In Vivo Induction of Glial Cell Proliferation and Axonal Outgrowth and Myelination by Brain-Derived Neurotrophic Factor

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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 *X. transgenesis* in combination with the POMC gene promoter to generate transgenic frogs overexpressing BDNF specifically and physiologically in vivo, the animal is black adapted. We used stable *X. transgenesis* in combination with the POMC gene promoter to generate transgenic frogs overexpressing BDNF specifically and physiologically inducible in the melanotrope cells. Intriguingly, an approximately 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.* after activation of transgene expression. The severity of the phenotype depended on the level of transgene expression, because the intermediate pituitaries from transgenic animals raised on a white background or from transgenics with only an approximately 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. (*Molecular Endocrinology* 20: 2987–2998, 2006)

**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 PNS (12–14) and by oligodendrocytes after spinal cord injury in the CNS (15). In addition, the neurotrophin may be involved in the proliferation of neuronal cells, because 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

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Abbreviations: BDNF, Brain-derived neurotrophic factor; CNS, central nervous system; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HE, hematoxylin/eosin; LFB, Luxol Fast Blue; NIL, neurointermediate lobe; PNS, peripheral nervous system; POMC, proopiomelanocortin; SP, signal peptide.

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the melanotrope cells is regulated by hypothalamic neurons that directly contact the melanotrope cells and release various neurochemical messengers (reviewed in Refs. 22 and 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) have 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.

**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-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).

To examine the amount of steady-state BDNF protein in the two transgenic lines 6 and 73, lysates of the pituitary neurointermediate lobe (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 approximately 5- and 25-fold higher levels of mature BDNF expression compared with wild-type NILs, respectively (Fig. 2). BDNF transgene expression in lines 6 and 73 was melanotrope cell specific because 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). Because 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.

**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 corti-
cotropic cells of the pars distalis showed that the localization of these cells in line-6 transgenic animals was comparable to that in wild-type animals (Fig. 3, A and B). Remarkably, in line-73 animals we observed a nodule that was approximately 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 line-73 transgenic pituitaries (Fig. 3G). The melanotrope cells of lines-6 and -73 transgenic animals showed higher expression levels of BDNF than wild-type cells (Fig. 3, D–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 antivasotocin antibody (specific for vasotocinergic neurons of the pars nervosa) and an antiprolactin 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 (Fig. 3, H–M). However, occasionally a number of vasotocinergic-positive fibers were found among the scattered melanotrope cells in the nodules of line-73 transgenic animals (Fig. 3J).

To characterize the additional tissue that was observed in the nodules of line-73 transgenic pituitaries, paraffin sections were stained with hematoxylin and eosin (HE) and Luxol Fast Blue (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 a glial fibrillary acidic protein (GFAP) antibody, showing a clear and specific glial staining in the nodules of line-73 transgenic pituitaries (Fig. 4B).

Line-6 animals have a relatively low level of transgene BDNF expression in the melanotrope cells. Ini-

![Fig. 2. BDNF Protein Expression in the Pituitary NIL of Wild-Type and BDNF-Transgenic Xenopus](image)

Western blot analysis of 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.

![Fig. 3. Pituitary Histology of Wild-Type Animals and Transgenic Xenopus Overexpressing BDNF in the Intermediate Pituitary Melanotrope Cells](image)

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 POMC/ACTH (A–C), BDNF (D–F), vasotocin (H–J), and prolactin (K–M). G. Cryosection of the pituitary of a line-73 transgenic animal showing direct GFP fluorescence. The arrow in J indicates an example of vasotocin-positive fibers localized in the nodule of a line-73 transgenic pituitary. pn, Pars nervosa; pi, intermediate pituitary; pd, pars distalis; n, nodule. Scale bars, 200 μm (A and B) and 250 μm (C).
Initially, we did not detect a pituitary phenotype in these transgenic animals, and we therefore wondered whether a relatively long time of adaptation of these animals to a black background (and thus a relatively long 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 more than 9 months, a relatively long adaptation period because animals are normally adapted for 3 wk. Pituitaries of long-term black-adapted line-6 animals and 3-wk black-adapted wild-type and line-73 animals were then prepared for microscopy analysis. Interestingly, in sections of long-term black-adapted line-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).

Together, these results show that the relatively high transgene BDNF expression in the line-73 pituitaries resulted in the formation of a nodule that was 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.

**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 dis-
sected the brain and pituitary of wild-type, line-6, and line-73 animals under a stereomicroscope (Fig. 6, B–E). In line-6 transgenic animals that were adapted to a black background for 3 wk (a relatively short period of adaptation), the morphology of the NILs was comparable to that of wild-type NILs (Fig. 6, B 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; Fig. 6, A and E). In addition, in transgenic line-6 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, because 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 nodules of line-73 transgenic Xenopus Line 73 originated. Staining of paraffin sections of line-73 pituitaries with an antimesotocin or antivasotocin antibody revealed in the nodule the presence of mesotocinergic and vasotocinergic fibers from the pituitary pars nervosa (Fig. 6, F 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.

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 \textit{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 d after 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 d after fertilization) we found that the line-73 intermediate pituitary was slightly disrupted by infiltrating tissue (Fig. 7B and data not shown). This phenotype progressively further developed (Fig. 7C and data not shown) and, after metamorphosis (stage 66; ~50 d after 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.

Physiological Induction of the Nodule in Transgenic \textit{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 low expression of the BDNF transgene. After metamorphosis, paraffin sec-

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**Fig. 7. Development of the Nodule Phenotype in BDNF-Transgenic \textit{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 transgenic line-73 tadpoles were fixed for serial paraffin sectioning and immunocytochemistry for POMC/ACTH and BDNF. The locations of the intermediate pituitary (p), 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 \( \mu \text{m} \) (A–C) and 200 \( \mu \text{m} \) (D).
tions were made of the brain and pituitary, and immunocytochemistry for POMC and BDNF, and HE and myelin stainings were also 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 line-73 transgenic 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 approximately 8-fold higher in black- compared with white-adapted line-73 animals (data not shown). After 9 wk of black-background adaptation, infiltrating glial cells were observed that displaced the intermediate pituitary melanotrope cells of the transgenic animals. After 15 wk of adaptation, a nodule was formed with the melanotrope cells intermingled throughout the glial cells (Fig. 9). When the 15-wk 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.

**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 Refs. 1–5 and 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 Refs. 22 and 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 line-73 transgenic 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), because we detected characteristics of the nodule phenotype in line-6 transgenic animals expressing untagged proBDNF and generated other transgenic lines expressing GFP alone or noncleavable 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 have been 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 approximately 18-fold increase in GFAP+ cells in line-73 nodules compared with 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), [3H]-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

**Fig. 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 transgenic line-73 frogs that were raised on a white background (WA). Stainings were performed for POMC/ACTH and BDNF. Scale bar, 200 μm.
The 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. Because 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 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 p75NTR may be differentially expressed in various cranial nerves. Quantitative RT-PCR analysis of TrkB and p75NTR 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 p75NTR 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 p75NTR (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 p75NTR 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 X. laevis, endogenous MSH-containing intermediate pituitary cells are found in tadpoles from stage 35/36 onward (42), and melanotrope cell-specific GFP transgene expression driven by the POMC transgene promoter has been detected from stage 40 onward (29). Thus, in line-73 embryos the transgene expression of BDNF from stage 40 onward 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

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**Fig. 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 transgenic line-73 animals grown on a white background were adapted to a black background for 4, 9, or 15 wk (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 POMC/ACTH and BDNF. Of 9 wk 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.
dependent on the level of BDNF transgene expression. First, initially line-6 transgenic animals with an approxi-
mately 5-fold overexpression of BDNF did not show nodule formation, but the phenotype was induced fol-
lowing a long adaptation of the animal to a black background, i.e. after 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, nonaffected line-73 frogs were adapted to a black background for several weeks, hyperplastic glial cells infiltrating the interme-
diate pituitary were observed, eventually leading to the nodule. The formation of the nodule was irreversible, because 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.

MATERIALS AND METHODS

Animals

South African claw-toed frogs, *X. 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 3 wk. For long-term black adaptation, line-6 transgenic animals were kept on a black background for more than 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.*, Santa Cruz, CA). POMC and ACTH were detected with an anti-POMC/ACTH antibody (generous gift of Dr. E. Roubos, Radboud University, Nijmegen, The Netherlands; Ref. 43), vasotocin and me-
sotocin with an antivasotocin and antimesotocin antibody, respectively (generous gifts of Dr. F. Vandesande and Dr. L. Arckens, respectively, Catholic University Leuven, Belgium; Refs. 44 and 45), and prolactin with an antiprolactin antibody (generous gift of Dr. F. Mattheij, Wageningen University, The Netherlands; Ref. 46). GFAP was detected with a polyclonal anti-GFAP antibody (DAKO, Glostrup, Denmark), and tubulin was detected 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 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/HindIII* Xenopus *BDNF* POMC gene A promoter sequence (29), a 0.65-kb *Sal/HindIII* fragment of the cardiac actin promoter (kindly provided by Paul Krieg, Department of Cell Biology and Anatomy, University of Arizona Health Sciences Center, Tucson, AZ), the GFP sequence, and the HSV-tk polyA signal obtained by PCR on the pIRE2-EGFP vector (Clontech, Mountain View, CA). To clone the BDNF sequence, total RNA was isolated from rat brain tissue according to the manufacturer’s instructions (*Life Technologies, Inc.*, Carlsbad, CA). The RNA was dissolved in 20 μl ribonuclease-free H2O, and the concentration was mea-
sured with a GeneQuant RNA/OSA/2000 microprocessor (Pharmacia, New York, NY). For RT-PCR, one μg total rat brain RNA was reverse transcription into cDNA according to the manufacturer’s instructions (*Life Technologies, Inc.*, Carlsbad, CA). BDNF was amplified by PCR in a reaction mixture containing *Pfu* buffer (Fermentas, Hanover, MD), 200 μM deoxynucleoside triphosphates, 0.5 mm 5' primer (5'-ggggagacttgtagcccacagtgaagatggatgag-3'), 0.5 mm 3' primer (5'-ggggaattctgcaagactgtttttctggctc-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 *HindIII* and EcoRI and cloned behind the POMC gene A promoter fragment in the vector pPCG(A)2+, resulting in the pPCG(A)+BDNF vector. Because the rat and *X. laevis* protein sequences of mature BDNF are highly conserved (amino acid sequence identity is 94%) and, in view of the pituitary nodule phenotype of the BDNF-transgenic animals, the transgene mature BDNF product was likely recognized by the frog receptors. For the construction of the pPOMC(A)+-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 BamHI and EcoRI into the pCS2+-A vector, thereby gener-
ing pcS2+-A SP-GFP. Finally, the cytomegalovirus pro-
moter was interchanged with the POMC promoter (29) using the *Sal* and *HindIII* 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 at 95 C, 1 min 50 C and 1.5 min 72 C), using 10 ng pPCG(A)+BDNF vector as input DNA, and the primers 5'-ggggagcta-
tgcgcggccactgaagcaagcaacc-3' and 5'-ggggtctagagcgcag-
gcttttctg-3'. The amplified product was digested with EcoRI and XbaI and cloned in frame behind the sequence of GFP in the pPOMC(A)+-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 (*PerkinElmer, Wellesley, MA*).

Generation of Xenopus Transgenic for BDNF

To generate transgenic frogs, linear 3577-bp and 2546-bp *Sal*/NotI DNA fragments were generated from the pPCG(A)+BDNF and pPOMC(A)+-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 analyze protein expression in wild-type and transgenic pituitary NILs (consisting of the pars nervosa and intermedi-
ate 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 deoxy-
cholate, 1 μM phenylmethylsulfonyl fluoride, 0.1 mg/ml soybean trypsin inhibitor). To analyze 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 CaCl2, 2 mg/ml glucose, and 0.3 mg/ml BSA] 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 (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol 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 antibody, and the antibody dilutions used were 1:1000 for anti-BDNF and 1:500 for antitubulin. The equivalent of approximately 40% of one NIL or pars distalis was loaded per lane. Signals were visualized with a BioChemi Imaging System, and relative quantitation 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-7 transgenic animals and of white- and black-adapted line-73 animals were measured and related to an approximately 47-kDa nonspecific 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) to reveal peroxidase activity. For myelin staining, paraffin sections of the pituitaries of wild-type and line-73 transgenic pituitaries, freshly dissected NILs of long-term black-adapted line-6 animals, and 3-wk 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. Next, tissues were rinsed with acetone, embedded in epon:acetate, dehydrated, and embedded in epon:acetate, and embedded in epoxy. 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 six (wild-type) and nine (line 73) unit areas (80 × 80 μm) in two different sections of the intermediate pituitary and node of wild-type and line-73 animals, respectively. Because in line-73 animals the GFAP+ cells were not evenly distributed throughout the node, 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 with those of wild-type animals. An unpaired t test was used for statistical analysis.

Real-Time Quantitative RT-PCR Analysis

To examine TrkB and p75NTR 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 (Bio-Rad, Hercules, CA). The following primer sets were used to amplify TrkB, p75NTR, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), respectively: 5′-acctctacgctgcagacagc-3′ and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse primer for p75NTR, respectively) and 5′-gagtaactctgccatgaaa-3′ (forward and reverse prime
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