incubation media of the transgenic melanotrope cells (Fig. 3) and with the reported subcellular localization of endogenous neuroserpin (8, 21).

Biosynthesis and processing of POMC and secretion of POMC-derived peptides by melanotrope cells of wild-type *Xenopus* or *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E)

To examine the effect of GFP-neuroserpin or GFP-neuroserpin(G392E) transgene expression on melanotrope cell functioning, and more specifically on the biosynthesis and processing of POMC and the secretion of POMC-derived peptides, we performed pulse-chase analyses on NILs of black-adapted wild-type and transgenic line-55 and -84 animals. After quantification of the amounts of newly synthesized 37-kDa and 18-kDa POMC produced in the NILs from wild-type and transgenic line-55 (GFP-neuroserpin) and line-84 (GFP-neuroserpin(G392E)) animals, we did not detect significant differences in the biosynthesis, processing and secretion of 37- and 18-kDa POMC by the melanotrope cells of line-55 or -84 compared with wild-type animals (Fig. 5).

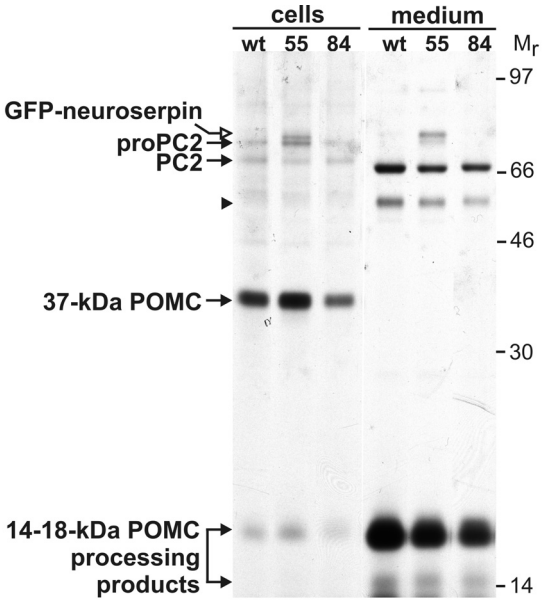


Figure 5: Newly synthesized proteins produced in the intermediate pituitary cells of wild-type *Xenopus* and *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E).
Neurointermediate lobes (cells) of black-adapted wild-type (wt) and transgenic line-55 (GFP-neuroserpin) and line-84 (GFP-neuroserpin(G392E)) *Xenopus* were pulse labeled for 30 min and chased for 180 min. Newly synthesized proteins extracted from the lobes (cells) and secreted into the incubation media (medium) were resolved by SDS-PAGE and visualized by fluorography. The amounts of newly synthesized 37-kDa POMC and of the 18-kDa POMC cleavage product were quantified by densitometry using a phosphoimager (n=3). Shown is a representative of three independent experiments. Abbreviations: (pro)PC2, (pro)prohormone convertase 2; POMC, proopiomelanocortin.

Since neuroserpin may play a role in synaptic plasticity and the transgene GFP-neuroserpin fusion products are secreted by the melanotrope cells (Fig. 3B), we wondered whether secreted GFP-neuroserpin had influenced the synaptic plasticity exerted by the hypothalamic fibers that directly innervate the melanotrope cells. We therefore performed pulse-chase analyses in the presence of the dopamine-agonist apomorphine; dopaminergic neurons of hypothalamic origin directly innervate and inhibit the regulated secretion by the melanotrope cells (27, 29). Furthermore, we examined melanotrope cells that were in different states of synaptic innervation by using line-55 animals that were adapted to a white or a black background for various time periods. We did not detect significant differences in the biosynthesis and processing of newly synthesized POMC and secretion of the POMC-derived peptides between wild-type and transgenic line-55 melanotrope cells (data not shown), indicating that the regulation of the biosynthetic activity of the transgenic cells was not affected.

Together, these results indicate that the biosynthetic and secretory processes in the transgenic melanotrope cells of lines 55 and 84 are not affected.

Steady-state expression of various secretory pathway proteins in the neurointermediate lobe of wild-type *Xenopus* and *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E)

Next, we examined whether the transgene expression of GFP-neuroserpin (line 55) or GFP-neuroserpin(G392E) (line 84) had an effect on the expression of secretory pathway proteins other than POMC. For this purpose, we used Western blotting to examine the steady-state expression levels of the endoplasmic reticulum (ER) chaperones calnexin and binding protein BiP, the prohormone convertase 2 (PC2), POMC, and p24$\delta_{1/2}$ (putative receptors for ER to Golgi cargo transport) in the NILs of wild-type and transgenic lines-55 and -84 animals. After quantification, we did not detect significant differences in the expression of these proteins between wild-type and transgenic line-55 or -84 animals (Fig. 6).

Ultrastructure of melanotrope cells from wild-type *Xenopus* or *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E)

Finally, to study any effects of GFP-neuroserpin or GFP-neuroserpin(G392E) transgene expression on the ultrastructure of the transgenic melanotrope cells, electron microscopy analysis was performed. After careful examination of the ultra-thin slices, the overall ultrastructures of the transgenic melanotrope cells of lines 55 and 84 were comparable with that of wild-type cells (Fig. 7). For example, no abnormalities were found in the nucleus, ER or secretory granules. However, in transgenic line-55 melanotrope cells more lysosomes were present, and in sections of the transgenic intermediate pituitaries of lines 55 and 84 more intercellular gaps were observed compared with wild-type intermediate pituitaries (Fig. 7).

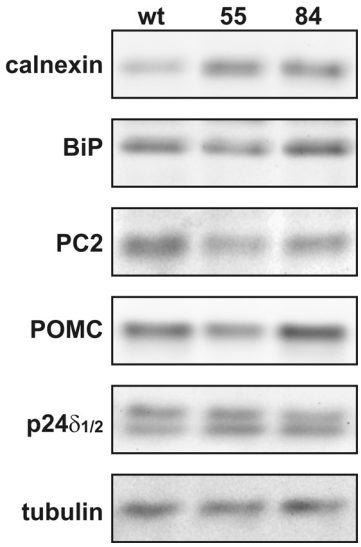


Figure 6: Steady-state levels of a number of secretory pathway proteins in the intermediate pituitary cells of wild-type *Xenopus* and *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E).
Western blot analysis of *Xenopus* neurointermediate lobe lysates of wild-type (wt) and transgenic line-55 (GFP-neuroserpin) and line-84 (GFP-neuroserpin(G392E)) animals using antibodies directed against calnexin, binding protein BiP, prohormone convertase 2 (PC2), proopiomelanocortin (POMC), p24$\delta_{1/2}$ and tubulin. The results shown are representatives of three independent experiments.

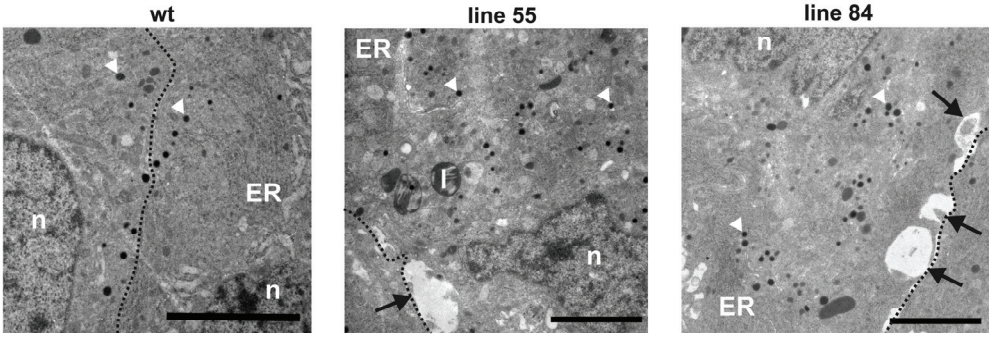


Figure 7: Ultrastructure of melanotrope cells from wild-type *Xenopus* and *Xenopus* transgenic for GFP-neuroserpin or GFP-neuroserpin(G392E).
Ultrastructural analysis by electron microscopy on melanotrope cells from wild-type (wt) and transgenic line-55 (GFP-neuroserpin) or line-84 (GFP-neuroserpin(G392E)) *Xenopus*. Examples of a number of intracellular structures are indicated, such as the nucleus (n), endoplasmic reticulum (ER), secretory granules (white arrow heads) and lysosomes (l). Intercellular gaps are indicated with black arrows and cell-cell boundaries with a dotted line. Scale bars: 5 μm (wt) and 2.5 μm (lines 55 and 84).

Discussion

Neuroserpin is a neuronal- and neuroendocrine-specific serpin (8-13) for which several physiological functions and target proteases have been suggested, but whose exact role remains elusive. A number of mutated forms of neuroserpin have been implicated in the pathophysiology of the dementia FENIB, and until now only *in vitro* cell systems have been used to study the cellular fate of the mutant neuroserpin proteins (42). In order to explore *in vivo* the physiological role of neuroserpin and the pathophysiological role of mutated neuroserpin, we used the intermediate pituitary neuroendocrine melanotrope cells of *Xenopus laevis* as a model system. To regulate the process of background adaptation of the frog, these cells and the hypothalamic neurons that contact them exhibit a high degree of plasticity, which can be observed at the level of proteins, subcellular structures and synapses (reviewed in (28, 29, 43). Via the technique of stable *Xenopus* transgenesis, frogs were generated with transgene expression of GFP-tagged neuroserpin or mutated neuroserpin(G392E) specifically in the melanotrope cells. In this way, we created transgenic line 55 with GFP-neuroserpin transgene expression and transgenic line 84 with GFP-neuroserpin(G392E) expression. In both transgenic lines, the transgene fusion products were secreted by the melanotrope cells.

Transgene expression of GFP-neuroserpin (line 55) or GFP-neuroserpin(G392E) (line 84) did not result in a clear effect on the transgenic melanotrope cells, as was examined via metabolic cell labeling and Western blot analyses. In addition, in transgenic line-84 animals, we have not detected the cell-biological characteristics observed in cells transfected with FENIB mutant neuroserpin. The histological characteristic of FENIB concerns the presence of neuroserpin inclusion bodies which accumulate in the ER and are formed by polymers of the mutated neuroserpin protein (14, 42). Electron microscopy and native gel electrophoresis did however not show neuroserpin inclusion bodies or polymer formation of the GFP-neuroserpin(G392E) protein in transgenic line-84 melanotrope cells (data not shown). Possibly, the expression levels of the transgene were too low in line 84 to induce such a phenotype. Alternatively, the time of adaptation of the transgenic animals to a black background (and therefore the time during which the melanotrope cells were exposed to the transgene product) has been too short. We have shown previously that the time of background adaptation of transgenic frogs can be important to create a phenotypic effect of transgene expression (37, 44). In transfected COS-7 cells, already 24 h post-transfection accumulation of the Portland neuroserpin mutant (S52R) has been observed (42). The neuroserpin(G392E) mutant that we have used in our studies is the most severely disrupting and 'polymerogenic' mutation thus far known, resulting in an early onset of the disease (7, 15, 45), suggesting that polymer formation of GFP-neuroserpin(G392E) would readily occur. Nevertheless, transgene expression of GFP-neuroserpin (line 55) and GFP-neuroserpin(G392E) (line 84) resulted in a phenotype at the ultrastuctural level, since in the two independently generated transgenic lines intercellular gaps between the transgenic

melanotrope cells were observed. Such gaps are normally not found in pituitaries from wild-type animals and also not in other transgenic intermediate pituitaries, such as for p24$\delta_{1/2}$ and the V-ATPase accessory subunit Ac45 (data not shown). These observations imply that the transgene expression of neuroserpin affected the structure of extracellular matrix (ECM) of the melanotrope cells, and therefore suggest a role for neuroserpin in the regulation of the integrity of the ECM. The molecular mechanism behind this observed effect remains elusive. Possibly, the expression of the neuroserpin transgene product resulted in an extensive inhibition of an extracellular target protease that is involved in the regulation of ECM integrity.

In conclusion, we report on the generation and analysis of *Xenopus laevis* with transgene expression of GFP-tagged neuroserpin and mutant neuroserpin(G392E) specifically in the intermediate pituitary melanotrope cells. Expression of the transgene fusion products did not have a clear effect on melanotrope cell functioning, but did result in an effect on the melanotrope cell ECM. These results raise the possibility that neuroserpin is part of a mechanism regulating the integrity of the ECM.

Acknowledgements

We would like to thank R. Engels for animal care and Drs. D.A. Lawrence, B. Wieringa, K. Geering, S. Tanaka and W. Van de Ven for providing antibodies.

References

1. Ye S, Goldsmith EJ. Serpins and other covalent protease inhibitors. Curr Opin Struct Biol 2001;11:740-5.
2. Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res 2000;10:1845-64.
3. Janciauskiene S. Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. Biochim Biophys Acta 2001;1535:221-35.
4. Silverman GA, Bird PI, Carrell RW, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem 2001;276:33293-6.
5. Huntington JA, Carrell RW. The serpins: nature's molecular mousetraps. Sci Prog 2001;84:125-36.
6. Gettins PG. Serpin structure, mechanism, and function. Chem Rev 2002;102:4751-804.
7. Lomas DA, Carrell RW. Serpinopathies and the conformational dementias. Nat Rev Genet 2002;3:759-68.
8. de Groot DM, Pol C, Martens GJ. Comparative analysis and expression of neuroserpin in Xenopus laevis. Neuroendocrinology 2005;82:11-20.
9. Hastings GA, Coleman TA, Haudenschild CC, et al. Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. J Biol Chem 1997;272:33062-7.
10. Krueger SR, Ghisu GP, Cinelli P, et al. Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J Neurosci 1997;17:8984-96.
11. Schrimpf SP, Bleiker AJ, Brecevic L, et al. Human neuroserpin (PI12): cDNA cloning and chromosomal localization to 3q26. Genomics 1997;40:55-62.
12. Stoeckli ET, Lemkin PF, Kuhn TB, Ruegg MA, Heller M, Sonderegger P. Identification of proteins secreted from axons of embryonic dorsal-root-ganglia neurons. Eur J Biochem 1989;180:249-58.
13. Hill RM, Coates LC, Parmar PK, Mezey E, Pearson JF, Birch NP. Expression and functional characterization of the serine protease inhibitor neuroserpin in endocrine cells. Ann N Y Acad Sci 2002;971:406-15.
14. Davis RL, Shrimpton AE, Holohan PD, et al. Familial dementia caused by polymerization of mutant neuroserpin. Nature 1999;401:376-9.
15. Davis RL, Shrimpton AE, Carrell RW, et al. Association between conformational mutations in neuroserpin and onset and severity of dementia. Lancet 2002;359:2242-7.
16. Wannier-Morino P, Rager G, Sonderegger P, Grabs D. Expression of neuroserpin in the visual cortex of the mouse during the developmental critical period. Eur J Neurosci 2003;17:1853-60.
17. Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. Embo J 1996;15:2944-53.
18. Osterwalder T, Cinelli P, Baici A, et al. The axonally secreted serine proteinase inhibitor, neuroserpin, inhibits plasminogen activators and plasmin but not thrombin. J Biol Chem 1998;273:2312-21.
19. Parmar PK, Coates LC, Pearson JF, Hill RM, Birch NP. Neuroserpin regulates neurite outgrowth in nerve growth factor-treated PC12 cells. J Neurochem 2002;82:1406-15.
20. Berger P, Kozlov SV, Cinelli P, Kruger SR, Vogt L, Sonderegger P. Neuronal depolarization enhances the transcription of the neuronal serine protease inhibitor neuroserpin. Mol Cell Neurosci 1999;14:455-67.
21. Hill RM, Parmar PK, Coates LC, Mezey E, Pearson JF, Birch NP. Neuroserpin is expressed in the pituitary and adrenal glands and induces the extension of neurite-like processes in AtT-20 cells. Biochem J 2000;345 Pt 3:595-601.
22. Yepes M, Lawrence DA. Neuroserpin: a selective inhibitor of tissue-type plasminogen activator in the central nervous system. Thromb Haemost 2004;91:457-64.
23. Yepes M, Sandkvist M, Coleman TA, et al. Regulation of seizure spreading by neuroserpin and tissue-type plasminogen activator is plasminogen-independent. J Clin Invest 2002;109:1571-8.
24. Yepes M, Lawrence DA. Tissue-type plasminogen activator and neuroserpin: a well-balanced act in the nervous system? Trends Cardiovasc Med 2004;14:173-80.
25. Zhang Z, Zhang L, Yepes M, et al. Adjuvant treatment with neuroserpin increases the therapeutic window for tissue-type plasminogen activator administration in a rat model of embolic stroke. Circulation 2002;106:740-5.

26. Lebeurrier N, Liot G, Lopez-Atalaya JP, et al. The brain-specific tissue-type plasminogen activator inhibitor, neuroserpin, protects neurons against excitotoxicity both in vitro and in vivo. Mol Cell Neurosci 2005;30:552-8.
27. Jenks B, Buzzi M, Dotman C, et al. The significance of multiple inhibitory mechanisms converging on the melanotrope cell of Xenopus laevis. Ann N Y Acad Sci 1998;839:229-34.
28. Roubos EW. Background adaptation by Xenopus laevis: a model for studying neuronal information processing in the pituitary pars intermedia. Comp Biochem Physiol A Physiol 1997;118:533-50.
29. Roubos EW, Scheenen WJ, Jenks BG. Neuronal, neurohormonal, and autocrine control of Xenopus melanotrope cell activity. Ann N Y Acad Sci 2005;1040:172-83.
30. Cuppen E, Wijers M, Schepens J, Fransen J, Wieringa B, Hendriks W. A FERM domain governs apical confinement of PTP-BL in epithelial cells. J Cell Sci 1999;112 (Pt 19):3299-308.
31. Beggah AT, Geering K. Alpha and beta subunits of Na,K-ATPase interact with BiP and calnexin. Ann N Y Acad Sci 1997;834:537-9.
32. Beggah A, Mathews P, Beguin P, Geering K. Degradation and endoplasmic reticulum retention of unassembled alpha- and beta-subunits of Na,K-ATPase correlate with interaction of BiP. J Biol Chem 1996;271:20895-902.
33. Berghs CA, Tanaka S, Van Strien FJ, Kurabuchi S, Roubos EW. The secretory granule and pro-opiomelanocortin processing in Xenopus melanotrope cells during background adaptation. J Histochem Cytochem 1997;45:1673-82.
34. van Eys GJ, van den Oetelaar P. Cytological localization of alpha-MSH, ACTH and beta-endorphin in the pars intermedia of the cichlid teleost Sarotherodon mossambicus. Cell Tissue Res 1981;215:625-33.
35. Creemers JW, Usac EF, Bright NA, et al. Identification of a transferable sorting domain for the regulated pathway in the prohormone convertase PC2. J Biol Chem 1996;271:25284-91.
36. Kuiper RP, Waterham HR, Rotter J, Bouw G, Martens GJ. Differential induction of two p24delta putative cargo receptors upon activation of a prohormone-producing cell. Mol Biol Cell 2000;11:131-40.
37. de Groot DM, Coenen, A.J.M, Verhofstad, A., van Herp, F., Martens, G.J.M. In vivo induction of glial cell proliferation and axonal outgrowth and myelination by brain-derived neurotrophic factor. Molecular Endocrinology 2006; in press.
38. Sparrow DB, Latinkic B, Mohun TJ. A simplified method of generating transgenic Xenopus. Nucleic Acids Res 2000;28:E12.
39. Kroll KL, Amaya E. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 1996;122:3173-83.
40. de Groot DM, Martens GJ. Expression of Neuroserpin Is Linked to Neuroendocrine Cell Activation. Endocrinology 2005.
41. Jansen EJ, Holling TM, van Herp F, Martens GJ. Transgene-driven protein expression specific to the intermediate pituitary melanotrope cells of Xenopus laevis. FEBS Lett 2002;516:201-7.
42. Miranda E, Romisch K, Lomas DA. Mutants of neuroserpin that cause dementia accumulate as polymers within the endoplasmic reticulum. J Biol Chem 2004;279:28283-91.
43. Kramer BM, Kolk SM, Berghs CA, et al. Dynamics and plasticity of peptidergic control centres in the retino-brain-pituitary system of Xenopus laevis. Microsc Res Tech 2001;54:188-99.
44. van Rosmalen JW, Martens GJ. Cell type-specific transgene expression of the prion protein in Xenopus intermediate pituitary cells. Febs J 2006;273:847-62.
45. Belorgey D, Sharp LK, Crowther DC, Onda M, Johansson J, Lomas DA. Neuroserpin Portland (Ser52Arg) is trapped as an inactive intermediate that rapidly forms polymers: implications for the epilepsy seen in the dementia FENIB. Eur J Biochem 2004;271:3360-7.
46. Bouw G, Van Huizen R, Jansen EJ, Martens GJ. A cell-specific transgenic approach in Xenopus reveals the importance of a functional p24 system for a secretory cell. Mol Biol Cell 2004;15:1244-53.

Generation and analysis of transgenic *Xenopus laevis* with cell-specific overexpression of proBDNF and mature BDNF in intermediate pituitary melanotrope cells

Dorien M. de Groot and Gerard J.M. Martens

Department of Molecular Animal Physiology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Institute for Neuroscience, Faculty of Science, Radboud University, Nijmegen, The Netherlands

Abstract

Brain-derived neurotrophic factor (BDNF) is a neuronal survival and differentiation factor, and a modulator of synaptic plasticity in the central and peripheral nervous systems. Recent studies have shown that besides mature BDNF also the noncleaved proprotein proBDNF can be released by cells and exert yet another biological effect, namely inducing cellular apoptosis. Here we studied functional aspects of pro- and mature BDNF by using the technique of stable transgenesis of the amphibian *Xenopus laevis.* A number of transgenic lines were generated that overexpressed proBDNF, mature BDNF or green fluorescent protein (GFP)-tagged (mutant) proBDNF fusion proteins specifically in the *Xenopus* intermediate pituitary melanotrope cells. These neuroendocrine cells endogenously produce both pro- and mature BDNF. The transgenic melanotrope cells overexpressing mature BDNF are discussed in chapter 6. In this chapter, we analysed transgenic melanotrope cells overexpressing intact proBDNF and GFP-proBDNF. We found that in the various transgenic lines the GFP-proBDNF protein was processed differently, giving rise to different levels of mature BDNF. Transgene expression of unprocessed GFP-proBDNF or a noncleavable GFP-proBDNF mutant fusion protein resulted in endoplasmic reticulum (ER) stress, probably due to misfolding of the transgene product, and an impaired transport of secretory proteins in these cells. In transgenic melanotrope cells expressing an untagged, noncleavable proBDNF mutant protein, we did not observe an effect on the biosynthesis, processing and secretion of newly synthesized proteins, presumably owing to the low levels of transgene expression. Together, our results suggest that intact GFP-proBDNF causes ER stress and impaired secretory protein transport in transgenic *Xenopus* melanotrope cells.

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that regulates neuronal survival and differentiation, and also modulates synaptic plasticity in the central and peripheral nervous systems (reviewed in 1-5). In addition, BDNF has recently been implicated in various other biological events such as neuronal and glial cell proliferation (6-8) and myelination (9-12).

Like other neurotrophin family members, BDNF is initially synthesized as a precursor of 249 amino acids (proBDNF). The N-terminal fragment of ~112 amino acids, or the 'pro-region', can then be proteolytically cleaved by furin or prohormone convertases PC1/PC2 to form mature BDNF (13, 14). BDNF can be secreted via either the regulated or constitutive pathway, depending on the available secretory pathways in a given cell (reviewed in 15). Furthermore, interaction between a motif in the tertiary structure of BDNF and the sorting receptor carboxypeptidase E directs BDNF mainly to the regulated secretory pathway for activity-dependent release in AtT20 cells and cortical neurons (16, 17). Besides this sorting motif in mature BDNF, the pro-sequence itself also influences

sorting, since a valine-to-methionine (val-66-met) substitution in the prodomain impaired proBDNF-sorting from the Golgi complex into secretogranin II-positive vesicles, which markedly reduced activity-dependent secretion of mature BDNF. This val-66-met substitution is a recognized polymorphism in the human *bdnf* gene that is associated with memory impairment and increased susceptibility to neuropsychiatric disorders (18-20).

Recently it was discovered that mature BDNF as well as proBDNF can be secreted, and the proteins appear to exert opposite biological effects by differential binding to two different receptors. Mature BDNF preferentially binds to and activates the TrkB receptor tyrosine kinase, inducing cell survival and differentiation signaling pathways, whereas proBDNF has a higher affinity for $p75^{NTR}$, leading to cellular apoptosis (21; reviewed in 3, 5). This finding increased the complexity of understanding the physiological role of BDNF in the nervous system, and the molecular and cellular mechanisms underlying this role in many cases remain elusive.

In our studies, we use the South-African claw-toed frog *Xenopus laevis* as a model system, and more specifically the melanotrope cells of the intermediate pituitary. These cells regulate the process of background adaptation of this amphibian by releasing pigment dispersing α-melanophore stimulating hormone (α-MSH), a cleavage product of the prohormone proopiomelanocortin (POMC) (reviewed in 22-24). In the *Xenopus* melanotrope cells, both proBDNF and mature BDNF are present (25, 26), and when secreted by these cells mature BDNF can stimulate in an autocrine manner the release of α-MSH and the biosynthesis of POMC (25).

To gain more insight into the physiological role of pro- and mature BDNF, we used *Xenopus* transgenesis as a tool to cell-specifically overexpress proBDNF, mature BDNF or proBDNF fused to green fluorescent protein (GFP). This chapter describes the generation and analysis of a number of transgenic lines. In transgenic melanotrope cells expressing a non-tagged, noncleavable proBDNF, we did not detect significant effects, probably because of the relatively low levels of transgene expression. However, transgene expression of unprocessed GFP-proBDNF resulted in endoplasmic reticulum (ER) stress, implying the importance of the prodomain for a proper folding of proBDNF. In addition, these transgenic lines showed an impaired processing of newly synthesized secretory pathway proteins in the melanotrope cells.

Materials and Methods

Animals

South African claw-toed frogs, *Xenopus laevis*, were reared in the Central Animal Facility of the Radboud University of Nijmegen (Nijmegen, The Netherlands). The animals were adapted to their background by keeping them in black buckets, with the lights turned on in cycles of 12 h. Experimental procedures were performed under the guidelines of the Dutch

law concerning animal welfare and permit RBD0166 (H10) to generate and house transgenic *Xenopus.*

Antibodies

An anti-BDNF antibody directed against the first 20 amino acid residues of mature BDNF (anti-BDNF; Santa Cruz) detected both proBDNF and mature BDNF on Western blots. GFP and tubulin were detected using a polyclonal anti-GFP antibody (anti-GFP; generous gift of Dr. B. Wieringa, Radboud University, Nijmegen, The Netherlands; 27) and the monoclonal anti-tubulin antibody E7 (Developmental Studies Hybridoma Bank, Rockland, Gilbertsville), respectively. *Xenopus* calnexin was detected with an anti-calnexin antibody (29), BiP with and anti-BiP antibody (29) (generous gifts of Dr. K. Geering, University of Lausanne, Lausanne, Switzerland), POMC with an anti-POMC antibody for Western blotting (ST62; generous gift of Dr. S. Tanaka, Shizuoka University, Japan; 30) or with an anti-adrenocorticotropic hormone (ACTH) antibody for immunocytochemistry (Biogenesis, Poole, UK), prohormone convertase PC2 with an anti-PC2 antibody against recombinant mature human PC2 (generous gift of Dr. W. Van de Ven, University of Leuven, Belgium; 31) and $p24\delta_2$ with anti-p24-antibody 1262N (32).

Cloning

A number of constructs were made to obtain the linear DNA fragments that were used for *Xenopus* transgenesis. First, a fusion construct with GFP fused to the C-terminus of proBDNF was generated. For this, PCR amplification of the rat BDNF sequence was carried out in a reaction mixture containing Tgo buffer (Roche), 0.8 units Tgo DNA polymerase (Roche), 250 μM dNTPs, 0.5 μM of each primer and 1 μl rat brain cDNA (5 amplication cycles: 1 min at 94°C, 1 min at 56°C and 1 min 30 sec at 72°C followed by 30 amplification cycles of: 1 min at 94°C, 1 min at 70°C and 1 min 30 sec at 72°C). The following forward and reverse primers were used: 5'- GGGGAAGCTTGTTCCACCAGGTG-AGAAGAGTGATG- 3' (containing a *Hind*III restriction site (underlined)) and 5'-GGGGGGATCC**CCGCTT**TCTTCCCCTTTTAATGGTCAG- 3' (containing an *Eco*RI restriction site (underlined) and an extra lysine and arginine codon (bold)). Subsequently, the amplified BDNF sequence was cloned via *Hind*III and *Eco*RI digestion into the pPOMC(A)2+-GFP vector (33), resulting in pPOMC-proBDNF-GFP. Secondly, fusion constructs were made in which GFP was fused to the N-terminus of proBDNF. For this, PCR amplification of the rat BDNF sequence was carried out in a reaction mixture containing Pfu buffer (Fermentas), 0.05 units/μl Pfu Turbo DNA polymerase (Fermentas), 200 μM dNTPs, 0.5 μM of each primer and 1 ng pPCG(A)2+-BDNF vector DNA (De Groot *et al*., 2006) (35 amplification cycles: 30 sec at 95°C, 1 min at 50°C and 1 min 30 sec at 72°C). The forward primer used was 5'- GGGGGAATTC**AAAAGG**GCGCCCATGAAAGAAGCAAACG-3' (containing an *Eco*RI restriction site (underlined) and an extra lysine and arginine codon (bold)) and the reverse primer 5'- GGGTCTAGAGCGCAAATGACTGTTTC-3' (containing an *Xba*I restriction site

(underlined)). The *Eco*RI- and *Xba*I- digested PCR fragment was cloned into the pPOMC(A)2+-SP-GFP vector (34), resulting in the pPOMC-SP-GFP-proBDNF construct (used to generate transgenic line 78). The construct that was used to generate transgenic line 73 has been described previously (34; pPOMC-SP-GFP-proBDNF), except that it does not contain the two extra lysine and arginine amino acids between the GFP and BDNF sequence. Third, to generate a noncleavable GFP-proBDNF fusion protein, the two arginine residues constituting the cleavage site were mutated into two alanines using the QuickChange Site-directed Mutagenesis Kit (Stratagene). For this, we used the pPOMC-SP-GFP-proBDNF construct described here as template DNA and primer 5'-CATGTCTATGAGGGTTGCCGCGCACTCCGACCCCGCC- 3' (nucleotides responsible for amino acid substitution are underlined) for PCR amplification, resulting in the pPOMC-SP-GFP-proBDNF(AA) construct, which was used to generate transgenic line 92. Fourth, double constructs were generated to overexpress untagged proBDNF or noncleavable proBDNF via *Xenopus* transgenesis. To generate transgenic line 6, we used construct pPCG-proBDNF described previously (34). The construct encoding the noncleavable proBDNF mutant protein expressed in transgenic line 111 was generated by cloning the *Ava*I- *Xba*I insert of pPOMC-SP-GFP-proBDNF(AA) into the pPCG-proBDNF vector (digested with *Ava*I and *Xba*I), resulting in the pPCG-proBDNF(AA) construct.

Generation of transgenic Xenopus

To create transgenic frogs, linear *Sal*I/*Not*I DNA fragments were generated from the constructs pPOMC-SP-GFP-proBDNF (2552 bp (line 78) or 2546 bp (line 73)), pPOMC-SP-GFP-proBDNF(AA) (2546 bp), pPCG-proBDNF (3577 bp) and pPCG-proBDNF(AA) (3577 bp) and a linear *Sal*I/*Pau*I fragment was generated from the construct pPOMC-BDNF-GFP (2349 bp). These linear fragments were used for stable *Xenopus* transgenesis (34-36). A number of injection rounds resulted in animals transgenic for the fusion proteins GFP-proBDNF (line 73 and 78) and GFP-proBDNF(AA) (line 92), and non-tagged proBDNF (line 6) and noncleavable proBDNF(AA) (line 111). To generate F1 offspring, the testes of male transgenic *Xenopus* frogs were isolated and used for *in vitro* fertilization of eggs harvested from wild-type *Xenopus* females.

Metabolic cell labeling

Pituitary NILs were metabolically labeled as described previously (37). NILs were pulsed for 30 min and chased for 180 min. The amounts of newly synthesized 37-kDa POMC and of the 18-kDa POMC cleavage product (total of cellular and secreted protein) were quantified by densitometry using a phosphoimager (BioRad). Relative processing of 37-kDa POMC was estimated by the ratio of 37-kDa to 18-kDa POMC and was presented in arbitrary units (n=3). An unpaired t test was used for statistical analysis and wild-type values were set at 1.

Western Blot analysis

To detect steady-state protein levels in neurointermediate lobes (NILs) of transgenic and wild-type animals, Western blot analysis was performed after metabolic cell labeling as described previously (37), using the anti-BDNF, anti-GFP, anti-tubulin, anti-calnexin, anti-BiP, anti-POMC, anti-p24 and anti-PC2 antibodies as primary antiserum. Signals were visualized with a BioChemi Imaging System, and relative quantification by densitometry was performed with Labworks 4.0 software (UVP BioImaging systems, Cambridge, UK). The densities of the various signals were normalized to the corresponding tubulin signals (n = 3). An unpaired t test was used for statistical analysis.

Immunocytochemistry

Paraffin sectioning and immunocytochemistry were performed as described previously (34). The anti-BDNF and anti-ACTH antibodies were used as primary antiserum.

TUNEL assay

To detect apoptotic cells, a TUNEL assay was performed on paraffin sections of NILs from wild-type and transgenic line-111 animals according to the manufacturer's instructions (*In Situ* Cell Death Detection Kit, POD; Roche). Treatment of the sections with DNaseI or performing the reaction without enzyme acted as a positive and negative control for the assay.

Results

Generation of *Xenopus* transgenic for GFP-tagged or non-tagged proBDNF and noncleavable proBDNF(AA)

We used various constructs to generate transgenic frogs that overexpress proBDNF, a noncleavable form of proBDNF (proBDNF(AA)) or these proteins fused to GFP (Fig. 1A). A *Xenopus* POMC gene promoter fragment was employed to express the transgene products specifically in the melanotrope cells of the intermediate pituitary. Transgenic tadpoles were identified by screening for GFP fluorescence in the pituitary (GFP-proBDNF and GFP-proBDNF(AA) fusion constructs) or in muscle tissue (non-tagged proBDNF and proBDNF(AA)). We created a number of transgenic *Xenopus* lines with overexpression of the GFP-proBDNF fusion protein (lines 73 and 78, of which transgenic line-78 animals had an extra arginine and lysine residue between the GFP and proBDNF sequence), GFP-proBDNF(AA) fusion protein (line 92), proBDNF (line 6) or proBDNF(AA) (line 111) in the intermediate pituitary melanotrope cells (Fig. 1B). For unknown reasons, we did not succeed in generating animals that were transgenic for the proBDNF-GFP fusion protein, while the sequence of the construct was correct and transfected cells showed expression and fluorescence of the proBDNF-GFP protein (data not shown).

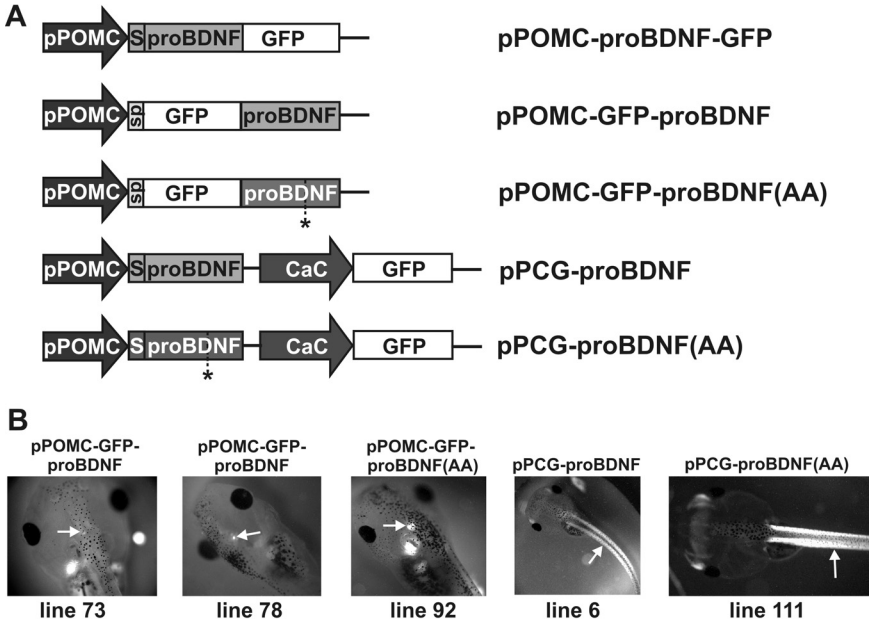


Figure 1: Generation of transgenic *Xenopus* with overexpression of GFP-tagged or non-tagged proBDNF or noncleavable proBDNF in the intermediate pituitary.
(A) Schematic representation of the linear DNA fragments that were used for *Xenopus* transgenesis. Abbreviations: pPOMC, promoter of the proopiomelanocortin gene; proBDNF, proform of brain-derived neurotrophic factor; proBDNF(AA), proform of brain-derived neurotrophic factor with a mutated cleavage site resulting in noncleavable proBDNF; S, signal peptide of proBDNF; sp, signal peptide cloned in front of GFP; GFP, green fluorescent protein; Cac, cardiac actin gene promoter. (B) GFP fluorescence in transgenic *Xenopus* tadpoles. Tadpoles transgenic for GFP-proBDNF or GFP-proBDNF(AA) were identified via screening of fluorescence in the intermediate pituitary, and transgenic for PCG-proBDNF or PCG-proBDNF(AA) by GFP fluorescence in muscle. The various transgenic lines that were generated are indicated below the photographs.

To examine the expression of the various transgene products in NIL lysates of adult transgenic animals, we performed Western blot analysis using anti-BDNF (Fig. 2A) and anti-GFP (Fig. 2B) antibodies. Using the anti-BDNF antibody, in transgenic line-73 NILs we detected the ~65-kDa fusion protein GFP-proBDNF and 14-kDa mature BDNF. In contrast, in transgenic NILs from line 78, we observed only expression of 65-kDa GFP-proBDNF. Thus, in various F0 animals with GFP-proBDNF expression the transgene was differentially processed, resulting in expression of either relatively high levels of mature BDNF (line 73) or the barely processed GFP-proBDNF fusion protein (line 78). In the NILs of transgenic line-92 animals, we detected besides the 65-kDa GFP-proBDNF(AA) fusion protein, a product of ~62 kDa, which probably corresponded to a non-glycosylated fusion protein. As expected, these line-92 mutant fusion proteins were not proteolytically cleaved. Remarkably, the amounts of the immunoreactive 46-kDa product that was reacting

nonspecfically with the antibody and that was detected in all NIL lysates were reduced in the NILs of line-78 and-92 animals, suggesting a possible effect of the uncleaved GFP-proBDNF fusion protein on protein expression. Finally, transgenic NILs from lines 6 and 111 showed a moderate transgene expression of ~14-kDa mature BDNF and 35-kDa proBDNF, respectively (Fig. 2A). In line with the results obtained with the anti-BDNF antibody, Western blot analysis with the anti-GFP antibody showed the 65-kDa GFP-proBDNF fusion protein in lines 73 and 78, and the two 62- and 65-kDa GFP-proBDNF fusion proteins in line 92. In addition, in line 73 we observed a product of ~45 kDa representing a cleavage product of the GFP-proBDNF product. As expected, with this antibody no products were detected in wild-type, and transgenic lines-6 and -111 NILs (Fig. 2B).

To examine whether the melanotrope cells also secreted the transgene products, NILs were incubated in media that were subsequently used for Western blot analysis using the anti-BDNF antibody. We found only secretion of mature BDNF in line 73, and of GFP-proBDNF(AA) in line 92. No secreted mature BDNF, proBDNF or GFP-proBDNF fusion protein was detected when NILs of wild-type animals or of animals of transgenic lines 78, 92 and 111 were incubated (Fig. 2C).

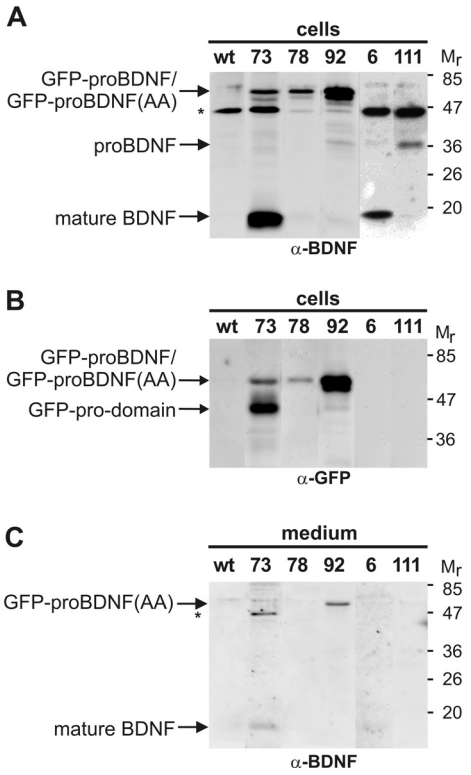


Figure 2: Steady-state protein expression in *Xenopus* intermediate pituitary cells transgenic for GFP-tagged or non-tagged proBDNF or noncleavable proBDNF.
Western blot analysis of neurointermediate (NIL) lobe lysates (cells) of adult wild-type (wt) and adult transgenic animals (lines 73, 78, 92, 6 and 111) using an anti-BDNF antibody (α-BDNF) (A) or an anti-GFP antibody (B). A non-specific immunoreactive ~46kDa product in A is indicated with an asterisk. (C) Western blot analysis of incubation media (medium) of NILs from adult wt and adult transgenic animals (lines 73, 78, 92, 6 and 111) using α-BDNF. NILs were incubated for 3 h.

Effect of transgene expression of intact proBDNF or GFP-proBDNF on the biosynthesis and processing of POMC and the secretion of POMC-derived peptides by the transgenic *Xenopus* intermediate pituitary cells

Next, the effects of transgene expression on melanotrope cell functioning, and more specifically the biosynthesis and processing of POMC and the secretion of POMC-derived peptides, were studied. The effects of the transgene expression of mature BDNF (lines 73 and 6) have been described elsewhere (34). Here we report on the findings in transgenic animals with expression of unprocessed GFP-proBDNF (line 78), noncleavable GFP-proBDNF(AA) (line 92) and noncleavable proBDNF(AA) (line 111). Metabolic labeling of NILs showed that following a 30-min pulse and a 180-min chase period in transgenic line-111 animals with expression of noncleavable proBDNF (proBDNF(AA)), the biosynthesis and processing of POMC and secretion of POMC-derived peptides did not differ significantly from that by NILs of wild-type animals (Figs. 3C and D). In contrast, we found in transgenic line-78 and line-92 animals significantly less processing of newly synthesized 37-kDa POMC into 18-kDa POMC (Figs. 3A and B). Quantification of the amounts of 37-kDa and 18-kDa POMC in wild-type and transgenic animals revealed ~15-fold and ~3-fold less processing in transgenic line-78 and -92 animals, respectively (Fig. 3D). In addition, the processing of pro-prohormone convertase 2 (proPC2), the pro-form of the enzyme involved in the proteolytic maturation of POMC, was also affected since in line-78 and -92 animals mainly the pro-forms of the enzyme were detected, whereas in wild-type NILs mature PC2 was present and secreted (Figs. 3A and B).

Effect of transgene expression of intact proBDNF or GFP-proBDNF on steady-state levels of various secretory pathway proteins in *Xenopus* melanotrope cells

To examine possible effects of the transgene expression of unprocessed GFP-proBDNF (line 78), noncleavable GFP-proBDNF(AA) (line 92) or noncleavable proBDNF (line 111) on the expression levels of a number of proteins in the melanotrope cells, Western blot analysis was performed on NIL lysates. We observed no differences in the steady-state levels of PC2, of the ER chaperone calnexin, of the putative ER-to-Golgi cargo receptor p24δ_2, and of POMC. In contrast, we found an ~10-fold and ~5-fold up-regulation of steady-state expression of the ER chaperone BiP in transgenic line-78 and -92 animals, respectively, compared with BiP expression in NILs from wild-type and transgenic line-111 animals (Figs. 4A and B).

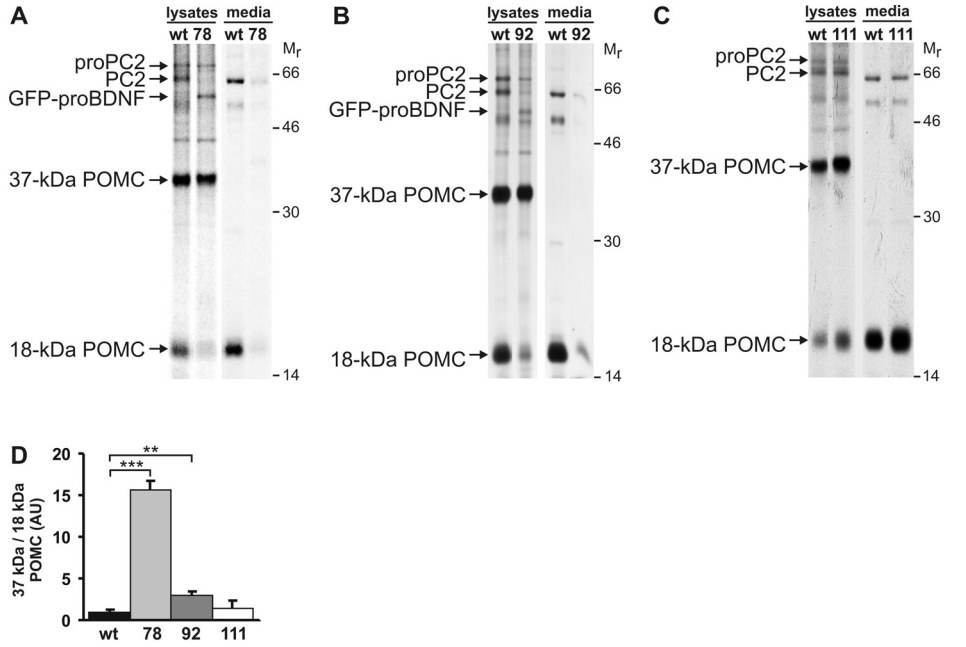


Figure 3: Newly synthesized proteins produced in intermediate pituitary cells of wild-type *Xenopus* and *Xenopus* transgenic for unprocessed or noncleavable GFP-proBDNF or noncleavable proBDNF.
Neurointermediate lobes (NILs) of black-adapted wild-type (wt) and transgenic line 78 (A), line 92 (B) or line 111 (C) animals were pulse labeled for 30 min and chased for 180 min. Newly synthesized proteins extracted from the lobes were resolved by SDS-PAGE and visualized by fluorography. Abbreviations: (pro)PC2, (pro)prohormone convertase 2; GFP-proBDNF, green fluorescent protein fused to the proform of brain-derived neurotrophic factor; POMC, proopiomelanocortin. (D) Quantification of the processing of 37-kDa POMC into 18-kDa POMC. Densities of 37-kDa and 18-kDa POMC signals were determined and the ratio of 37-kDa to 18-kDa is given in arbitrary units (AU). Values are expressed as means ± SEM (n=3). Significant differences are indicated (**: $p<0.01$; ***: $p<0.001$).

Effect of transgene expression of intact proBDNF on cellular apoptosis

To detect any possible effects of proBDNF(AA) transgene expression (line 111) on cellular apoptosis, we used a TUNEL assay on paraffin sections of pituitaries of both wild-type and transgenic line-111 animals. We first performed immunocytochemistry to detect BDNF and POMC as a marker for the intermediate pituitary melanotrope cells. We found strong POMC staining in the intermediate pituitaries of both wild-type and transgenic line-111 animals (Fig. 5A and B, respectively) and detected clearly more BDNF staining in the intermediate pituitaries of transgenic line-111 animals compared to wild-type animals, confirming the proBDNF(AA) transgene expression in line 111 (Fig. 5D and C, respectively). However, using the TUNEL assay we were not able to detect significant differences in the number of apoptotic cells in the pituitaries of wild-type (Fig. 5E)

compared to transgenic animals (Fig. 5F). Treatment of the sections with DNaseI or performing the reaction without enzyme acted as a positive and negative control for the assay, respectively, which indicated that the assay had worked properly (Figs. 5G and H).

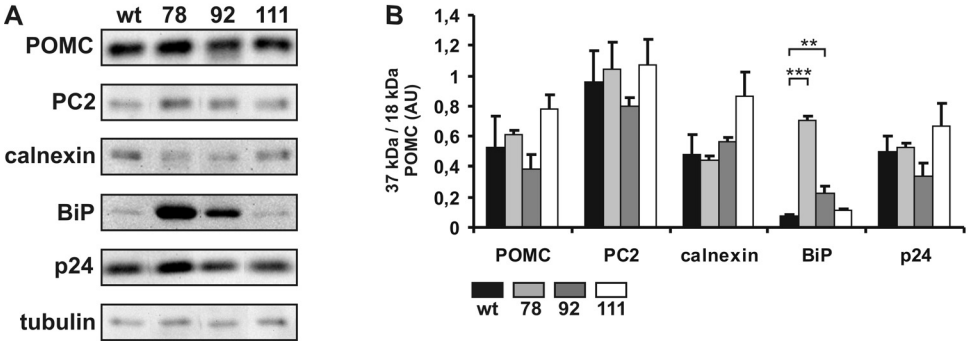


Figure 4: Steady-state levels of various secretory pathway proteins in intermediate pituitary cells of wild-type *Xenopus* and *Xenopus* transgenic for unprocessed or noncleavable GFP-proBDNF or noncleavable proBDNF.

(A) Western blot analysis of neurointermediate lobes of wild-type (wt) and transgenic line-78, -92 and -111 animals using antibodies directed against proopiomelanocortin (POMC, prohormone convertase 2 (PC2), calnexin, BiP, p24δ_2 and tubulin. The results shown are representatives of three independent experiments. (B) Quantification of the relative protein expression levels of POMC, PC2, calnexin, BiP and p24δ_2 in wt and transgenic lines-78, -92 and -111 neurointermediate lobes. Values (in arbitrary units (AU)) are expressed as means ± SEM (n=3). Significant differences are indicated (**: $p<0.01$; ***: $p<0.001$).

Discussion

The neurotrophin family member BDNF is a well-known neuronal survival and differentiation factor that has also been implicated in the process of synaptic plasticity (reviewed in 1-5). Like its family members, BDNF is produced as a proprotein that can be cleaved in the secretory pathway after which the mature protein is released from the cells (13, 14). However, in recent years the prodomain of neurotrophins has gained interest because exciting new data have shown that besides the mature form of BDNF also proBDNF can be released and that, compared with mature BDNF, the proform exerts an opposite effect on cells, namely the induction of apoptosis or facilitation of hippocampal long-term depression (18, 20, 38-40). In addition, the prodomain appears to be involved in the sorting of proBDNF to the proper subcompartment of the secretory pathway (15, 17).

In our study, we used the intermediate pituitary melanotrope cells of *Xenopus laevis* as a model system to study functional aspects of pro- and mature BDNF. For this, we

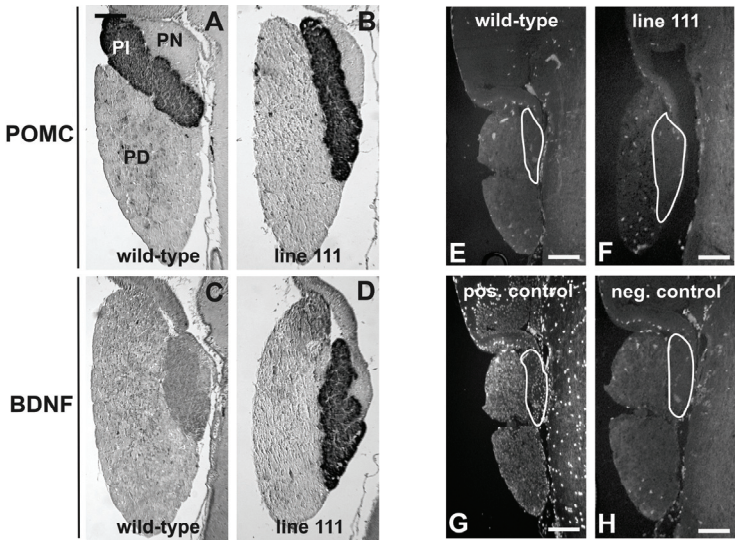


Figure 5: TUNEL assay on the pituitaries of wild-type *Xenopus* and *Xenopus* transgenic for noncleavable proBDNF.
Immunocytochemistry on paraffin sections of wild-type (A, C) and transgenic line-111 (B, D) pituitaries to detect proopiomelanocortin (POMC; A, B), and brain-derived neurotrophic factor (BDNF; C, D). Abbreviations: pn, pars nervosa; pi, intermediate pituitary and pd, pars distalis. Scale bar = 100 μm. (E-H) Photographs of fluorescence of paraffin sections after a TUNEL assay to detect apoptotic cells in the pituitaries of wild-type (E) and transgenic line-111 (F) animals. Wild-type pituitaries were also used to perform a positive (G) and negative (H) control for the assay, by treating the sections with DNaseI or performing the reaction without enzyme, respectively. The intermediate pituitaries are encircled in white. Scale bar = 200 μm.

generated transgenic *Xenopus* with expression of pro- or mature BDNF, or of GFP-tagged versions of these proteins specifically in the melanotrope cells. First, in transgenic line-111 animals with transgene expression of noncleavable proBDNF(AA) in the pituitary melanotrope cells, we did not detect secreted proBDNF(AA) and did not find significant effects on the biosynthesis and processing of POMC and the secretion of POMC-derived peptides or on cellular apoptosis. Possibly, these findings are due to the relatively low levels of transgene expression in line 111.

Remarkably, we found that in various independently generated F0 animals transgenic for GFP-proBDNF the fusion protein was processed with different efficiencies. For example, transgenic line-73 animals showed effective GFP-proBDNF processing resulting in transgene expression of high amounts of mature BDNF, whereas line-78 animals expressed a not efficiently processed intact fusion protein. This differential processing efficiency was observed in more independently generated transgenic F0 animals with GFP-proBDNF expression (data not shown). Possibly, in different transgenic animals

the transgenes are translated in different subcompartments of the ER, resulting in differences in protein folding and processing efficiencies.

Interestingly, in transgenic animals with transgene expression of non-processed or uncleaved GFP-proBDNF (lines 78 and 92) we found an up-regulation of the ER chaperone BiP. In general, upregulation of BiP is considered to be a hallmark of the unfolded protein response (UPR) (reviewed in 41-43). The UPR is a collective name for adaptive signaling mechanisms that the ER has developed to cope with accumulation of unfolded or misfolded proteins in the ER lumen, which can be caused by a number of biochemical and physiological stimuli imposing stress to the ER. The observed up-regulation of BiP in the transgenic melanotropes of our line-78 and line-92 animals implies that in these animals the folding of the fusion protein is impaired, leading to an accumulation of unfolded transgene product and ER stress. Possibly, the GFP moiety of the GFP-proBDNF fusion protein impaired a proper folding of the transgene protein in the ER. The prodomain of NGF, and thus presumably also of BDNF, is important for a proper folding of the proneurotrophin (44, 45), and therefore GFP N-terminally fused to BDNF might interfere with a proper intramolecular chaperone function of the prodomain. Remarkably, the GFP-proBDNF transgene product was processed highly efficient in the melanotrope cells of line-73 animals. A C-terminally GFP-tagged BDNF fusion protein expressed in injected cortical neurons has been shown to be biologically active, mimicking the releasing characteristics of untagged BDNF (46, 47). Accordingly, we have tried to generate transgenic frogs with transgene expression of proBDNF-GFP, but unfortunately for unknown reasons we did not succeed in generating such transgenic animals (data not shown).

In the transgenic melanotrope cells of lines 78 and 92, the ER stress also resulted in an impaired processing of POMC and of the processing enzyme proPC2. Previous studies have shown that overexpression of transgene proteins in cell lines, *e.g.* blood coagulation factor VIII (48, 49), or antitrombin III (50) can cause exhaustion of the capacity of the protein folding machinery, resulting in the accumulation of unfolded, aggregated proteins in the ER and activation of the UPR. However, in our case such an effect seems not to result from our transgenic approach per se, since in our lab we have thus far generated multiple transgenic lines with overexpression of other transgene products that were directed to the secretory pathway, like the serine protease inhibitor neuroserpin (chapter 4) or prion protein (51), in which we have not observed a UPR.

The accumulation and lack of secretion of unprocessed GFP-proBDNF by the melantorope cells of our transgenic line-78 animals reminded us of transfection studies dealing with cellular aspects of the BDNF val-66-met polymorphism. In these studies, trafficking of $proBDNF_{MET}$ was altered from the regulated secretory pathway to a more concentrated distribution of the protein in the cell body of polarized neurosecretory cells, and a decrease in regulated BDNF secretion (18, 20). We now find that unprocessed proBDNF can induce ER stress and a block of protein transport and processing of other secretory pathway proteins, which implies that intact BDNF affects neuroendocrine cell

functioning. We propose that the impaired intracellular trafficking of proBDNF$_{MET}$ also imposes stress to the ER, resulting in a reduced transport, processing and secretion of other neuronal secretory pathway proteins. Eventually, this may lead to multiple effects of proBDNF$_{MET}$ on neuronal cell functioning, possibly explaining some aspects of the wide variety of phenotypes observed in individuals carrying the BDNF$_{MET}$ polymorphism.

In conclusion, we generated and analysed transgenic *Xenopus* with expression of pro- and mature BDNF, and GFP-tagged versions of these proteins to study aspects of the physiological role of pro- and mature BDNF in the *Xenopus* intermediate pituitary melanotrope cells. We found that transgene expression of noncleavable proBDNF(AA) did not result in a clear phenotype, presumably due to the low levels of transgene expression. However, transgene expression of intact GFP-proBDNF resulted in ER stress and a block in secretory protein transport. These results imply that intact proBDNF can affect the transport of proteins through the (regulated) secretory pathway, resulting in an impaired functioning of neuroendocrine cells.

Acknowledgements

We gratefully thank T. Coenen for the sectioning and immumnocytochemistry, Drs. B. Wieringa, K. Geering, S. Tanaka and W. Van de Ven for providing antibodies, and R. Engels for animal care.

References

1. Binder DK, Scharfman HE. Brain-derived neurotrophic factor. Growth Factors 2004;22:123-31.
2. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;4:299-309.
3. Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 2001;11:272-80.
4. Poo MM. Neurotrophins as synaptic modulators. Nat Rev Neurosci 2001;2:24-32.
5. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 2000;10:381-91.
6. Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 2001;21:6706-17.
7. Lambert WS, Clark AF, Wordinger RJ. Effect of exogenous neurotrophins on Trk receptor phosphorylation, cell proliferation, and neurotrophin secretion by cells isolated from the human lamina cribrosa. Mol Vis 2004;10:289-96.
8. Zhang J, Geula C, Lu C, Koziel H, Hatcher LM, Roisen FJ. Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. Exp Neurol 2003;183:469-81.
9. Chan JR, Cosgaya JM, Wu YJ, Shooter EM. Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci U S A 2001;98:14661-8.
10. Cosgaya JM, Chan JR, Shooter EM. The neurotrophin receptor p75NTR as a positive modulator of myelination. Science 2002;298:1245-8.
11. Tolwani RJ, Cosgaya JM, Varma S, Jacob R, Kuo LE, Shooter EM. BDNF overexpression produces a long-term increase in myelin formation in the peripheral nervous system. J Neurosci Res 2004;77:662-9.
12. McTigue DM, Horner PJ, Stokes BT, Gage FH. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci 1998;18:5354-65.
13. Lu B. Pro-region of neurotrophins: role in synaptic modulation. Neuron 2003;39:735-8.
14. Seidah NG, Benjannet S, Pareek S, Chretien M, Murphy RA. Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett 1996;379:247-50.
15. Lessmann V, Gottmann K, Malcangio M. Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 2003;69:341-74.
16. Lou H, Kim SK, Zaitsev E, Snell CR, Lu B, Loh YP. Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase e. Neuron 2005;45:245-55.
17. Thomas K, Davies A. Neurotrophins: a ticket to ride for BDNF. Curr Biol 2005;15:R262-4.
18. Egan MF, Kojima M, Callicott JH, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 2003;112:257-69.
19. Hariri AR, Goldberg TE, Mattay VS, et al. Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. J Neurosci 2003;23:6690-4.
20. Chen ZY, Patel PD, Sant G, et al. Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. J Neurosci 2004;24:4401-11.
21. Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science 2001;294:1945-8.
22. Kramer BM, Kolk SM, Berghs CA, et al. Dynamics and plasticity of peptidergic control centres in the retino-brain-pituitary system of Xenopus laevis. Microsc Res Tech 2001;54:188-99.
23. Roubos EW. Background adaptation by Xenopus laevis: a model for studying neuronal information processing in the pituitary pars intermedia. Comp Biochem Physiol A Physiol 1997;118:533-50.
24. Roubos EW, Scheenen WJ, Jenks BG. Neuronal, neurohormonal, and autocrine control of Xenopus melanotrope cell activity. Ann N Y Acad Sci 2005;1040:172-83.
25. Kramer BM, Cruijsen PM, Ouwens DT, et al. Evidence that brain-derived neurotrophic factor acts as an autocrine factor on pituitary melanotrope cells of Xenopus laevis. Endocrinology 2002;143:1337-45.
26. Wang LC, Meijer HK, Humbel BM, Jenks BG, Roubos EW. Activity-dependent dynamics of coexisting brain-derived neurotrophic factor, pro-opiomelanocortin and alpha-melanophore-stimulating hormone in melanotrope cells of Xenopus laevis. J Neuroendocrinol 2004;16:19-25.

27. Cuppen E, Wijers M, Schepens J, Fransen J, Wieringa B, Hendriks W. A FERM domain governs apical confinement of PTP-BL in epithelial cells. J Cell Sci 1999;112 (Pt 19):3299-308.
28. Beggah A, Mathews P, Beguin P, Geering K. Degradation and endoplasmic reticulum retention of unassembled alpha- and beta-subunits of Na,K-ATPase correlate with interaction of BiP. J Biol Chem 1996;271:20895-902.
29. Beggah AT, Geering K. Alpha and beta subunits of Na,K-ATPase interact with BiP and calnexin. Ann N Y Acad Sci 1997;834:537-9.
30. Berghs CA, Tanaka S, Van Strien FJ, Kurabuchi S, Roubos EW. The secretory granule and pro-opiomelanocortin processing in Xenopus melanotrope cells during background adaptation. J Histochem Cytochem 1997;45:1673-82.
31. Creemers JW, Usac EF, Bright NA, et al. Identification of a transferable sorting domain for the regulated pathway in the prohormone convertase PC2. J Biol Chem 1996;271:25284-91.
32. Kuiper RP, Bouw G, Janssen KP, Rotter J, van Herp F, Martens GJ. Localization of p24 putative cargo receptors in the early secretory pathway depends on the biosynthetic activity of the cell. Biochem J 2001;360:421-9.
33. Collin RW, Martens GJ. The coding sequence of amyloid-beta precursor protein APP contains a neural-specific promoter element. Brain Res 2006; 1087:41-51.
34. De Groot DM, Coenen, A.J.M., Verhofstad, A., van Herp, F., Martens, G.J.M. In vivo induction of glial cell proliferation and axonal outgrowth and myelination by brain-derived neurotrophic factor. Mol Endocrinology 2006; in press.
35. Kroll KL, Amaya E. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 1996;122:3173-83.
36. Sparrow DB, Latinkic B, Mohun TJ. A simplified method of generating transgenic Xenopus. Nucleic Acids Res 2000;28:E12.
37. de Groot DM, Martens GJ. Expression of neuroserpin is linked to neuroendocrine cell activation. Endocrinology 2005;146:3791-9.
38. Mowla SJ, Farhadi HF, Pareek S, et al. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J Biol Chem 2001;276:12660-6.
39. Teng HK, Teng KK, Lee R, et al. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 2005;25:5455-63.
40. Woo NH, Teng HK, Siao CJ, et al. Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 2005;8:1069-77.
41. Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annu Rev Biochem 2005;74:739-89.
42. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res 2005;569:29-63.
43. Ma Y, Hendershot LM. ER chaperone functions during normal and stress conditions. J Chem Neuroanat 2004;28:51-65.
44. Suter U, Heymach JV, Jr., Shooter EM. Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. Embo J 1991;10:2395-400.
45. Rattenholl A, Ruoppolo M, Flagiello A, et al. Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. J Mol Biol 2001;305:523-33.
46. Kohara K, Kitamura A, Morishima M, Tsumoto T. Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. Science 2001;291:2419-23.
47. Kojima M, Takei N, Numakawa T, et al. Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J Neurosci Res 2001;64:1-10.
48. Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. J Biol Chem 1988;263:6352-62.
49. Dorner AJ, Wasley LC, Kaufman RJ. Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. J Biol Chem 1989;264:20602-7.
50. Schroder M, Schafer R, Friedl P. Induction of protein aggregation in an early secretory compartment by elevation of expression level. Biotechnol Bioeng 2002;78:131-40.
51. van Rosmalen JW, Martens GJ. Cell type-specific transgene expression of the prion protein in Xenopus intermediate pituitary cells. Febs J 2006;273:847-62.

6

In vivo induction of glial cell proliferation and axonal outgrowth and myelination by brain-derived neurotrophic factor

Dorien M. de Groot[1], Anton J.M. Coenen[1], Albert Verhofstad[2], François van Herp[1] and Gerard J.M. Martens[1]

[1]Department of Molecular Animal Physiology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Institute for Neuroscience, Faculty of Science, Radboud University, Nijmegen, The Netherlands [2]Department of Pathology, Radboud University Medical Centre, Nijmegen, The Netherlands

Molecular Endocrinology (2006) 20(11): 2987-2998

Abstract

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family of neuronal cell survival and differentiation factors, but is thought to be involved in neuronal cell proliferation and myelination as well. To explore the role of BDNF *in vivo*, we employed the intermediate pituitary melanotrope cells of the amphibian *Xenopus laevis* as a model system. These cells mediate background adaptation of the animal by producing high levels of the prohormone proopiomelanocortin (POMC) when the animal is black adapted. We used stable *Xenopus* transgenesis in combination with the POMC gene promoter to generate transgenic frogs overexpressing BDNF specifically and physiologically inducible in the melanotrope cells. Intriguingly, an ~25-fold overexpression of BDNF resulted in hyperplastic glial cells and myelinated axons infiltrating the pituitary, whereby the transgenic melanotrope cells became located dispersed among the induced tissue. The infiltrating glial cells and axons originated from both peripheral and central nervous system sources. The formation of the phenotype started around tadpole stage 50 and was induced by placing white-adapted transgenics on a black background, i.e. following activation of transgene expression. The severity of the phenotype depended on the level of transgene expression, since the intermediate pituitaries from transgenic animals raised on a white background or from transgenics with only an ~5-fold BDNF overexpression were essentially not affected. In conclusion, we show in a physiological context that, besides its classical role as neuronal cell survival and differentiation factor, *in vivo* BDNF can also induce glial cell proliferation as well as axonal outgrowth and myelination.

Introduction

Similar to its neurotrophin family members neurotrophin-3 and -4/5, brain-derived neurotrophic factor (BDNF) was originally identified as a neuronal survival and differentiation factor. BDNF is now also known as a modulator of neurotransmission and synaptic plasticity in the central and peripheral nervous systems (CNS and PNS, respectively), and for its role in neurite growth (1-11). In addition to these classical functions, evidence is emerging that BDNF has a broader role in nervous system functionality. For example, BDNF likely functions as a regulator of myelination in that it enhances myelin formation by Schwann cells in the peripheral nervous system (12-14) and by oligodendrocytes after spinal cord injury in the central nervous system (15). In addition, the neurotrophin may be involved in the proliferation of neuronal cells, since infusion of BDNF into the lateral ventricle of adult rat led to new neurons in various brain regions (16) and bacterial meningitis in mice caused an increase in BDNF expression resulting in neurogenesis (17). Finally, BDNF may stimulate the proliferation of glial cells as well (15, 18-20).

For a study on the physiological role of BDNF, the melanotrope cells of the intermediate pituitary of the amphibian *Xenopus laevis* constitute an attractive cell model system. BDNF has been found to be expressed in the *Xenopus* melanotrope cells (21). The melanotropes play an essential role in the process of background adaptation of *Xenopus*. On a black background, the melanotrope cells are biosynthetically very active, synthesize large amounts of the prohormone proopiomelanocortin (POMC) and release the POMC-derived peptide α-melanophore stimulating hormone (α-MSH), which causes pigment dispersion in skin melanophores. In contrast, on a white background the cells are biosynthetically inactive and α-MSH release is inhibited. Thus, the biosynthetic and secretory activity of the melanotrope cells can be physiologically manipulated by placing the animals on either a black or a white background. At the cellular level, the activity of the melanotrope cells is regulated by hypothalamic neurons that directly contact the melanotrope cells and release various neurochemical messengers (reviewed in 22, 23).

Previously, effects of BDNF overexpression have been examined in transgenic mice, in which an increase in myelination (14), dendrite complexity in hippocampal dentate gyrus (24), learning and excitability (25), and sensory innervation and neuron number (26) has been observed. In the present study, we combined the unique properties of the *Xenopus* melanotrope cell with the technique of stable *Xenopus* transgenesis (27, 28) to explore the role of BDNF *in vivo*. Transgene expression of BDNF was driven by a *Xenopus* POMC promoter fragment, allowing expression of the transgene to be physiologically inducible and specific to the melanotrope cells (29), and leaving the integrity of the regulatory hypothalamic neurons intact. Analysis of the transgenic animals revealed that *in vivo* BDNF can induce Schwann cell and glial cell proliferation, as well as axonal outgrowth and myelination.

Materials and Methods

Animals

South African claw-toed frogs, *Xenopus laevis*, were reared in green containers (unless stated otherwise) in the Central Animal Facility of the Radboud University of Nijmegen (Nijmegen, The Netherlands) with a light/dark cycle of 12 h. The animals were adapted to their background by keeping them in either white or black buckets for at least three weeks. For long-term black adaptation, line-6 transgenic animals were kept on a black background for >9 months. Experimental procedures were performed under the guidelines of the Dutch law concerning animal welfare.

Antibodies

Pro- and mature BDNF were detected with an anti-BDNF antibody directed against the first 20 amino acids of mature BDNF (Santa Cruz Biotechnology, Inc., California). POMC and

adrenocorticotropic hormone (ACTH) were detected with an anti-POMC/ACTH antibody (generous gift of Dr. E. Roubos, Radboud University, Nijmegen, The Netherlands; 43), vasotocin and mesotocin with an anti-vasotocin and anti-mesotocin antibody, respectively (generous gifts of Dr. F. Vandesande and Dr. L. Arckens, respectively, Catholic University Leuven, Belgium; 44, 45), and prolactin with an anti-prolactin antibody (generous gift of Dr. J. Mattheij, Wageningen University, The Netherlands; 46). Glial fibrillary acidic protein (GFAP) was detected with a polyclonal anti-GFAP antibody (DAKO, Glostrup, Denmark), and tubulin with the monoclonal E7 anti-tubulin antibody (Developmental Studies Hybridoma Bank, Rockland, Gilbertsville).

Generation of DNA constructs encoding BDNF

Two different constructs encoding BDNF (pPCG(A)+-BDNF) and a green fluorescent protein (GFP)-BDNF fusion protein (pPOMC(A)+-SP-GFP-BDNF) were generated. For the construction of pPCG(A)+-BDNF, first the pPCG(A)2+ vector was made, consisting of the pCS2+ backbone (47), a 540 bp *Sal*I-*Hind*III *Xenopus* POMC gene A promoter sequence (29), a 0.65 kb *Sal*I-*Hind*III fragment of the cardiac actin promoter (kindly provided by Paul Krieg, University of Arizona Health Sciences Center, Tucson, AZ, USA), the GFP sequence and the HSV-tk polyA signal obtained by PCR on the pIRES2-EGFP vector (ClonTech, Mountain View, CA, USA). To clone the BDNF sequence, total RNA was isolated from rat brain tissue according to the manufacturer's instructions (Life Technologies, Inc., Carlsbad, CA, USA). The RNA was dissolved in 20 µl RNase-free H_2O and the concentration was measured with a GeneQuant RNA/DNA calculator (Pharmacia, New York, New York, USA). For RT-PCR, one µg total rat brain RNA was reverse transcribed into cDNA according to the manufacturer's instructions (Life Technologies, Inc.). BDNF was amplified by PCR in a reaction mixture containing Pfu buffer (Fermentas, Hanover, MD, USA), 200 µM dNTPs, 0.5 mM 5' primer (5'- GGGGAAGCTTGTTCCACCAGG TGAGAAGAGTGATG -3'), 0.5 mM 3' primer (5'- GGGGGAATTCTGCGCAAATGACTGTTTCTTTC TGGTC -3'), 0.01 U Pfu Turbo DNA polymerase (Fermentas) and 1 µl cDNA (35 amplification cycles: 30 sec at 95°C, 1 min at 56°C and 1.5 min at 72°C). The amplified product was digested with *Hind*III and *Eco*RI, and cloned behind the POMC gene A promoter fragment in the vector pPCG(A)2+, resulting in the pPCG(A)+-BDNF vector. Since the rat and *Xenopus laevis* protein sequences of mature BDNF are highly conserved (amino acid sequence identity is 94%), and in view of the phenotype of the BDNF-transgenic animals, the transgene product was likely recognized by the frog receptors. For the construction of the pPOMC(A)2+-SP-GFP vector, the coding sequence of GFP was amplified via PCR and fused in the correct reading frame downstream of the signal peptide (SP) sequence of *Xenopus* Ac45. The SP-GFP fusion was subcloned with *Bam*HI and *Eco*RI into the pCS2+(A) vector, thereby generating pCS2+(A) SP-GFP. Finally, the CMV promoter was interchanged with the POMC promoter (29) using the *Sal*I and *Hind*III restriction sites. Next, to generate the GFP-BDNF fusion protein, the sequence of BDNF

without the signal peptide was amplified via PCR (35 amplification cycles; 30 sec 95°C, 1 min 50°C and 1 min and 30 sec 72°C), using 10 ng pPCG(A)+-BDNF vector as input DNA, and the primers 5'- GGGGGAATTCGCGCCCACTGAAAGAAGCAAACG -3' and 5'- GGGGTCTAGAGCGCAAATGACTGTTTC -3'. The amplified product was digested with *EcoR*I and *Xba*I, and cloned in frame behind the sequence of GFP in the pPOMC(A)2+-SP-GFP vector. This resulted in the pPOMC(A)+-SP-GFP-BDNF vector. The generated constructs were checked by cycle sequencing using the Big Dye Ready Reaction system (Perkin Elmer, Wellesley, MA, USA).

Generation of Xenopus transgenic for BDNF

To generate transgenic frogs, linear 3577-bp and 2546-bp *Sal*I/*Not*I DNA fragments were generated from the pPCG(A)2+-BDNF and pPOMC(A)2+-SP-GFP-BDNF constructs, respectively. These linear fragments were used for stable *Xenopus* transgenesis (27, 28). A number of injection rounds resulted in animals transgenic for BDNF (line 6) and the fusion protein GFP-BDNF (line 73). To generate F1 and F2 offspring, the testes of male transgenic *Xenopus* frogs were isolated and used for *in vitro* fertilization of eggs harvested from wild-type *Xenopus* females.

Western blot analysis

To analyse protein expression in wild-type and transgenic pituitary neurointermediate lobes (NILs; consisting of the pars nervosa and intermediate pituitary) and pars distalis, tissue homogenates were made in lysis buffer (50 mM HEPES, 140 mM NaCl, 0.1% Triton X-100, 1% Tween 20, 1 mM EDTA, 1 mg/ml deoxycholate, 1 μM phenylmethylsulfonyl fluoride, 0.1 mg/ml soybean tryspin inhibitor). To analyse secreted proteins by NILs, lobes were incubated in Ringer's medium (112 mM NaCl, 15 mM Hepes (pH 7.4), 2 mM KCl, 2 mM $CaCl_2$, 2 mg/ml glucose and 0.3 mg/ml bovine serum albumine) for 3 h after which media were collected and NILs were homogenized. After a 20-min incubation on ice, samples were centrifuged for 20 min (15,300 rpm, 4°C) and sample buffer (50 mM TrisCl (pH6.8), 100 mM dithiothreitol, 2% sodiumdodecylsulphate, 0.1% bromephenol blue, 10% glycerol) was added to the supernatant. Samples were heated for 5 min at 100°C and Western blot analysis was performed as described previously (48). Blots were incubated overnight with primary antiserum and the antibody dilutions used were 1:1000 for anti-BDNF and 1:500 for anti-tubulin. The equivalent of ~40% of one NIL or pars distalis was loaded per lane. Signals were visualized with a BioChemi Imaging System, and relative quantification by densitometry was performed with Labworks 4.0 software (UVP BioImaging systems, Cambridge, UK). Densities of mature BDNF signals in NILs of wild-type, line-6 and line-73 transgenic animals and of white- and black-adapted line-73 animals were measured and related to an ~47-kDa non-specific immunoreactive signal as an internal control or to tubulin expression, respectively. An unpaired t test was used for statistical analysis.

Immunocytochemistry

For cryosectioning, *Xenopus* brains with pituitary glands attached were immersed in 10% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 16 h at 4°C, and subsequently frozen in Tissue-Tek (Sakura, Tokyo, Japan). Sagittal sections (20 μm) were mounted on poly-L-lysine-coated slides and dried at 37°C for 16 h. Finally, direct GFP fluorescence was viewed under a fluorescence microscope. Alternatively, *Xenopus* brains with pituitary glands attached were frozen in liquid N_2 immediately after dissection. Next, sagittal sections (5 μm) were mounted on uncoated slides and dried at room temperature for 12 h. For immunocytochemistry, sections were blocked in 0.2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min and subsequently incubated with primary antiserum (1:100 anti-GFAP) for 1 h and secondary antiserum (1:200 goat anti-rabbit Alexa 488) for 1 h.

For paraffin sectioning, *Xenopus* brains with pituitary glands attached were fixed for 4-12 h in Bouin-Hollande solution, dehydrated and embedded in paraffin. Serial sagittal 5 μm sections were mounted on gelatine-coated glass slides. After deparaffinization and rehydration, sections were blocked with 20% normal goat serum in Tris-buffered saline (TBS) for 10 min, and then incubated with primary antiserum (1:300 anti-BDNF, 1:2000 anti-POMC/ACTH, 1:4000 anti-vasotocin and anti-mesotocin, and 1:500 anti-prolactin) for 16 h, with goat anti-rabbit immunoglobulin G (1:100; Nordic Immunology, Tilburg, The Netherlands) for 1 h, and finally with rabbit peroxidase-antiperoxidase (1:800; Nordic Immunology) for 1 h. To detect BDNF, sections were incubated with anti-BDNF antibody for 24 h, subsequently with biotinylated goat anti-rabbit immunoglobulin (1:200; Vectastain, Vector Laboratories, Burlingame, CA, USA) for 2 h, and finally with ABC reagent (1:100; Vectastain) for 30 min. Finally, after antibody incubations and washing in TBS without saline, sections were treated with 0.025% 3,3'-diaminobenzidine tetrahydrochloride (Sigma), 0.25% nickel ammonium sulphate and 0.01% hydrogen peroxide in 0.05M Tris-HCl, pH 7.6 to reveal peroxidase activity. For myelin staining, paraffin-embedded sections were incubated in Luxol fast blue (LFB) solution (0.1% LFB MBS (Chroma, Muenster, Germany) 0.05% acetic acid in 96% ethanol) for 16 h, followed by a differentiation of 1 min in 0.1% lithium carbonate and 3-5 min in 70% ethanol. Finally, the sections were stained with hematoxylin and eosin (HE).

Microscopy analysis

For microscopy analysis of *Xenopus* wild-type and transgenic pituitaries, paraffin sections of the tissues were examined in a Leitz Dialux 22 microscope (Leica Microsystems, Wetzlar, Germany) and photographs were taken with a Nikon Coolpix 900 camera (Nikon, Tokyo, Japan). Cryosections were directly viewed under a Leica DM RA fluorescent microscope and photographs were taken with a Cohu High Performance CCD Camera (Cohu, Inc., San Diego, USA) using the Leica Q Fluoro software. Dissection of *Xenopus* brain and pituitary was performed under a Leica MZ FLIII fluorescent stereomicroscope

and photographs were taken with a Leica DC200 color camera using the Leica DCviewer software. For a more detailed microscopy analysis of *Xenopus* wild-type and transgenic pituitaries, freshly dissected NILs of long-term black-adapted line-6 animals, and 3-weeks black-adapted wild-type and line-73 *Xenopus* were used. Whole lobes were fixed by high-pressure freezing (Leitz) and substituted by automatic freeze substitution using acetone and 2% osmium tetroxide. After this, tissues were rinsed with acetone, incubated in epon:acetate, and embedded in Epon 812. One-micron thick sections were cut and stained with toluidine blue. Subsequently, the sections were examined under a Leica DM6000B microscope, and pictures were taken using a Leica DFC480 camera and Leica IM500 Image manager software.

Quantification of GFAP+ cells

To estimate the number of GFAP+ cells in GFAP-stained cryosections of the pituitaries of wild-type and line-73 transgenic animals, the number of GFAP+ cells was counted in 6 (wild-type) and 9 (line 73) unit areas (~80 x 80 μm) in two different sections of the intermediate pituitary and nodule of wild-type and line-73 animals, respectively. Since in line-73 animals the GFAP+ cells were not evenly distributed throughout the nodule, unit areas mainly consisting of glial cells were chosen. Cell counts were summed for each animal and the mean for line 73 animals was compared to that of wild-type animals. An unpaired t test was used for statistical analysis.

Real-time quantitative RT-PCR analysis

To examine TrkB and $p75^{NTR}$ neurotrophin receptor mRNA expression levels in the NIL and cranial nerves III, V and VII, real-time quantitative RT-PCR analysis was performed as described previously (48), using the iTaq SYBR Green Supermix with ROX kit (BioRad, CA, USA). The following primer sets were used to amplify TrkB, $p75^{NTR}$ and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), respectively: 5'- ACCTCTACCGCGA GCAAGAC- 3' and 5'- GAGTAACTCTGCTTCCCGATGAA- 3' (forward and reverse primer for TrkB, respectively), 5'- GGGAAAGTCTGAGCTTGCTG- 3'and 5'- CACTATCTGTGAGGACGGTG- 3' (forward and reverse primer for $p75^{NTR}$, respectively) and 5'-GCCGTGTATGTGGTGG AATCT-3' and 5'-AAGTTGTCGTTGATGACCTTTGC-3' (forward and reverse primer for GAPDH, respectively).

Results

Generation of transgenic *Xenopus* with overexpression of BDNF specifically in the intermediate pituitary melanotrope cells

To generate frogs transgenic for BDNF, we made and used two different DNA constructs, one construct driving transgene expression of BDNF to the melanotrope cells as well as

green fluorescent protein (GFP) to muscle cells for identification of the transgenics (resulting in transgenic line 6; Fig. 1A), and a second construct encoding BDNF fused to the C-terminus of GFP (GFP-BDNF) and driving expression to the melanotrope cells (transgenic line 73; Fig. 1B). Melanotrope cell-specific transgene expression was accomplished by using constructs in which the BDNF- and GFP-BDNF-encoding sequences were cloned behind a POMC gene promoter fragment. The transgenic tadpoles were identified via direct screening for GFP fluorescence in muscle (in tadpoles from line 6; Fig. 1A) or in the intermediate pituitary (in line 73 tadpoles; Fig. 1B).

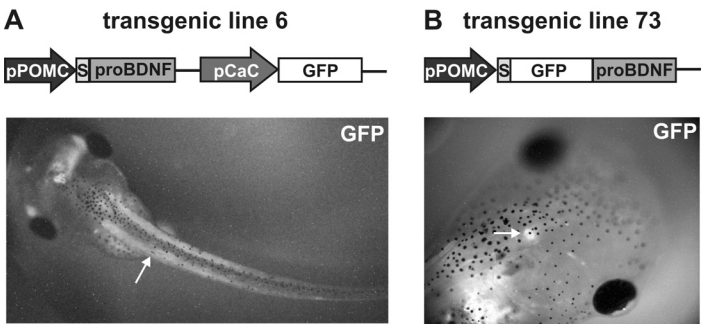


Figure 1: Generation of transgenic *Xenopus laevis* with BDNF overexpression specifically in intermediate pituitary melanotrope cells.
Schematic representation of the two different constructs that were used to generate transgenic lines 6 (A) and 73 (B). Transgenic tadpoles were identified via screening for GFP fluorescence in muscle (line 6; A, white arrow) or in the intermediate pituitary (line 73; B, white arrow). Abbreviations used are: pPOMC, proopiomelanocortin gene promoter; proBDNF, pro-brain-derived neurotrophic factor; pCaC, cardiac actin gene promoter; S, signal peptide; GFP, enhanced green fluorescent protein.

To examine the amount of steady-state BDNF protein in the two transgenic lines 6 and 73, lysates of the pituitary NIL and pars distalis of adult wild-type and transgenic frogs were used for Western blot analysis with an anti-BDNF antibody. NILs from transgenic lines 6 and 73 had ~ 5- and ~25-fold higher levels of mature BDNF expression compared to wild-type NILs, respectively (Fig. 2). BDNF transgene expression in lines 6 and 73 was melanotrope cell specific since the transgene was not expressed in the pars distalis (Fig. 2). Analysis of the incubation media showed the secretion of the mature transgene BDNF-product by line-73 NILs. We did not detect secreted BDNF from wild-type or line-6 transgenic NILs (Fig. 2, right panel). Since secreted BDNF is known to have a relatively short half-life (30, 31), the absence of BDNF in the media is probably caused by protein degradation. We thus generated transgenic lines 6 and 73 with moderate and relatively high overexpression of mature BDNF specifically in the intermediate pituitary melanotrope cells, respectively.

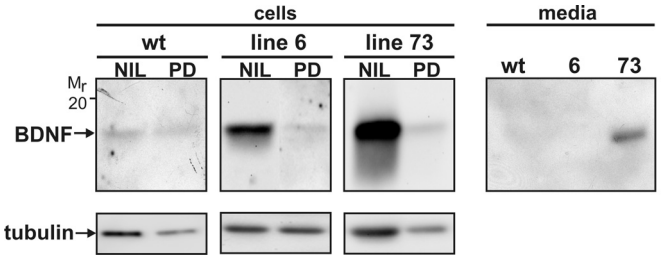


Figure 2: BDNF protein expression in the pituitary neurointermediate lobe of wild-type and BDNF-transgenic *Xenopus*.
Western blot analysis of neurointermediate lobe (NIL) and pars distalis (PD) cell lysates and media of wild-type (wt) and lines-6 and -73 transgenic animals using an anti-BDNF antibody. Tubulin expression served as a loading control.

Effect of BDNF transgene expression in the *Xenopus* melanotrope cells on the anatomy and histology of the pituitary

To examine the effect of BDNF overexpression in the transgenic melanotrope cells on the histology of the pituitary, cryo- and paraffin sections were made of wild-type and lines-6 and -73 pituitaries, and immunocytochemistry screening of the sections was performed using a number of antibodies. Staining with an anti-POMC/ACTH antibody to identify the melanotrope cells of the intermediate pituitary and the corticotropic cells of the pars distalis showed that the localization of these cells in transgenic line 6 animals was comparable to that in wild-type animals (Figs. 3A and B). Remarkably, in line 73 animals we observed a nodule that was ~10-15 times larger than the intermediate pituitary of wild-type animals, and in which the transgenic melanotrope cells were intermingled in tissue that is normally not found in the intermediate pituitary (Fig. 3C). Thus, the intermediate pituitary of line 73 transgenic animals was not intact anymore. Distributed melanotrope cells were also observed in sections stained with an anti-BDNF antibody (Fig. 3F) and via direct GFP fluorescence in cryosections of transgenic line 73 pituitaries (Fig. 3G). The melanotrope cells of lines 6 and 73 transgenic animals showed higher expression levels of BDNF than wild-type cells (Figs. 3D to F), in line with the western blot results (Fig. 2A).

We then wondered what the effect of melanotrope BDNF overexpression would be on the histology of the pituitary pars nervosa and pars distalis. Immunocytochemistry screening using an anti-vasotocin antibody (specific for vasotocinergic neurons of the pars nervosa) and an anti-prolactin antibody (specific for lactotropic cells of the pars distalis) showed that in transgenic lines 6 and 73 the two pituitary lobes were similar to those of wild-type animals (Figs. 3H-M). However, occasionally a number of vasotocinergic-positive fibers were found among the scattered melanotrope cells in the nodules of transgenic line 73 animals (Fig. 3J).

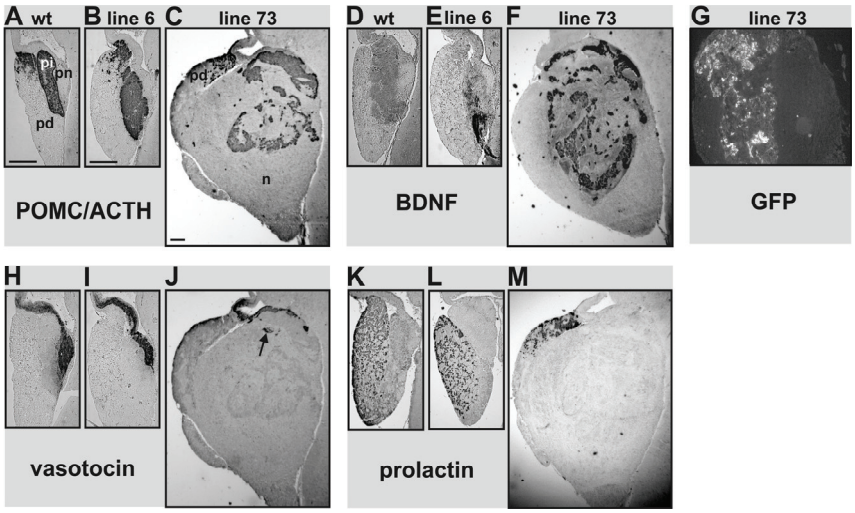


Figure 3: Pituitary histology of wild-type animals and transgenic *Xenopus* overexpressing BDNF in the intermediate pituitary melanotrope cells.
Serial sagittal paraffin sections were made of the pituitary of wild-type (wt) and lines-6 and -73 transgenic animals, and the sections were stained for proopiomelanocortin/ adrenocorticotropic hormone (POMC/ACTH; A, B, C), BDNF (D, E, F), vasotocin (H, I, J) and prolactin (K, L, M). (G) Cryosection of the pituitary of a line-73 transgenic animal showing direct green fluorescent protein (GFP) fluorescence. The arrow in J indicates an example of vasotocin-positive fibers localized in the nodule of a line-73 transgenic pituitary. Abbreviations used are: pn, pars nervosa; pi, intermediate pituitary; pd, pars distalis and n, nodule. Scale bars: 200 μm (A and B) and 250 μm (C).

In order to characterize the additional tissue that was observed in the nodules of transgenic line 73 pituitaries, paraffin sections were stained with HE and LFB solution (staining for myelin). We found that in the nodules the melanotrope cells were surrounded by hyperplastic Schwann cells and axons of PNS origin, and hyperplastic glial cells and axons of CNS origin. Furthermore, a significant part of the various axons was myelinated (Fig. 4A). Additional evidence for the presence of Schwann/glial cells was provided by immunocytochemistry with anti-GFAP antibody, showing a clear and specific glial staining in the nodules of transgenic line 73 pituitaries (Fig. 4B).

Line-6 animals have a relatively low level of transgene BDNF expression in the melanotrope cells. Initially, we did not detect a pituitary phenotype in these animals, and we therefore wondered whether a relatively long time of adaptation of these animals to a black background (and thus a relatively long time period during which cells had been exposed to the transgene product) may cause a pituitary phenotype. For this purpose, line-6 animals were adapted to a black background for >9 months, a relatively long adaptation period since animals are normally adapted for three weeks. Pituitaries of long-term black-adapted line-6 animals and 3-weeks black-adapted wild-type and line-73 animals were then

prepared for microscopy analysis. Interestingly, in sections of long-term black-adapted lin-6 animals we detected the same phenotypic characteristics (namely Schwann and glial cells, and myelinated axons) as were observed in the nodules of line-73 animals (Fig. 5). Thus, in line-6 transgenic animals a prolonged exposure to the transgene BDNF product induced a phenotype similar to that observed in line-73 animals. Furthermore, the fact that two independent BDNF transgenic animals (lines 6 and 73) display a similar phenotype excludes the possibility that the phenotype would be due to a position effect (i.e. due to the integration of the transgene).

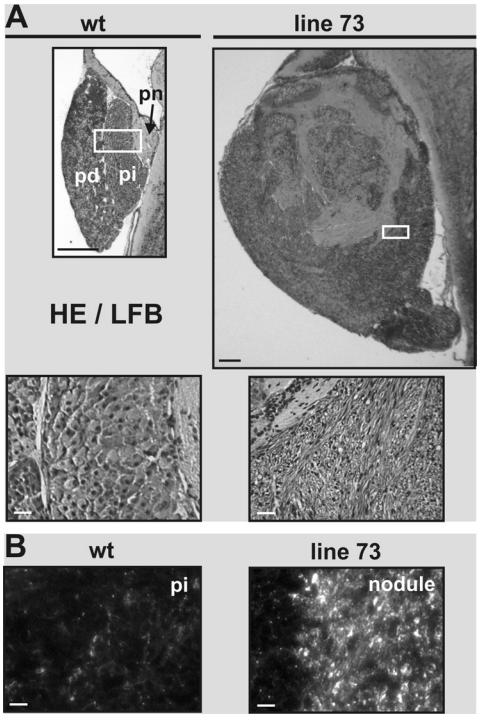


Figure 4: Characterization of tissues in the nodules of BDNF-transgenic *Xenopus* line 73. (A) Hematoxylin/eosin (HE) and luxol fast blue (LFB) staining for myelin (blue) of sagittal paraffin sections of the pituitaries of wild-type (wt) and line-73 transgenic animals. The upper panels represent overviews of the whole pituitary, consisting of the pars nervosa (pn), intermediate pituitary (pi) and pars distalis (pd). The boxed areas are enlarged in the lower panels. Scale bars: 200 and 250 μm (upper panels, wt and line 73, respectively) and 25 μm (lower panels). (B) Glial fibrillary acidic protein (GFAP) staining of cryosections of the pituitaries of wt and line-73 transgenic animals. Shown are details of a wt intermediate pituitary (left panel) and of a line-73 nodule (right panel). Scale bar: 25 μm.

Together, these results show that relatively high transgene BDNF expression in the line-73 pituitaries resulted in the formation of nodules that were 10-15 times larger than wild-type intermediate pituitaries. Pituitaries from line-6 transgenic animals that had been black adapted for a relatively short time period were morphologically comparable to wild-type pituitaries, whereas long-term black adaptation of these animals resulted in characteristics of the nodule phenotype. In the nodules of line-73 animals, the melanotrope cells were intermingled among proliferated Schwann cells and axons from the PNS and glial cells and axons of CNS origin, a significant part of which was myelinated.

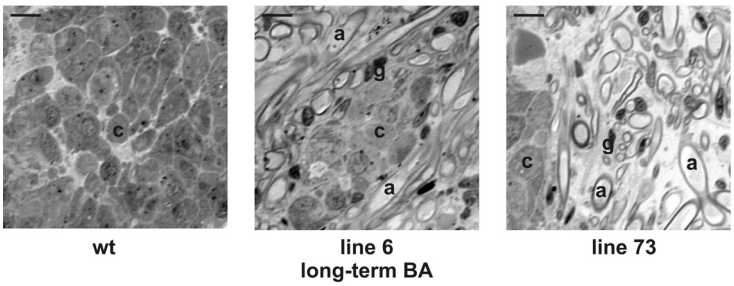


Figure 5: **Light-microscopical pictures of one-µm epon sections of the pituitaries of relatively long-term black-adapted (BA) line-6 transgenic *Xenopus* and 3-weeks BA wild-type and line-73 transgenic animals.** Shown are details of the intermediate pituitaries of wild-type (wt) *Xenopus* and lines-6 and -73 transgenic *Xenopus*. c: melanotrope cell, a: myelinated axon and g: Schwann/glial cell. Scale bar: 10 µm.

Origin of the nodule in transgenic *Xenopus* line 73

We then wanted to determine the origin of the induced tissues in the nodules of line-73 transgenic animals. The amphibian pituitary is surrounded by a number of brain areas and cranial nerves that potentially could form the source of the infiltrating tissues (Fig. 6A). First, to examine the source of the observed hyperplastic Schwann cells and axons, we carefully dissected the brain and pituitary of wild-type, line-6 and line-73 animals under a stereomicroscope (Figs. 6B to E). In line-6 transgenic animals that were adapted to a black background for three weeks (a relatively short period of adaptation), the morphology of the NILs was comparable to that of wild-type NILs (Figs. 6B and C). In contrast, the nodule-containing pituitaries of line-73 transgenic animals were anatomically connected to two cranial nerves, i.e. cranial nerves V and VII (the trigeminal and facial nerves, respectively; Figs. 6A and E). In addition, in line-6 transgenic animals that were adapted to a black background for 9 months (a relatively long adaptation period) the NILs were also connected to these cranial nerves (Fig. 6D). Thus, since the nodules in line-73 transgenic pituitaries were anatomically connected to the facial and trigeminal nerves, the infiltrating PNS-derived Schwann cells and myelinated axons that were present in the nodules probably originated from these cranial nerves.

We next examined in more detail from which CNS region the hyperplastic glial cells and axons in the line-73 nodules originated. Staining of paraffin sections of line-73 pituitaries with an anti-mesotocin or anti-vasotocin antibody revealed in the nodule the presence of mesotocinergic and vasotocinergic fibers from the pituitary pars nervosa (Figs. 6F and G). These results indicated that the glial cells and axons with CNS characteristics found in the nodules of line-73 pituitaries most likely originated from the pituitary pars nervosa.

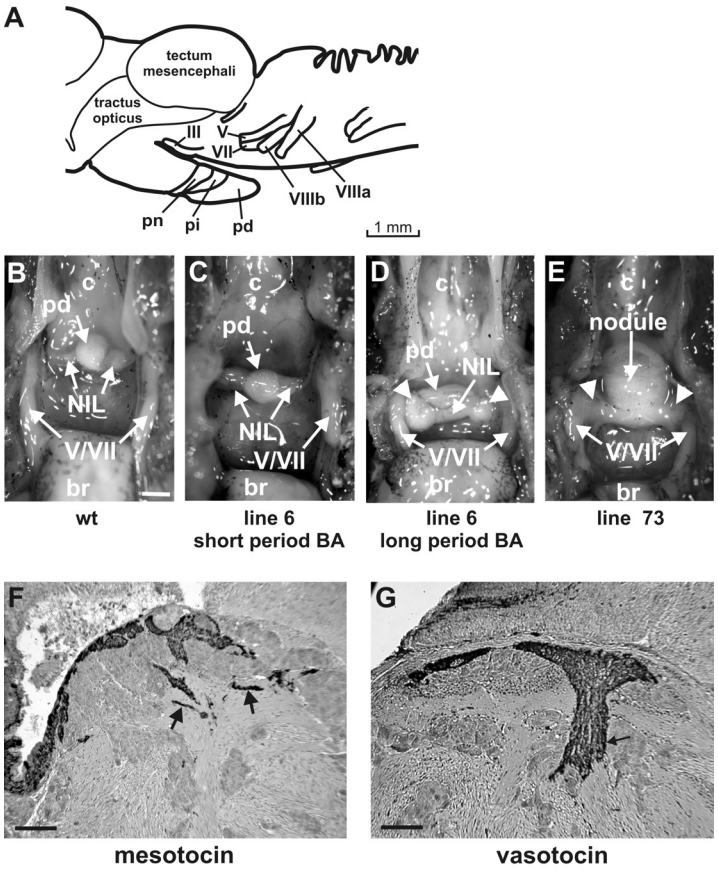


Figure 6: Origins of the hyperplastic Schwann cells, glial cells and axons in the nodules of BDNF-transgenic *Xenopus* line 73.
(A) Schematic lateral view of a frog brain. Figure was derived from Nieuwenhuys *et al*. (49). Indicated are: VIIIa, nervus vestibulo-cochlearis, posterior root; VIIIb, nervus vestibulo-cochlearis, anterior root; VII, nervus facialis; V, nervus trigeminus; III, nervus oculomotorius; pn, pars nervosa; pi, intermediate pituitary and pd, pars distalis. (B, C, D, E) Dorsal view of the brain cavity (c) after removal of the brain (br) of a wild-type (wt) animal (B), line-6 transgenic animals (short- and long-term black-adapted (BA); C and D, respectively) and line-73 transgenic animal (E). The pituitary pars distalis (pd) and neurointermediate lobe (NIL) are visible lying on the floor of the brain cavity. The numbers V/VII indicate the trigeminal and facial nerves, and the white arrowheads indicate connections between these nerves and the NIL (line 6) or nodule (line 73). Scale bar used is 1 mm. (F, G) Sagittal paraffin sections of a line-73 pituitary stained with anti-mesotocin (F) or anti-vasotocin (G) antibody. Examples of mesotocinergic- or vasotocineric-positive fibers in the intermediate pituitary are indicated with arrows. Scale bar: 200 μm.

Altogether, our study concerning the origin of the PNS-derived hyperplastic Schwann cells and axons, and CNS-derived glial cells and axons in the nodules of line-73 transgenic animals indicated that the PNS- and CNS-derived infiltrating tissues originated from the cranial nerves V and VII, and the pituitary pars nervosa, respectively.

Development of the nodule in transgenic *Xenopus* line 73

We were also interested at what time during development of the line-73 transgenic animals the pituitary nodule appeared. For this reason, at a number of developmental stages black-adapted wild-type and line-73 tadpoles were fixed for paraffin sectioning and staining (HE

and myelin), and immunocytochemistry for POMC and BDNF. Up to stage 49 (~12 days post-fertilization), no differences were found between the developing pituitaries of wild-type and transgenic tadpoles (Fig. 7A and data not shown). In contrast, in stage 50-51 tadpoles (~15-17 days post-fertilization) we found that the intermediate pituitary of transgenic line 73 was slightly disrupted by infiltrating tissue (Fig. 7B and data not shown). This phenotype progressively further developed (Fig. 7C and data not shown) and following metamorphosis (stage 66; ~50 days post-fertilization), clearly enlarged nodules were present in the pituitaries of line-73 frogs, with the melanotrope cells intermingled throughout glial cells (Fig. 7D and data not shown). Thus, the phenotype in line-73 transgenic animals was observed for the first time in stage 50-51 tadpoles and became gradually more pronounced during further development of the transgenic animal.

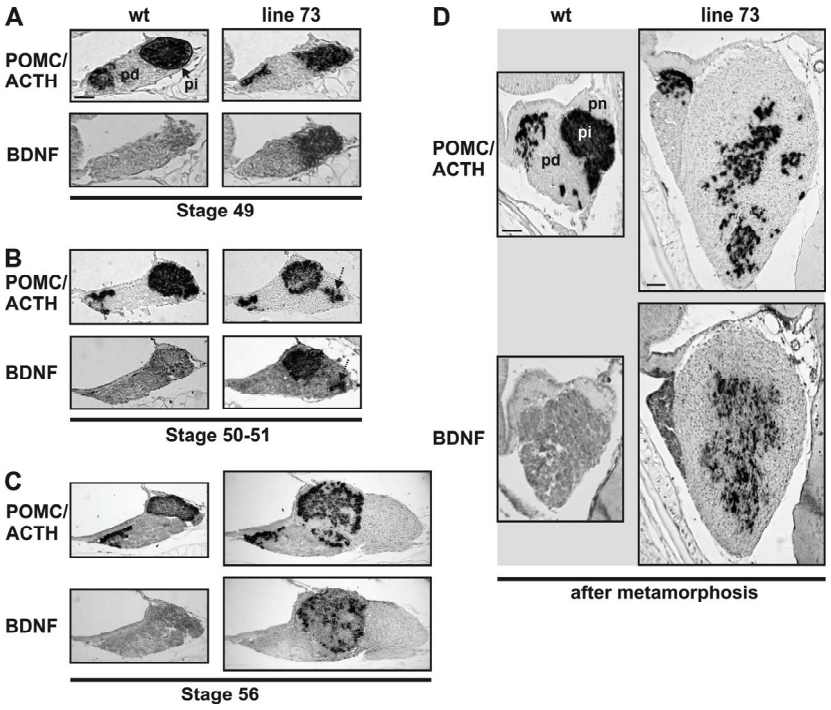


Figure 7: Development of the nodule phenotype in BDNF-transgenic *Xenopus* line 73.
At various developmental stages (stage 49 (A), stage 50-51 (B), stage 56 (C) and immediately after metamorphosis (D)) wild-type (wt) and line-73 transgenic tadpoles were fixed for serial paraffin sectioning and immunocytochemistry for proopiomelanocortin/ adrenocorticotropic hormone (POMC/ACTH), and BDNF. The locations of the intermediate pituitary (pi), pars distalis (pd) and pars nervosa (pn) are indicated. The dotted arrows in B indicate displaced intermediate pituitary melanotrope cells in line-73 transgenic animals. Scale bars: 50 μm (A, B and C) and 200 μm (D).

Physiological induction of the nodule in transgenic *Xenopus* line 73

Next, we wondered whether increasing the level of BDNF transgene expression in the melanotrope cells of tadpoles and frogs with a low level of transgene expression would induce the formation of the nodule. For this purpose, we took advantage of our model system by first growing line-73 transgenic tadpoles on a white background, giving lower expression of the BDNF transgene. After metamorphosis, paraffin sections were made of the brain and pituitary, and immunocytochemistry for POMC and BDNF, as well as HE and myelin stainings were performed. We found that pituitaries of white-adapted transgenic frogs did not contain nodules and were similar to wild-type pituitaries from animals adapted to a white background (Fig. 8). We then performed a longitudinal study by adapting fully white-adapted transgenic line-73 frogs to a black background for various time periods, thus increasing transgene BDNF expression in a physiological way. Western blot analysis indeed showed that transgene BDNF expression was ~8-fold higher in black-compared with white-adapted line-73 animals (data not shown). Following 9 weeks of black-background adaptation, infiltrating glial cells were observed that displaced the intermediate pituitary melanotrope cells of the transgenic animals. After 15 weeks of adaptation, a nodule was formed with the melanotrope cells intermingled throughout the glial cells (Fig. 9). When the 15-weeks-black-adapted line-73 animals were placed back on a white background again, the induced nodule remained (data not shown). Thus, the nodule phenotype of the transgenic *Xenopus* line-73 pituitary was irreversibly induced by enhancing BDNF transgene expression.

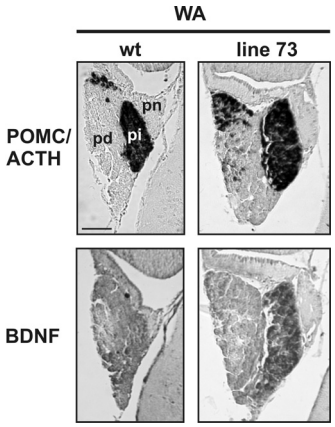


Figure 8: Pituitary histology of white-adapted wild-type *Xenopus* and transgenic *Xenopus* line 73.
Serial paraffin sections were made of the brain and pituitary (consisting of the pars nervosa (pn), intermediate pituitary (pi) and pars distalis (pd)) of wild-type (wt) and line-73 transgenic frogs that were raised on a white background (WA). Stainings were performed for proopiomelanocortin/ adrenocorticotropic hormone (POMC/ACTH), and BDNF. Scale bar: 200 μm.

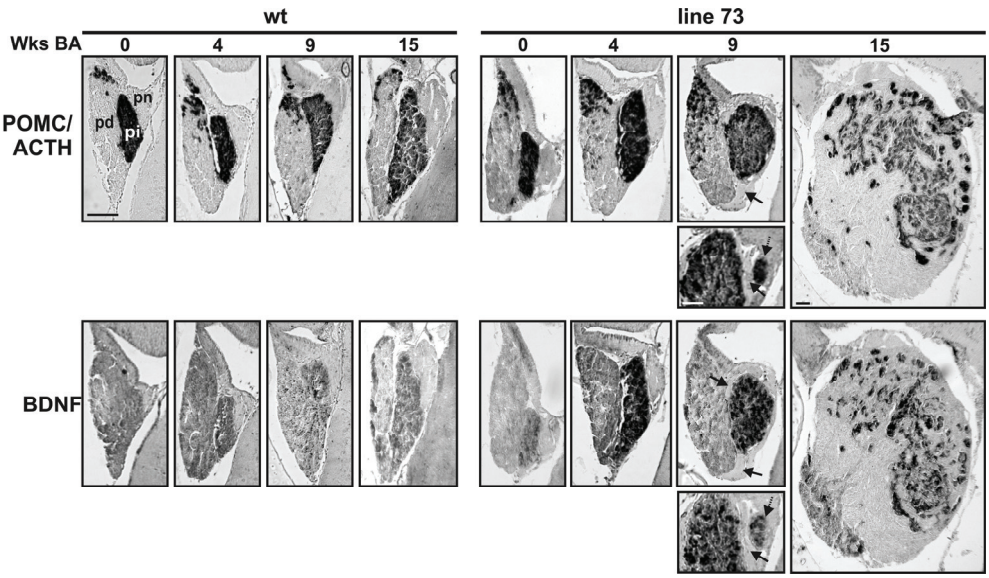


Figure 9: ***In vivo*** **induction of nodule formation in transgenic** ***Xenopus*** **line 73 pituitaries by the physiological activation of BDNF transgene expression.**
Wild-type (wt) and line-73 transgenic animals grown on a white background were adapted to a black background for four, nine or fifteen weeks (wks BA). After fixation and serial paraffin sectioning of the pituitary (consisting of the pars nervosa (pn), intermediate pituitary (pi) and pars distalis (pd)), immunocytochemistry was performed for proopiomelanocortin/ adrenocorticotropic hormone (POMC/ACTH), and BDNF. Of 9 wks BA line-73 animals, two sections are shown for the POMC/ACTH as well as the BDNF staining, with the lower panel showing a higher magnification of part of the pi and pn. Black and dotted arrows indicate infiltrating tissue and displaced melanotrope cells, respectively. Scale bar: 200 μm.

Discussion

BDNF is a well-known neurotrophic growth factor that plays an important role in neuronal cell survival, differentiation, growth and synaptic plasticity (*e.g.* reviewed in 1-5, 11). However, BDNF appears to have more physiological functions than previously thought, *e.g.* it has been implicated also in the formation of myelin in the central and peripheral nervous systems (12-15, 32, 33), and may play a role in the proliferation of neuronal cells (16, 17). We have examined functional aspects of BDNF using the *Xenopus* intermediate pituitary melanotrope cells as a model system. The melanotrope cells regulate the process of background adaptation of the frog via the production and release of the pigment-dispersing hormone α-MSH by the biosynthetically highly active cells when the animals are on a black background, whereas on a white background the cells are inhibited and biosynthetically inactive (reviewed in 22, 23).

In this study, we generated via stable *Xenopus* transgenesis frogs with overexpression of BDNF specifically in the intermediate pituitary melanotrope cells. Two independent transgenic lines were created, one with a relatively low (~5-fold; transgenic line 6) and a second with a high (~25-fold; transgenic line 73) level of transgene expression of mature BDNF. Intriguingly, in transgenic line-73 animals with high levels of BDNF transgene expression we found infiltrating hyperplastic Schwann cells, glial cells and axons that intermingled with the melanotrope cells of the intermediate pituitary, resulting in a nodule with a size 10-15 times larger than a wild-type intermediate pituitary. This phenotype was a specific effect of transgene expression of mature BDNF and not of other transgene products (*e.g.* GFP or the prodomain of BDNF), since we detected characteristics of the nodule phenotype in line-6 transgenic animals expressing untagged proBDNF, and generated other transgenic lines expressing GFP alone or non-cleavable GFP-proBDNF that did not show the pituitary phenotype (data not shown). In wild-type *Xenopus* intermediate pituitaries, only a limited number of GFAP+ folliculo-stellate cells are found (34, 35). To estimate the increase in Schwann and glial cells in the line-73 nodules, GFAP+ cells per unit area were counted, revealing an ~18-fold increase in GFAP+ cells in line-73 nodules compared to wild-type intermediate pituitaries (data not shown). A proliferative effect of BDNF on glial cells has thus far been described only in a limited number of studies, *e.g.* BDNF increased the cell number of two microglial cell lines (19), [^{3}H]-thymidine incorporation in microglia cells (20), the cell number of optic nerve head astrocytes (18), and oligodendrocyte proliferation in the contused adult rat spinal cord (15). We show now that *in vivo* BDNF stimulates the proliferation of both glial cells and Schwann cells, providing evidence that the neurotrophin may regulate glial cell functioning in the nervous system. Furthermore, the presence of many axons in the nodules of line 73 transgenic animals suggests that BDNF also stimulates axonal outgrowth.

Upon closer examination of the origin of the infiltrating tissues in the nodules of line-73 pituitaries, we observed both CNS-derived glial cells and axons, and PNS-derived Schwann cells and axons. The mesotocin- and vasotocin-stained paraffin sections of the nodules and the HE-stained sections of the early stages of nodule formation in line-73 transgenic animals showed that the glial cells and axons with CNS characteristics most likely originated from the pituitary pars nervosa. The main glial cells that are present in the wild-type pituitary pars nervosa form a specialized group of glial cells, called pituicytes (36-40), and it is therefore possible that in our line-73 transgenic animals BDNF stimulated the proliferation of the pituicytes. Since the nodules were anatomically tightly connected to the cranial nerves V and VII (the trigeminal and facial nerves, respectively), the PNS-derived Schwann cells and axons most likely originated from these cranial nerves. The question then arose why only the cranial nerves V and VII and not other also nearby situated cranial nerves, such as cranial nerve III, were affected by the overexpression of BDNF in the transgenic melanotrope cells. We therefore considered the possibility that the BNDF receptors TrkB or p75^{NTR} may be differentially expressed in various cranial nerves.

Quantitative RT-PCR analysis of TrkB and $p75^{NTR}$ mRNA expression showed no significant differences in the mRNA levels of these receptors between cranial nerves III, V and VII of wild-type animals (data not shown), indicating that the TrkB and $p75^{NTR}$ receptors are probably not responsible for the effect of the BDNF transgene product specifically on cranial nerves V and VII. A more likely explanation for the specific induction of the observed phenotype may well be the fact that cranial nerves V and VII are localized in close proximity of the pituitary, making them prime targets for the BDNF protein that was overexpressed in the intermediate pituitary melanotrope cells.

A large portion of the infiltrated axons of the line 73 nodules was myelinated, indicating that overexpression of BDNF may stimulate myelin formation. Neuronal BDNF has previously been shown to stimulate the expression of myelin protein by Schwann cells (41), presumably via its interaction with $p75^{NTR}$ (12-15, 32, 33). In the induced nodule of line-73 animals, the PNS-derived Schwann cells and axons appeared more sensitive to the myelinating effect of BDNF than the CNS-derived glial cells and axons from the pars nervosa, possibly because PNS tissue was found to contain higher $p75^{NTR}$ mRNA expression levels than CNS tissue (data not shown).

Our longitudinal study on the development of the nodule phenotype in line-73 transgenic animals revealed in tadpoles of stage 50/51 the first cells infiltrating the intermediate pituitary. In *Xenopus laevis*, endogenous MSH-containing intermediate pituitary cells are found in tadpoles from stage 35/36 onwards (42), and melanotrope cell-specific GFP transgene expression driven by the POMC transgene promoter has been detected from stage 40 onwards (29). Thus, in line-73 embryos the transgene expression of BDNF from stage 40 onwards apparently resulted in a detectable phenotype in transgenic embryos of stage 50/51, followed by a gradual formation of the nodule during further development of the transgenic animals.

A number of observations suggest that the severity of the nodule phenotype of the transgenic frogs was dependent on the level of BDNF transgene expression. First, initially transgenic line-6 animals with an ~5-fold overexpression of BDNF did not show nodule formation, but the phenotype was induced following a long adaptation of the animal to a black background, *i.e.* following extensive exposure of the tissue surrounding the intermediate pituitary cells to the transgene product. Second, the intermediate pituitary of line-73 transgenic frogs that were continuously grown on a white background did not show abnormalities, whereas black-background-adapted line-73 frogs had a clearly affected intermediate pituitary. Third, when white-adapted, non-affected line-73 frogs were adapted to a black background for several weeks, hyperplastic glial cells infiltrating the intermediate pituitary were observed, eventually leading to the nodule. The formation of the nodule was irreversible, since the phenotype of black animals remained when the animals were placed on a white background.

Altogether, the results of our transgenic studies in a physiological context show that, besides its classical role as neuronal survival and differentiation factor, *in vivo* BDNF can induce axonal outgrowth and myelination, and stimulate glial cell proliferation.

Acknowledgements

We gratefully thank Drs. P. Wesseling and P. Jap for their helpful advice concerning neuroanatomical and morphological aspects, T. Hafmans for help with the cryosectioning and electron microscopy analysis, and Dr. P. Jap for critical reading of the manuscript. Furthermore, we would like to thank Drs. F. Vandesande, L. Arckens, J. Mattheij and E. Roubos for providing antibodies, and R. Engels for animal care.

References

1. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 2000;10:381-91.
2. Poo MM. Neurotrophins as synaptic modulators. Nat Rev Neurosci 2001;2:24-32.
3. Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 2001;11:272-80.
4. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;4:299-309.
5. Binder DK, Scharfman HE. Brain-derived neurotrophic factor. Growth Factors 2004;22:123-31.
6. Lu P, Jones LL, Tuszynski MH. BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. Exp Neurol 2005;191:344-60.
7. Kim JH, Sung DK, Park CW, et al. Brain-derived neurotrophic factor promotes neurite growth and survival of antennal lobe neurons in brain from the silk moth, Bombyx mori in vitro. Zoolog Sci 2005;22:333-42.
8. Salie R, Steeves JD. IGF-1 and BDNF promote chick bulbospinal neurite outgrowth in vitro. Int J Dev Neurosci 2005;23:587-98.
9. Cronin AS, Horan TL, Spergel DJ, Brooks AN, Hastings MH, Ebling FJ. Neurotrophic effects of BDNF on embryonic gonadotropin-releasing hormone (GnRH) neurons. Eur J Neurosci 2004;20:338-44.
10. McAllister AK. Cellular and molecular mechanisms of dendrite growth. Cereb Cortex 2000;10:963-73.
11. Davies AM. Neurotrophins: neurotrophic modulation of neurite growth. Curr Biol 2000;10:R198-200.
12. Chan JR, Cosgaya JM, Wu YJ, Shooter EM. Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci U S A 2001;98:14661-8.
13. Cosgaya JM, Chan JR, Shooter EM. The neurotrophin receptor p75NTR as a positive modulator of myelination. Science 2002;298:1245-8.
14. Tolwani RJ, Cosgaya JM, Varma S, Jacob R, Kuo LE, Shooter EM. BDNF overexpression produces a long-term increase in myelin formation in the peripheral nervous system. J Neurosci Res 2004;77:662-9.
15. McTigue DM, Horner PJ, Stokes BT, Gage FH. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci 1998;18:5354-65.
16. Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 2001;21:6706-17.
17. Tauber SC, Stadelmann C, Spreer A, Bruck W, Nau R, Gerber J. Increased expression of BDNF and proliferation of dentate granule cells after bacterial meningitis. J Neuropathol Exp Neurol 2005;64:806-15.
18. Lambert WS, Clark AF, Wordinger RJ. Effect of exogenous neurotrophins on Trk receptor phosphorylation, cell proliferation, and neurotrophin secretion by cells isolated from the human lamina cribrosa. Mol Vis 2004;10:289-96.
19. Zhang J, Geula C, Lu C, Koziel H, Hatcher LM, Roisen FJ. Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. Exp Neurol 2003;183:469-81.
20. Elkabes S, DiCicco-Bloom EM, Black IB. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 1996;16:2508-21.
21. Kramer BM, Cruijsen PM, Ouwens DT, et al. Evidence that brain-derived neurotrophic factor acts as an autocrine factor on pituitary melanotrope cells of Xenopus laevis. Endocrinology 2002;143:1337-45.
22. Kolk SM, Kramer BM, Cornelisse LN, Scheenen WJ, Jenks BG, Roubos EW. Multiple control and dynamic response of the Xenopus melanotrope cell. Comp Biochem Physiol B Biochem Mol Biol 2002;132:257-68.
23. Roubos EW, Scheenen WJ, Jenks BG. Neuronal, neurohormonal, and autocrine control of Xenopus melanotrope cell activity. Ann N Y Acad Sci 2005;1040:172-83.
24. Tolwani RJ, Buckmaster PS, Varma S, et al. BDNF overexpression increases dendrite complexity in hippocampal dentate gyrus. Neuroscience 2002;114:795-805.
25. Croll SD, Suri C, Compton DL, et al. Brain-derived neurotrophic factor transgenic mice exhibit passive avoidance deficits, increased seizure severity and in vitro hyperexcitability in the hippocampus and entorhinal cortex. Neuroscience 1999;93:1491-506.
26. LeMaster AM, Krimm RF, Davis BM, et al. Overexpression of brain-derived neurotrophic factor enhances sensory innervation and selectively increases neuron number. J Neurosci 1999;19:5919-31.

27. Kroll KL, Amaya E. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 1996;122:3173-83.
28. Sparrow DB, Latinkic B, Mohun TJ. A simplified method of generating transgenic Xenopus. Nucleic Acids Res 2000;28:E12.
29. Jansen EJ, Holling TM, van Herp F, Martens GJ. Transgene-driven protein expression specific to the intermediate pituitary melanotrope cells of Xenopus laevis. FEBS Lett 2002;516:201-7.
30. Poduslo JF, Curran GL. Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. Brain Res Mol Brain Res 1996;36:280-6.
31. Pardridge WM, Kang YS, Buciak JL. Transport of human recombinant brain-derived neurotrophic factor (BDNF) through the rat blood-brain barrier in vivo using vector-mediated peptide drug delivery. Pharm Res 1994;11:738-46.
32. Hempstead BL, Salzer JL. Neurobiology. A glial spin on neurotrophins. Science 2002;298:1184-6.
33. Notterpek L. Neurotrophins in myelination: a new role for a puzzling receptor. Trends Neurosci 2003;26:232-4.
34. Borst JG, Lodder JC, Roubos EW, Kits KS. In situ recordings of presumed folliculo-stellate cells in the intermediate lobe of the pituitary gland of Xenopus laevis. Neurosci Lett 1996;209:61-4.
35. Jenks BG, Leenders, H.J., Martens, G.J.M., Roubos, E.W. Adaptation physiology: the functioning of pituitary melanotrope cells during background adaptation of the amphibian *Xenopus laevis*. Zoolog Sci 1993;10:1-11.
36. Virard I, Coquillat D, Bancila M, Kaing S, Durbec P. Oligodendrocyte precursor cells generate pituicytes in vivo during neurohypophysis development. Glia 2006;53:294-303.
37. de Rijk EP, Jenks BG, Vaudry H, Roubos EW. GABA and neuropeptide Y co-exist in axons innervating the neurointermediate lobe of the pituitary of Xenopus laevis--an immunoelectron microscopic study. Neuroscience 1990;38:495-502.
38. Wittkowski W. Tanycytes and pituicytes: morphological and functional aspects of neuroglial interaction. Microsc Res Tech 1998;41:29-42.
39. Hatton GI. Pituicytes, glia and control of terminal secretion. J Exp Biol 1988;139:67-79.
40. Hatton GI, Perlmutter LS, Salm AK, Tweedle CD. Dynamic neuronal-glial interactions in hypothalamus and pituitary: implications for control of hormone synthesis and release. Peptides 1984;5 Suppl 1:121-38.
41. Pruginin-Bluger M, Shelton DL, Kalcheim C. A paracrine effect for neuron-derived BDNF in development of dorsal root ganglia: stimulation of Schwann cell myelin protein expression by glial cells. Mech Dev 1997;61:99-111.
42. Ogawa K, Suzuki E, Taniguchi K. Immunohistochemical studies on the development of the hypothalamo-hypophysial system in Xenopus laevis. Anat Rec 1995;241:244-54.
43. van Eys GJ, van den Oetelaar P. Cytological localization of alpha-MSH, ACTH and beta-endorphin in the pars intermedia of the cichlid teleost Sarotherodon mossambicus. Cell Tissue Res 1981;215:625-33.
44. Vandesande F, Dierickx K. Immunocytochemical demonstration of separate vasotocinergic and mesotocinergic neurons in the amphibian hypothalamic magnocellular neurosecretory system. Cell Tissue Res 1976;175:289-96.
45. Vandesande F, Dierickx K, De Mey J. The origin of the vasopressinergic and oxytocinergic fibres of the external region of the median eminence of the rat hypophysis. Cell Tissue Res 1977;180:443-52.
46. Mattheij JA, Stroband HW, Kingma FJ. The cell types in the adenohypophysis of the cichlid fish Cichlasoma biocellatum Regan, with special attention to its osmoregulatory role. Z Zellforsch Mikrosk Anat 1971;118:113-26.
47. Turner DL, Weintraub H. Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev 1994;8:1434-47.
48. de Groot DM, Martens GJ. Expression of neuroserpin is linked to neuroendocrine cell activation. Endocrinology 2005;146:3791-9.
49. Nieuwenhuys R, Ten Donkelaar, H.J., Nicholson, C. The Central Nervous System of Vertebrates. Heidelberg: Springer-Verlag, 1998.

General Discussion

In ancient times, the old Egyptians were not interested in brains: using a spoon the skull of a deceased pharaoh was emptied through the nose. Later in time, when it was appreciated that in fact our head houses our intellect, not the brain itself but rather the ventricles within our brain were thought to be involved. Currently, science is discovering more and more about our brain and how it works, and at the same time it appears that the brain is far more complicated than we could have ever imagined. One intriguing aspect is the fact that the brain is a highly plastic structure, which can adapt or modify itself in response to experiences and the environment. This is for example reflected in our capacity to learn and memorize enormous amounts of skills and data. Much of the brains ability to change itself lies in the plasticity of synaptic connections between neuronal cells, both short- and long term. Substantial evidence indicates that the number and strength of synapses can be changed by neuronal activity (1-3), and it is now widely accepted that activity-dependent modulation of synapse strength is critical for brain development as well as many cognitive functions in the adult. However, the molecular mechanisms that translate patterns of neuronal activity into specific changes in the structure and function of synapses and other brain parts remain largely unknown. To solve a tiny part of this puzzle, we set out to study physiological aspects of two proteins that are presumably involved in neuronal plasticity, namely neuroserpin and brain-derived neurotrophic factor (BDNF). In our studies, we have used as a model system the well-defined neuroendocrine intermediate pituitary melanotrope cells of the amphibian *Xenopus laevis*, which exert plasticity at both the cellular and neuronal level. We have explored the endogenous expression of neuroserpin in *Xenopus,* and the effects of neuroserpin and (pro)BDNF transgene expression on melanotrope cell functioning. In this final chapter, the results of these studies will be summarized and placed into a broader context.

Neuroserpin

Neuroserpin was first described 17 years ago by the group of Sonderegger (4), and ever since interest in the protein has steadily increased. A number of observations make neuroserpin an interesting protein for more detailed functional analysis. First, during development and in adult life neuroserpin has been proposed to play a role in neurite outgrowth and synaptogenesis in the nervous system (4-13). Second, neuroserpin appears to protect the brain from ischemic injury by inhibiting tissue-type plasminogen activator (t-PA) (14-18). Third, mutations in the neuroserpin gene can cause the autosomal dominant dementia Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB; 19, 20). The studies described in chapters 2, 3 and 4 examine in more detail the physiological role of neuroserpin in the *Xenopus* melanotrope cells.

Neuroserpin in Xenopus laevis

Since thus far neuroserpin has been described only in a limited number of vertebrate species, we first set out to identify and clone neuroserpin in our model system *Xenopus* (chapter 2). Subsequent characterization of neuroserpin mRNA and protein expression revealed that in adult *Xenopus* this serpin is predominantly expressed in neuronal and neuroendocrine tissues. In addition, the developmental expression patterns were determined in *Xenopus* tadpoles, revealing expression of both neuroserpin mRNA and protein throughout development. These results were in line with findings by others in human, mice and rat (7, 8, 12, 21, 22), and imply a role for neuroserpin in nervous system functionality, both during development and in later life. Remarkably, we also found neuroserpin mRNA and protein expression already in *Xenopus* developmental stage 0 (unfertilized egg) (chapter 2), which suggested additional functions of neuroserpin during early development and possibly fertilization. The *Xenopus* oocyte is surrounded by an extracellular matrix (ECM) forming a dynamic filamentous structure, which is of biological significance since it is known to play a vital role in sperm binding, polyspermy block and protection of the embryo. The distinctive layers that comprise the ECM overlay the plasma membrane and undergo specific ultrastructural and biochemical modifications during oogenesis and maturation (23). These modifications are for a large part dependent on proteases and protease inhibitors (24). For example, the serine protease ovochymase is released from *Xenopus* eggs after fertilization and is involved in the conversion of the ECM to block polyspermy and physically 'harden' the ECM for embryo protection (25, 26). In addition, recently a key role was demonstrated for serpin-27A as an inhibitor of the serine protease Easter in pattern formation during embryonic development of *Drosophila* (27-29). In view of these observations, neuroserpin may play a role as serine protease inhibitor in the ECM during oocyte development, fertilization and early development of *Xenopus*.

Physiological target of neuroserpin in Xenopus intermediate pituitary

We have examined in more detail the expression and functional aspects of neuroserpin in the *Xenopus* pituitary (chapters 2 and 3). Remarkably, we observed various forms of the neuroserpin protein in the different parts of the pituitary, namely a 55-kDa form in the pars nervosa, which was also detected in brain and therefore appeared to be a neuronal form of the protein, products of 44-, 47- and 50 kDa in the intermediate pituitary, and a 46-kDa product in the pars distalis. In rat pituitary, different forms of the neuroserpin protein have also been detected in the intermediate pituitary and pars distalis, with molecular weights of 55- and 53 kDa, respectively (12). These multiple forms of neuroserpin probably represent post-translationally modified products, since we found that the 47-, 50-kDa and 46-kDa *Xenopus* neuroserpin proteins were N-linked glycosylated, whereas the 44-kDa and 55-kDa products were not (chapters 2 and 3). The 44-kDa neuroserpin protein most likely

represents the primary translation and still not-modified form, whereas the observed 55-kDa product may contain a post-translational modification that has not yet been characterized. Since protein modifications can considerably increase the information content and functional repertoire of proteins, possibly the various forms of the neuroserpin protein exert different functions in the distinct pituitary cell types.

Intriguingly, we also detected an SDS-stable 130-kDa neuroserpin-containing protein complex in the intermediate pituitary melanotrope cells of black-adapted *Xenopus*. Since the complex was dynamically expressed during background adaptation of *Xenopus*, it may play a role in the cellular and synaptic plasticity observed in the intermediate pituitary during the adaptation process of the frog (chapter 3). However, a number of questions concerning the composition and functional significance of the complex still remain. For instance, since serpins are known to form SDS-stable covalent interactions with target serine proteases (reviewed in 30, 31), the 130-kDa product may represent a complex of newly synthesized neuroserpin with its target protease. Comparison of the *Xenopus* neuroserpin amino acid sequence with the known vertebrate neuroserpin sequences revealed an overall identity of 58%, whereas the sequences of the reactive center loop (RCL) were 91% identical (chapter 2). The high degree of conservation of the RCL indicates a trypsin-like substrate specificity of neuroserpin in *Xenopus*. The inhibitory activity of recombinant purified neuroserpin has been assessed *in vitro* by the formation of SDS-stable complexes with putative target proteases, and in this way mouse, chicken and human neuroserpin have been found to inhibit t-PA, urokinase-type plasminogen activator (u-PA), plasmin and thrombin (6-8). However, in view of the acidic nature of the 130-kDa complex (pI of ~5.4) and its molecular weight it is unlikely that in *Xenopus* these proteases are part of the complex. Several other candidate target serine proteases with trypsin-like substrate specificity may be proposed, such as the family of tissue kallikreins, of which kallikrein-6 and -8 (also named neuropsin) appear the most interesting since they are mainly expressed in neuronal tissues (32, 33). Other possible candidates may be found among members of the family of type II transmembrane serine proteases (TTSPs) that form a large new family of membrane-anchored serine proteases of which most members are still poorly characterized (34-37). Examples of inhibitory complexes of TTSPs with serpins exist, like mouse DESC1 with plasminogen activator inhibitor-1 and protein C inhibitor (38, 39) or mouse matriptase-3 with an array of serpins, including plasminogen activator inhibitor-1, protein C inhibitor, α1-proteinase inhibitor, α2-antiplasmin and antithrombin III (40). Two TTSPs cloned from *Xenopus*, namely *Xenopus* embryonic serine protease (Xesp)-1 and a homolog of human MT-SP1 (*Xenopus* MT-SP1), have been shown to play a role during early *Xenopus* development (41). Interestingly, these TTSPs have predicted molecular weights and pIs that would give an acidic complex of ~130 kDa with *Xenopus* neuroserpin, similar to the complex we observed in the *Xenopus* intermediate pituitary cells.

A second issue to be considered concerns a more detailed analysis of the fate and (extra)cellular localization of neuroserpin and the 130-kDa complex, for example by immuno-electron microscopy. Such an analysis will help to better understand its physiological role. We found in our pulse-chase analyses that newly synthesized 47- and 50-kDa neuroserpin were secreted by the melanotrope cells, whereas the 130-kDa product was not (chapter 3). These results imply that the complex is localized intracellularly or in the ECM of the melanotropes. At a certain point of time, the 130-kDa complex may be endocytosed by the melanotrope cells followed by intracellular degradation, which would constitute a clearance mechanism to remove the complex from its site of action. A candidate receptor for this process might be the low density lipoprotein (LDL) receptor-related protein (LRP). LRP has been suggested to be a multifunctional scavenger receptor for which numerous ligands already have been identified (42-45) and that has been proposed to mediate the internalization of active neuroserpin and neuroserpin/t-PA complexes by mouse cortical neurons and embryonic fibroblasts (46). Alternatively, the 130-kDa complex may be degraded extracellularly, or neuroserpin might be cleaved by its target protease resulting in inactive neuroserpin and active protease. *In vitro*, after neuroserpin/t-PA complex formation, neuroserpin was indeed cleaved by t-PA, after which cleaved neuroserpin and active t-PA were detected (47).

Altogether, the elusive target protease will likely determine the physiological effect of neuroserpin in the *Xenopus* intermediate pituitary. Via inhibition of this target protease, neuroserpin may affect the integrity of the ECM or the cleavage of transmembrane or ECM-related signalling proteins.

Functional studies on neuroserpin

To study the physiological role of neuroserpin in an *in vivo* context, we used the technique of stable *Xenopus* transgenesis to overexpress neuroserpin and a mutated form of neuroserpin specifically and physiologically inducible in the intermediate pituitary melanotrope cells (chapter 4). Previously, neuroserpin-transgenic and knock-out mice have been generated, which have been analyzed at the behavioural but not at the cell-biologial level (48, 49). Phenotypic characterization of these mice has shown that altered expression of neuroserpin is associated with deficient exploratory behaviour and abnormal reaction to novelty, indicating a role of neuroserpin in the regulation of emotional state (49). In our transgenic *Xenopus*, transgene expression of GFP-neuroserpin (line 55) or mutated GFP-neuroserpin(G392E) (line 84) did not affect endogenous neuroserpin protein levels and melanotrope cell functioning was not detectably altered, as determined by metabolic cell labeling and Western blot analysis of various secretory pathway proteins. Remarkably, in transgenic line-84 animals, we have not detected the cell-biological characteristics observed in cells transfected with FENIB mutant neuroserpin, namely neuroserpin inclusion bodies which accumulate in the endoplasmic reticulum (ER) and are formed by polymers of the

mutated neuroserpin protein (20, 50). This may be due to the fact that in our transgenic animals the level of overexpression of GFP-neuroserpin(G392E) was relatively low when compared with that in the transfected cells. Because of the higher expression levels in the transfected cells, polymerization and accumulation of the mutant neuroserpin may have been observed in these cells. Alternatively, the time of adaptation of the transgenic animals to a black background (and therefore the time during which the melanotrope cells were exposed to the transgene product) has been too short. We have shown before that the time of background adaptation of transgenic frogs can be important to create a phenotypic effect of transgene expression (51, 52). Interestingly, the neuroserpin- and mutant neuroserpin(G392E)-transgenic *Xenopus* melanotrope cells did show a difference at the ultrastructural level, namely the presence of intercellular gaps, which were not present among wild-type melanotrope cells or cells transgenic for proteins other than neuroserpin (chapter 4 and data not shown). These gaps may imply a change in the ECM of the transgenic cells, and therefore suggest a role for neuroserpin in the regulation of the integrity of the ECM. This would be in line with the previously proposed function of neuroserpin as an inhibitor in the ECM (12, 21).

Thus, to investigate functional aspects of neuroserpin we have used *Xenopus* transgenesis to generate transgenic frogs with overexpression of neuroserpin or mutant neuroserpin. Further studies are necessary to examine the role of neuroserpin in manipulating the melanotrope ECM.

BDNF

The second protein of which in this thesis functional aspects were explored in more detail concerns BDNF. Following the discovery of NGF in the early 1950s, BDNF was the second member of the neurotrophin (NT) family to be identified in 1982 (53). Since then, interest in BDNF has only increased, because studying this NT continues to provide new insights into neuronal function. Initially, BDNF was characterized as a neuronal survival- and differentiation factor (reviewed in 54, 55), but currently it is also known to play a role in axonal and dendritic growth and guidance (reviewed in 56-59), neurogenesis (60-63), long term potentiation (LTP) and synaptic plasticity (reviewed in 55, 64-67). In addition, the protein has been implicated in numerous pathological diseases, such as epilepsy, neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's disease, and neuropsychiatric disorders, like depression, schizophrenia and bipolar disorder (reviewed in 55). However, despite the obvious wide role of BDNF in nervous system functionality, in many cases the underlying molecular and cellular mechanisms remain elusive. In addition, the recent discovery that the proforms of NTs also exert a physiological role that appears opposite to that of mature NTs adds an additional complication to the understanding of the physiological role of NTs in the nervous system.

The studies described in chapters 5 and 6 examined *in vivo* physiological aspects of pro- and mature BDNF functioning. As a model system, we again used the intermediate pituitary melanotrope cells of *Xenopus laevis*.

Physiological aspects of proBDNF

Until recently, little attention has been paid to the NT-proregions, which were originally thought to be readily degraded. Now evidence is accumulating that proNTs, and in particular proBDNF, can also be secreted by cells (68-74) and subsequently have a physiological role, such as facilitating long-term depression and neuronal apoptosis, via their high-affinity binding to the $p75^{NTR}$ receptor (75-77). In addition, extracellular cleavage by plasmin or selective matrix metalloproteases can convert extracellular proBDNF in mature BDNF, and as such may represent a new way to control synaptic functioning of BDNF (67, 72, 78, 79).

In an attempt to explore the physiological role of proBDNF in more detail, we generated transgenic *Xenopus laevis* with overexpression of proBDNF or GFP-tagged proBDNF specifically in the intermediate pituitary melanotrope cells. Thus far, analyses of transgenic lines with expression of untagged proBDNF did not reveal a clear phenotype, possibly due to the relatively low levels of transgene expression (chapter 5 and unpublished observations). In contrast, analyses of transgenic melanotrope cells with expression of the unprocessed GFP-proBDNF fusion protein revealed ER stress and a block of transport of other secretory proteins through the secretory pathway (chapter 5). Since the observed ER stress implies an accumulation of misfolded proteins, these results indicate that the GFP moiety hampered the prodomain to properly act as an intramolecular chaperone for proBDNF. Correct proNGF folding also depends on its prodomain (80, 81). Furthermore, the prodomain may control the intracellular trafficking and secretion of proBDNF, since a single-nucleotide polymorphism (SNP) in the pro-region of proBDNF (valine instead of methionine at residue 66) has been found to affect synaptic targeting and activity-dependent secretion of the protein (74). This val66met SNP is a recognized polymorphism of the human BDNF gene which is associated with memory impairment and increased susceptibility to neuropsychiatric disorders (73). Interestingly, the intracellular perinuclear accumulation and lack of secretion of GFP-proBDNF we observed in the GFP-proBDNF-transgenic melantorope cells (chapter 5) is reminiscent of the concentrated distribution of proBDNF(met) in the cell body and the decrease in regulated BDNF secretion by polarized proBDNF(met)-transfected neurosecretory PC12 cells and rat cortical neurons (73, 74). We therefore hypothesize that the impaired intracellular trafficking of BDNF(met) may also impose stress to the ER, resulting in a reduced transport, processing and secretion of other neuronal secretory pathway proteins. Eventually, this may lead to multiple effects of BDNF(met) on neuronal cell functioning, possibly explaining some aspects of the wide variety of phenotypes observed in individuals carrying the BDNF(met) polymorphism.

Physiological aspects of mature BDNF: role in glial cell physiology

To study the physiological role of mature BDNF, we generated transgenic *Xenopus laevis* that overexpressed mature BDNF specifically in the intermediate pituitary melanotrope cells. Intriguingly, analysis of these animals revealed that BDNF induced proliferation of both central nervous system (CNS)-derived glial cells and peripheral nervous system (PNS)-derived Schwann cells (chapter 6).

Various types of glial cells exist, such as astrocytes and oligodendrocytes in the CNS and Schwann cells in the PNS, each with their own physiological role and specific characteristics. For example, while $p75^{NTR}$ and truncated TrkB expression has been detected in oligodendrocytes, astrocytes and Schwann cells, full-length TrkB appears to be expressed only in oligodendrocytes (82), and $p75^{NTR}$ mediates the action of NTs differently in basal forebrain oligodendrocytes and Schwann cells (83). In our model system, we found a proliferating effect of BDNF on both peripheral Schwann cells from cranial nerves V and VII, and glial cells with CNS characteristics that most likely originated from the pituitary pars nervosa (chapter 6). Since the pituicytes are the main group of glial cells in the pituitary pars nervosa (84-87), BDNF presumably affected these cells. Remarkably, we did not observe an effect of BDNF on another glial-like cell group in the intermediate and distal lobe of the pituitary, namely the folliculo-stellate (FS) cells (88-92). This apparent difference in sensitivity to BDNF by the two different types of glial cells may be explained by differences in $p75^{NTR}$ and TrkB expression levels in pituicytes and FS cells. Schwann cells have been shown to express the $p75^{NTR}$ and truncated TrkB receptors (reviewed in 82). In agreement with this, we detected in cranial nerves V and VII of wild-type *Xenopus* relatively high $p75^{NTR}$ mRNA expression levels and relatively low TrkB mRNA expression levels (unpublished observations), indicating that in our model system BDNF might induce a proliferating signal via the $p75^{NTR}$ and/or possibly the truncated TrkB receptor (we did not examine the expression levels of truncated TrkB). However, the intracellular signaling pathways that ultimately induced glial cell proliferation remain unknown.

In addition to the proliferating effect of BDNF on glial and Schwann cells, we also observed in our transgenic *Xenopus* a stimulating effect of overexpressed mature BDNF on axonal outgrowth and myelination (chapter 6). These findings are in line with the results of previous studies showing a stimulating effect of BDNF on neuronal growth in a number of other systems as well (reviewed in 56-59). Furthermore, evidence is accumulating that BDNF plays a stimulatory role in the myelination process in both the PNS and CNS (93-99). The formation of myelin sheets is achieved by Schwann cells in the PNS, whereas oligodendrocytes are the myelinating cells in the CNS. The mechanisms of myelin formation, and membrane wrapping and maintenance are dependent on complex interactions between axons and the myelinating glial cells (reviewed in 100-102). Although BDNF has been found to stimulate myelin formation in both the CNS and PNS, thus far only a molecular mechanism has been proposed for its effect on peripheral Schwann cells,

where BDNF appears to stimulate the formation of myelin via $p75^{NTR}$ (reviewed in 97, 98). Presumably, another mechanism is used by oligodendrocytes, as in the basal forebrain the effect of BDNF on myelination appeared to be dependent on the actions of the trkB and not the $p75^{NTR}$ receptor (83). The emerging role of BDNF in PNS and CNS myelination may provide new therapeutic opportunities for treating diseases of the nervous system in which the myelinating process is affected, such as multiple sclerosis. Possibly, overexpression of BDNF in affected CNS and PNS regions, by transplanted genetically modified BDNF-producing cells or by retroviral infection, may stimulate remyelination of demyelinated axons.

Altogether, besides its well-known role in neuronal survival and differentiation, BDNF appears to be also involved in glial cell physiology. Glial cells have traditionally been viewed as myelinating cells (oligodendrocytes and Schwann cells) and support cells for neurons by regulating ion concentrations, taking up neurotransmitters and providing substrates for energy metabolism (astrocytes). Recent studies have however shown that glial cells can actively control the structural and functional plasticity of synapses in developing and adult organisms (reviewed in 103-106), indicating active intercellular signaling between glial cells and neurons (reviewed in 107-109). It is tempting to speculate that NTs, and especially BDNF, are involved in such signaling. For example, BDNF has been shown to induce calcium signaling in glial cells (110), and glial cell calcium signaling may play an important role in glial-glial and neuron-glial communication (reviewed in 108, 111-113).

Neuroserpin and BDNF in neuroendocrine and neuronal plasticity: a model

In our studies, we have used the neuroendocrine melanotrope cells of the *Xenopus* intermediate pituitary as a model system to study aspects of the physiological roles of neuroserpin and BDNF. The results presented in this thesis allow us to propose the following models for the functions of these proteins in neuroendocrine and neuronal plasticity.

First, we consider the possible role of neuroserpin (Figure 1). This protein forms a complex with an up-to-now unidentified serine protease in the melanotropes of black- but not of white-adapted animals, indicating that the formation of this complex is linked to melanotrope cell activation (chapter 3). More specifically, in black animals adapting to a white background we found a relatively fast decrease (already after 1 day of adaptation) in the amount of complex, implying that the protease and thus proteolytic activity in the melanotrope ECM becomes readily available after transfer to a white background. Since clearance of the complex occurs within one day, in the first 24 h of white-background adaptation the contacting hypothalamic neurons from the suprachiasmatic nuclues that inhibit the melanotrope cells (SMINs) may be activated, resulting in a fast initial inhibition of melanotrope cell activity. To sustain this melanotrope inhibition, the initial phase is

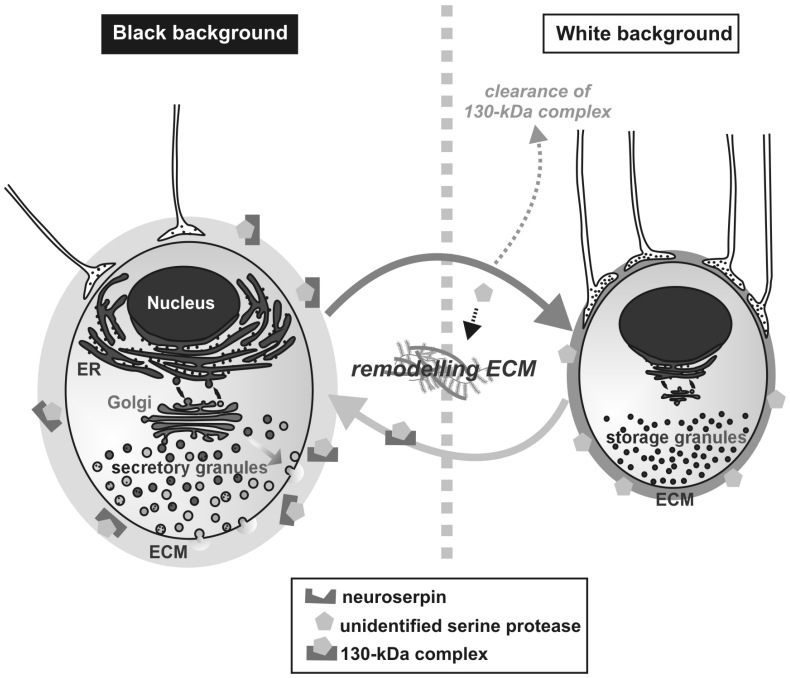


Figure 1: **Model for the role of neuroserpin in neuroendocrine plasticity**.
Neuroserpin is produced and secreted by *Xenopus* intermediate pituitary melanotrope cells and is proposed to inhibit a target serine protease, thereby influencing the remodelling of the extracellular matrix during black- and white-background adaptation of the animal. See text for details. Abbreviations: ER, endoplasmic reticulum; ECM, extracellular matrix.

immediately followed by elaborate synaptic changes (namely an increase in size and number) of the SMINs. This plasticity is made possible by changes in the melanotrope ECM, as the ECM is thought to play an important role in synaptic plasticity (114, 115). For maintenance of the ECM, a proper balance between matrix proteases and their inhibitors is essential. Therefore, in black animals that are put on a white background, rapid clearance of the complex and increase in protease activity immediately after adaptation will likely result in a net breakdown of the ECM, regulating the plasticity of the SMINs during the initial stages of white-background adaptation. In contrast, in white animals put on a black background, the neuronal activity of the SMINs may be shut down immediately, resulting in loss of inhibitory signal and an instantaneous release of α-melanophore stimulating hormone (α-MSH) that is stored in the storage granules of the melanotropes. In white animals adapting to a black background, the neuroserpin-protease complex was detected only after five days of adaptation. This allows protease activity in the cell matrix to remain present during the first days of adaptation, regulating ECM integrity and therefore plasticity

of the SMINs (namely a decrease in SMIN number and size). Protease activity will then be gradually inhibited by neuroserpin, which will lead to a change in the balance of protease and inhibitor in the ECM, resulting in a change in architecture of the ECM. The ECM may become denser, since the number and sizes of contacting SMINs are smaller in the intermediate pituitary of black animals.

In the second model, a possible role of BDNF in neuroendocrine plasticity is considered (Figure 2). In *Xenopus,* both pro- and mature BDNF have been detected in the melanotrope cells (116, 117). Analysis of BDNF-transgenic *Xenopus* showed that a high level of secreted mature BDNF exerts a proliferating effect on nearby glial cells from the pituitary pars nervosa and cranial nerves V and VII, and a stimulatory effect on the growth and myelination of axons (chapter 6). Notably, this phenotype was detected only in transgenic animals with relatively high expression levels of mature BDNF (line 73) or in long-term black-adapted transgenic animals with relatively low expression levels (line 6). Thus, BDNF can have an effect on glial cell proliferartion and myelination, and therefore on glial cell physiology. However, for melanotrope cell functioning in wild-type *Xenopus* it is most likely that other functional aspects of pro- and mature BDNF are important. For example, *in vitro* mature BDNF can stimulate the biosynthesis of proopiomelanocortin (POMC) and release of α-MSH in an autocrine manner (117). It remains to be established whether these effects also occur *in vivo*. Furthermore, the proBDNF protein may act on the melanotrope cells in an autocrine manner, thereby opposing the effect of mature BDNF. In addition, both mature and proBDNF may affect the synaptic varicosities of the hypothalamic neurons contacting the melanotrope cells, thus modulating the plasticity of these neurons. The balance between mature and proBDNF in the extracellular space and the relative BDNF-receptor and co-receptor expression levels on both the melanotrope cells and contacting hypothalamic neurons will therefore ultimately determine the physiological effect of mature and proBDNF on the *Xenopus* neurointermediate pituitary.

Finally, in the third model we propose that a functional link exists between neuroserpin and BDNF in species or cell systems other than the *Xenopus* intermediate pituitary (Figure 3). In other systems, t-PA has been suggested to be a main target protease of neuroserpin (6, 8, 15). Interestingly, t-PA is known to cleave plasminogen into plasmin (17) and, in turn, plasmin can cleave ECM components involved in neuronal plasticity, such as laminin (118) or the cell-adhesion molecule L1 (119). Plasmin has been shown to also cleave proBDNF (67, 72), providing an additional mechanism for the control of the extracellular balance between pro- and mature BDNF. Cleavage of proBDNF by t-PA/plasmin is essential for long-term hippocampal plasticity in mice (67, 120). Thus, by interacting with t-PA, neuroserpin may have an effect on the extracellular cleavage of proBDNF into mature BDNF. It remains to be established whether neuroserpin has an effect on plasmin or proBDNF in the *Xenopus* intermediate pituitary.

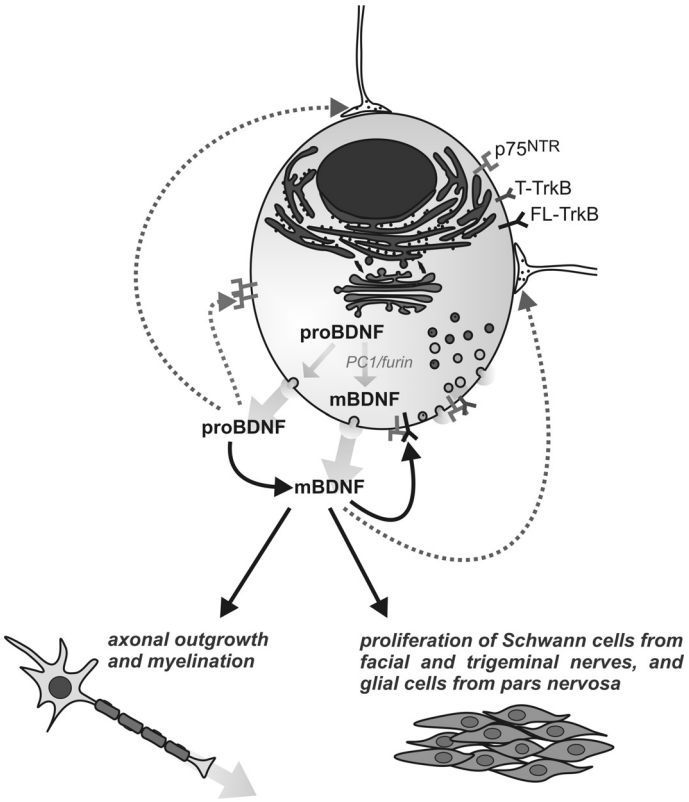


Figure 2: Model for the role of BDNF in neuronal and neuroendocrine plasticity.
Pro- and mature BDNF are produced and secreted by the *Xenopus* intermediate pituitary melanotrope cells and are proposed to have an effect on the melanotrope cells themselves and on the innervating hypothalamic neurons. In addition, transgene mature BDNF expression was shown to stimulate the proliferation of glial cells, and the myelination and outgrowth of axons. See text for details. Abbreviations: (pro)BDNF, (pro) brain-derived neurotrophic factor; mBDNF, mature brain-derived neurotrophic factor; PC1, prohormone convertase 1; $p75^{NTR}$, p75 neurotrophin receptor; T-TrkB, truncated TrkB receptor; FL-TrkB, full-length TrkB receptor.

Future prospects

It is almost a 'rule' in science that a research project ends with more questions than initially raised, and the studies described in this thesis are not an exception to this apparent rule. Concerning the physiological role of neuroserpin, an important question is the identity of the putative target substrate of neuroserpin in the 130-kDa complex. To identify this target, we have used a number of experimental approaches, such as MALDI-MS and LC/ESI-MS, pull-down experiments using recombinant GST-neuroserpin, and Western blot analysis with antibodies against suspected candidate targets (data not shown). Unfortunately, these

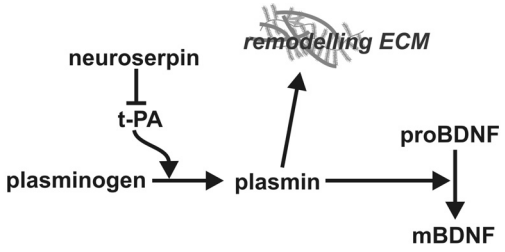


Figure 3: Model of a functional link between neuroserpin and BDNF.
In other species or cell systems than the *Xenopus* intermediate pituitary, neuroserpin has been proposed to inhibit the serine protease t-PA, which, in turn, is involved in the cleavage of plasminogen into plasmin. Plasmin plays a role in ECM remodelling and as such is involved in plasticity. In addition, plasmin can extracellularly cleave proBDNF, thus providing a mechanism to control the extracellular balance between pro- and mature BDNF. See text for details.

attempts have thus far been unsuccessful, probably due to the low expression levels of the endogenous complex. Furthermore, the expression of the TTSPs Xesp-1 and *Xenopus* MT-SP1 in the *Xenopus* intermediate pituitary should be examined to consider the possibility that these proteases interact with neuroserpin. For this purpose, Western blotting, pull-down experiments and co-immunoprecipitation analyses can be performed.

Our neuroserpin-transgenic *Xenopus* have to be further analyzed in detail to learn more about the physiological aspects of neuroserpin, in particular the consequences of its apparent effect on the ECM. In addition, it might be worthwhile to generate transgenic animals with expression levels of mutant neuroserpin(G392E) that are higher than those in the presently available lines. Also, generation of lines with transgene expression of untagged neuroserpin will be valuable to avoid possible interference of the GFP-moiety on the biological activity of the exogenous neuroserpin. Furthermore, the technique of RNA interference may be employed to specifically knock-down endogenous neuroserpin expression. This technique has been used before in *Xenopus* to knock-down GFP in GFP-transgenic embryos (121, 122) and an endogenous protein named Xlim-1 in non-transgenic embryos by injection of double stranded Xlim-1 RNA in 4-cell stage embryos (123).

Regarding the physiological role of pro- and mature BDNF in our model system, an important next step will be the identification and characterization of the various BDNF (co-) receptors, such as TrkB, truncated TrkB, $p75^{NTR}$, and sortilin, in the *Xenopus* intermediate pituitary. For this purpose, *in situ* hybridization experiments, immunocytochemistry, immuno-electron microscopy, quantitative RT-PCR analysis or the use of specific receptor antogonists may be helpful. Furthermore, receptor modulation can also influence receptor functioning and should therefore be considered. For instance, regulated intramembrane proteolysis of the $p75^{NTR}$ by the alpha- and gamma-secretase enzymes has been shown to modulate $p75^{NTR}$ association with the TrkA receptor (124).

Preliminary results suggest that multiple $p75^{NTR}$ co-receptors can modulate p75 processing by either inducing novel p75 ectodomain shedding (by an as yet unidentified serine protease) or inhibiting p75 proteolysis (personal communication with Elissa L. Ash, Columbia University, New York, USA). In addition, in *Xenopus* a novel gene encoding a protein with substantial sequence identity with $p75^{NTR}$, named neurotrophin receptor homolog 1 (NRH-1) has recently been identified (125). NRH-1 has been shown to undergo multiple proteolytic cleavages that ultimately released cytoplasmic fragments with possible signalling capacities (126, 127). Thus, *Xenopus* NRH-1 expression and its possible involvement in melanotrope cell functioning should be examined. Furthermore, it will be interesting to study the intracellular signaling pathways in the melanotrope cells that may be affected by pro- and mature BDNF. In this respect, especially intracellular calcium signaling should be investigated, since intracellular calcium is known to play an important role in melanotrope cell signaling (128, 129) and BDNF has been shown to induce calcium signaling in glial cells (110).

For further functional characterization of pro- and mature BDNF, a continuation of the use of *Xenopus* transgenesis may be helpful. For instance, new transgenic lines with higher expression levels of intact proBDNF should be generated. The transgenic animals with transgene expression of mature BDNF (transgenic lines 6 and 73) should be further examined to obtain more detailed insights into its effects on melanotrope cell functioning. Thus far, we have not found an effect of mature BDNF transgene expression on the biosynthesis and processing of POMC or secretion of POMC-derived peptides, or on steady-state POMC protein expression levels (data not shown). This finding was unexpected since *in vitro* studies have shown a stimulatory effect of recombinant BDNF on the biosynthesis of POMC and release of α-MSH by the melanotrope cells (117). Finally, since the balance between proBDNF and mature BDNF expression appears important for the eventual biological effect, the possibility should be considered that plasmin plays a role in the extracellular processing of proBDNF in the *Xenopus* intermediate pituitary. Additionally, a transgenic line with overexpression of mature BDNF may be crossed with a line with transgene expression of proBDNF, to generate new double-transgenic lines with varying ratios of pro- and mature BDNF. It will be interesting to see whether these double-transgenic animals show differences in for example melanotrope cell activity or in background-adaptation capacity.

Since we found in *Xenopus* that mature BDNF can induce glial cell proliferation, it will be highly interesting to examine such a role in other species, for example human. A role in glial cell proliferation has also been suggested on the basis of the fact that BDNF increased the cell number of two microglial cell lines (130), [^{3}H]-thymidine incorporation in microglia cells (131), the cell number of optic nerve head astrocytes (132), and oligodendrocyte proliferation in the contused adult rat spinal cord (94). Furthermore, glial cells not only function as support cells for neurons but actually play an important role in nervous system functionality by modulating neuronal plasticity and communicating with

neurons, a process in which BDNF is possibly involved (110). However, the molecular mechanisms underlying these effects remain to be elucidated, *e.g.* which receptor complex and intracellular signaling pathway are involved. In addition, the emerging role of BDNF in PNS and CNS myelination should be examined in more detail, since the exact molecular mechanism of its stimulating effect remains unknown. Obviously, a more detailed knowledge of these functional aspects of BDNF might prove very useful for the development of therapeutic strategies against various disorders of the nervous system, such as neurodegenerative and/or neurodevelopmental demyelination disorders or glial tumors.

In conclusion, the data presented in this thesis have provided new insights into the physiological roles of neuroserpin and BDNF in neuronal and neuroendocrine plasticity. Through our continuously increasing knowledge about functional roles of and interactions between proteins, a more detailed picture of neuronal plasticity is emerging, but at the same time the puzzle is gaining complexity. Ultimately, a better understanding of the molecular mechanisms underlying plasticity will not only increase our knowledge about brain functioning, but it will also broaden therapeutic perspectives to combat neurodegenerative and neurodevelopmental disorders.

References

1. Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 1993;361:31-9.
2. Malenka RC, Nicoll RA. Long-term potentiation--a decade of progress? Science 1999;285:1870-4.
3. Waites CL, Craig AM, Garner CC. Mechanisms of vertebrate synaptogenesis. Annu Rev Neurosci 2005;28:251-74.
4. Stoeckli ET, Lemkin PF, Kuhn TB, Ruegg MA, Heller M, Sonderegger P. Identification of proteins secreted from axons of embryonic dorsal-root-ganglia neurons. Eur J Biochem 1989;180:249-58.
5. Osterwalder T, Contartese J, Stoeckli ET, Kuhn TB, Sonderegger P. Neuroserpin, an axonally secreted serine protease inhibitor. Embo J 1996;15:2944-53.
6. Osterwalder T, Cinelli P, Baici A, et al. The axonally secreted serine proteinase inhibitor, neuroserpin, inhibits plasminogen activators and plasmin but not thrombin. J Biol Chem 1998;273:2312-21.
7. Hastings GA, Coleman TA, Haudenschild CC, et al. Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. J Biol Chem 1997;272:33062-7.
8. Krueger SR, Ghisu GP, Cinelli P, et al. Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J Neurosci 1997;17:8984-96.
9. Parmar PK, Coates LC, Pearson JF, Hill RM, Birch NP. Neuroserpin regulates neurite outgrowth in nerve growth factor-treated PC12 cells. J Neurochem 2002;82:1406-15.
10. Berger P, Kozlov SV, Cinelli P, Kruger SR, Vogt L, Sonderegger P. Neuronal depolarization enhances the transcription of the neuronal serine protease inhibitor neuroserpin. Mol Cell Neurosci 1999;14:455-67.
11. Wannier-Morino P, Rager G, Sonderegger P, Grabs D. Expression of neuroserpin in the visual cortex of the mouse during the developmental critical period. Eur J Neurosci 2003;17:1853-60.
12. Hill RM, Parmar PK, Coates LC, Mezey E, Pearson JF, Birch NP. Neuroserpin is expressed in the pituitary and adrenal glands and induces the extension of neurite-like processes in AtT-20 cells. Biochem J 2000;345 Pt 3:595-601.
13. Yepes M, Lawrence DA. Neuroserpin: a selective inhibitor of tissue-type plasminogen activator in the central nervous system. Thromb Haemost 2004;91:457-64.
14. Lebeurrier N, Liot G, Lopez-Atalaya JP, et al. The brain-specific tissue-type plasminogen activator inhibitor, neuroserpin, protects neurons against excitotoxicity both in vitro and in vivo. Mol Cell Neurosci 2005;30:552-8.
15. Yepes M, Lawrence DA. Tissue-type plasminogen activator and neuroserpin: a well-balanced act in the nervous system? Trends Cardiovasc Med 2004;14:173-80.
16. Yepes M, Sandkvist M, Coleman TA, et al. Regulation of seizure spreading by neuroserpin and tissue-type plasminogen activator is plasminogen-independent. J Clin Invest 2002;109:1571-8.
17. Yepes M, Lawrence DA. New functions for an old enzyme: nonhemostatic roles for tissue-type plasminogen activator in the central nervous system. Exp Biol Med (Maywood) 2004;229:1097-104.
18. Zhang Z, Zhang L, Yepes M, et al. Adjuvant treatment with neuroserpin increases the therapeutic window for tissue-type plasminogen activator administration in a rat model of embolic stroke. Circulation 2002;106:740-5.
19. Davis RL, Shrimpton AE, Carrell RW, et al. Association between conformational mutations in neuroserpin and onset and severity of dementia. Lancet 2002;359:2242-7.
20. Davis RL, Shrimpton AE, Holohan PD, et al. Familial dementia caused by polymerization of mutant neuroserpin. Nature 1999;401:376-9.
21. Hill RM, Coates LC, Parmar PK, Mezey E, Pearson JF, Birch NP. Expression and functional characterization of the serine protease inhibitor neuroserpin in endocrine cells. Ann N Y Acad Sci 2002;971:406-15.
22. Schrimpf SP, Bleiker AJ, Brecevic L, et al. Human neuroserpin (PI12): cDNA cloning and chromosomal localization to 3q26. Genomics 1997;40:55-62.
23. Hedrick JL, Nishihara T. Structure and function of the extracellular matrix of anuran eggs. J Electron Microsc Tech 1991;17:319-35.
24. Basbaum CB, Werb Z. Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. Curr Opin Cell Biol 1996;8:731-8.
25. Lindsay LL, Yang JC, Hedrick JL. Ovochymase, a Xenopus laevis egg extracellular protease, is translated as part of an unusual polyprotease. Proc Natl Acad Sci U S A 1999;96:11253-8.
26. Lindsay LL, Hedrick JL. Isolation and characterization of ovochymase, a chymotrypsin-like protease released during Xenopus laevis egg activation. Dev Biol 1995;167:513-6.

27. Rushlow C. Dorsoventral patterning: a serpin pinned down at last. Curr Biol 2004;14:R16-8.
28. Ligoxygakis P, Roth S, Reichhart JM. A serpin regulates dorsal-ventral axis formation in the Drosophila embryo. Curr Biol 2003;13:2097-102.
29. Hashimoto C, Kim DR, Weiss LA, Miller JW, Morisato D. Spatial regulation of developmental signaling by a serpin. Dev Cell 2003;5:945-50.
30. Ye S, Goldsmith EJ. Serpins and other covalent protease inhibitors. Curr Opin Struct Biol 2001;11:740-5.
31. Gettins PG. Serpin structure, mechanism, and function. Chem Rev 2002;102:4751-804.
32. Magklara A, Mellati AA, Wasney GA, et al. Characterization of the enzymatic activity of human kallikrein 6: Autoactivation, substrate specificity, and regulation by inhibitors. Biochem Biophys Res Commun 2003;307:948-55.
33. Obiezu CV, Diamandis EP. Human tissue kallikrein gene family: applications in cancer. Cancer Lett 2005;224:1-22.
34. Wu Q. Type II transmembrane serine proteases. Curr Top Dev Biol 2003;54:167-206.
35. Hooper JD, Clements JA, Quigley JP, Antalis TM. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. J Biol Chem 2001;276:857-60.
36. Szabo R, Wu Q, Dickson RB, Netzel-Arnett S, Antalis TM, Bugge TH. Type II transmembrane serine proteases. Thromb Haemost 2003;90:185-93.
37. Netzel-Arnett S, Hooper JD, Szabo R, et al. Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. Cancer Metastasis Rev 2003;22:237-58.
38. Hobson JP, Netzel-Arnett S, Szabo R, et al. Mouse DESC1 is located within a cluster of seven DESC1-like genes and encodes a type II transmembrane serine protease that forms serpin inhibitory complexes. J Biol Chem 2004;279:46981-94.
39. Behrens M, Bufe B, Schmale H, Meyerhof W. Molecular cloning and characterisation of DESC4, a new transmembrane serine protease. Cell Mol Life Sci 2004;61:2866-77.
40. Szabo R, Netzel-Arnett S, Hobson JP, Antalis TM, Bugge TH. Matriptase-3 is a novel phylogenetically preserved membrane-anchored serine protease with broad serpin reactivity. Biochem J 2005;390:231-42.
41. Yamada K, Takabatake T, Takeshima K. Isolation and characterization of three novel serine protease genes from Xenopus laevis. Gene 2000;252:209-16.
42. Gaultier A, Salicioni AM, Arandjelovic S, Gonias SL. Regulation of the composition of the extracellular matrix by low density lipoprotein receptor-related protein-1: activities based on regulation of mRNA expression. J Biol Chem 2006;281:7332-40.
43. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest 2001;108:779-84.
44. Strickland DK, Ranganathan S. Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. J Thromb Haemost 2003;1:1663-70.
45. Gonias SL, Wu L, Salicioni AM. Low density lipoprotein receptor-related protein: regulation of the plasma membrane proteome. Thromb Haemost 2004;91:1056-64.
46. Makarova A, Mikhailenko I, Bugge TH, List K, Lawrence DA, Strickland DK. The low density lipoprotein receptor-related protein modulates protease activity in the brain by mediating the cellular internalization of both neuroserpin and neuroserpin-tissue-type plasminogen activator complexes. J Biol Chem 2003;278:50250-8.
47. Barker-Carlson K, Lawrence DA, Schwartz BS. Acyl-enzyme complexes between tissue-type plasminogen activator and neuroserpin are short-lived in vitro. J Biol Chem 2002;277:46852-7.
48. Cinelli P, Madani R, Tsuzuki N, et al. Neuroserpin, a neuroprotective factor in focal ischemic stroke. Mol Cell Neurosci 2001;18:443-57.
49. Madani R, Kozlov S, Akhmedov A, et al. Impaired explorative behavior and neophobia in genetically modified mice lacking or overexpressing the extracellular serine protease inhibitor neuroserpin. Mol Cell Neurosci 2003;23:473-94.
50. Miranda E, Romisch K, Lomas DA. Mutants of neuroserpin that cause dementia accumulate as polymers within the endoplasmic reticulum. J Biol Chem 2004;279:28283-91.
51. van Rosmalen JW, Martens GJ. Cell type-specific transgene expression of the prion protein in Xenopus intermediate pituitary cells. Febs J 2006;273:847-62.
52. de Groot DM, Coenen, A.J.M., Verhofstad, A., van Herp, F., Martens G.J.M. In vivo induction of glial cell proliferation and axonal outgrowth and myelination by brain-derived neurotrophic factor. Molecular Endocrinology 2006; in press.
53. Barde YA, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. Embo J 1982;1:549-53.

54. Chao MV. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 2003;4:299-309.
55. Binder DK, Scharfman HE. Brain-derived neurotrophic factor. Growth Factors 2004;22:123-31.
56. Davies AM. Neurotrophins: neurotrophic modulation of neurite growth. Curr Biol 2000;10:R198-200.
57. Tucker KL. Neurotrophins and the control of axonal outgrowth. Panminerva Med 2002;44:325-33.
58. Cohen-Cory S, Lom B. Neurotrophic regulation of retinal ganglion cell synaptic connectivity: from axons and dendrites to synapses. Int J Dev Biol 2004;48:947-56.
59. Horch HW. Local effects of BDNF on dendritic growth. Rev Neurosci 2004;15:117-29.
60. Katoh-Semba R, Asano T, Ueda H, et al. Riluzole enhances expression of brain-derived neurotrophic factor with consequent proliferation of granule precursor cells in the rat hippocampus. Faseb J 2002;16:1328-30.
61. Zigova T, Pencea V, Wiegand SJ, Luskin MB. Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Mol Cell Neurosci 1998;11:234-45.
62. Benraiss A, Chmielnicki E, Lerner K, Roh D, Goldman SA. Adenoviral brain-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. J Neurosci 2001;21:6718-31.
63. Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 2001;21:6706-17.
64. Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog Neurobiol 2005;76:99-125.
65. Poo MM. Neurotrophins as synaptic modulators. Nat Rev Neurosci 2001;2:24-32.
66. Lu B. Acute and long-term synaptic modulation by neurotrophins. Prog Brain Res 2004;146:137-50.
67. Pang PT, Lu B. Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Res Rev 2004;3:407-30.
68. Mowla SJ, Farhadi HF, Pareek S, et al. Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J Biol Chem 2001;276:12660-6.
69. Mowla SJ, Pareek S, Farhadi HF, et al. Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 1999;19:2069-80.
70. Heymach JV, Jr., Shooter EM. The biosynthesis of neurotrophin heterodimers by transfected mammalian cells. J Biol Chem 1995;270:12297-304.
71. Fahnestock M, Michalski B, Xu B, Coughlin MD. The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease. Mol Cell Neurosci 2001;18:210-20.
72. Lee R, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. Science 2001;294:1945-8.
73. Egan MF, Kojima M, Callicott JH, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 2003;112:257-69.
74. Chen ZY, Patel PD, Sant G, et al. Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. J Neurosci 2004;24:4401-11.
75. Teng HK, Teng KK, Lee R, et al. ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 2005;25:5455-63.
76. Woo NH, Teng HK, Siao CJ, et al. Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 2005;8:1069-77.
77. Chao MV, Bothwell M. Neurotrophins: to cleave or not to cleave. Neuron 2002;33:9-12.
78. Lu B. Pro-region of neurotrophins: role in synaptic modulation. Neuron 2003;39:735-8.
79. Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat Rev Neurosci 2005;6:603-14.
80. Rattenholl A, Ruoppolo M, Flagiello A, et al. Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. J Mol Biol 2001;305:523-33.
81. Suter U, Heymach JV, Jr., Shooter EM. Two conserved domains in the NGF propeptide are necessary and sufficient for the biosynthesis of correctly processed and biologically active NGF. Embo J 1991;10:2395-400.
82. Althaus HH, Richter-Landsberg C. Glial cells as targets and producers of neurotrophins. Int Rev Cytol 2000;197:203-77.
83. Du Y, Fischer TZ, Clinton-Luke P, Lercher LD, Dreyfus CF. Distinct effects of p75 in mediating actions of neurotrophins on basal forebrain oligodendrocytes. Mol Cell Neurosci 2006;31:366-75.
84. Allen BG, Weeks DL. Transgenic Xenopus laevis embryos can be generated using phiC31 integrase. Nat Methods 2005;2:975-9.

85. de Rijk EP, Jenks BG, Vaudry H, Roubos EW. GABA and neuropeptide Y co-exist in axons innervating the neurointermediate lobe of the pituitary of Xenopus laevis--an immunoelectron microscopic study. Neuroscience 1990;38:495-502.
86. Wittkowski W. Tanycytes and pituicytes: morphological and functional aspects of neuroglial interaction. Microsc Res Tech 1998;41:29-42.
87. Virard I, Coquillat D, Bancila M, Kaing S, Durbec P. Oligodendrocyte precursor cells generate pituicytes in vivo during neurohypophysis development. Glia 2006;53:294-303.
88. Inoue K, Couch EF, Takano K, Ogawa S. The structure and function of folliculo-stellate cells in the anterior pituitary gland. Arch Histol Cytol 1999;62:205-18.
89. Borst JG, Lodder JC, Roubos EW, Kits KS. In situ recordings of presumed folliculo-stellate cells in the intermediate lobe of the pituitary gland of Xenopus laevis. Neurosci Lett 1996;209:61-4.
90. Mudrick-Donnon LA, Williams PJ, Pittman QJ, MacVicar BA. Postsynaptic potentials mediated by GABA and dopamine evoked in stellate glial cells of the pituitary pars intermedia. J Neurosci 1993;13:4660-8.
91. Allaerts W, Carmeliet P, Denef C. New perspectives in the function of pituitary folliculo-stellate cells. Mol Cell Endocrinol 1990;71:73-81.
92. Allaerts W, Vankelecom H. History and perspectives of pituitary folliculo-stellate cell research. Eur J Endocrinol 2005;153:1-12.
93. Pruginin-Bluger M, Shelton DL, Kalcheim C. A paracrine effect for neuron-derived BDNF in development of dorsal root ganglia: stimulation of Schwann cell myelin protein expression by glial cells. Mech Dev 1997;61:99-111.
94. McTigue DM, Horner PJ, Stokes BT, Gage FH. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci 1998;18:5354-65.
95. Chan JR, Cosgaya JM, Wu YJ, Shooter EM. Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci U S A 2001;98:14661-8.
96. Cosgaya JM, Chan JR, Shooter EM. The neurotrophin receptor p75NTR as a positive modulator of myelination. Science 2002;298:1245-8.
97. Hempstead BL, Salzer JL. Neurobiology. A glial spin on neurotrophins. Science 2002;298:1184-6.
98. Notterpek L. Neurotrophins in myelination: a new role for a puzzling receptor. Trends Neurosci 2003;26:232-4.
99. Tolwani RJ, Cosgaya JM, Varma S, Jacob R, Kuo LE, Shooter EM. BDNF overexpression produces a long-term increase in myelin formation in the peripheral nervous system. J Neurosci Res 2004;77:662-9.
100. Dubois-Dalcq M, Ffrench-Constant C, Franklin RJ. Enhancing central nervous system remyelination in multiple sclerosis. Neuron 2005;48:9-12.
101. Sherman DL, Brophy PJ. Mechanisms of axon ensheathment and myelin growth. Nat Rev Neurosci 2005;6:683-90.
102. Garbay B, Heape AM, Sargueil F, Cassagne C. Myelin synthesis in the peripheral nervous system. Prog Neurobiol 2000;61:267-304.
103. Allen NJ, Barres BA. Signaling between glia and neurons: focus on synaptic plasticity. Curr Opin Neurobiol 2005;15:542-8.
104. Stevens B. Glia: much more than the neuron's side-kick. Curr Biol 2003;13:R469-72.
105. Ullian EM, Christopherson KS, Barres BA. Role for glia in synaptogenesis. Glia 2004;47:209-16.
106. Freeman MR. Glial control of synaptogenesis. Cell 2005;120:292-3.
107. Haydon PG. GLIA: listening and talking to the synapse. Nat Rev Neurosci 2001;2:185-93.
108. Carmignoto G. Reciprocal communication systems between astrocytes and neurones. Prog Neurobiol 2000;62:561-81.
109. Araque A, Carmignoto G, Haydon PG. Dynamic signaling between astrocytes and neurons. Annu Rev Physiol 2001;63:795-813.
110. Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A. Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature 2003;426:74-8.
111. Newman EA. New roles for astrocytes: regulation of synaptic transmission. Trends Neurosci 2003;26:536-42.
112. Perea G, Araque A. Synaptic regulation of the astrocyte calcium signal. J Neural Transm 2005;112:127-35.
113. Perea G, Araque A. Glial calcium signaling and neuron-glia communication. Cell Calcium 2005;38:375-82.
114. Wright JW, Harding JW. The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning, and memory. Prog Neurobiol 2004;72:263-93.

115. Dityatev A, Schachner M. The extracellular matrix and synapses. Cell Tissue Res 2006.
116. Wang LC, Meijer HK, Humbel BM, Jenks BG, Roubos EW. Activity-dependent dynamics of coexisting brain-derived neurotrophic factor, pro-opiomelanocortin and alpha-melanophore-stimulating hormone in melanotrope cells of Xenopus laevis. J Neuroendocrinol 2004;16:19-25.
117. Kramer BM, Cruijsen PM, Ouwens DT, et al. Evidence that brain-derived neurotrophic factor acts as an autocrine factor on pituitary melanotrope cells of Xenopus laevis. Endocrinology 2002;143:1337-45.
118. Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci 2000;20:2003-10.
119. Nayeem N, Silletti S, Yang X, et al. A potential role for the plasmin(ogen) system in the posttranslational cleavage of the neural cell adhesion molecule L1. J Cell Sci 1999;112 (Pt 24):4739-49.
120. Pang PT, Teng HK, Zaitsev E, et al. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 2004;306:487-91.
121. Dirks RPH, Bouw, G., Van Huizen, R., Jansen, E.J.R., Martens, G.J.M. Functional genomics in Xenopus laevis: Towards Transgene-Driven RNA Interference and Cell-Specific Transgene Expression. Current Genomics 2003;4:699-711.
122. Li M, Rohrer B. Gene silencing in Xenopus laevis by DNA vector-based RNA interference and transgenesis. Cell Res 2006;16:99-105.
123. Nakano H, Amemiya S, Shiokawa K, Taira M. RNA interference for the organizer-specific gene Xlim-1 in Xenopus embryos. Biochem Biophys Res Commun 2000;274:434-9.
124. Jung KM, Tan S, Landman N, et al. Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. J Biol Chem 2003;278:42161-9.
125. Hutson LD, Bothwell M. Expression and function of Xenopus laevis p75(NTR) suggest evolution of developmental regulatory mechanisms. J Neurobiol 2001;49:79-98.
126. Sasai N, Nakazawa Y, Haraguchi T, Sasai Y. The neurotrophin-receptor-related protein NRH1 is essential for convergent extension movements. Nat Cell Biol 2004;6:741-8.
127. Kanning KC, Hudson M, Amieux PS, Wiley JC, Bothwell M, Schecterson LC. Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J Neurosci 2003;23:5425-36.
128. Roubos EW, Scheenen WJ, Jenks BG. Neuronal, neurohormonal, and autocrine control of Xenopus melanotrope cell activity. Ann N Y Acad Sci 2005;1040:172-83.
129. Jenks BG, Roubos EW, Scheenen WJ. Ca2+ oscillations in melanotropes of Xenopus laevis: their generation, propagation, and function. Gen Comp Endocrinol 2003;131:209-19.
130. Zhang J, Geula C, Lu C, Koziel H, Hatcher LM, Roisen FJ. Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. Exp Neurol 2003;183:469-81.
131. Elkabes S, DiCicco-Bloom EM, Black IB. Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 1996;16:2508-21.
132. Lambert WS, Clark AF, Wordinger RJ. Effect of exogenous neurotrophins on Trk receptor phosphorylation, cell proliferation, and neurotrophin secretion by cells isolated from the human lamina cribrosa. Mol Vis 2004;10:289-96.

Summary

An intriguing characteristic of the brain is its ability to continuously change its structural organization to adapt to new situations that may emerge from a change in developmental and environmental circumstances as well as from injuries. This adaptive capacity is called neuronal plasticity, and is for example reflected in the processes of learning and memory formation. Despite its obvious importance, the molecular mechanisms underlying neuronal plasticity are still far from being understood. In this thesis, we set out to explore functional aspects of two proteins that are thought to play a role in neuronal plasticity, namely the serine protease inhibitor neuroserpin and the brain-derived neurotrophic factor BDNF. In **chapter 1**, a brief outline is given concerning these two proteins. During development and in adult life, neuroserpin has been proposed to play a role in neurite outgrowth and synaptogenesis. BDNF regulates neuronal survival and differentiation, and modulates synaptic plasticity. In addition, BDNF appears to have more physiological functions than previously thought, *e.g.* it has been implicated in the formation of myelin and may play a role in the proliferation of neuronal cells. In our studies, we have used the well-defined, neuroendocrine intermediate pituitary melanotrope cells of the amphibian *Xenopus laevis* as a model system. These cells exert plasticity at both the cellular and neuronal level. *In vivo*, the melanotropes are responsible for the process of background adaptation of *Xenopus* by regulating the release of the hormone α-melanophore stimulating hormone (α-MSH), which causes pigment dispersion in skin melanophores. On a black background, the melanotrope cells are very active, producing vast amounts of proopiomelanocortin (POMC), the prohormone of α-MSH. In contrast, on a white background, cellular activity is inhibited by hypothalamic neurons that make direct synaptic contacts with the melanotrope cells. This inhibition results in biosynthetically inactive melanotropes that do not release α-MSH. Thus, the biosynthetic and secretory activity of these neuroendocrine

cells and their regulation by hypothalamic neurons can be manipulated in a physiological manner by placing the animal on a black or a white background.

Since neuroserpin has thus far been described only in a limited number of vertebrate species, we first set out to identify and clone this serpin in *Xenopus* (**chapter 2**). Subsequent studies revealed that throughout *Xenopus* development neuroserpin mRNA and protein are expressed, and in adult *Xenopus* predominantly in neuronal and neuroendocrine tissues. In addition, we found a high degree of amino acid sequence conservation, especially of the reactive centre loop, between the *Xenopus*, mammalian and chicken neuroserpin sequences, suggesting conserved substrate specificity. Thus, *Xenopus* neuroserpin may play an important physiological role as a serine protease inhibitor for proper neuronal and neuroendocrine cell functioning. To explore the role of neuroserpin in more detail, we examined neuroserpin expression in our model system, the neuroendocrine *Xenopus* melanotrope cells (**chapter 3**). We found that the expression of neuroserpin was linked to melanotrope cell activation. Specifically, neuroserpin mRNA and protein levels were upregulated in melanotrope cells of black-adapted animals, and neuroserpin formed a stable complex with an as yet unidentified target substrate, but only in the active cells. This target substrate may well be a serine protease exerting proteolytic activity in the extracellular matrix (ECM) of the melanotrope cell, and we thus speculate that by inhibiting the target, neuroserpin is involved in remodelling of the ECM during background adaptation and thereby plays a role in neuroendocrine plasticity.

To perform *in vivo* studies on the physiological role of neuroserpin, we generated transgenic *Xenopus* in which a POMC gene promoter fragment caused overexpression of neuroserpin or mutant neuroserpin(G392E) specifically in the melanotrope cells (**chapter 4**). The mutant neuroserpin(G392E) protein is linked to Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB), an autosomal dominant dementia. Analyses of the transgenic *Xenopus* showed that while overexpression of neuroserpin or neuroserpin(G392E) does not have a clear effect on the biosynthetic activity of the transgenic melanotrope cells, it does appear to affect the morphology of the melanotrope ECM. The mechanism presumably involves a prolonged inhibition of an extracellular target protease that regulates ECM integrity.

In the second part of this thesis, we have examined physiological aspects of pro- and mature BDNF. Although BDNF has been described extensively as a neuronal survival- and differentiation factor and as a modulator of synaptic plasticity, interest in the protein has increased over recent years, since pro- and mature BDNF have been found to play an even broader role in nervous system functionality. To investigate aspects of the role of pro- and mature BDNF in neuroendocrine plasticity, we generated transgenic *Xenopus* with overexpression of pro- and mature BDNF, and of a number of their variants specifically in the melanotrope cells (**chapter 5**). We observed that in various transgenic *Xenopus* lines with overexpression of proBDNF that the transgene product was not properly processed and secreted. Furthermore, in the proBDNF-transgenic melanotrope cells we detected

cellular stress and a block of transport of a number of secretory pathway proteins, similar to what others have observed in cells expressing a mutated form of human proBDNF. This mutation is a recognized polymorphism in the human *bdnf* gene that is associated with memory impairment and increased susceptibility to neuropsychiatric disorders.

In **chapter 6,** we analyzed transgenic *Xenopus* with melantrope cell-specific overexpression of mature BDNF. In the pituitary of these animals, we observed the formation of a nodule consisting of glial cells and axons of which a significant part was myelinated. Thus, mature BDNF may induce glial cell proliferation and axonal outgrowth and myelination, and as such is involved in neuronal plasticity and possibly glial cell physiology. A more detailed knowledge of these functional aspects of BDNF may prove to be useful for the development of therapeutic strategies against certain disorders of the nervous system, such as neurodegenerative and/or neurodevelopmental demyelination disorders.

Finally, in **chapter 7** the findings described in this thesis are summarized and placed into a broader perspective. Based on our results, we present two models proposing roles for neuroserpin and BDNF in neuroendocrine and neuronal plasticity, and a third model of an indirect interaction between the two proteins.

In conclusion, the results described in this thesis enhance our understanding of the physiological roles of neuroserpin and BDNF in neuroendocrine and neuronal plasticity. A better understanding of the complex molecular mechanisms underlying plasticity will ultimately improve our knowledge of brain functioning in health and disease.

Samenvatting

Summary in Dutch

Een fascinerende eigenschap van het brein is diens capaciteit om zijn structurele organisatie continu aan te passen aan nieuwe situaties. Deze nieuwe situaties ontstaan door veranderingen die optreden tijdens de ontwikkeling, in de omgeving, of door verwondingen. Dit aanpassingsvermogen wordt neuronale plasticiteit genoemd. Neuronale plasticiteit ligt bijvoorbeeld ook ten grondslag aan de processen van leren en geheugenvorming. Ondanks het enorme belang van deze plasticiteit voor het goed functioneren van het brein, zijn de moleculaire mechanismen die hiervan het uitgangspunt vormen nog lang niet geheel begrepen. In dit proefschrift staat het onderzoek beschreven dat is uitgevoerd naar de rol van twee eiwitten in het proces van neuronale plasticiteit, namelijk van de serine protease remmer neuroserpin en de neurotrofe groeifactor 'brain-derived neurotrophic factor' (BDNF). In **hoofdstuk 1** worden deze twee eiwitten kort geïntroduceerd. Neuroserpin is mogelijk betrokken bij de groei van zenuwuitlopers en de vorming van synapsen zowel tijdens de ontwikkeling als in een volwassen stadium. BDNF is belangrijk voor het overleven en de differentiatie van zenuwcellen, en speelt een modulerende rol bij synaptische plasticiteit. Daarnaast lijkt BDNF meer fysiologische functies te vervullen dan voorheen werd aangenomen; het is bijvoorbeeld ook betrokken bij de vorming van myeline en speelt mogelijk een rol bij de proliferatie van zenuwcellen. Voor ons onderzoek hebben we gebruik gemaakt van een goed gedefinieerd model systeem, namelijk de neuroendocriene melanotrope cellen van de hypofyse-middenkwab van de amfibie *Xenopus laevis*. Deze cellen vertonen zowel op cellulair als neuronaal niveau een grote mate van plasticiteit. *In vivo* zijn de melanotrope cellen verantwoordelijk voor het proces van achtergrondadaptatie van *Xenopus* doordat ze de afgifte van het α-melanofoor stimulerende hormoon (α-MSH) reguleren. α-MSH veroorzaakt de dispersie van pigment in huidcellen. Op een zwarte achtergrond zijn de melanotrope cellen zeer actief en

produceren grote hoeveelheden proopiomelanocortine (POMC), het prohormoon van α-MSH. Op een witte achtergrond daarentegen wordt de activiteit van de melanotrope cellen geremd door zenuwcellen uit de hypothalamus die direct contact maken met de cellen. Deze remming resulteert in biosynthetisch inactieve cellen die geen α-MSH meer afgeven. Dus de biosynthetische en afgifte activiteit van deze neuroendocriene melanotrope cellen en hun regulerende hypothalame zenuwcellen kunnen op een natuurlijke manier gereguleerd worden door het dier eenvoudigweg op een witte of zwarte achtergrond te plaatsen.

Aangezien neuroserpin tot nu toe alleen is beschreven in een beperkt aantal soorten vertebraten, zijn we begonnen om deze serine protease remmer in *Xenopus* te identificeren en kloneren (**hoofdstuk 2**). Daarop volgende studies hebben laten zien dat neuroserpin mRNA en eiwit tijdens de hele ontwikkeling van *Xenopus* tot expressie komen, en in een volwassen dier met name in neuronaal- en neuroendocrien weefsel. Daarnaast hebben we gevonden dat de aminozuursequentie van *Xenopus* neuroserpin, en met name de sequentie van de reactieve center loop, in hoge mate overeenkomt met die van neuroserpin in zoogdieren en kippen. Dit suggereert dat het substraat van neuroserpin geconserveerd is. Al met al zijn dit aanwijzingen voor een mogelijk belangrijke fysiologische rol van *Xenopus* neuroserpin als serine protease remmer voor het goed functioneren van neuronale en neuroendocriene cellen. Om de rol van neuroserpin in meer detail te onderzoeken, hebben we de expressie van neuroserpin ook onderzocht in ons modelsysteem, de *Xenopus* neuroendocriene melanotrope cellen (**hoofdstuk 3**). We hebben gevonden dat de expressie van neuroserpin gekoppeld was aan de activatie van deze cellen. Namelijk, neuroserpin mRNA en eiwit kwamen hoger tot expressie in melanotrope cellen van zwart-geadapteerde dieren. Daarnaast vormde neuroserpin specifiek in actieve melanotrope cellen een stabiel complex met een tot nu toe niet geïdentificeerd substraat. Dit substraat is mogelijkerwijs een serine protease met proteolytische activiteit in de extracellulaire matrix (ECM) van de melanotrope cel. We speculeren dan ook dat neuroserpin, door remming van dit substraat, betrokken is bij de herstructurering van de ECM gedurende de achtergrondadaptatie van *Xenopus* en zodoende een rol speelt in neuroendocriene plasticiteit.

Om *in vivo* studies uit te voeren naar de fysiologische rol van neuroserpin, hebben we transgene *Xenopus* gegenereert, waarin een POMC-gen promoter fragment zorgde voor een overexpressie van neuroserpin of mutant neuroserpin(G392E) specifiek in de melanotrope cellen (**hoofdstuk 4**). Het mutant neuroserpin(G392E) eiwit is betrokken bij een autosomale dominante dementie genaamd ‘Familial Encephalopathy with Neuroserpin Inclusion Bodies’ (FENIB). Analyses van de transgene dieren liet zien dat overexpressie van neuroserpin of neuroserpin(G392E) niet direct een effect had op de biosynthetische activiteit van de transgene melanotrope cellen, maar mogelijk wel op de morfologie van de ECM. Dit effect kan wellicht verklaard worden door een langere remming van een extracellulaire protease die de structuur van de ECM reguleert.

In het tweede deel van dit proefschrift hebben we fysiologische aspecten van pro- en matuur BDNF onderzocht. Alhoewel BDNF reeds uitvoerig is beschreven als een

neuronale overlevings- en differentiatiefactor, en als een modulator van synaptische plasticiteit, is de interesse in het eiwit de laatste jaren alleen maar toegenomen, omdat pro- en matuur BDNF een bredere rol lijken te hebben in het functioneren van het zenuwstelsel. Om aspecten van de rol van pro- en matuur BDNF in neuroendocriene plasticiteit te onderzoeken, hebben we transgene *Xenopus* gegenereert met overexpressie van pro- en matuur BDNF, en een aantal varianten hiervan, in de melanotrope cellen (**hoofdstuk 5**). We hebben gevonden dat in verschillende transgene lijnen met overexpressie van proBDNF het transgen product niet goed verwerkt en afgegeven werd. Daarnaast detecteerden we in proBDNF-transgene melanotrope cellen cel stress en een blokkade in het transport van verschillende eiwitten van de secretieroute. Deze bevindingen lijken op de resultaten van anderen, die gevonden zijn in cellen met overexpressie van een gemuteerde vorm van humaan proBDNF. Deze mutatie is een erkend polymorfisme in het humaan *bdnf* gen dat geassocieerd wordt met afwijkingen in geheugenvorming en een grotere vatbaarheid voor neuropsychiatrische aandoeningen.

In **hoofdstuk 6** hebben we transgene *Xenopus* geanalyseerd met overexpressie van matuur BDNF specifiek in de melanotrope cellen. In de hypofyse van deze dieren werd een nodus gevormd die bestond uit glia cellen en axonen, waarvan een aanzienlijk deel gemyeliniseerd was. Dus matuur BDNF induceert mogelijk de proliferatie van glia cellen en de uitgroei en myelinisatie van axonen, en speelt zodoende een rol in neuronale plasticiteit en glia cel fysiologie. Een gedetailleerde kennis van deze functionele aspecten van BDNF kan nuttig zijn voor de ontwikkeling van therapeutische strategieën voor bepaalde aandoeningen van het zenuwstelsel, zoals neurodegeneratieve en/of demyelinisatie aandoeningen tijdens de ontwikkeling.

Tenslotte worden in **hoofdstuk 7** de bevindingen die in dit proefschrift zijn beschreven samengevat en in een bredere context geplaatst. Gebaseerd op onze resultaten presenteren we twee modellen waarin een rol voor neuroserpin en BDNF in neuroendocriene en neuronale plasticiteit voorgesteld wordt, en een derde model betreffende een indirecte interactie tussen de twee eiwitten.

Samengevat, de resultaten beschreven in dit proefschrift vergroten onze kennis over de fysiologische rol van neuroserpin en BDNF in neuroendocriene en neuronale plasticiteit. Een beter begrip van de comlexe moleculaire mechanismen die ten grondslag liggen aan plasticiteit zal uiteindelijk onze kennis over het functioneren van het brein in ziekte en gezondheid vergroten.

Dankwoord

Acknowledgements

En dan nu het allerlaatste en waarschijnlijk meest gelezen onderdeel .....het dankwoord! Ik heb er heel lang naar uitgekeken, en nu is het dan eindelijk zover: míjn boekje is af! Terugblikkend op m'n AIO-tijd kan ik zeggen dat het vooral een hele leerzame en plezierige periode geweest is. Zoals voor velen zal gelden, is ook mijn promotietraject gepaard gegaan met de nodige bergen en dalen; van het euforische gevoel wanneer je eerste artikel gepubliceerd wordt tot aan het uithuilen wanneer je na een half jaar onderzoek absoluut nul resultaat hebt... Toch zijn ook de 'dalen' voor mij heel leerzaam geweest, waardoor het - achteraf gezien - misschien maar goed was dat ze er waren. En uiteindelijk is het allemaal helemaal goed gekomen! Dit niet in de laatste plaats door de steun van veel mensen. Graag wil ik dan ook iedereen die op de een of andere manier een bijdrage heeft geleverd aan mijn promotie hartstikke bedanken! Van deze mensen kan ik er een aantal niet onvermeld laten.

Op de eerste plaats Gerard, mijn promotor. Bedankt dat ik onder jouw hoede heb mogen promoveren, en voor je steun en vertrouwen die mij telkens toch weer de motivatie gaven om het daadwerkelijk tot een goed einde te brengen. Ik heb veel van je geleerd. François, bedankt voor je bijdrage aan mijn onderzoek en de gezellige samenwerking. Ik heb onze inhoudelijke discussies altijd erg gewaardeerd.

Daarnaast een groot woord van dank aan alle (ex-)moldiertjes: Eric, Karen, Jos, Jeroen, Jessica, Nick, Martine, Bart, Astrid, Gerrit, Rob, Marcel, Jacopo, Ron D., Rick, Ron E. en Tony, bedankt voor de gezelligheid, technische hulp en mentale bijstand tijdens en na het werk! Ik denk met veel plezier terug aan alle borrels, film-avondjes, kerstdiners en weekendjes Ardennen. Karel, het leven gaat verder, maar met je nagedachtenis waak je nog steeds over de netheid van het lab. Jij ook bedankt voor je bijdrage aan dit proefschrift.

Verder kan ik natuurlijk niet onze ‘buren’ van celbiologie en ‘overburen’ van cellulaire dierfysiologie vergeten. Bedankt voor de goede sfeer, alle gezelligheid en hulp bij experimenten! Dit geldt natuurlijk ook voor ‘mijn’ studenten die me vol enthousiasme geholpen hebben. Ties, Christine en Martin, bedankt voor jullie inzet.

Buiten de werkvloer zijn er veel mensen die (misschien onbewust) voor mij ook heel belangrijk zijn geweest om dit project te voltooien. Fleur, Koen, Douwe en Kelly, ontzettend bedankt voor jullie luisterend oor, adviezen en alle steun van de afgelopen jaren. Ik hoop dat we elkaar - ook in de toekomst - heel vaak zullen blijven zien! Hanneke, Kim, Stan, Vera en Manon, plus al jullie aanhang natuurlijk, altijd kon ik bij jullie m’n ei kwijt of konden we juist het werk helemaal achter ons laten en genieten van andere dingen... Heerlijk! Fleur en Stan, we kennen elkaar al behoorlijk lang en ik vind het super dat jullie mijn paranimfen willen zijn; we gaan er een hartstikke leuke dag van maken!

Lieve familie en schoonfamilie. Pap, mam, Marlon en Laurens, bedankt voor jullie liefde, onvoorwaardelijke steun en nimmer aflatende interesse. Het is niet altijd makkelijk geweest om precies uit te leggen waar ik de afgelopen 5 jaar toch steeds zo druk mee bezig was, maar ik hoop dat dit boekje een aardige indruk geeft! Laurens, nogmaals bedankt voor al je directe hulp wanneer ik weer eens ruzie had met de computer. Jan, Els, Liselotte, Annemieke en Jasper, jullie ook hartstikke bedankt voor alle steun, interesse en gezelligheid!

Tenslotte, Joris, mijn liefie! Wat ben ik blij dat we nu sámen in Amsterdam wonen; ik geniet er nog steeds iedere dag van. Bedankt voor al je liefde, steun en - heel belangrijk - relativeringsvermogen. Ik hoop dat we in de toekomst samen nog vele mooie avonturen mogen beleven. Hou van jou!

Liefs, Dorien

Curriculum vitae

Dorien Martine de Groot werd geboren op 24 september 1978 in Buchten. In 1996 behaalde zij *(summa cum laude)* haar Gymnasium diploma aan het College Sittard in Sittard, waarna ze in september startte met de studie (medische) Biologie aan de Radboud Universiteit Nijmegen (toen nog de Katholieke Universiteit Nijmegen geheten). Tijdens deze studie heeft ze stage gelopen op de afdeling Moleculaire Dierfysiologie (onder leiding van prof. dr. Gerard J.M. Martens) van de Radboud Universiteit Nijmegen, het 'Laboratoire de Neuroendocrinology Cellulaire et Moléculaire' (onder leiding van prof. dr. Hubert Vaudry) van de Universiteit van Rouen in Frankrijk, en de afdeling Celbiologie (onder leiding van prof. dr. Bé Wieringa) van het Universitair Medisch Centrum St. Radboud te Nijmegen. In augustus 2001 werd deze studie succesvol afgesloten met het *(cum laude)* behalen van het doctoraal diploma. In oktober 2001 startte Dorien als assistent in opleiding, en later als junior onderzoeker, met haar promotieonderzoek op de afdeling Moleculaire Dierfysiologie aan de Radboud Universiteit, onder leiding van prof. dr. Gerard J.M. Martens. Het onderzoek op deze afdeling naar functionele aspecten van de serine protease remmer neuroserpin en de neurotrofe groeifactor BDNF staat beschreven in dit proefschrift. De resultaten zijn ondermeer gepresenteerd op verschillende nationale en internationale congressen. Tevens werd in deze periode een bijdrage geleverd aan het biologie-onderwijs door het begeleiden van een aantal studenten en het practicum 'Ontwikkelingsfysiologie'.

List of publications

De Groot DM, Coenen AJM, Verhofstad A, van Herp F, Martens GJM, *In vivo* induction of glial cell proliferation and axonal outgrowth and myelination by brain-derived neurotrophic factor*, Molecular Endocrinology, 2006, 20: 2987-2998.*

De Groot DM, Pol C and Martens GJM, Comparative analysis and expression of neuroserpin in *Xenopus laevis, Neuroendocrinology, 2005, 82: 11-20.*

De Groot DM and Martens GJM, Expression of neuroserpin is linked to neuroendocrine cell activation*, Endocrinology, 2005, 146: 3791-9.*

Sicard F., de Groot DM, Gras M, Leprince J, Conlon JM, Roubos EW, Vaudry H, Delarue C., Neuroendocrine regulation of frog adrenocortical cells by neurotensin, *Ann N Y Acad Sci. 2005, 1040: 200-5.*