Regulation of brain-derived neurotrophic factor in the adult mouse brain

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by

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Abstract

In the adult central nervous system (CNS) brain-derived neurotrophic factor (BDNF) has been implicated in neuroprotection and synaptic plasticity among other functions. However, relatively little is known of its regulation. In this thesis, we attempted to learn more about BDNF regulation by means of: an *in situ* hybridization study of the four distinct untranslated exons in the adult mouse brain; use of transgenic animals to define BDNF promoter regions; and use of comparative genomics to identify evolutionarily conserved regions of BDNF. The *in situ* hybridization study suggests that the four distinct BDNF promoters are differentially regulated and that neighboring promoters are coregulated. Also it appears that all four promoters function in most of the same nuclei of the adult CNS. Inspite of the large size of the transgenic constructs used in this study specific to exons 1/2 and 3/4 (11.4 kb and 16 kb respectively), they were insufficient to mediate endogenous-like BDNF expression in the adult CNS.

However, this study suggests that these regions may drive endogenous-like expression in a subset of nuclei (random chance integration cannot however be ruled out). The bioinformatics study revealed 9 highly conserved elements that are good candidates for cis-regulatory elements of BDNF. In conclusion, the regulation of the BDNF gene appears far more complicated than was previously predicted.

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Chapter 1. Introduction

The neurotrophin family

Neurotrophic factors are a small family of dimeric, secreted proteins, that play important roles in the vertebrate peripheral and central nervous systems (Bibel and Barde, 2000; Davies, 1994; Huang and Reichardt, 2001; Lewin and Barde, 1996; Murer et al., 2001; Poo, 2001; Schinder and Poo, 2000). In mammals, four members of the neurotrophin family of molecules have been identified, namely, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These factors are highly related, both structurally and functionally. Neurotrophins function through two different classes of receptor, the high-affinity receptor tyrosine kinase (Trk) and the low-affinity p75 receptor, a member of the tumor necrosis factor (TNF) receptor superfamily. TrkA is the receptor for NGF, TrkB for BDNF and NT-4/5, and Trk-C for NT-3, although NT-3 was shown to activate TrkA and TrkB receptors in certain cell types. The central tenet of the neurotrophic factor hypothesis is that during the development of the vertebrate peripheral nervous system, targets of innervation secrete limiting amounts of survival factors, and hence neurons must compete for these neurotrophic factors (Levi-Montalcini, 1987). Neurons that cannot successfully compete for limiting neurotrophins die by apoptosis, and this results in a balance between the size of the innervating neurons and their target. It appears that all sensory neurons require the support of at least one neurotrophin during development and each neurotrophin supports the survival of distinct as well as overlapping groups of neurons (Davies, 1994). In addition to their functions in neuronal survival, neurotrophins were shown to regulate cell proliferation, neuronal differentiation, axon and dendritic outgrowth, synaptogenesis and neurotransmission (Bibel and

Barde, 2000; Davies, 1994; Huang and Reichardt, 2001; Lewin and Barde, 1996; Murer et al., 2001; Poo, 2001; Schinder and Poo, 2000).

BDNF Expression

Expression studies have shown that BDNF mRNA and protein is widely expressed throughout the peripheral and central nervous systems (Ernfors et al., 1992; Ernfors et al., 1990b; Furukawa et al., 1998; Katoh-Semba et al., 1998; Kawamoto et al., 1999; Maisonpierre et al., 1990; Yan et al., 1997). In line with the neurotrophic hypothesis, BDNF is expressed in the peripheral targets of BDNF-dependent sensory ganglia (Ernfors et al., 1990a; Ernfors et al., 1992; Maisonpierre et al., 1990) Also BDNF is expressed in the sensory ganglia themselves, but not until after their axons contact neurotrophins produced by the target tissue: it has been shown for adult DRG neurons that BDNF functions in an autocrine fashion to support the survival of a subpopulation of these neurons (Acheson et al., 1995). In the rat embryo, the BDNF mRNA level is relatively low and displays a dramatic rise between E11 and E12 which coincides with the onset of neurogenesis (peripheral and central) (Maisonpierre et al., 1990). BDNF reaches its maximal level around 2 weeks postnatally and remains high throughout adulthood (Furukawa et al., 1998; Katoh-Semba et al., 1998; Kawamoto et al., 1996; Maisonpierre et al., 1990; Yan et al., 1997). In addition to neurons throughout the adult brain, including the hippocampus, amygdala, cortex, olfactory bulb, thalamus, hypothalamus, and brainstem, BDNF appears to be expressed in both embryonic and adult neural progenitors (Barnabe-Heider and Miller, 2003). Notably, BDNF mRNA is absent from the septum and striatum. BDNF mRNA was shown to be highest in the hippocampus, and second highest in the cerebellum of the major brain regions (Maisonpierre et al., 1990). While BDNF is predominantly expressed in neurons, evidence suggests that BDNF is also weakly expressed in oligodendrocytes, astrocytes and microglia (Davies, 1994; Dreyfus et al., 1999; Furukawa et al., 1998; Kawamoto et al., 1999; Maisonpierre et al., 1990). It is also noteworthy, that in the adult animal it was

shown that there is abundant expression of BDNF by the visceral epithelia, where BDNF likely functions as a neuroprotective factor for adult peripheral neurons (Lommatzsch et al., 1999). In fact, BDNF levels in several regions including the urinary bladder, lung, colon and heart are significantly higher than in the brain.

BDNF clearly has a complex spatial and temporal expression pattern. Early in development, it is expressed in the peripheral targets of sensory neurons, while in the adult it is expressed in the sensory ganglia themselves. It is expressed both in embryonic and adult neural progenitors, and in neurons and glia throughout development. Furthermore, in the adult, it is expressed in numerous organs besides the brain at relatively high levels. This complex expression pattern points towards a complex regulation of BDNF.

Regulation of BDNF expression: calcium and the four BDNF noncoding exons

It appears likely that the complex organization of the BDNF gene, with its multiple promoters and transcripts is necessary for the numerous, widespread functions of this fascinating gene.

In the mouse, the BDNF gene spans close to 50kb of genomic DNA, and until recently was believed to be comprised of five exons, with exon 5 encoding the entire prepro-BDNF protein (Fig. 1) (Timmusk et al., 1993). The 5' flanking region of each of the first four untranslated exons has a putative promoter, and the 3' end of each exon has a splicing donor site. Exon 5 has a splicing acceptor site at its 5' end and two alternative polyadenylation sites at its 3' end. As a result of alternative splicing and use of polyadenylation sites, at least 8 different BDNF transcripts are possible. All BDNF transcripts appear to encode the same BDNF protein. Available evidence suggests that the untranslated exons serve as promoter-specific reporters, that is if a majority of transcripts obtained are of the exon 3-type, this means that promoter 3 is the major promoter being activated. A fifth untranslated exon of BDNF has only recently been identified and maps

about 700bp downstream of exon 2 (NCBI entry AY057907; (Hong et al., 2005)).

In the CNS, BDNF can be synthesized and secreted in an activity-dependent manner (Murer et al., 2001; Poo, 2001; Schinder and Poo, 2000). Stimuli that regulate BDNF expression include: visual and tactile sensory stimuli, seizures, hypoxia-ischemia, and certain electrical stimuli that induce long term potentiation (LTP) (Murer et al., 2001). Also a wide variety of hormones regulate BDNF expression (Murer et al., 2001). The calcium influx associated with synaptic activity induces the transcription of BDNF, along with numerous other genes, and is critical for both activity-dependent neuronal survival and long term potentiation (LTP) (West et al., 2001). Studies of cultured neurons, suggest that the major mechanism driving activity-dependent BDNF transcription is the influx of calcium through L-VSCC; while calcium entry through NMDA-Rs is a relatively inefficient means of BDNF induction (Ghosh et al., 1994; West et al., 2001). One hypothesis regarding the differential induction of the four major BDNF transcripts is that the timing, source and concentration of intracellular calcium determines which transcript(s) is expressed (West et al., 2001). So why does the entry of calcium through certain channels lead to activation of gene transcription, while calcium entry through other calcium channels does not? The prevailing hypothesis is that certain calcium channels are associated with specific signaling molecules, that allow them to couple calcium influxes with gene transcription (West et al., 2001). Interestingly, it has been shown that calcium entry through synaptic NMDA receptors induces BDNF gene expression; while calcium entry through extrasynaptic NMDA receptors activates a pathway that turns off CREB and blocks BDNF expression (Hardingham et al., 2002). The association of different signaling pathways with different L-VSCC, while appearing likely, has yet to be demonstrated. To date a role for the amplitude or kinetics of the intracellular calcium rise as opposed to the source of calcium in regulating BDNF expression has not been shown (Bradley and Finkbeiner, 2002).

After testing numerous physiological and synthetic transmitter substances, kainic acid, a glutamate receptor agonist was shown to be by far the most potent inducer of BDNF mRNA expression in vitro, and its potent effects were also observed in vivo (Zafra et al., 1990). The effects of kainic acid were shown to be mimicked by calcium ionophores, blocked by the voltage-gated L-VSCC antagonist, nifedipine, and mediated by the calcium/calmodulin-dependent protein kinase (Zafra et al., 1992). Since a variety of neurotransmitters and neuropeptides regulate cAMP, it is noteworthy, that forskolin, an activator of adenylate cyclase was shown to markedly potentiate the effects of kainic acid on BDNF mRNA, while forskolin alone had only a slight effect on BDNF expression (Zafra et al., 1992). It appears that a balance between glutamatergic and GABAergic systems regulates the levels of BDNF in the hippocampus, which is supported by the fact that bicuculline, a $GABA_A$ receptor antagonist, in addition to kainic acid increases BDNF mRNA levels in hippocampal neurons (Zafra et al., 1991).

Several reports have demonstrated similarities in the stimulus-temporal regulation of exons 1 and 2, as well as that of exons 3 and 4, suggesting that neighboring promoters share regulatory elements. Interestingly, exon 3 and 4 transcripts, unlike exon 1 and 2 transcripts were shown to be regulated by neuronal activity as immediate early genes, that is without the requirement for protein synthesis (Lauterborn et al., 1996). In response to kainate treatment, the expression timecourse of exon 1 is similar to that of exon 2, and the expression timecourse of exon 3 is similar to that of exon 4 in the hippocampus and cortex (Metsis et al., 1993). Furthermore, the negative effects of glucocorticoids on activity-dependent BDNF expression in the hippocampus are largely mediated by exon 1 and 2 transcripts as opposed to exon 3 and 4 transcripts (Lauterborn et al., 1998). Additionally, exercise appears to predominantly regulate exon 1 and 2 transcripts (Russo-Neustadt et al., 2000).

A number of studies have shown that the four main types of BDNF transcripts are differentially expressed in a stimulus-specific, brain region-specific, and developmental timepoint-specific manner. Most of these studies

have focused on regions of high synaptic plasticity, such as the hippocampus, neocortex, piriform cortex and amygdala, where all four exons are expressed. It has been shown that the exon1 transcript can be induced in the absence of exon 2 transcript induction and that the exon 1 and 2 transcripts can be induced by markedly different timecourses (Adlard et al., 2004; Gall et al., 2003; Russo-Neustadt et al., 2000; Tsukahara et al., 1998). Similarly, the exon 3 transcript can be induced with no or minimal effects on the exon 4 transcript (Berchtold et al., 1999; Tsukahara et al., 1998). An early report used quantitation by RPA of the 4 exons at different times in development in several different brain regions to show that the 4 exons have distinct regulation patterns (Timmusk et al., 1994). Another noteworthy in vivo study, used specific glutamate receptor antagonists to show that the four BDNF exons are differentially regulated in a brain region-specific manner by activation of different subtypes of glutamate receptors (Metsis et al., 1993). Of all the 4 major BDNF transcripts, exon 1 and 3 transcripts are the most highly upregulated and exon 4 is least upregulated in response to several seizure paradigms, including kainate, bicuculline, and pilocarpine-mediated seizures as well as certain insults such as ischemia and hypoglycemic coma (Kokaia et al., 1994; Metsis et al., 1993; Tetsuya Tsukahara, 1998). However, the regional expression pattern of exon 1 is apparently distinct from that of exon 3, and in the case of kainate seizures only the induction of exon 1 is blocked by the CamKII/IV inhibitor KN-62 (Murray et al., 1998). Furthermore, one study suggests that in rat cortical neurons, BDNF promoter 3 is responsive to calcium entry through either NMDA-R or L-VDCC; while promoter 1 is only responsive to calcium entry through L-VSCC (Tabuchi et al., 2000). This report implicates promoter 3 as opposed to promoter 1 in long term potentiation.

To date, BDNF promoters 1 and 3 are the best characterized (Chen et al., 2003; Shieh et al., 1998; Tabuchi et al., 2002; Tao et al., 1998; Tao et al., 2002). Using rat cortical cultures, calcium-responsive elements were found in distal and proximal regions of BDNF promoter 1, and both CREB and a USF family member was shown to bind in the proximal region, which appears to be largely accountable for promoter 1 activation (Tabuchi et al., 2002). In the case of

promoter 3, it was shown that KCL depolarization of cultured cortical neurons resulted in the induction of primarily exon 3-type BDNF transcripts via L-type VSCC (~80%), and each of the other BDNF transcripts was at least weakly induced (Shieh et al., 1998; Tao et al., 1998). Three calcium response elements (CaREs) were found within 80bp of the exon 3 transcriptional start site, and the transcription factors that bind these elements were identified (Chen et al., 2003; Shieh et al., 1998; Tao et al., 1998; Tao et al., 2002). Interestingly, a CREB family member was shown to bind the most proximal CaRE in a calcium-dependent manner, and CaM kinase IV was implicated in the signaling pathway upstream of CREB (Shieh et al., 1998; Tao et al., 1998). The calcium response element closest to the CREB binding site, is an E box and was shown to bind USF1/2 (Chen et al., 2003). The transcriptional activity mediated by the "ubiquitously" expressed USF1/2 was shown to be regulated by calcium signaling. The calcium responsive element 5' to the E box, was shown to bind a novel transcription factor, CaRF, which appears to function in a calcium and neuron-specific fashion (Tao et al., 2002). Mutational studies suggest that activation of all three promoter elements is necessary for the transcriptional activation of BDNF (Shieh et al., 1998; Tao et al., 1998). Additionally, recent studies have suggested that DNA methylation and related chromatin remodeling function in the activity-dependent expression of BDNF (Chen et al., 2003; Martinowich et al., 2003). Decreased methylation of several CpGs islands close to the 5' region of BDNF promoter 3 was correlated with the activity-dependent increase in BDNF expression (in cultured cortical neurons), and the decreased binding of the methyl – CpGs island binding protein MeCP2. It was shown, that following depolarization, the MeCP2 repressor is phosphorylated and dissociates from BDNF promoter 3, thereby allowing transcription to proceed.

There have been other elements identified with respect to the 4 BDNF promoters. One noteworthy study demonstrated that promoters 1 and 2 were regulated by a neuron-restrictive silencer element (NRSE), which is involved in the repression of basal and kainic acid-mediated expression from these promoters in certain populations of CNS neurons and has no effect on nonneural

cells (Timmusk et al., 1999). A zinc finger family member REST/NRSF has been shown to bind NRSE. As far as promoter 4 is concerned, CAM KII was shown to regulate the exon 4 transcript and the relevant cis-regulatory elements were identified (Takeuchi et al., 2002). Furthermore, C/EBP was shown to enhance promoter function and CaM KII increased C/EBP activity. MEKK and PKA were also shown to have an effect although CAM KIV had no effect on exon 4 expression. In addition to these elements, a motif resembling an estrogen response element was identified at the junction of the fourth intron and the fifth (coding) exon (Sohrabji et al., 1995).

It is hypothesized that the four major BDNF transcripts are differentially regulated not only at the level of transcription, but also at the level of mRNA stability and translatability on account of their differing 5' regions. In light of the fact that BDNF mRNA was shown to be transported into dendrites, it is also an interesting speculation, that BDNF transcripts tagged by the 4 different exons are differentially trafficked within the neuron (Tongiorgi et al., 1997). Recent evidence suggests that the exon 4 transcript is trafficked to the dendrites, in addition to being present in the soma, while the exon 3 transcript is only present in the soma (Pattabiraman et al., 2005). This suggests differential functions of the 5' UTRs of the two distinct transcripts.

The central rational for the present study is that the complex organization of the BDNF gene, with its multiple promoters and transcripts is necessary for the numerous, widespread functions of this fascinating gene.

Gene Regulation

The major control point of gene expression is at the level of transcription, and approximately 5-10% of the coding mammalian genome is believed to encode gene regulatory proteins. Unlike prokaryotic gene expression which often uses a single transcription factor for the initiation of transcription, eukaryotic transcriptional regulation is generally combinatorial: it involves the coordination of multiple transcription factors, integrates many signaling pathways and each

transcription factor contributes to the control of many genes (Carey M., 2000). Transcription factors possess an activating or repressing domain which interacts directly or indirectly with different components of the basal transcription machinery, described below, thereby activating or repressing transcription. They bind to DNA elements (~5-15bp) located in promoter, enhancer, or repressor regions, described below. A fascinating aspect of transcription factors is that while 2 factors may have low affinity for binding one another, they may cooperate to bind a DNA sequence that neither can bind alone, and form a surface to recruit other factors to create a complex that will function in stimulating transcription. Coactivators and corepressors are gene regulatory factors that do not interact with DNA, but assemble on DNA bound transcription factors.

A common core promoter consists of DNA sequences between –40 and +50 in relation to the transcriptional start site (Carey M., 2000). Core promoter elements: regulate the assembly of RNA Polymerase II, general transcription factors and coactivators; position the transcriptional start site; control the direction of transcription; and respond to activators and repressors. The preinitiation complex is comprised of general transcription factors, which includes TFIID, coactivators, corepressors as well as RNA Polymerase II. TFIID is special in that it is the only general transcription factor that binds the core promoter DNA specifically. TFIID is a multisubunit protein consisting of many TAFs and TBP. Other general transcription factors bind cooperatively with TFIID and one another to form a stable complex, known as a holoenzyme. Around 30bp upstream of the transcriptional start site is commonly found a TATA motif which binds TBP directly and appears to direct preinitiator complex formation and determine transcriptional start site location. The commonly occuring initiator element (Inr) overlaps the transcription start site, and appears to have a similar function as the TATA box. In TATA-less promoters, a commonly found element, approximately 30bp downstream of the initiation site is DPE, and it appears to function in specific transcriptional initiation. There are clearly promoters that lack TATA, Inr and DPE. Besides general transcription factors that constitute the holoenzyme, there are promoter-proximal transcription factors that bind at further

distances -100-200bp - upstream of the transcriptional start site. Some of these transcription factors are unregulated, ubiquitous, and increase the efficiency of transcriptional initiation. Others are regulated and responsible for tissue and time-specific transcription. Relatively more distal factors involved in transcriptional initiation bind elements known as enhancers. Enhancer or repressor elements are orientation independent regulatory elements that can be located upstream, downstream or even within a coding region. They are usually located close to the 5' end of the promoter, but can be located hundreds of kb away from the promotor. For example, the wing margin enhancer of the Drosophila cut locus resides 85 kb upstream of its promoter (Blackwood and Kadonaga, 1998).

The transcriptional regulatory region of a given gene often consists of many cis-regulatory element modules (Carey M., 2000). Available evidence suggests that each module serves a specific function, such as activation of its gene in a specific cell type or developmental timepoint, or in response to a specific stimulus. The same appears to be true of repressors except that they repress as opposed to enhance gene expression. Many diverse DNA-binding proteins - activator proteins - bind directly with sequences in the enhancer and in turn numerous coactivators interact with these proteins, and together these interact with components of the basal transcription machinery to form a large transcription complex at the promoter. In essence, activator proteins make the assembly of the holoenzyme energetically favorable. Activators (and repressors) function not only by acting directly on the basic transcriptional machinery, but also by altering the chromatin structure around the promoter, through covalent histone modifications and nucleosome remodeling. Many transcriptional activators and coactivators have histone acetyltransferase activity (while transcriptional corepressors have been shown to exhibit histone deacetylase activity) and ATP-dependent chromatin remodeling complexes. Thereby, transcriptional activators allow greater accessibility of DNA to general transcription factors and the RNA polymerase holoenzyme at the promoter as well as allowing for the binding of additional regulatory proteins.

While the precise mechanism of enhancer or repressor function has not

been elucidated, a popular model is known as “facilitated tracking” (Carey M., 2000). In this model, a complex of transcription factors and coactivators that binds the enhancer or repressor, “tracks” (takes small steps) along the chromatin until it reaches its promoter, at which point it forms a stable “loop structure”.

The murine immunoglobulin μ gene enhancer is a breakthrough in the area of gene regulation as it was the first enhancer to be discovered (Carey M., 2000; Ernst and Smale, 1995). Four major ideas regarding transcriptional regulation emerged from the study of this gene: genes are regulated by distant enhancers; an enhancer element contains multiple transcription factor binding sites; multiple mechanisms can achieve tissue-specific expression of a gene; and chromatin structure is important for gene regulation. For simplicity, only the rearranged Igμ gene will be discussed. The rearranged Igμ gene consists of a core promoter, a regulatory promoter, a proximal 250bp intronic enhancer and matrix attachment regions (MARS) – all common for a mammalian gene. The intronic enhancer together with the MARs is refered to as a locus control region or LCR. LCRs appear to function as both enhancers and insulators in that they mediate tissue-specific and physiological levels of expression on linked genes and activate transgene transcription in a position-independent, copy number-dependent fashion. The Igμ gene LCR is responsible for the B-cell specific expression levels and the pattern of a fully rearranged Igμ gene. The regulation of Igμ has been studied since 1983, and yet its transcriptional regulatory mechanisms remain unsatisfactorily defined. This can be explained by the vast array of proteins that is clearly required for appropriate Igμ expression and the large size of the transcription factor families of these proteins. Furthermore, there is considerable redundancy of elements within the Igμ gene enhancer. The enhancer contains both B-cell-specific elements and elements that appear to be necessary for optimal function of cell-specific elements, but do not contribute to cell-specific expression in and of themselves. The promoter and LCR appear to be sufficient for endogenous like levels and expression pattern in transgenic mice, although mutation of individual enhancer elements appear to only partially decrease enhancer activity. B-cell-specific transcription can be achieved either when the

Igμ promoter is attached to a heterologous enhancer or when the Igμ enhancer is attached to a non-endogenous promoter. To date, it has been shown that either of three enhancer elements contribute substantially to B-cell specificity of Igμ transcription, and a complex interplay between positive and negative regulators of Igμ expression was shown to restrict Igμ expression to B cells. Studies suggest that this gene serves as a good example of combinatorial gene regulation: it was suggested that B-cell specificity can be achieved by proteins that are each individually expressed in many other cell lines so long as the expression overlap of these proteins was unique to the B-cell lineage.

One of the best characterized complex mammalian regulatory regions is found in the human β-globin gene, which is only expressed in red blood cells at a specific developmental timepoint (Cao and Moi, 2002; Harju et al., 2002). A variety of activators and repressors regulate the expression of this gene. While some of the regulatory proteins are found cell-wide, others are present in relatively few cell types and hence are thought to contribute to the specificity of gene expression. The activities of many of the gene regulatory proteins are believed to vary during development and only a specific combination of these proteins appears to lead to gene transcription. The human β -globin gene is part of a globin gene cluster, which contains 4 other genes that are arranged spatially in the order of their expression during development: each gene is transcribed only at a specific developmental timepoint in erythroid cells of different organs. Each gene has a unique set of regulatory proteins that mediate its complex expression pattern, although promoters of all globin genes share considerable homology. TATA, CAAT and CACCC boxes were shown to be required for all globin gene expression. One breakthrough in the field was the discovery of transcription factors that bind with higher affinity to one globin promoter than another, hence preferentially activating that promoter. Together with the locus control region discussed below, they provide an explanation for stage specific expression of globin genes.

In addition to the unique regulation of each globin gene, the entire globin gene cluster is under the regulation of a locus control region (LCR). The β-globin

LCR lies 50kb upstream of the gene cluster and appears to regulate chromatin condensation. The LCR was discovered when DNAse1 sensitivity assays were performed to assess the chromatin conformation of the beta-globin locus. In cells where globin genes are not expressed, the chromatin is highly packed; meanwhile in erythroid cells, the chromatin is decondensed enabling the access of regulatory proteins to the DNA. The individual genes of the globin locus are developmentally regulated via regions approximately 500 bp 5' of their promoters, but position effects in transgenic mice are usually overcome and high levels of expression achieved, only by linkage to the LCR. In certain cases, data indicate that the developmental switch from one globin gene to another is regulated by promoter competition for the LCR. That is the γ and β-globin genes are developmentally regulated only when the LCR and both genes are present in the construct. Otherwise, innappropriate expression occurs for the β-globin gene at fetal stages and for the γ gene at adult stages. The ε globin gene is unique among the β-globin genes in that it maintains appropriate developmental regulation even when it is associated by itself with the LCR. Transgenic mouse experiments have shown that a 3.7 kb DNA fragment that contains the ε globin gene has activating sequences that lie within 200bp of the promoter and a silencer further upstream of the promoter and is sufficient for developmental expression of the ε globin gene.

Recently, there have been several studies that used minimal enhancer identification and mutational analysis approaches to identify key regulatory elements of the neurotrophins and their receptors. The strategy to identify a minimal enhancer in a live organism involves first identifying a large genomic fragment that faithfully reproduces the expression of the given gene in the transient transgenic organism of interest. Enhancer sequences are often highly conserved across species, and hence alignments for sequence homology (comparative genomics applied in chapter 5) can aid in the minimal enhancer search. The next step is to design progressively smaller fragments, based on the transient transgenic injection results of the prior fragment, such that you are able to "rule in" and "rule out" specific regions as containing or not the minimal

enhancer. Mutational analysis refers to mutating specific key elements within an enhancer of interest to determine their function. This may be based on a transcription factor identification program as discussed in chapter 5.

In our laboratory, Long et al. (2000) employed the minimal enhancer determination approach using mice to identify a minimal 457bp enhancer for TrkA, (the NGF receptor), 380bp upstream of the TrkA promoter (Ma et al., 2000). This minimal enhancer was sufficient to drive endogenous-like TrkA expression in sensory and sympathetic neurons. Further mutational analysis was performed based on consensus transcription factor binding sites, and this revealed the importance of these sites to TrkA expression (Ma et al., 2000). This minimal enhancer was highly conserved in mouse, human and chicken. Meanwhile, in the case of TrkB, deletional analysis in N2a mouse neuroblastoma cells was used to identify two alternative promoters for TrkB, P1 and P2 (Barettino et al., 1999). Using transient transfection of cortical neurons with TrkB luciferase constructs, calcium was shown to inhibit P1, but activate P2 (Kingsbury et al., 2003). Mutational analysis was used to show that calcium regulation of TrkB requires two adjacent, non-identical CRE sites within P2 (Kingsbury et al., 2003). CRE sites are binding sites for members of the CREB family of transcription factors, which were shown to function in calcium-dependent transcription of many genes including BDNF. As discussed in a previous section, deletional and mutational analysis of the region proximal to BDNF promoter 3 in cortical neurons identified three calcium response elements (within 80bp of the exon 3 transcriptional start site). Also, promoter analysis and mutational analysis of the region proximal to the exon 1 BDNF promoter identified two regions of calcium responsive elements. Due to the apparent complexity of BDNF expression and regulation, it is perhaps not surprising, that no study of the kind described for TrkA has yet been performed for BDNF. The transgenic study outlined below, and the studies discussed in this thesis take the first step towards identifying a genomic fragment that recapitulates some aspect of endogenous BDNF expression.

The transgenic approach

Transgenic animals have been the golden standard for studies such as this one, that strive to identify cis-acting elements responsible for the regulation of genes, that cannot be studied *in vitro*. It is inappropriate to use cultured neurons for this promoter-analysis study, since the BDNF regulatory elements of interest regulate BDNF expression throughout the brain. However, the complexity of BDNF regulation aside, it has been shown time and again that transgenes simply do not behave *in vitro* as they do *in vivo* (Jaenisch, 1988; Nagy a., 2003; Picciotto and Wickman, 1998). While many studies using transgenics have been successful, there are several caveats. Most cis-acting regulatory elements appear to reside close to the 5' end of the promoter, while others reside close to the 3' end or even in the gene itself. However, there are numerous examples of cis-acting elements functioning at long distances from their promoter (see above). Position effects are a major problem with transgenics (although fragments greater than 50kb suffer considerably less from these effects) (Jaenisch, 1988; Nagy a., 2003; Picciotto and Wickman, 1998). Position effects result from the random integration of a transgene into the mouse genome. Transcriptional enhancers and repressors in the vicinity of the transgene can have significant effects on its pattern and levels of expression. Furthermore, a transgene may integrate in the vicinity of a region of condensed chromatin or heterochromatin, resulting in transgene silencing. Yet another problem with transgenics, is that of the copy number of integrated transgenes, which usually ranges from 2 to 50. This often results in varying levels of expression in different founders generated from the same construct. Other inherent problems with the transgenic technique include rearangment of host sequences at the site of transgene integration, which can naturally disrupt host gene function, as well as mosaicism, which occurs when transgene integration occurs after the one-cell stage. In an attempt to ameliorate some of the problems described above in this study, a heterologous intron, and the chicken B-globin insulator were used in the transgenic constructs. Studies suggest that the inclusion of a heterologous intron in a transgenic construct can increase transcription levels by as much as 5- to 300-fold, which is not surprising

in light of recent research showing that introns can influence almost every step of mRNA metabolism (Choi et al., 1991; Le Hir et al., 2003). Insulators are a family of DNA elements that protect a given gene from inappropriate surrounding signals (West et al., 2002). Insulators such as the chicken β-globin insulator - that was used in this study- possess two distinct functions: firstly they block the action of an inappropriate enhancer or repressor on a promotor; and secondly they act as "barriers" to condensed chromatin near the gene of interest, hence preventing the silencing of the gene's expression. The mechanisms of insulator action are incompletely understood and the level of their beneficial properties appears to vary from transgenic to transgenic. Based on results of previous studies, the transgenics in the present study showed no obvious benefits of using a heterologous intron and insulator elements.

Previous transgenic study with BDNF promoter regions

There has so far been only one transgenic study, that attempted to define the promoter regions necessary for mediating brain-region specific and neuronal activity-induced expression of BDNF (Timmusk et al., 1995). Two transgenic constructs gave endogenous BDNF-like expression in the brain: the first construct (Exon 1+2-CAT) consisted of a fragment spanning from ~7kb of genomic sequence 5' of exon 1 to ~0.7kb of sequence 3' of exon 2 hooked up to a chloramphenicol acetyl transferase (CAT)-BDNF fusion protein; the second construct (Exon 3+4-CAT) spanned from ~6.5kb of sequence 5' of exon 3 to ~0.7kb of sequence 3' of exon 4 hooked up to CAT-BDNF. Both constructs had splice donor and acceptor sites for exon 5, 0.7kb of genomic fragment covering the 5' flanking region of exon 5, and the 3' UTR of BDNF, including endogenous polyadenylation signals. The exon 1/2 and exon 3/4–specific transgenic constructs used by Timmusk et al. were each longer on the 5' end by approximately 1kb, and shorter on the 3' end by several kb as compared to the constructs of the present study. In their study, they used CAT as a reporter for transgenic expression and showed that high levels of transgenic expression were present in the major brain regions of high endogenous BDNF expression, with

minor differences. CAT mRNA and endogenous BDNF mRNA were quantitated for several lines, and it was shown that transgenic mRNA was equivalent or higher than endogenous BDNF mRNA in the hippocampus. For most of the lines, transgenic expression appeared to be highest in the hippocampus of all brain regions, which is true of endogenous BDNF expression. Surprisingly, the Timmusk study showed that most founder lines for each of the constructs described above gave very similar expression patterns, suggesting the presence of an insulator-like element in their constructs. In the present study – unlike the study by Timmusk et al. – it was of interest to identify promoter regions that gave expression in the correct nuclei, not merely the correct major brain regions. In summary, this study shows that promoter regions similar in size to those used in the Timmusk study do not in fact give the faithful expression throughout the brain that may be expected based on the Timmusk study. Clearly, the reproducibly high levels seen in most major brain regions in the Timmusk study, were not obtained in the present study.

The authors of the Timmusk study, also tested smaller sized constructs that lacked the mini intron prior to exon 5. An exon 1-CAT construct that had ~2kb of sequence 5' of exon 1, and terminated at the Cla1 site within exon 1 was shown to be insufficient to drive brain-specific expression. An exon 2-CAT construct that had the same 5' end as exon 1-CAT, but extended ~1.2kb 3' also appeared insufficient to drive brain-specific expression. An exon 3-CAT construct that had ~4kb of sequence 5' of exon3, and terminated at the Xba1 site within exon 3 appeared to possess some elements needed for brain-specific expression. Finally, an exon 4-CAT construct with the same 5' end as exon 3-CAT, but extended ~1.1kb 3' also appeared to express some elements needed for brain-specific expression. Furthermore, it was shown that exon 4-CAT was sufficient to drive lesion-induced expression of CAT in the sciatic nerve. Kainate was shown to upregulate CAT mRNA in two out of two Exon 1+2-CAT lines, and 4 out of 4 Exon 3+4-CAT lines, as well as 2 out of 2 exon 4-CAT lines, but no CAT mRNA upregulation was seen in exon 1-CAT, exon 2-CAT or exon 3-CAT lines. Also spreading depression, caused by KCL depolarization of

cortical neurons was shown to increase CAT mRNA for lines of Exon 1+2-CAT, Exon 3+4-CAT, and exon 4-CAT. Interestingly, it was shown for Exon 1+2-CAT and exon 4-CAT that certain animals in which CAT was induced by kainate, CAT was not induced by spreading depression, suggesting that kainate and spreading depression elements are different. Moreover, it is noteworthy, that only 1 out of 6 Exon 1+2-CAT and 1 out of 4 Exon 3+4-CAT showed an increase in CAT activity upon kainate treatment. Perhaps this is due to some mechanism that functions at the translational level to prevent BDNF from reaching inappropriately high levels.

Hypothesis and Strategy

Aside from the studies described above, nothing is known of the molecular mechanisms regulating tissue-specific, developmental-timepoint specific or stimulus-specific expression of BDNF. The goal of the studies in this thesis was to learn more about the promoter regions that regulate the expression of BDNF in the mouse CNS, and possibly PNS as well. The hypothesis is that the four different BDNF promoters have distinct functions in different cell types as well as having distinct functions in the same cell type. At first, a detailed *in situ* hybridization study was conducted, to determine the expression of each promoter-specific exon in the adult mouse CNS. The conclusion of this study was that the BDNF untranslated exons are differentially expressed in the adult CNS, although the four exons are present for the most part in the same major brain regions. Furthermore, large transgenic constructs specific to the four BDNF promoters were designed, each with BDNF fused to the GFP reporter. The exon 1/2 transgenic constructs have ~6.2kb of 5' sequence (to the left of exon1), and ~3.5kb of 3' sequence (to the right of exon 2). The exon 3/4 transgenic constructs have ~5.5kb of 5' sequence (to the left of exon3), and ~9kb of 3' sequence (to the right of exon 4). Each of these constructs also contains a 5' heterologous intron, and all our constructs except the exon-3-specific construct contain insulators. As discussed above, these constructs are clearly different from the "large CAT"

constructs that appeared to give high endogenous-like brain expression in the study by Timmusk et al. (1995). However, the regions for the smaller CAT constructs in the study by Timmusk et al. are encompassed in the constructs of the present study, and hence based on that study, it was predicted, that at least the exon 3 and exon 4-specific constructs would contain brain-specific regulatory elements (the smaller Timmusk CAT constructs specific to exon1/2 did not appear to contain brain-specific elements). Unfortunately, none of the transgenic constructs of this study were sufficient to reproduce endogenous-like expression in transgenic animals.

Before embarking on the transgenic study, it was shown that the BDNF-GFP fusion protein was functional (Table 2, Fig. 12). Several different directions this study could take were considered. In the short-term, the plan was to do the following. First it was planned to characterize the transgenic mice in detail by *in situ* hybridization or preferably GFP immunohistochemistry. Once the lines were characterized, only lines with endogenous-like expression would be kept for further studies. If lines with faithful, and relatively high endogenous-like expression were obtained, it would be determined if the BDNF-GFP fusion protein is being made using Western blotting before proceeding with further studies, such as rescue experiments with BDNF mutant animals.

Bioinformatics study

With the increase in the amount of genome sequence data for a variety of species, comparative genomics, also referred to as phylogenetic footprinting is becoming a powerful tool for identifying the elements regulating gene expression in multicellular eukaryotes (Miller et al., 2004). This approach involves the alignment of multispecies, orthologous regulatory sequences to identify highly conserved elements. To date, at least partial genomic sequences are available for two types of pufferfish, the zebrafish, the frog, the rat, the mouse and the human. The underlying assumption of comparative genomics is that functional sequences (genes and cis-regulatory elements), are more conserved evolutionarily than

nonfunctional regions, as functional sequences tend to evolve much slower than nonfunctional sequences. Naturally, in vivo data is necessary to validate this approach. Comparative genomics was used successfully for identifying regulatory elements of genes that include ε-globin, γ-globin, cystic fibrosis conductance regulator, tumor necrosis factor-α and interleukins 4, 5 and 13 (Blanchette and Tompa, 2002).

Key to the success of using comparative genomics is the selection of appropriate species for comparison (Miller et al., 2004). About 40% of the human genome aligns with the mouse genome, and since only about 5 % of the human genome is believed to be functional (consist of coding regions and cis-regulatory elements), it appears that comparing mouse and human sequences for conservation, gives many regions of high conservation that are nonfunctional. While many alignments of mouse and human sequences have been highly informative, when mouse-human sequence conservation is too high and this alignment alone is not ideal, it is often useful to align the mouse sequences with more divergent organisms, such as the chicken, frog, or even the fish. In general, conserved sequences between mice and birds or say mice and frogs, are more likely to be functional than those between humans and mice. This is because most neutrally evolving sequences in say mice and birds will have diverged more than those between mice and humans. While the majority of conserved orthologous sequences between mice and fish are coding sequences, thousands of conserved sequences appear to be noncoding. Nevertheless, such comparisons will inevitably miss the many cis-regulatory elements not conserved over such vast phylogenetic distances. Since many processes during the development of the embryo are highly conserved in vertebrates, it is possible, that the cis-regulatory elements identified in comparisons of such divergent species as say mice and frogs, will be more likely to yield elements that function in embryonic development, than comparisons between more related species such as mice and humans.

rVISTA is one of the best tools for identifying transcription factor binding sites (TFBS) (Loots and Ovcharenko, 2004). The rVISTA algorithm consists of

the detection of TFBS matches in each sequence using the TRANSFAC database; the identification of locally aligned TFBS; and the selection of TFBS in highly conserved sequences. The TRANSFAC database is the most comprehensive transcription factor database. It uses position weight matrices (PWM) to describe the binding site of each transcription factor. The PWM has a matrix position for each base and each position in the transcription factor binding site that gives the probability of a given base occurring in a given position. Thus, each binding site receives a score based on the sum of the matrix probabilities. One obvious problem with the PWM and other available computational tools is that it is unable to find binding sites of unknown transcription factors. Also only a fraction of predicted TFBS are functionally relevant as many TFBS appear frequently in the genome. Another pitfall is that the PWM cannot take into consideration the fact that many transcription factors interact with each other and hence bind to DNA differently than if either transcription factor was binding alone. Furthermore, the PWM cannot account for the fact that the contribution of one position of a binding site may effect another position.

Comparing orthologous sequences of appropriate species is clearly a powerful method of identifying ECRs. rVISTA, inspite its limitations, can also be a very useful tool for guiding further studies. As discussed above, there are many examples where comparative genomics was successful in identifying gene enhancers. Within 100kb flanking regions of the BDNF gene, and the gene itself included, 7 ECRs between mouse, human and chick were identified, three of which are partially conserved in the frog. Three of these ECRs are within the BDNF gene itself, two of which are within the transgenic construct 3/4 of this thesis. Two ECRs are within the 3'UTR of BDNF, and another 2 ECR are relatively far upstream of exon 1 (25kb and 50kb respectively). Yet another two ECRs are conserved between the mouse, human and frog, but not the chick, and overlap with BDNF exons 1 and 3.

Chapter 2. Materials and Methods

Transgenic Constructs

In order to design BDNF promoter-specific reporter genes, a mouse 129/Sv bacteriophage λ genomic library was screened using probes based on the four untranslated BDNF exons and appropriately sized clones were isolated. Screening was performed according to the protocols in Current Protocols in Molecular Biology (Ausubel, 1995a). To maximize the chances of each clone having all the necessary regulatory elements for driving endogenous-like brain-wide expression of the exon of interest, clones with the largest 5' and 3' regions flanking each exon were selected. The selected clone that contains exon 1 and exon 2 is approximately 11.4kb, and the selected clone with exon3 and exon4 is approximately 16kb. The exon 1/2 transgenic constructs have ~6.2kb of 5' sequence, and ~3.5kb of 3' sequence. The exon 3/4 transgenic constructs have ~5.5kb of 5' sequence, and ~9kb of 3' sequence. For the exon 1 and exon 2-specific constructs, Pme1 adapters were cloned for transgene excision on either side of our exon-1/exon-2 genomic fragment in PBSK. The unique Cla1 site in exon 1 and the unique Apa1 site ~ 1kb downstream of the end of exon 2 were employed to design a PCR mutagenesis strategy to engineer a unique Asc1 site at the end of exon 1, just prior to the splicing signal (see Fig.1). This Asc1 site is used for cloning the exon-specific reporter. A similar strategy was used in the exon 2-specific construct to design a unique Asc1 site behind exon 2. For the exon 3 and exon 4-specific constructs, Xho1 transgene excision sites were engineered on either side of the exon 3/exon 4 genomic fragment in PBSK. For the purpose of cloning the exon-specific reporter behind exon 3 and exon 4 for exon 3 and exon 4-specific constructs respectively, two distinct PCR mutagenesis strategies were used to engineer a Sal1 site behind respective exons (see Fig. 2). In the case of the exon 3-specific construct a semi-unique Nhe1 site in exon 3 and a unique Asc1 site in exon 4 was used. For the exon 4-specific construct, the

unique Asc1 site within exon 4 and a unique Cla1 site ~ 1.5 kb downstream of exon 4 were used. Behind each exon, in each respective construct, an intron-BDNF-GFP cassette was cloned. The BDNF-GFP fusion has been described previously (Kojima et al., 2001). The rat insulin-II-intron A was kindly provided by Dr. Bob Hammer, Dallas, TX (Palmiter et al., 1991).

Generation of transgenic mice

The DNA for pronuclear injection was first digested with Pme1 in the case of exon 1 and exon 2-specific constructs, and with Xho1 in the case of exon 3 and exon 4-specific constructs. The DNA was purified from 0.4% agarose gels by electroelution using the protocol in Current Protocols in Molecular Biology (Ausubel, 1995b). The DNA was further purified using the Elutip kit from Schleicher and Schuell, according to manufacturers instructions, before using for pronuclear injection. Pronuclear injection and oviduct transplantation of injected embryos was performed as described previously (Nagy a., 2003). After injection of B6D2F1 eggs, the eggs were transferred into the oviducts of pseudopregnant CD2 female mice for development. Once pups reached 10 days of age, they were genotyped. Once transgenic pups reached breeding age, they were mated with CD1 mice. The transgenic progeny of this mating were screened by RT-PCR to determine if GFP mRNA was being synthesized. Transgenic littermates of all RT-PCR positive pups were screened by *in situ* hybridization to assess the expression pattern of GFP.

Genotyping of transgenic mice

Genotyping of transgenic mice was performed using the following PCR conditions: 94C, 30sec; 59C, 30sec; 72C, 1min; 35 cycles. The primers for GFP are: F5'-GAGCTGGACGGCGACGTAAAC-3' and R5'-CGTTGTGGCTGTTGTAGTTGTAC-3'

RT-PCR analysis of transgenic expression

RNA was isolated from the cortex and hippocampus regions combined using the Invitrogen TRIzol reagent according to the Invitrogen protocol. The SuperScript II Reverse Transcriptase kit from Invitrogen was used to synthesize cDNA. PCR for GFP was performed using the same conditions and primers described above. For the RT-PCR positive control the following beta actin primers were used: F5'-CCTAGGCACCAGGGTGTGAT-3' and R5'-TCACGGTTGGCCTTAGGGTT-3'. The same PCR conditions as for GFP were used. For RT-PCR of exons 1-5 in wild-type brains, the same PCR cycling conditions as described above and the following primers were used. Exon 1 primers used are: F5'-TTACCTTCCTGCATCTGTTGG-3' and R5'-TGTCCGTGGACGTTTACTTCT-3'. Exon 2 primers used are: F5'-AGCTCCGGGTTGGTATACTG-3' and R5'-TGTCCGTGGACGTTTACTTCT-3'. Exon 3 primers used are F5'-GAGCAGCTGCCTTGATGTTT-3' and R5'-ACACCTGGGTAGGCCAAGTT-3': Exon 4 primers used are: F5'-GACCAGAAGCGTGACAACAAT-3' and R5'-ACACCTGGGTAGGCCAAGTT-3'. Exon 5 primers used are: F5'-ACTGCAGTGGACATGTCTGG-3' and R5'-GATTGGGTAGTTCGGCATTG-3'.

***In situ* hybridization for expression study**

The Ambion MAXIscript kit was used to generate riboprobes for BDNF exons 1-5. For exons 1-4, the entire length of each exon was used to generate each corresponding probe. For exon 5 a 368bp probe that corresponds to the mouse BDNF gene region 185-552bp was used. Probes were labeled with [^{35}S]CTP as described in the Ambion MAXIscript manual. 7-9 week old male 129Sv mouse brains were dissected from unfixed animals and cryoembedded. 20uM thick frozen coronal and sagital brain sections were cut on a cryostat at –19ºC. The *in situ* hybridization protocol of Nef S. et al. (1996) (Nef et al., 1996) was followed with some modifications, as described below. Cryostat sections were first incubated for 1 hour at 37ºC and then fixed in 4% paraformaldehyde (in 1X PBS) for 30 min. Sections were then rinsed 3 X 5 min

in 1X PBS followed by an incubation with 1ug/ml proteinase K at 37ºC for 15 min. Sections were fixed again in 4% PFA for 10 min, washed once in 0.1M triethanolamine (pH8), and then acetylated by treating in 1ml of acetic anhydride in 400ml of 0.1M triethanolamine (pH8) for 10 min. Sections were washed 2 X 5 min in 1X PBS and dehydrated in ethanol. Sections were next hybridized with 30000 cpm/ul of probe in hybridization solution (0.3M NaCl, 0.02M Tris-HCL (pH 8), 5mM EDTA, 10% Dextran sulfate, 1X Denhardt's, 0.5ug/ml tRNA, 0.1M DTT, 50% formamide) for 16 hours at 60ºC. Post hybridization, sections were washed in 1.4g DTT/Liter of 4X SSC for 4 X 15 min at room temperature. Next, sections were washed with stringent solution (0.15M NaCL, 0.02M Tris-HCL (pH 7.5), 5mM EDTA, 0.1M DTT, 50% formamide) for 30 min at 60ºC. Sections were rinsed in RNAse buffer (0.04M NaCl, 1mM Tris-HCL (pH 7.6), 0.5mM EDTA) and then treated with 20ug/ml RNAse Type II-A (Sigma) in RNAse buffer for 15 min at 37^{0}C. Sections were rinsed in 2X SSC and then washed in 2X SSC for 15 min at 60ºC, followed by a wash in 0.1X SSC for 15 min at 60ºC. Finally, sections were dehydrated in ethanol. Once dry, sections were emulsion coated. Sections for exons 1-5 were developed at 9,10,8,8,and 10 days respectively. With each probe, *in situ* hybridization was performed on coronal sections from 2 brains. *In situ* hybridization was performed on sagital sections using 5 brains in total, and for each brain, probes for BDNF exons 1-5 were hybridized on consecutive sections. For each probe, signal intensities in each brain region are reported as relative labeling of cells with silver grains, from very high (++++) to absent (-).

In situ hybridization for GFP transgenics

The same *in situ* hybridization protocol as described above was used. The GFP riboprobe corresponds to bases 651-930 of the Clontech pEGFP map.

Materials and Methods for Bioinformatics

NCBI

NCBI was used to locate BDNF on the mouse chromosome and identify known neighboring genes. It was also used to pull out sequences for constructs in Figs. 9 and 10.

Ensembl

Ensembl was used to extract orthologous sequences for mouse, human, chicken, frog and zebrafish BDNF (Ovcharenko et al., 2004b). The extracted sequences spanned 100kb on either side of the BDNF gene.

zPicture

zPicture (a program based on blastZ) was used for pairwise alignments of sequences for mouse, human, chicken, frog and zebrafish. The zPicture settings used were at least 70% homology and a minimum length of 100 bp or more. These criteria have been used in previous successful studies (Ovcharenko et al., 2004a).

rVISTA

rVISTA was used to identify putative transcription factor binding sites in the chosen evolutionarily conserved region. The standard 0.85 matrix similarity setting was used (Loots and Ovcharenko, 2004).

Chapter 3. Expression of four untranslated BDNF exons in the adult mouse brain

Introduction

Given the scope of BDNF function and the breadth of its expression, it is likely that the complex organization of its promoters and untranslated regions are crucial for BDNF regulation. The organization of the BDNF gene is shown in figure 9. The present study was designed to shed light on the potential functions of the four untranslated BDNF exons through the determination of their expression patterns in the adult mouse brain. This study provides the first detailed analysis of the four BDNF untranslated exons throughout the adult mouse brain under baseline conditions. The strategy was to use radioactive *in situ* hybridization, which is a powerful tool, that is more sensitive than non-radioactive *in situ* hybridization and has been used successfully for studying the expression of a wide variety of genes. This study supports previous work, and provides insight into the regulation of BDNF.

Results

To examine the expression of BDNF exons 1-5 in the adult mouse brain, radioactive *in situ* hybridization was performed on adjacent 20 micron cryostat sections with exon specific probes.

This study focused on brain regions expressing moderate to high levels of the translated BDNF exon 5. Table 1 summarizes the results of this study. Overall, it was found that while all BDNF promoters function widely, the relative activity of each promoter can differ substantially between brain regions. The specificity of the probes is supported by the fact that the sum of the expression patterns for the four untranslated exons appears to correspond to that of BDNF

exon 5. Regions such as the striatum, septum and arcuate nucleus (three regions which do not express BDNF exon 5) serve as good negative controls.

Telencephalon

Hippocampus

BDNF exon 5 expression is high in all regions of the hippocampus (Fig. 2, Fig. 3).

As shown in Figures 2 and 3, exon 1 and 2 expression is highest in the CA3 region and lowest in the CA1 and CA2. Exons 3 and 4 apparently account for the majority of BDNF expression in the CA1 and CA2 in addition to being high in the CA3. In the dentate gyrus, exons 3 and 4 account for the majority of expression while exons 1 and 2 are expressed at relatively low levels and apparently in only a subset of cells. Thus, in the hippocampus, BDNF expression is high, but regional expression is differentially regulated.

Cerebral cortex

BDNF exon 5 expression in this region is moderate in layers 2-3 and 5-6, and below detection in layer 4 (Fig. 2, Fig. 4). Hybridization to exons 1-4 is present in the same layers as that to exon 5, however the relative levels of signal are distinct (Fig. 2, Fig. 4). Exons 1 and 2 are apparently expressed in a relatively small subset of cortical neurons while Exon 3 and 4 signal reflects a very similar pattern to that of exon 5. Hence, this region provides another example of differential exon expression.

Piriform cortex and Amygdala

Exon 5 expression is moderately high in the piriform cortex (Fig. 5). Hybridization to exons 1-4 is graded as equivalent in this region, however, as compared to exons 2-4, exon 1 signal is apparently present in a smaller subset of cells and consists of higher intensity puncta.

Exon 5 expression is moderate in the basomedial, basolateral, and medial amygdaloid nuclei (Fig. 5, data not shown). Hybridization to exons 1-4 is graded as equivalent in all three nuclei. Exon 2-4 signal closely mimics the pattern of exon 5 expression, however exon 1 signal is apparently present in a smaller subset of cells and consists of higher intensity puncta.

Olfactory bulb

Exon 5 expression is low in the mitral, granule and periglomerular layers (Fig. 6). Hybridization to exons 1-4 is graded low in all these regions and mimics the expression pattern of exon 5. However, exon 1 appears to be the least dominant species in these regions.

Septum/ striatum

These negative control regions shows no expression of exons 1-5 (Fig. 2, data not shown).

Diencephalon

Thalamus

Exon 5 expression is moderate high in the paraventricular thalamic nucleus (Fig. 7a). Exons 1, 2 and 4 appear to be the dominant species in this region, while hybridization of exon 3 is relatively low. Exons 1 and 2 appear to be present in a smaller subset of cells than exons 3 and 4. This is apparently true for all the examined thalamic nuclei. Exon 5 expression in the central medial thalamic nucleus is moderate. In this nucleus, exons 2 and 4 appear to be the dominant species, while exons 1 and 3 are expressed at relatively low levels (Fig. 7a). Exon 5 expression is moderate in the parafascicular thalamic nucleus (Fig. 7b). Exon 4 apparently accounts for the vast majority of expression in this nucleus, and mimics the pattern of exon 5 expression. Exon 5 expression is moderate in the anterodorsal thalamic nucleus (data not shown). Exon 4 appears to be the dominant species in this nucleus. Exon 2 expression is significantly higher than

that of exons 1 and 3 in this nucleus. Hence, all the examined thalamic nuclei show differential exon expression.

Hypothalamus

Exon 5 expression is moderate in most examined hypothalamic nuclei, with the exception of the lateroanterior hypothalamic nucleus, the premammillary nucleus and the medial mammillary nucleus, where expression is moderately high (Fig. 8, data not shown). Exons 1-4 are all expressed in each of the hypothalamic nuclei examined (Fig. 8, data not shown). Exon 3 is apparently the least abundant species in all examined nuclei. Exon 1,2, and 4 expression levels are graded as equivalent in the medial preoptic area, paraventricular nucleus, ventromedial nucleus, and posterior hypothalamic area. However, exons 1 and 2 are apparently present in a smaller subset of cells than exons 3 and 4. This is apparently true of all examined hypothalamic nuclei. In contrast to the other nuclei, in the lateroanterior nucleus, exon 4 expression is lower than that of exons 1 and 2. Exons 2 and 4 appear to be the dominant species in the premammillary nucleus and the medial mammillary body. Hence, all nuclei of the hypothalamus show differential exon expression.

Mesencephalon

Exon 5 expression is moderate in the periaqueductal gray area, the only mesencephalic region that was examined (data not shown). Exons 1 and 2 appear to be the dominant species in this region. Exon 4 is present at much lower levels, but apparently in a larger subset of cells than exons 1 and 2. Exon 3 is below detection levels in this region. As all the regions above, the periaqueductal gray region shows differential exon expression.

Rombencephalon

Cerebellum

Exon 5 expression is moderate in the cerebellar granule cell layer (Fig. 9). Exon1 is absent in this region, while exons 2-4 are expressed at equivalent levels in a pattern that mimics exon 5 expression. This is yet another region of differential exon expression.

Conclusion

The regulation of the four major BDNF transcripts is clearly highly complicated. The differential expression of the four untranslated exons in multiple brain regions suggests that distinct signaling pathways function upstream of the four BDNF promoters. Besides providing evidence for the differential regulation of each of the four promoters, this study implies the coregulation of neighboring promoters. It also suggests that all four BDNF promoters function in most of the same nuclei of the adult brain.

Area	BDNF Ex5	Ex1	Ex2	Ex3	Ex4
Telencephalon					
Hippocampus:					
CA1	++++ po	+ p	+ po	++++ po	++++ u
CA2	++++ po	+ p	+ po	++++ po	++++ u
CA3	++++ po	++++ p	++++ po	++++ po	++++ po
DG	++++ u	++ po	+++ u	++++ u	++++ u
Cerebral cortex:					
Layers II-III (somatosens.)	++ po	+ p	+ po	++ po	++ po
Layers V-VI (somatosens.)	++ po	+ p	+ po	++ po	++ po
Amygdala:					
Medial amygdaloid nu.	++ po	++ p	++ po	++ po	++ po
Basomedial nu.	++ po	++ p	++ po	++ po	++ po
Basolateral nu.	++ po	++ p	++ po	++ po	++ po
Piriform cortex	+++ po	+++ p	+++ po	+++ po	+++ po
Olfactory bulb:					
Periglomerular layer	+ u	+ u	+ u	+ u	+ u
Mitral cell layer	+ u	+ u	+ u	+ u	+ u
Granule cell layer	+ u	+ u	+ u	+ u	+ u
Striatum	-	-	-	-	-
Diencephalon					
Thalamus:					
Paraventricular nu.	+++ po	+++ p	+++ po	++ po	+++ po
Central medial thalamic nu.	++ po	+ p	++ po	+ po	++po
Parafascicular nu.	++ po	+ p	+po	+ po	+++ po
Anterodorsal nu.	++ po	+ p	++ po	+ po	+++ po
Hypothalamus:					
Medial preoptic area	++ po	++ p	++ po	+ po	++ po
Paraventricular nu.	++ po	++ p	++ po	+ po	++ po
Lateroanterior hypoth. area	+++ po	+++ p	+++ po	+ po	++ po
Ventromedial hypoth nu.	++ po	++ p	++ po	+ po	++ po
Arcuate nu.	-	-	-	-	-
Posterior hypoth. area	++ po	++ p	++ po	+ po	++ po
Premammillary nu.	+++ po	+ u	+++ po	+ u	+++ po
Medial mammillary nu.	+++ po	+ u	+++ po	+ u	+++ po
Mesencephalon					
Periaqueductal gray	++ po	++ p	++ po	-	+ u
Rombencephalon					
Cerebellum:					
Granule cell layer	++ u	-	++ u	++ u	++ u

Table 1. Summary of *in situ* hybridization data. For each probe, signal intensities in each brain region are reported as relative labeling of cells with silver grains, from high (++++) to absent (-). "p" refers to punctate expression with no underlying lower grade signal. "po" refers to punctate expression with underlying lower grade expression. "u" refers to uniform expression.

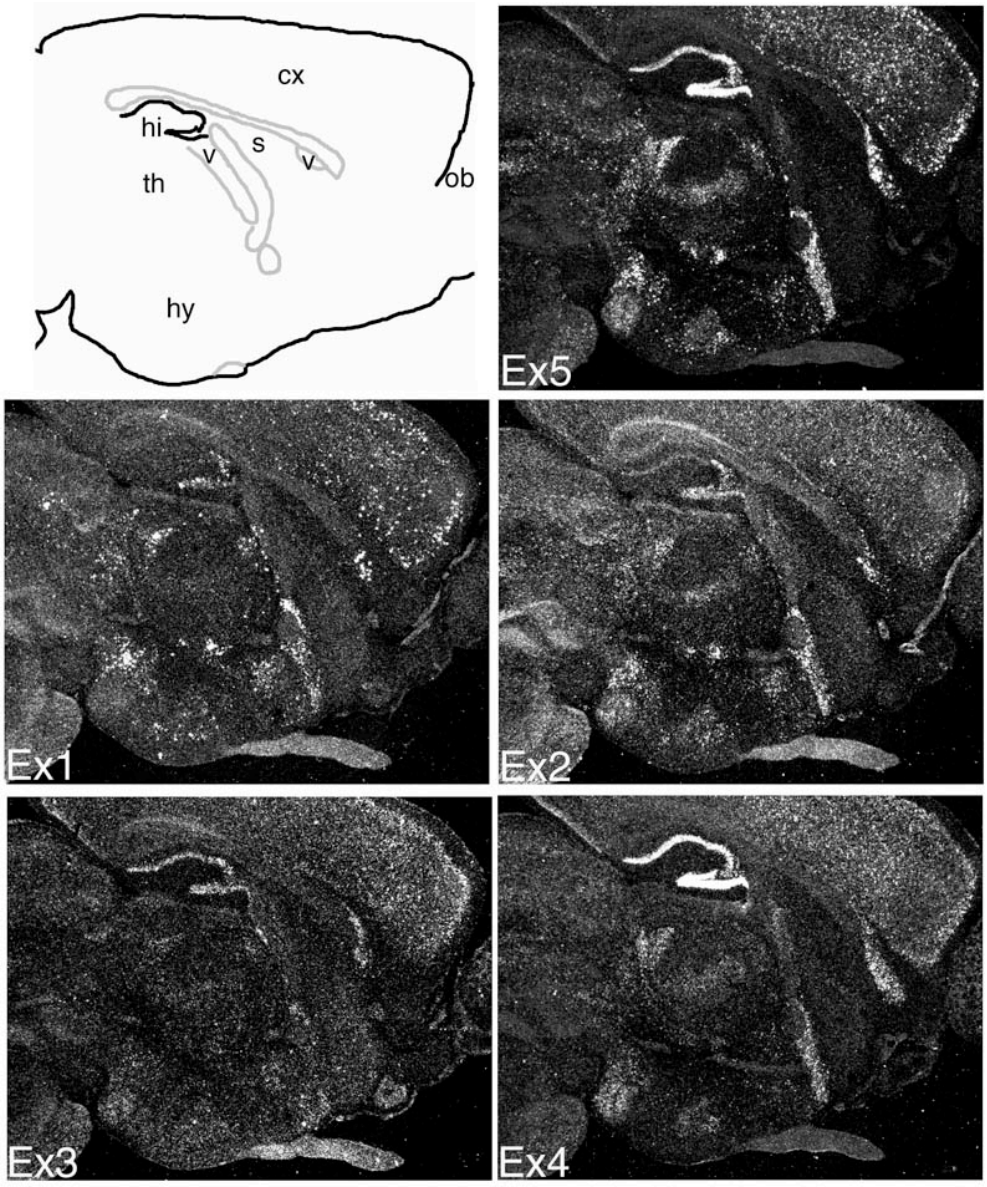

Fig. 1a. Sagittal sections showing the expression of BDNF exons 1-5 by *in situ* hybridization. Hi, hippocampus; cx, cortex; s, septum; th, thalamus; hy, hypothalamus; ob, olfactory bulb; v, ventricle. Mice are 7-9 weeks of age.

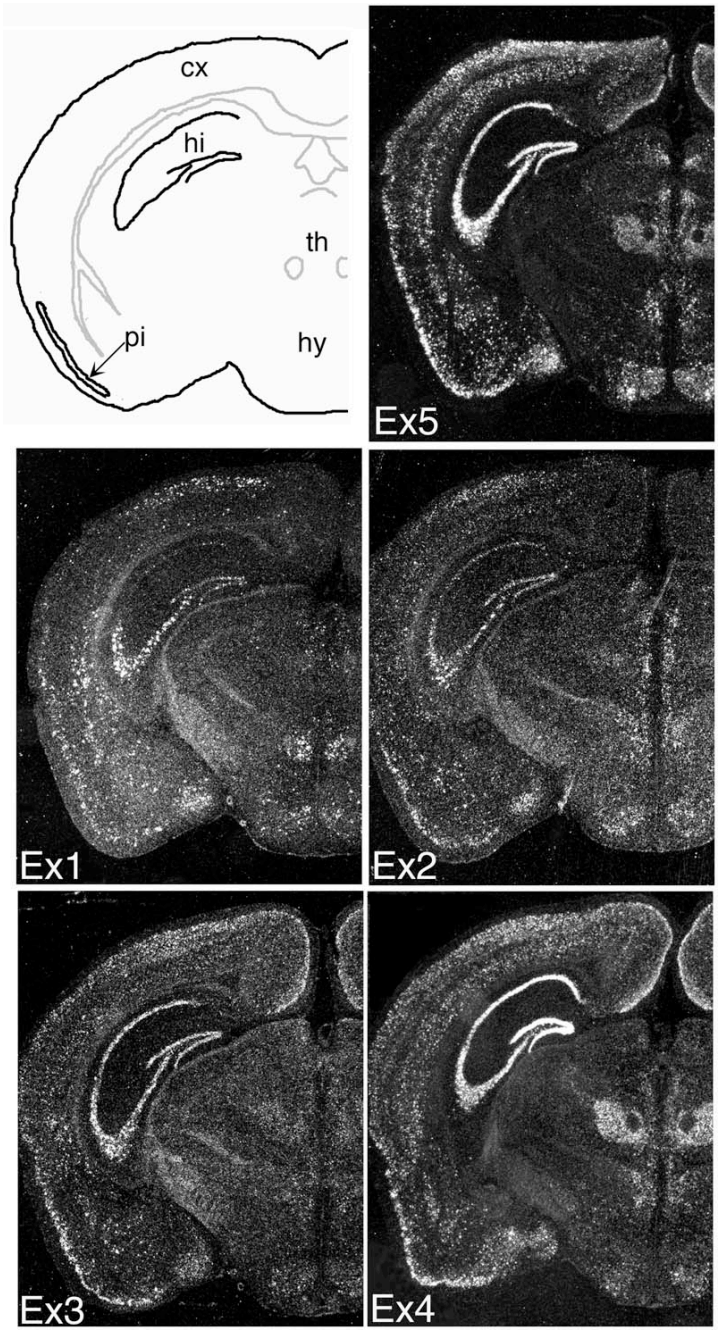

Fig. 1b. Coronal sections showing the expression of BDNF exons 1-5 by *in situ* hybridization. Hi, hippocampus; cx, cerebral cortex; th, thalamus; hy, hypothalamus; pi, piriform cortex. Mice are 7-9 weeks of age.

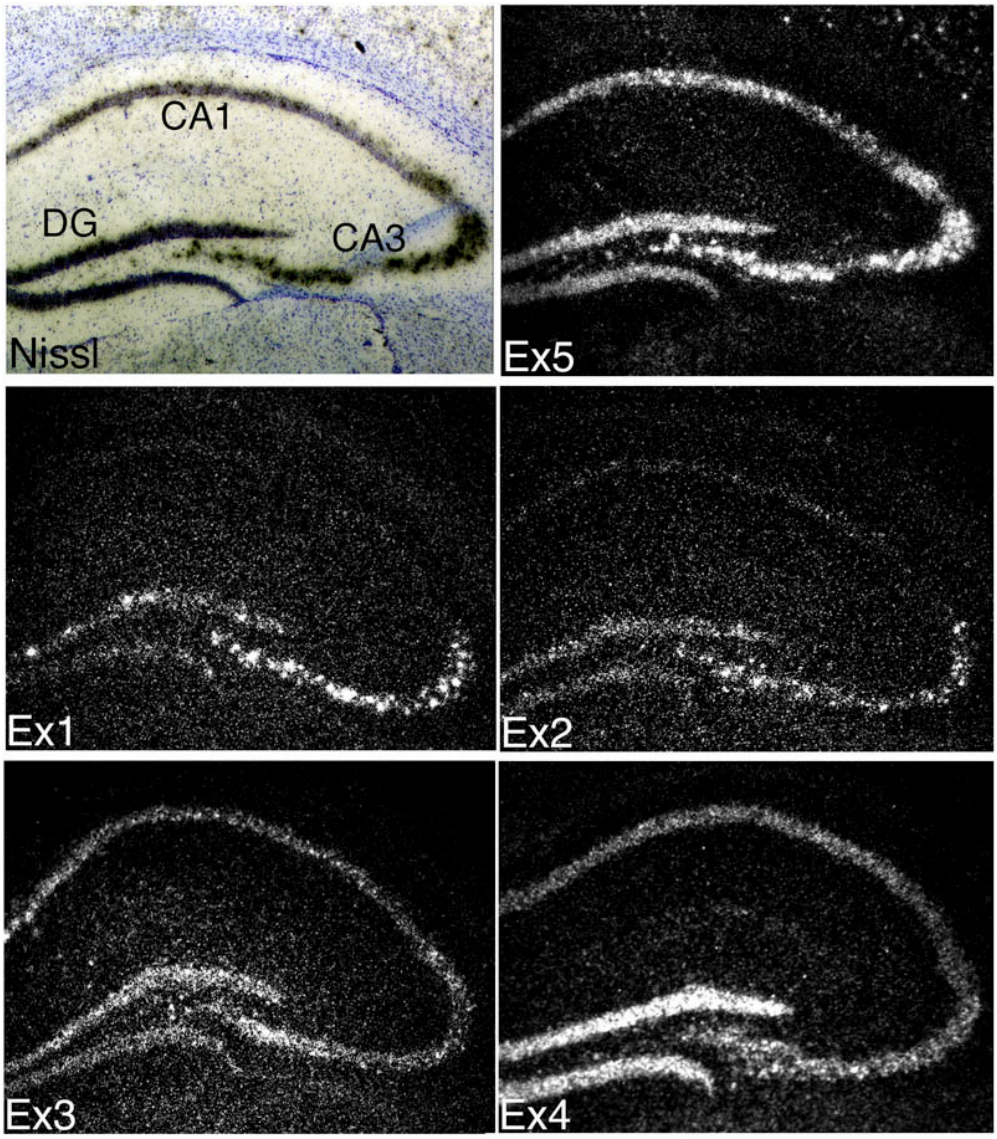

Fig. 2. Sections of the hippocampus showing the expression of BDNF exons 1-5 by *in situ* hybridization. DG, dentate gyrus. Mice are 7-9 weeks of age.

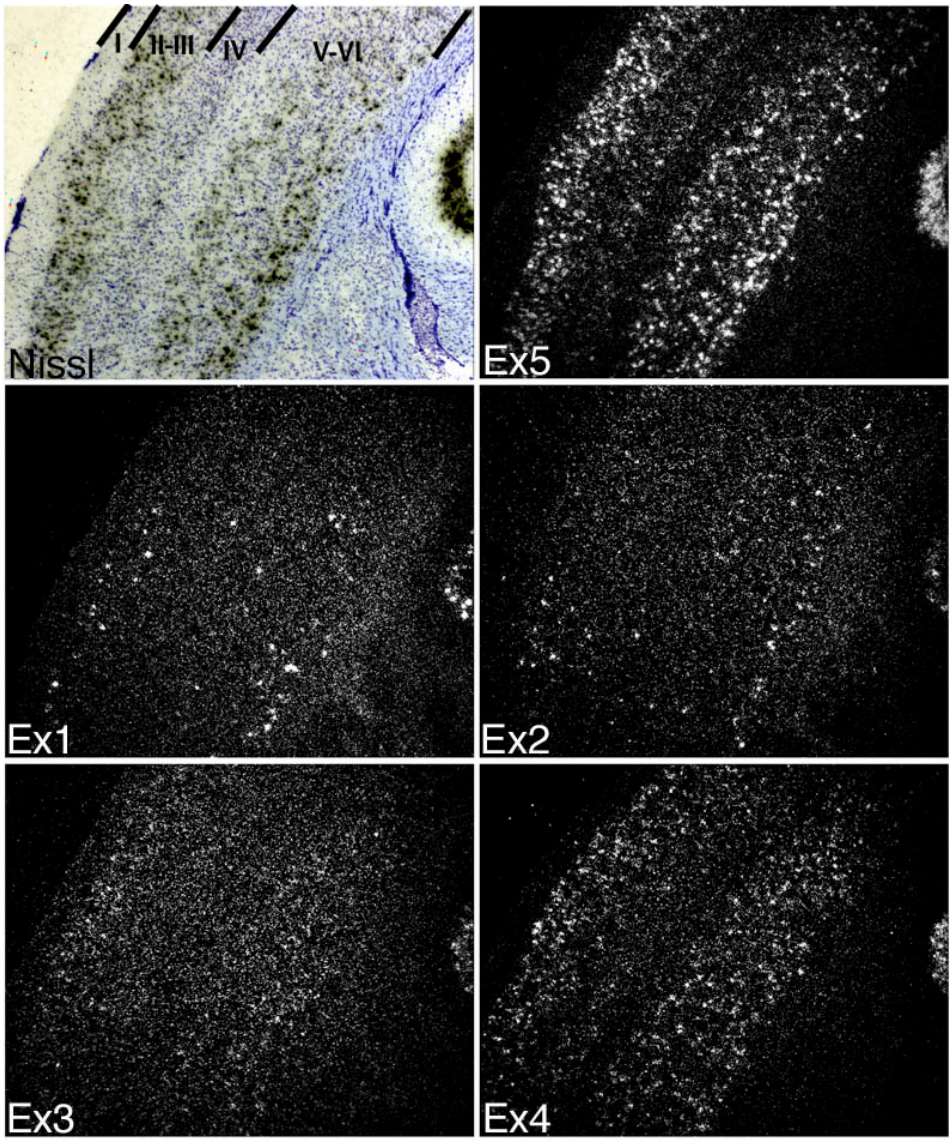

Fig. 3a. Sections of the primary and secondary somatosensory cortex showing the expression of BDNF exons 1-5 by *in situ* hybridization. I-VI, six layers of the cortex. Mice are 7-9 weeks of age.

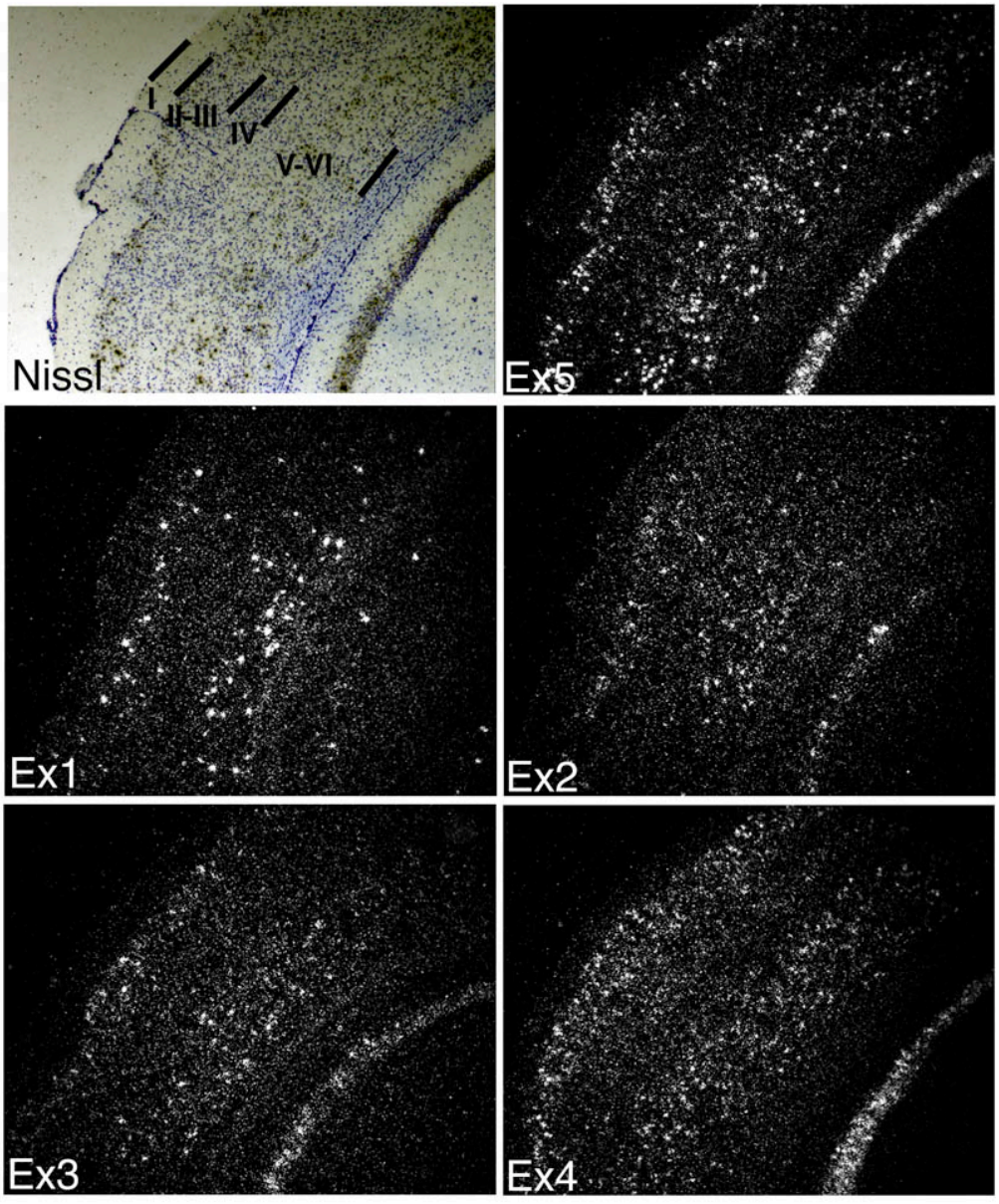

Fig. 3b. Sections of the dorsal auditory cortex showing the expression of BDNF exons 1-5 by *in situ* hybridization. I-VI, six layers of the cortex. Mice are 7-9 weeks of age.

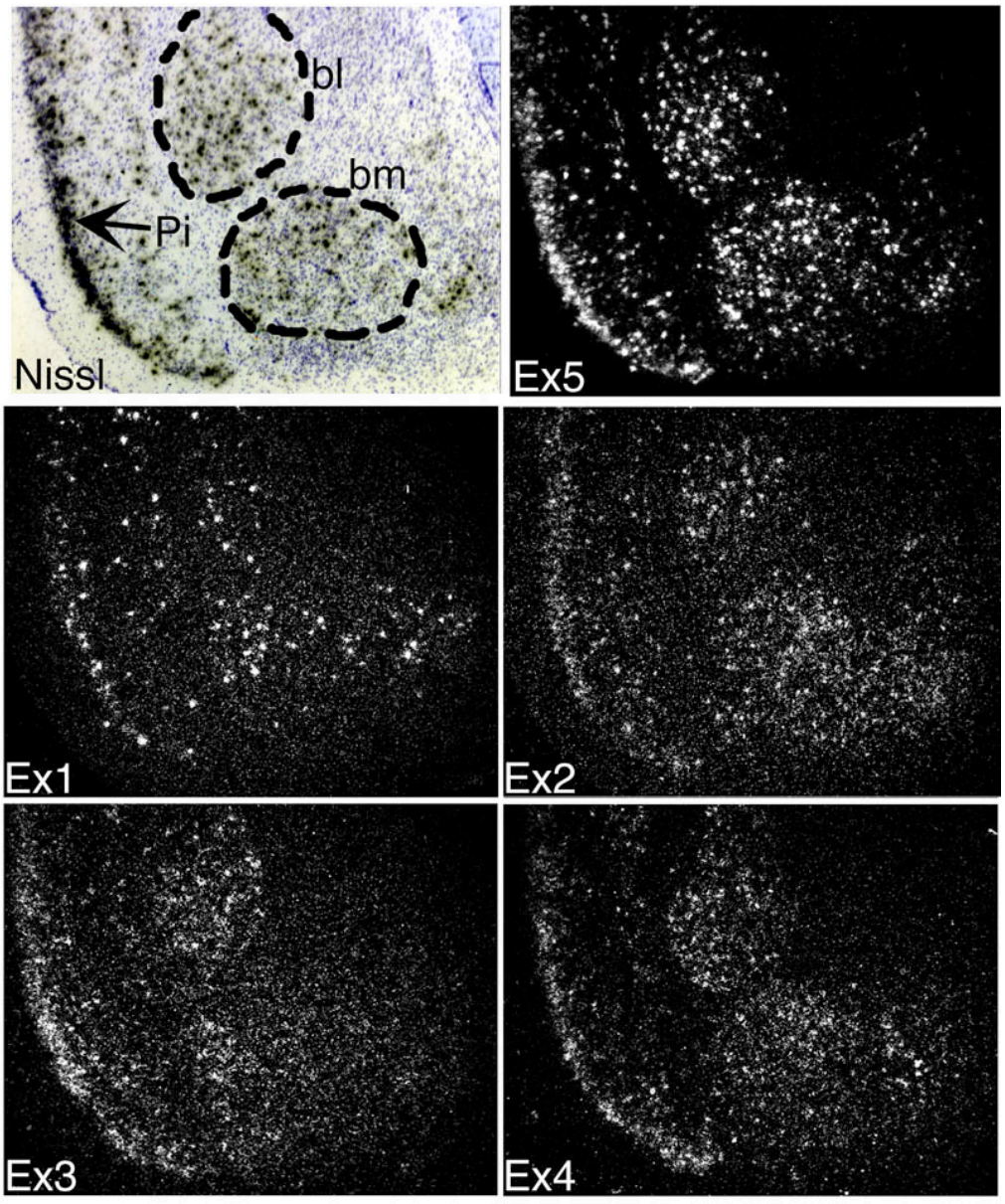

Fig. 4. Sections of the piriform cortex (pi) and the basomedial (bm) and basolateral (bl) amygdala showing the expression of BDNF exons 1-5 by *in situ* hybridization. Mice are 7-9 weeks of age.

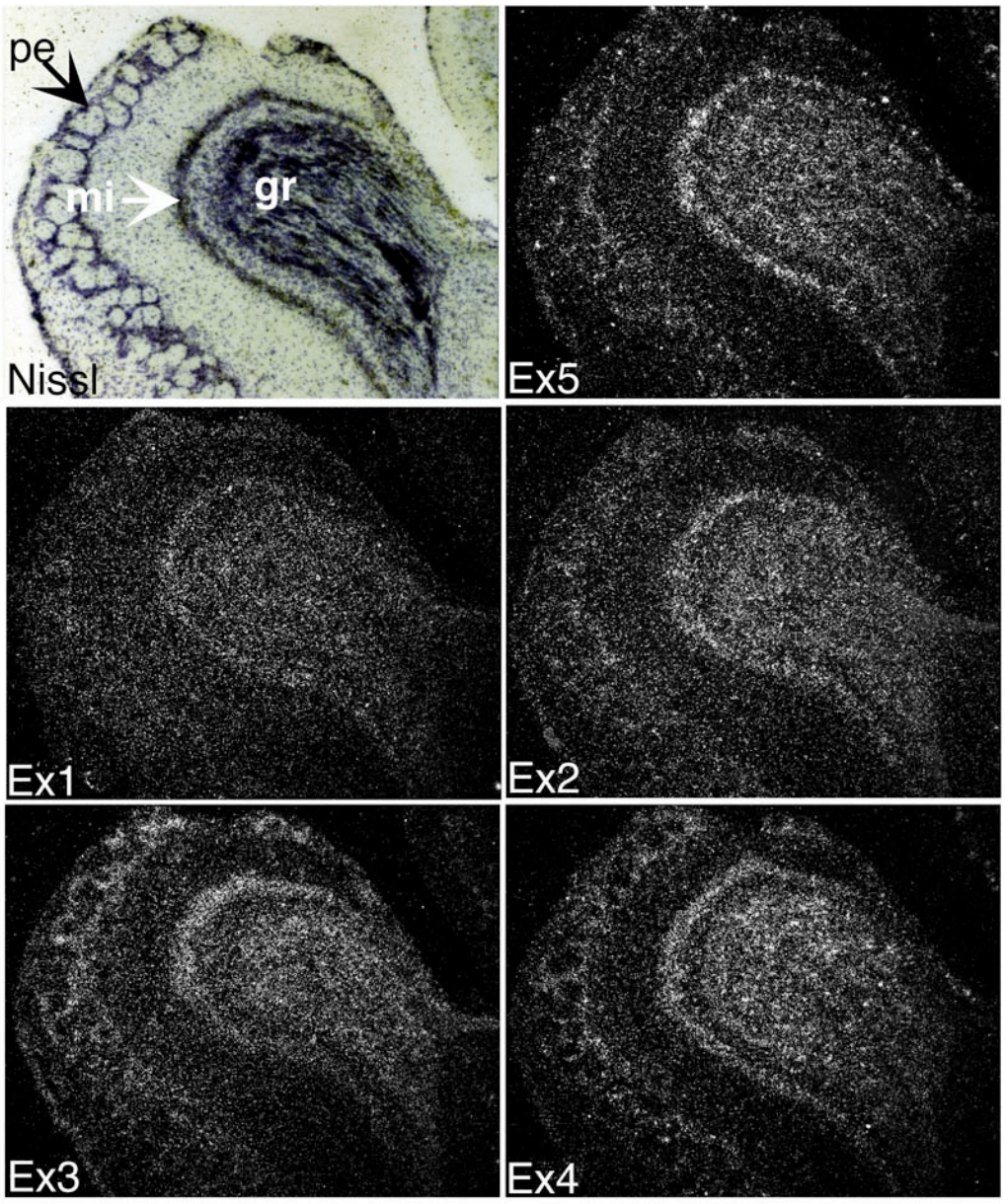

Fig. 5. Sections of the olfactory bulb showing the expression of BDNF exons 1-5 by *in situ* hybridization. Gr, granule cell layer; mi, mitral cell layer; pe, periglomerular cell layer. Mice are 7-9 weeks of age.

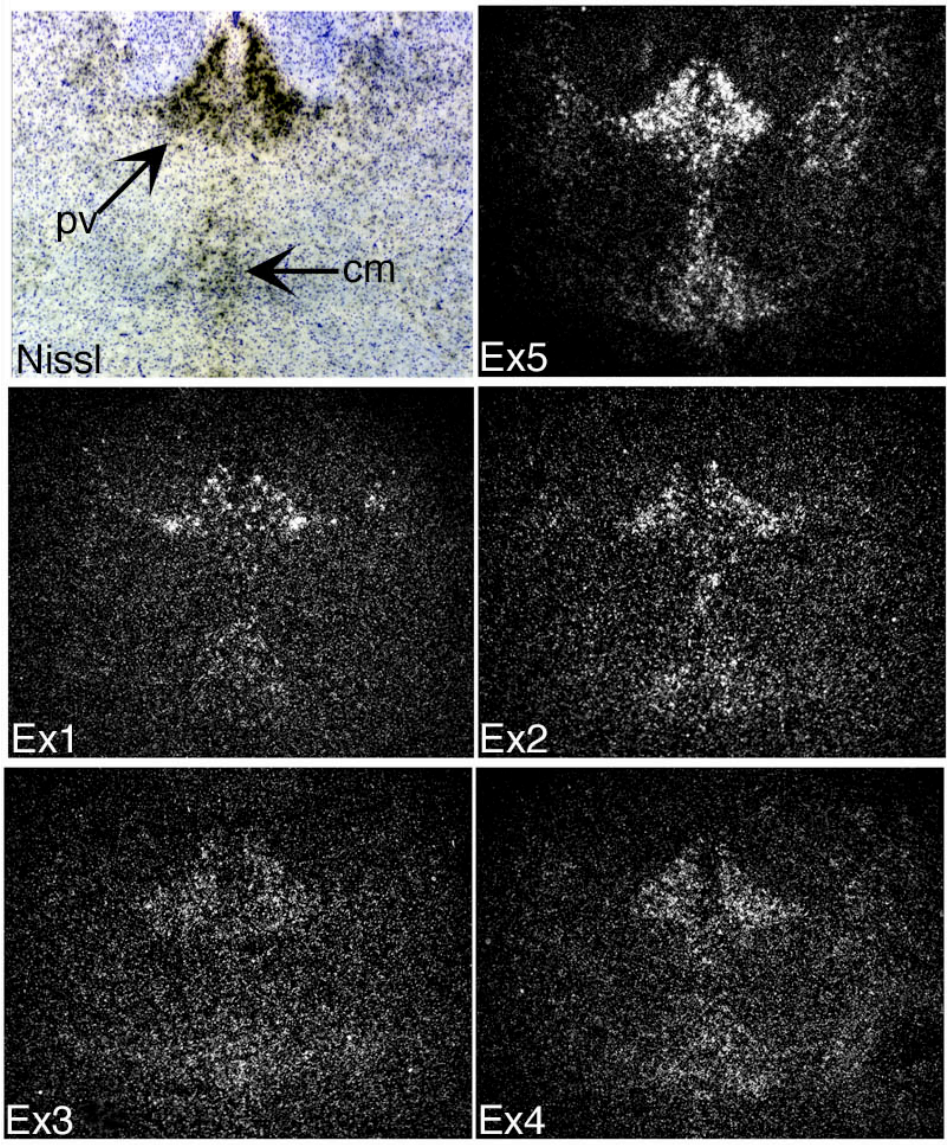

Fig. 6a. Sections of the paraventricular (pv) and central medial (cm) thalamic nuclei showing the expression of BDNF exons 1-5 by *in situ* hybridization. Mice are 7-9 weeks of age.

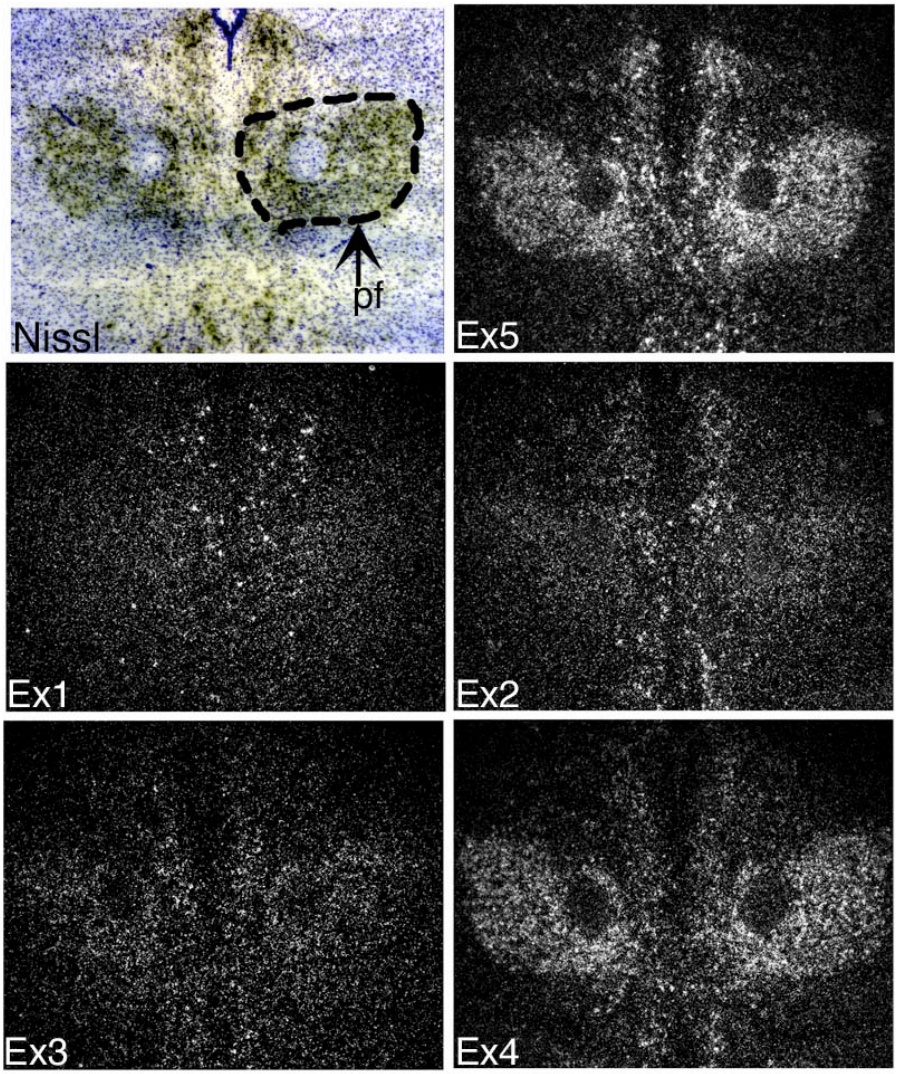

Fig. 6b. Sections of the parafascicular thalamic nucleus (pf) showing the expression of BDNF exons 1-5 by in siu hybridization. Mice are 7-9 weeks of age.

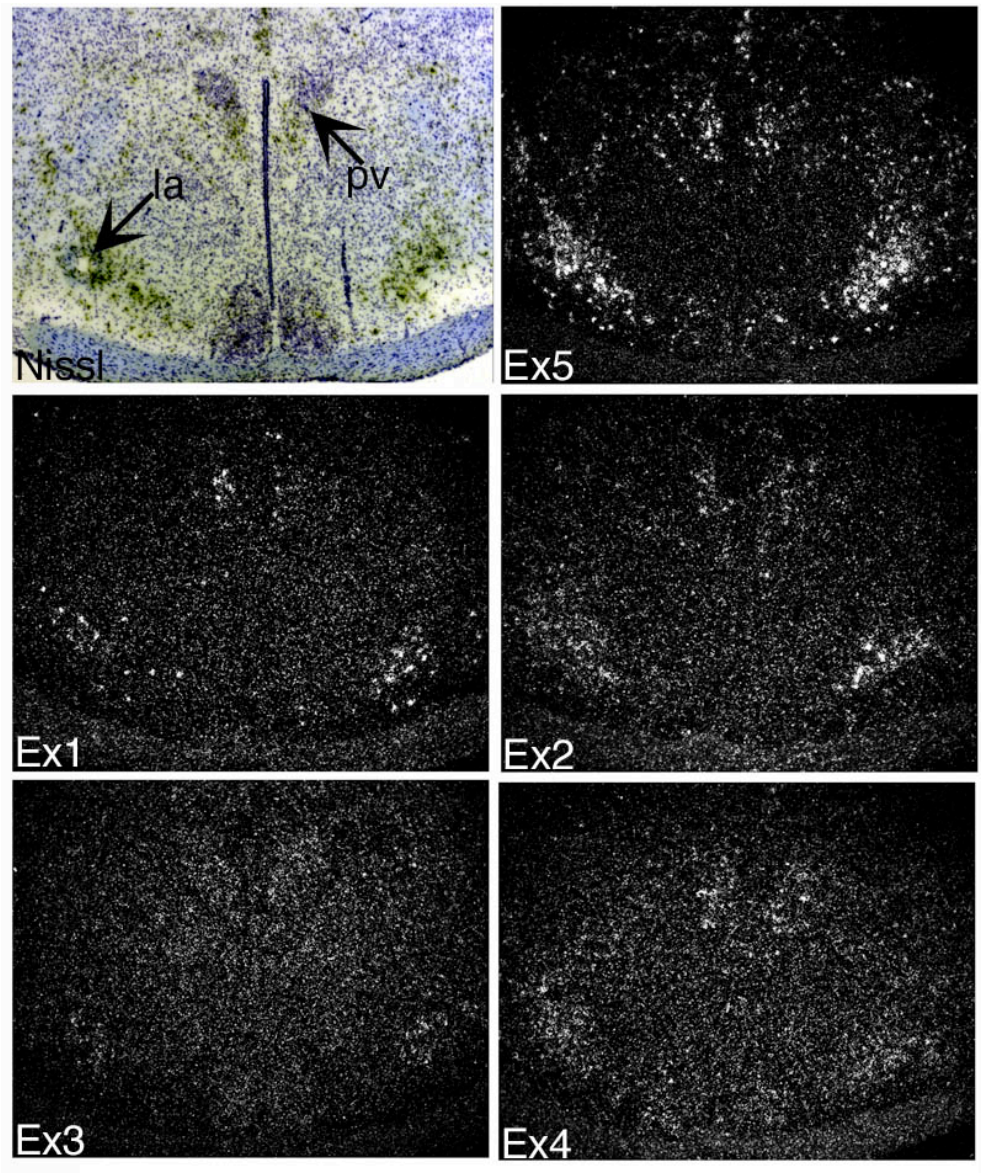

Fig. 7a. Sections of the paraventricular (pv) and lateroanterior (la) hypothalamic nuclei showing the expression of BDNF exons 1-5 by *in situ* hybridization. Mice are 7-9 weeks of age.

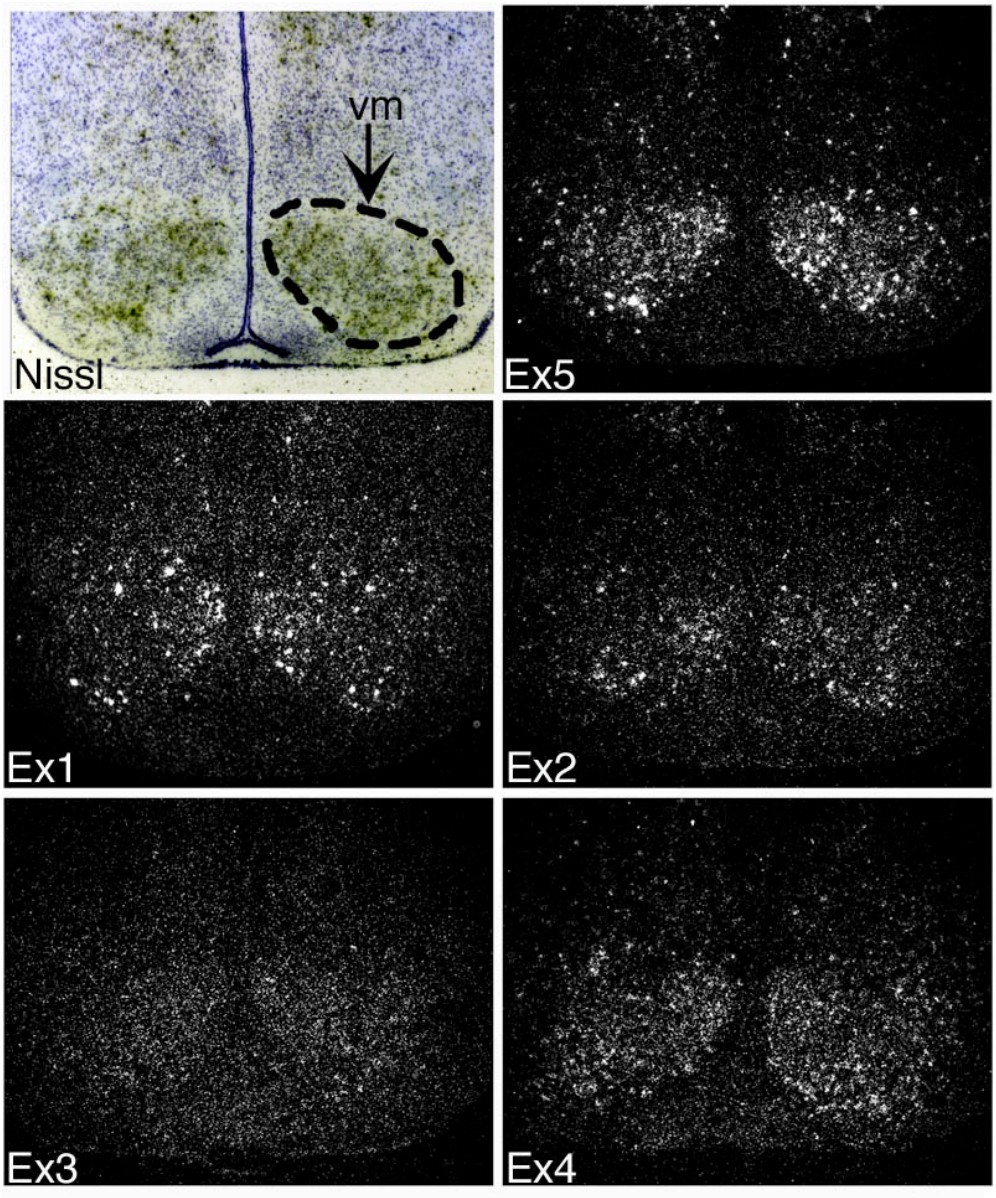

Fig. 7b. Sections of the ventromedial hypothalamic nucleus (vm) showing the expression of BDNF exons 1-5 by *in situ* hybridization. Mice are 7-9 weeks of age.

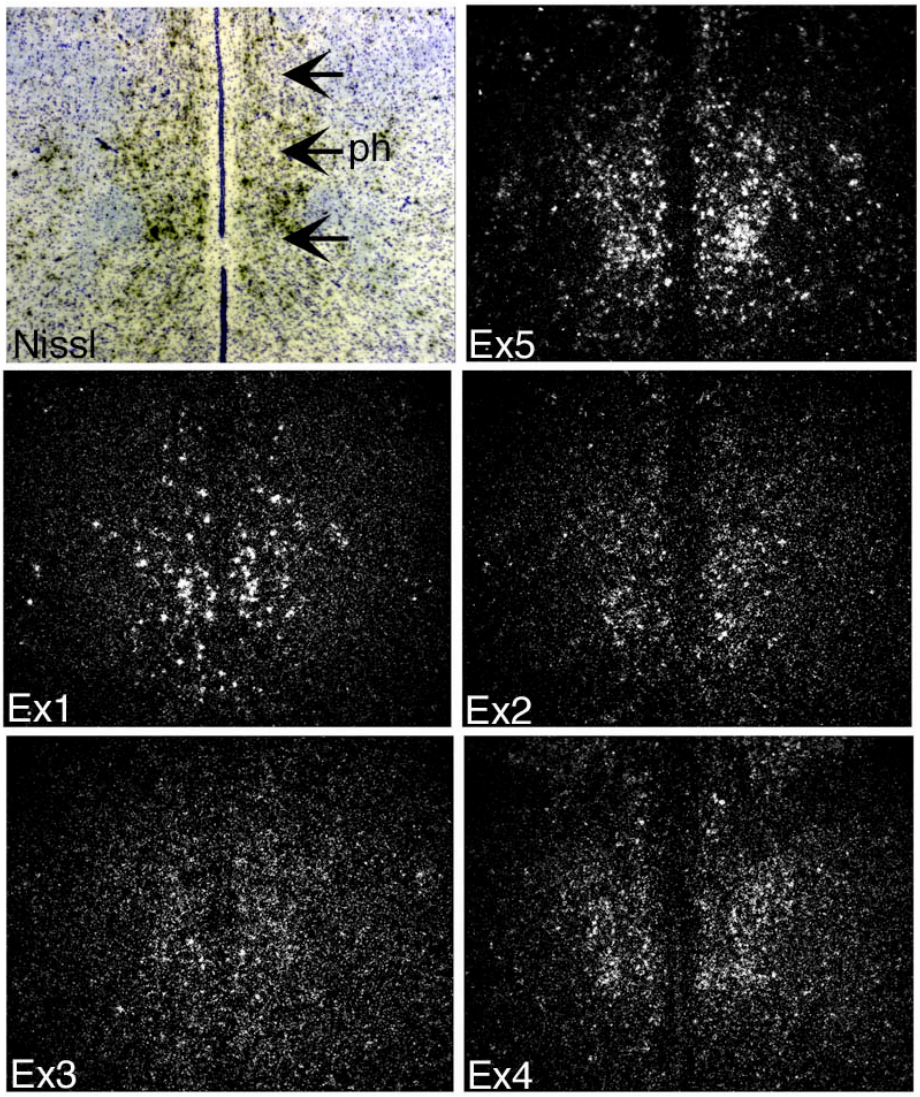

Fig. 7c. Sections of the posterior hypothalamic area (ph) showing the expression of BDNF exons 1-5 by *in situ* hybridization. Mice are 7-9 weeks of age.

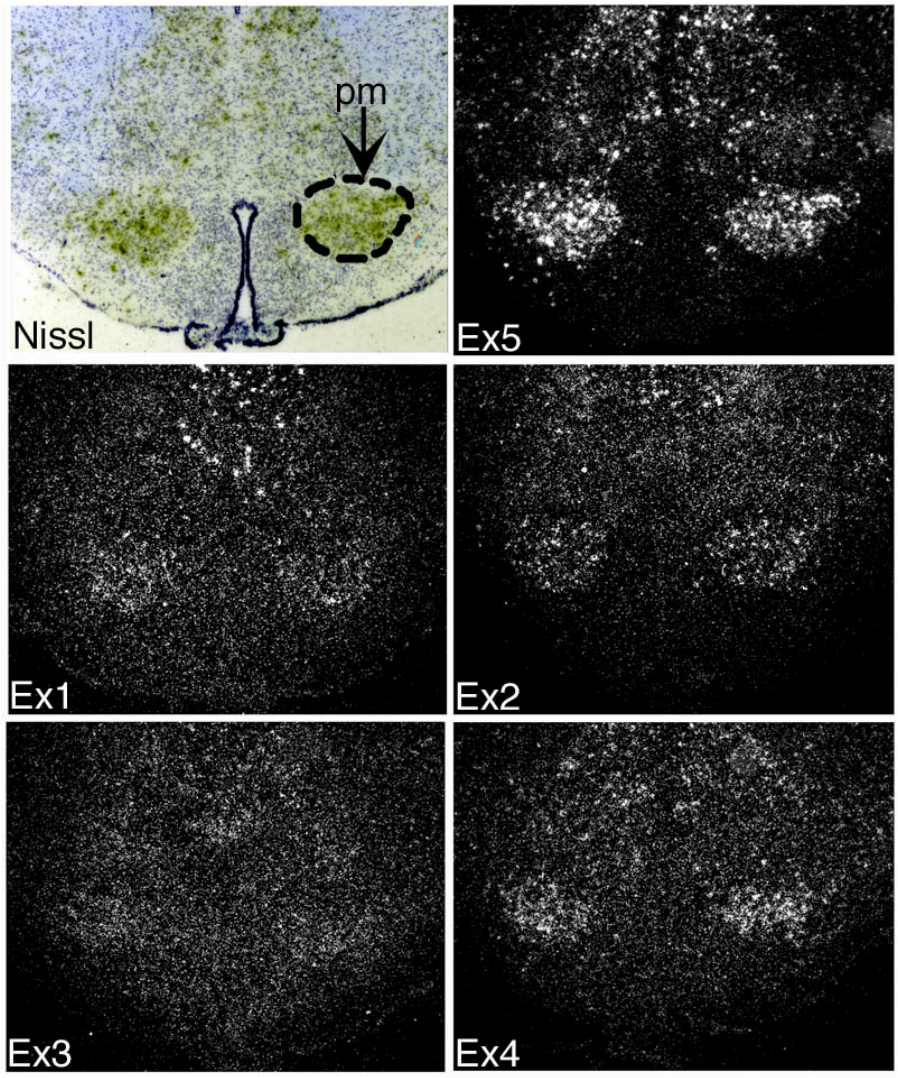

Fig. 7d. Sections of the premammillary hypothalamic nucleus (pm) showing the expression of BDNF exons 1-5 by *in situ* hybridization. Mice are 7-9 weeks of age.

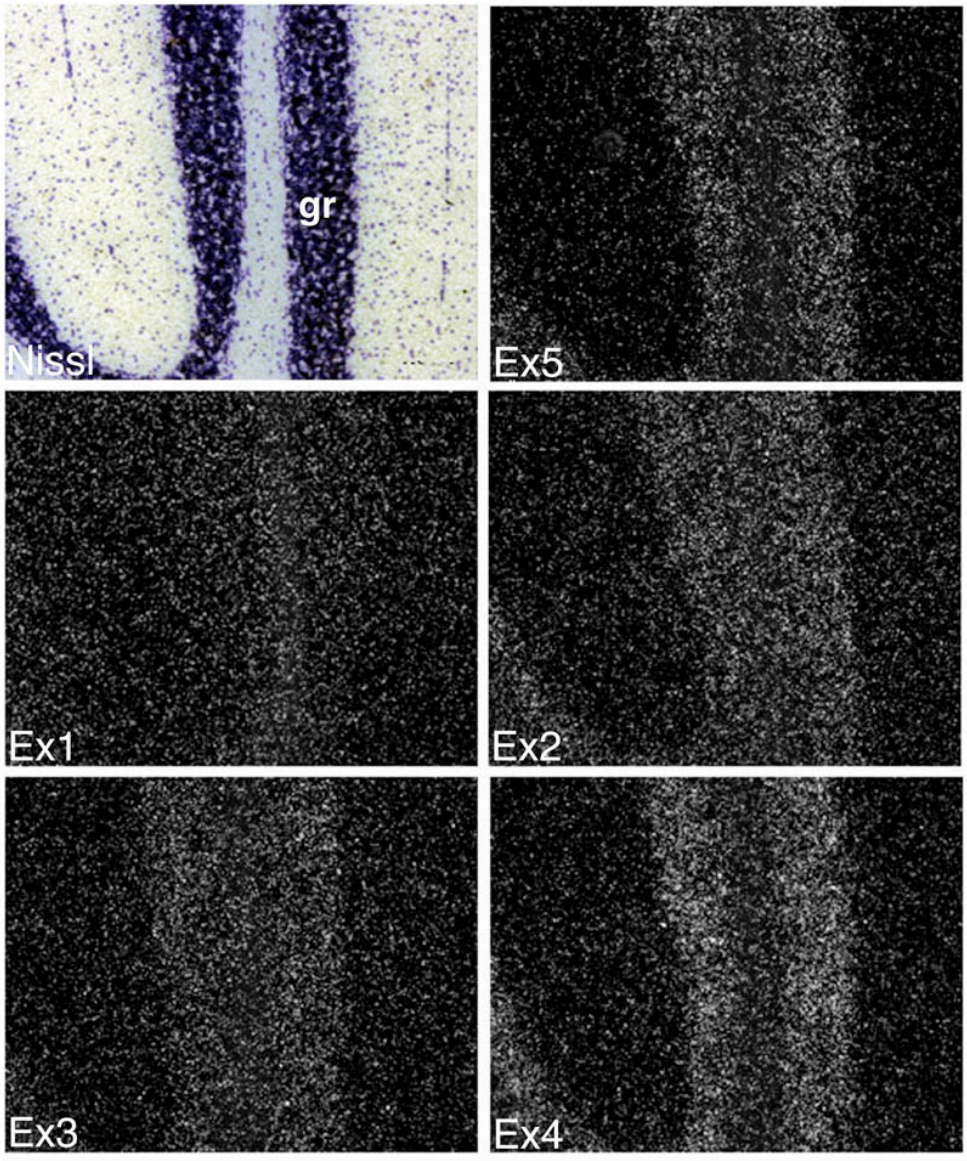

Fig. 8. Sections of the cerebellum showing the expression of BDNF exons 1-5 by *in situ* hybridization in the granule cell layer (gr). Mice are 7-9 weeks of age.

Chapter 4. Transgenic study to identify brain-derived neurotrophic factor promoter regions mediating expression in distinct brain areas

Introduction

The preceding chapter provides evidence to suggest that the four promoters of BDNF show differential functions. To examine this in more detail and to identify regulatory elements within the four promoters, a transgenic strategy was employed. The benefits of using transgenic animals were discussed in chapter 1. Four transgenic constructs specific to each of the four BDNF promoters were made and used to generate four types of transgenic animals. With a GFP reporter, it was possible to localize the transgenic signal by *in situ* hybridization to specific groups of cells. The rationale behind this study was that if several transgenic lines generated from the same transgenic construct showed endogenous-like expression, this could be the first step to identifying regulatory elements for the given promoter. By first screening adult mouse brains by RT-PCR for GFP and then performing *in situ* hybridization on RT-PCR positive lines, it was possible to identify seven lines – for comparison -that had expression in several regions of BDNF expression. For analysis, exon 1 and exon 2-specific lines were grouped. Also exon 3 and exon 4-specific lines were grouped. The prediction was that neighboring promoters 1 and 2 would share regulatory elements and hence corresponding transgenic lines would share expression patterns. The same was predicted of promoter 3 and 4-specific transgenic lines.

Results

RT-PCR on brains of transgenic animals specific to BDNF promoters 1-4

A total of 14 transgenic lines was generated using constructs specific to BDNF promoters 1/2 (Fig. 10, Table 3). 13 lines were generated using constructs specific to BDNF promoters 3-4 (Fig. 11, Table 3). Lines were analysed by RT-

PCR and ISH, with the objective of identifying lines that were present in the largest number of brain regions. To determine whether transgenic expression was present in the brain, RT-PCR (using GFP primers) was performed on tissue of the entire brain, lacking the cerebellum and olfactory bulb. For transgenic constructs 1-4, 7 out of 11; 3 out of 3; 6 out of 6; and 4 out of 7 RT-PCR positive lines respectively were obtained. ISH (using a GFP probe) on RT-PCR positive transgenic lines- specific to constructs 1-4 yielded 2, 1, 2, and 2 lines respectively that were expressing at relatively high levels above background in multiple brain regions. For these 7 lines, transgenic expression was also analysed in nonneural tissues by RT-PCR, as discussed in our final results section below.

Detailed examination of seven transgenic lines specific to BDNF promoters 1-4 in the brain

For the 7 selected transgenic lines, ISH was performed on sagittal and in several cases coronal sections, and GFP expression was examined in detail in major regions of endogenous expression. Results are summarized in Tables 4-6. The transgenic expression of each line is compared and contrasted to that of endogenous exons 1-4. For the analysis of exon 1/2-specific transgenics, expression is compared to that of both endogenous exons 1 and 2, as neighboring promoters 1 and 2 are likely to share regulatory elements. By similar logic, expression of exon 3/4-specific transgenic animals is compared to that of endogenous exons 3 and 4. Hence when endogenous levels for exon1/2-specific transgenics are spoken of, this refers to endogenous levels of either exon 1 or exon 2.

Transgenic lines Tg1a,Tg1b and Tg2

For Tg1a, one animal at 2 months of age was examined by sagittal sections, and two animals at 2 months of age were examined by coronal sections. For Tg1b, one animal at one month of age and another animal at 2 months of age were examined by sagital sections. For Tg2, 2 animals at 2 months of age were examined by sagital sections, and 2 animals at 2 months of age were examined by

coronal sections. With the exception of the cortex, the only regions discussed are those for which at least two animals show relatively strong endogenous like expression, or no expression in the case of negative control regions. The cortex provides an example of aberrant expression, and we picked this region specifically because it is a major region and because it has high endogenous expression. In general the lines show expression only in regions of BDNF expression, and fail to show expression in regions where BDNF is absent. Results are summarized in tables 4-5.

Hippocampus

In the hippocampus, exons 1 and 2 show highest expression in the CA3, lowest expression in the CA1, and intermediate level expression in the dentate gyrus. Tg1a and Tg2 show moderate-high expression in the dentate gyrus and CA3 (Fig. 13). Tg1a shows low expression in the CA1, while Tg2 shows high expression in this region. Hence, the pattern of Tg1a and Tg2 expression in this region is not substantially different from endogenous expression.

Cerebral cortex

In the cortex, exon 1 and exon 2 expression is moderate in layers 2-3 and 5-6 and below detection in layer 4. Expression for both exons appears somewhat higher in the rostral cortex than in the region of the retrosplenial granular and agranular cortices (caudal cortex). Tg1a expression is high and mimics the pattern of endogenous expression in the cortex, with the exception that expression is higher in the rostral than in the caudal cortices (Fig. 14). Tg1b expression is aberrantly high in the retrosplenial granular, prelimbic, infralimbic, dorsal peduncular, and dorsal tenia tecta cortices. Tg1b expression is abnormally low in layers 2-3 and 5-6 throughout the rest of the cortex. Therefore, neither Tg1a nor Tg2 expression recapitulates endogenous expression.

Piriform cortex

Exon 1 and exon 2 expression is moderate high in the piriform cortex. While Tg1a expression is low, and appears to be in only a subset of cells, it is clearly visible above background and in the pattern of endogenous expression (Fig. 13). Tg2 expression is moderate-high, clearly visible above background, and also mimics the pattern of endogenous expression. Hence, for this region, it appears that Tg1a and Tg2 expression to a significant degree recapitulates endogenous expression.

Olfactory bulb

Endogenous exon 1 and 2 expression is low in the olfactory granule cell layer. Tg1b and Tg2 expression is moderate in the granule cell layer and shares the uniform quality of endogenous expression (Fig. 15). Hence, Tg1b and Tg2 expression to a significant degree mimics endogenous expression.

Septum/Striatum

In line with BDNF exon 5 expression, Tg1a, Tg1b, and Tg2 show no expression above background in these (negative control) regions (Fig. 14).

Hypothalamus

Exon 1 and 2 expression is moderately high in the lateroanterior hypothalamic nucleus. Tg1a and Tg1b expression is moderate and high respectively in this region and while it mimics the pattern of endogenous expression, it appears to be present in a larger subset of cells (Fig. 16).

Cerebellum

Exon 2 expression in the cerebellar granule cell layer is moderate and uniform; however, exon 1 is absent from this region. Tg1a and Tg2 expression is moderate and moderate-high respectively and of the same uniform quality as exon 2 expression in this region (Fig. 17)**.**

Transgenic lines Tg3a, Tg3b, Tg4a, Tg4b

For Tg3a, 2 animals at 2 months of age were analysed by sagittal sections, and 2 animals at 2 months of age were analysed by coronal sections. For Tg3b, one animal at 1 month of age was analysed by sagittal sections. For Tg4a, one animal at 1 month of age was analysed by sagittal sections. For Tg4b, one animal at 1 month of age was analysed by sagittal sections. Results are summarized in tables 4 and 6.

Hippocampus

Exon 3 and exon 4 expression is high in the CA1, CA2, CA3, and dentate gyrus. Tg3a, Tg3b and Tg4b all show moderately high expression in the dentate gyrus (Fig. 18). Tg4a also shows definitive signal above background in the dentate gyrus.

Cerebral cortex

In the cortex, exon 3 and 4 expression is moderate in layers 2-3 and layers 5-6 and absent in layer 4. Expression for both exons appears somewhat higher in the rostral cortex than in the region of the retrosplenial granular and agranular cortices (caudal cortex). Tg3a expression is present in layers 5-6 of the cortex, and is particularly high in the frontal cortex; while it is absent from layers 2-3 (Fig. 19). Tg3b expression is present in layers 2-3 and 5-6, but is abnormally low in layers 2-3, and inappropriately high in layer 5. Tg4b expression is present at very low levels in layers 2-3 and 5-6, with highest expression in layer 5. The expression of none of the four exon 3/4 lines appears to recapitulate endogenous expression.

Olfactory bulb

Exon 3 and 4 expression is low in the granule cell layer of the olfactory bulb. Tg3b, Tg4a and Tg4b expression is moderate and has the uniform quality of endogenous expression in this region (Fig. 20).

Septum/Striatum

Tg3a, Tg3b, and Tg4b show no expression in these negative control regions (Fig. 19, data not shown); however line Tg4a shows high expression in these regions.

Thalamus

Exon 3 expression is low, while exon 4 expression is moderately high in the parafascicular thalamic nucleus. Tg4a and Tg4b expression is moderately high and high respectively and expressed in a pattern that mimics the endogenous in this region (Fig. 21). Tg4b expression appears to be present in a larger subset of cells than endogenous expression.

Hypothalamus

Exon 3 and 4 expression is low in the lateroanterior hypothalamic nucleus. Tg3a, and Tg3b expression is moderate and in a pattern that mimics the endogenous in this nucleus (Fig. 22).

Exon 3 and 4 expression is low and moderate respectively in the ventromedial hypothalamic nucleus. Tg3a expression is low and apparently present in a smaller subset of cells than endogenous expression, although clearly visible above background. Tg4a expression is moderate and appears to mimic the pattern of endogenous expression (Fig. 23).

Cerebellum

Exon 3 and 4 expression is moderate in the cerebellum. Tg3b, Tg4a and Tg4b expression is moderately high and has the uniform quality of endogenous expression in this region (Fig. 24).

Expression of seven transgenic lines in non-neural tissues by RT-PCR

It was of interest to see whether any of the seven transgenic lines recapitulated endogenous expression in nonneural tissues. RT-PCR was performed on 6 different tissues, namely the heart, kidney, liver, lung, spleen and muscle (table

7). It was shown that endogenous exon 5 is present in all tissues except for the spleen, as are endogenous exons 3 and 4. However, endogenous exons 1 and 2 are not present in any of the tissues examined. In agreement with the results for endogenous exons 1 and 2, Tg1a and Tg1b show no transgenic expression in any of the tissues examined. Meanwhile, contrary to endogenous expression, Tg2 shows expression in all tissues. Tg3a, Tg3b and Tg4a all show expression in the kidney and lung; Tg3a, and Tg4a also show expression in the heart and muscle; Tg4b shows expression only in the kidney.

Conclusion

This study indicates that the organization of the regulatory elements of the BDNF gene is far more complicated than was previously envisioned. It was shown that an 11.4 kb genomic region covering BDNF promoters 1 and 2 is insufficient to drive transgenic expression in an endogenous-like manner. The same is true of a 16 kb region covering BDNF promoters 3 and 4. However, the results of this study suggest that these regions may drive expression in a subset of nuclei of endogenous BDNF expression. Nevertheless, random chance integration cannot be ruled out as an explanation for these results, due to the small number of transgenic animals obtained. In support of a certain level of fidelity to BDNF expression, most regions that normally lack BDNF expression, such as the septum and striatum, lacked expression in the majority of transgenic lines. Also 20 out of 27 transgenic brains were positive for GFP by RT-PCR. In nonneural tissues, two exon 1-specific lines recapitulated endogenous expression, and 4 exon 3/4-specific transgenic lines showed expression in the kidney (where exons 3 and 4 are normally expressed). Hence, these results suggest that key cell-specific regulatory elements necessary for endogenous-like neural and nonneural BDNF expression lie further 5', 3' or both of the genomic sequences used in the constructs of the present study. The long distance of these regulatory elements from their promoters and the likelihood of multiple elements functioning in concert, makes it a considerable challenge for the future to identify these elements.

A previous study by Timmusk et al., 1995 (discussed in chapter 6) gave promising results showing that constructs of similar size to those used in this study gave high levels of transgenic expression in major regions of BDNF expression. Possible reasons for the substantial discrepancy between the present study and the Timmusk study could include the following differences in their constructs: the presence of the endogenous BDNF 3'UTR, a 1kb longer 5' region, an endogenous 0.7kb intron and the usage of a CAT-BDNF fusion.

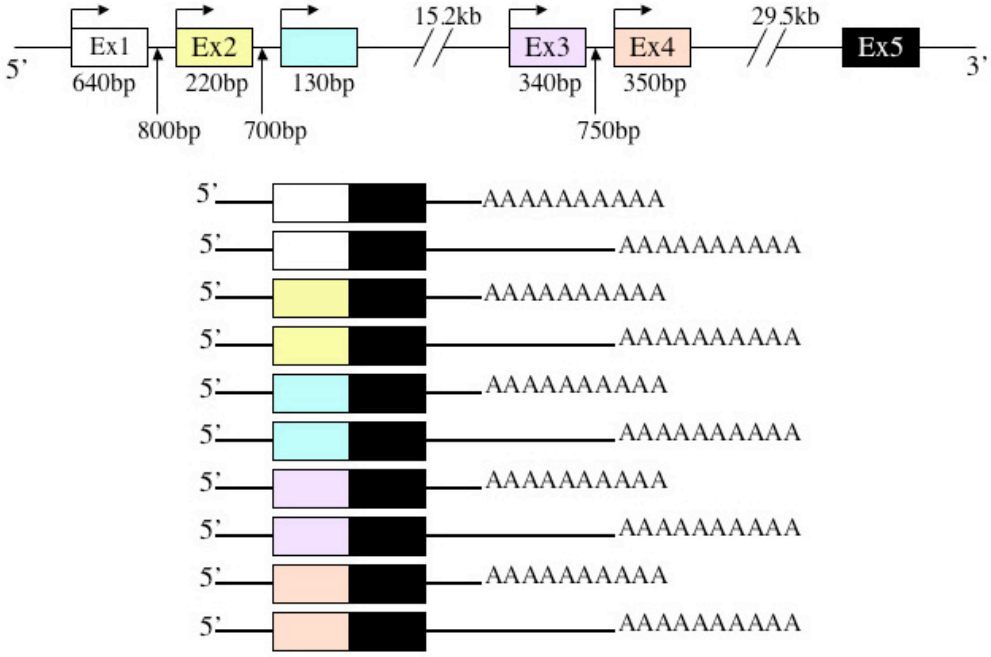

Fig. 9. Organisation of the BDNF gene. Each of the first five untranslated exons is associated with a promoter. The unlabelled exon is newly discovered (NCBI entry AY057907). Exon 5 encodes the entire BDNF protein. Alternative splicing and usage of polyadenylation sites results in at least 10 transcripts

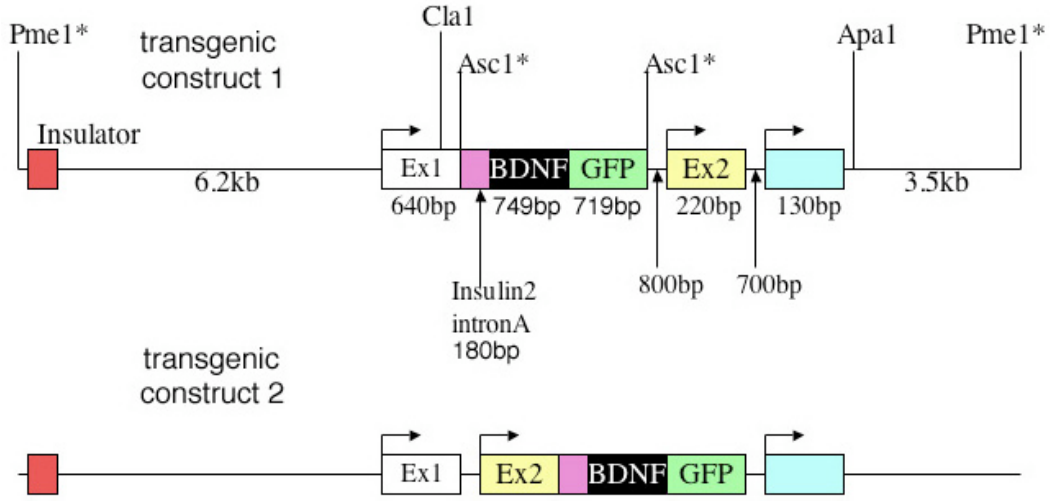

Fig. 10. Diagrams of BDNF promoter 1/2-specific constructs (~11.4kb) used to generate transgenic animals specific to BDNF promoters 1/2. The nomenclature of untranslated exons 1 and 2 is from the Timmusk study (1993). The unnamed pale blue exon was recently identified (NCBI entry AY057907). EGFP functions as a transgenic reporter for all RT-PCR and *in situ* hybridization studies. The 5' insulin 2 intron A was previously shown to highly increase transgene transcription levels. Chicken beta-globin insulator regions were previously shown to protect against position effects as well as transgene silencing due to nearby condensed chromatin. The diagrams show restriction enzyme sites important for the cloning strategy: sites with stars are engineered; unique endogenous sites lack stars.

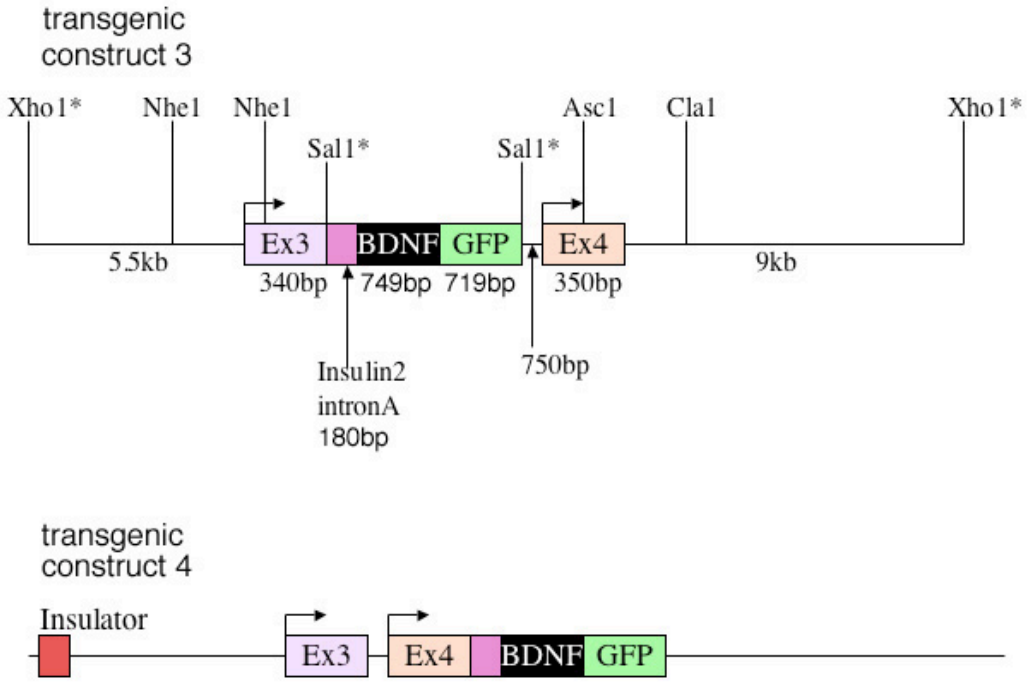

Fig. 11. Diagrams of BDNF promoter 3/4-specific constructs (~16kb) used to generate transgenic animals specific to BDNF promoters 3/4. The nomenclature of untranslated exons 3 and 4 is from the Timmusk study (1993). The unamed pale blue exon was recently identified (NCBI entry AY057907). EGFP functions as a transgenic reporter for all RT-PCR and *in situ* hybridization studies. The 5' insulin 2 intron A was previously shown to highly increase transgene transcription levels. Chicken beta-globin insulator regions were previously shown to protect against position effects as well as transgene silencing due to nearby condensed chromatin. The diagrams show restriction enzyme sites important for the cloning strategy: sites with stars are engineered; unique endogenous sites lack stars.

PC12 Neurites equal to and longer than 3 cell bodies counted in a region with 400 total PC12 cells in each of 3 (6-well) plates: counts made 3 days after addition of supernatant or commercial BDNF			
GFP supernatant	4	3	3
Intron-BDNF-GFP Supernatant 0.5ng/ml BDNF	112	104	116
BDNF Commercial 10ng/ml BDNF	96	105	98

Table 2. Testing the BDNF-GFP fusion protein used in transgenic constructs 1-4. Neurite outgrowth of PC12 cells (stably transfected with TrkB) after application of Cos-7 cell media containing the putative functional BDNF-GFP fusion protein. (unpaired T test $p<0.0001$)

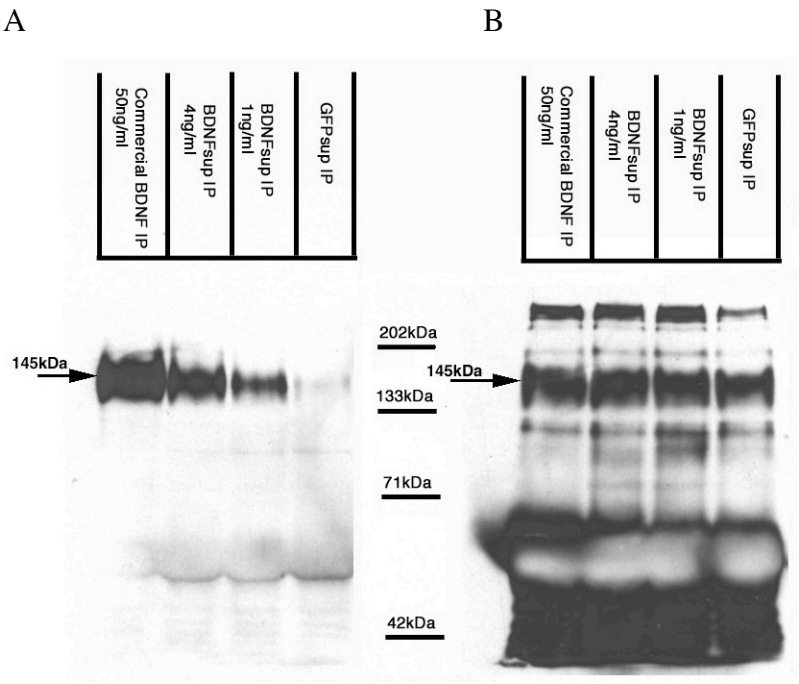

Fig. 12. Testing the BDNF-GFP fusion protein used in transgenic constructs 1-4. A. Immunoprecipitation with panTrk203 (for 145kDa TrkB) and immunoblotting with antiphosphotyrosine (4G10) of lysate from TrkB expressing PC12 cells, treated with Cos-7 supernatant containing putative functional BDNF-GFP fusion protein. BDNFsup is supernatant from Cos-7 cells transfected with pcDNA 3.1-intron-BDNF-GFP. GFPsup is supernatant from Cos-7 cells transfected with pcDNA 3.1-GFP. Commercial BDNF at 50ng/ml was used as a positive control. B. Immunoprecipitation with panTrk203 and immunoblotting with panTrk203 of lysate from TrkB expressing PC12 cells treated with supernatant from Cos-7 cells containing putative functional BDNF-GFP (loading control for Fig. 12A)

Transgenic line Specific to:	Positive by RT-PCR	Expression by *in situ*	Names of lines used in study
Promoter 1	7/11	2	Tg1a, Tg1b
Promoter 2	3/3	1	Tg2
Promoter 3	6/6	2	Tg3a, Tg3b
Promoter 4	4/7	2	Tg4a, Tg4b

Table 3. Selection by RT-PCR and *in situ* hybridization of promoter 1-4-specific transgenic lines that were used for further analysis. Transgenic lines express a BDNF-GFP reporter. All animals used were 4-8 weeks of age.

	Ex.1-5	Tg.1/2	Tg.3/4
hippocampus	+	+	+
cortex	+	+	+
olfactory b.	+	+	+
septum	-	-	-
thalamus	+	+	+
hypothalamus	+	+	+
midbrain	+	+	+
cerebellum	+	+	+

Table 4. Summary of *in situ* hybridization data for exon 1/2-specific and exon 3/4-specific transgenic lines. A GFP riboprobe was used for *in situ* hybridization. There are 3 exon 1/2-specific transgenic lines, and 4 exon 3/4-specific transgenic lines. A + signifies that at least two transgenic lines express in a nucleus of that region (with endogenous expression), even if the signal is weak. A – signifies that at least two transgenic lines do not express in a negative control region. All animals are 4-8 weeks of age.

	Exon5	Exon 1	Exon 2	Tg1a	Tg1b	Tg2
Hippocampus						
CA1-CA2	++++ po	+ p	+ p	+ p	++ p	+++ u
CA3,	++++ po	++++ p	++++ po	+++ p	++ p	+++ u
Dentate gyrus	++++ u	++ po	+++ u	+++ p	++ p	+++ u
Piriform cortex	+++ pu	+++ p	+++ po	+ p	NA	+++ u
Olfactory bulb						
Granule cell layer	+ u	+ u	+ u	-	++ u	++ u
Septum/Striatum	-	-	-	-	-	-
Hypothalamus						
Lateroanterior nu.	++ po	++ p	++ po	++ p	++++ p	-
Cerebellum						
Granule cell layer	++ u	-	++ u	++ u	- u	+++ u

Table 5. Summary of *in situ* hybridization data for exon 1/2-specific transgenic lines. Tg1a and Tg1b are promoter 1-specific transgenic lines. Tg2 is a promoter 2-specific transgenic line. Transgenic *in situ* hybridization was performed with a GFP riboprobe. Transgenic expression is compared to that of endogenous BDNF exon 1 and exon 2. Exon 1, 2 and 5 riboprobes were used for *in situ* hybridization on wild-type animals. The number of pluses signifies the relative level of cell labeling with silver grains for a specific probe in a given region as compared to the same probe in other regions. ++++ corresponds to high cell labeling with silver grains; +++ to moderate high labeling; ++ to moderate labeling; + to low labeling; and – to no detectable labeling. "p" stands for *in situ* hybridization signal that appears punctate. "po" stands for signal that appears punctate, and appears to overly a lower grade signal. "u" stands for signal that appears uniform. The four transgenic animals used were 4-8 weeks of age. The multiple wild-type animals used were 7-9 weeks of age.

	Exon 5	Exon 3	Exon 4	Tg.3a	Tg.3b	Tg.4a	Tg.4b
Hippocampus							
CA1-CA2	++++ po	++++ po	++++ u	+ u	+ u	++ u	+ u
CA3	++++ po	++++ po	++++ po	+ u	+ u	-	+ u
Dentate gyrus	++++ u	++++ u	++++ u	+++ u	+++ u	++ u	+++ u
Olfactory bulb							
Granule cell layer	+ u	+ u	+ u	+ u	++ u	++ u	++ u
Septum/striatum	-	-	-	-	-	++ po	-
Thalamus							
Parafascicular nu.	++ po	+ po	+++ po	-	-	+++ po	++++ p
Hypothalamus							
Lateroanterior nu.	++ po	+ po	+ po	++p	++ p	-	+p
Ventromedial nu.	++ po	+ po	++ po	+ p	-	++ po	-
Cerebellum							
Granule cell layer	++ u	++ u	++ u	+ u	+++ u	+++ u	+++ u

Table 6. Summary of *in situ* hybridization data for exon 3/4-specific transgenic lines. Tg3a and Tg3b are promoter 3-specific transgenic lines. Tg4a and Tg4b are promoter 4-specific transgenic lines. Transgenic *in situ* hybridization was performed with a GFP riboprobe. Transgenic expression is compared to that of endogenous BDNF exon 3 and exon 4. Exon 3, 4 and 5 riboprobes were used for *in situ* hybridization on wild-type animals. The number of pluses signifies the relative level of cell labeling with silver grains for a specific probe in a given region as compared to the same probe in other regions. ++++ corresponds to high cell labeling with silver grains; +++ to moderate high labeling; ++ to moderate labeling; + to low labeling; and – to no detectable labeling. "p" stands for *in situ* hybridization signal that appears punctate. "po" stands for signal that appears punctate, and appears to overly a lower grade, signal. "u" stands for signal that appears uniform. The four transgenic animals used were 4-8 weeks of age. The multiple wild-type animals used were 7-9 weeks of age.

Wild-type exon or exon-specific transgenic line	RT-PCR results for 6 different nonneural regions dissected from wild-type or transgenic animals					
	heart	kidney	liver	lung	spleen	muscle
Exon 5 (wt)	+	+	+	+	-	+
Exon 1 (wt)	-	-	-	-	-	-
Exon 2 (wt)	-	-	-	-	-	-
Exon 3 (wt)	+	+	+	+	-	+
Exon 4 (wt)	+	+	+	+	-	+
Tg1a	-	-	-	-	-	-
Tg1b	-	-	-	-	-	-
Tg2	+	+	+	+	+	+
Tg3a	+	+	-	+	-	+
Tg3b	-	+	-	+	-	-
Tg4a	+	+	-	+	-	+
Tg4b	-	+	-	-	-	-

Table 7. Summary of RT-PCR data comparing expression of GFP in transgenic animals specific to BDNF exons 1-4 with expression of endogenous BDNF exons 1-4 respectively in wild-type animals in 6 non-neural tissues. Tg1a-Tg4b are 6 distinct lines specific to the promoter indicated by the number following the "Tg." Transgenic RT-PCR was performed with GFP primers. Primers for endogenous BDNF exons 1-5 were used for RT-PCR on wild-type animals. 2 animals of 4-8 weeks of age were used per transgenic line, and 2 wild-type animals were used.

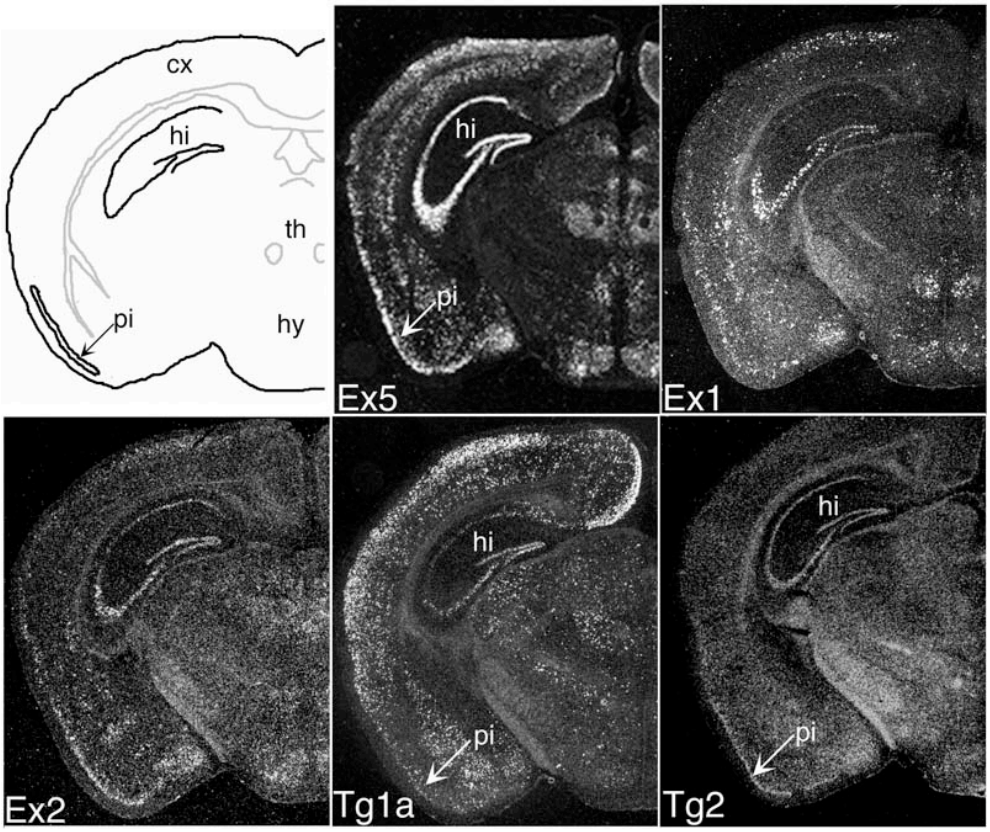

Fig. 13. Coronal sections of BDNF promoter 1/2-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the hippocampus (hi) and piriform cortex (pi). Tg1a and Tg2 are promoter 1 and promoter 2-specific transgenic animals respectively. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 1 (Ex1) or exon 2 (Ex2). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

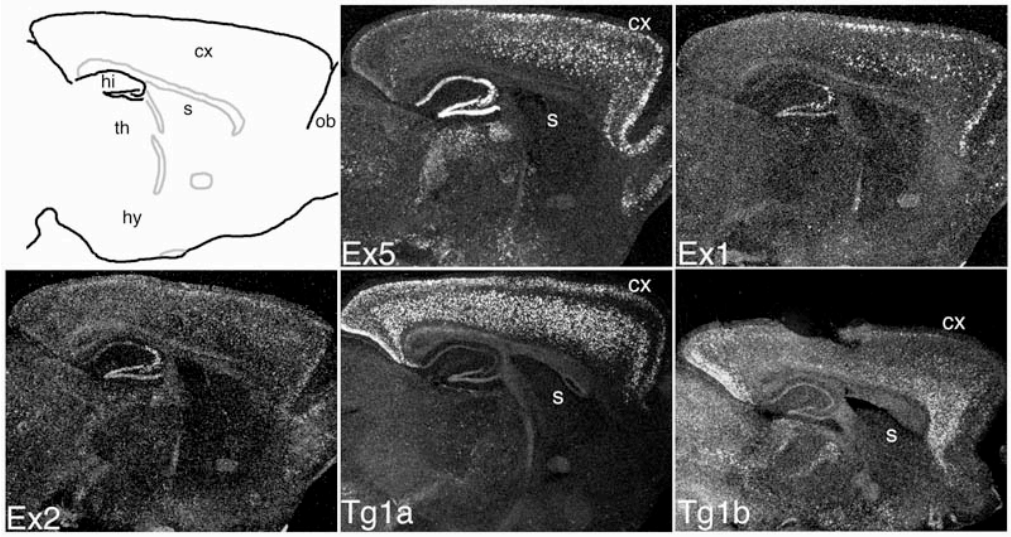

Fig. 14. Sagittal sections of BDNF promoter 1-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the cortex (cx). The septum (s) is a negative control region. Tg1a and Tg1b are promoter 1-specific transgenic animals from two distinct transgenic lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 1 (Ex1) or exon 2 (Ex2). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

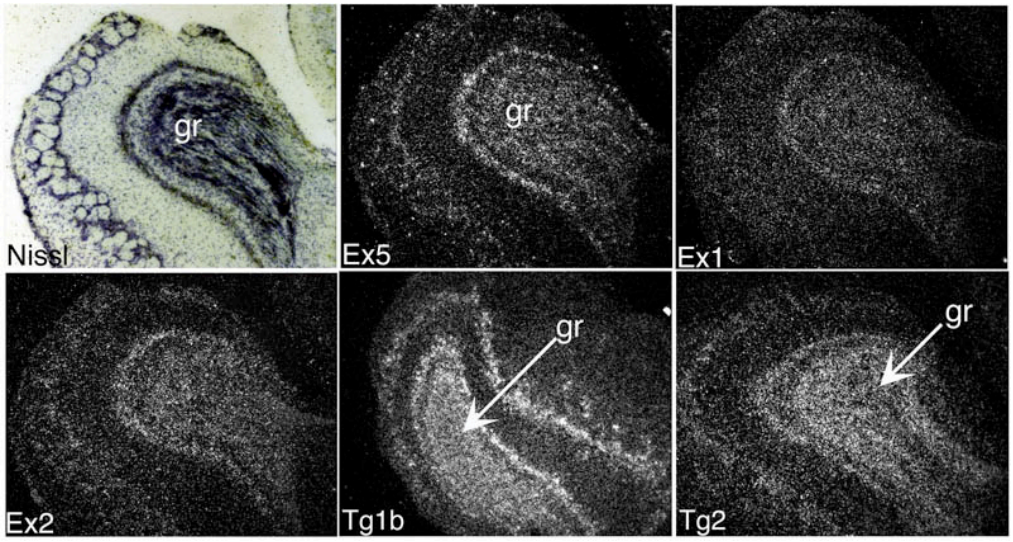

Fig. 15. Sagittal sections of BDNF promoter 1/2-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the olfactory bulb granule cell layer (gr). Tg1b and Tg2 are promoter 1 and promoter 2-specific transgenic animals. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 1 (Ex1) or exon 2 (Ex2). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

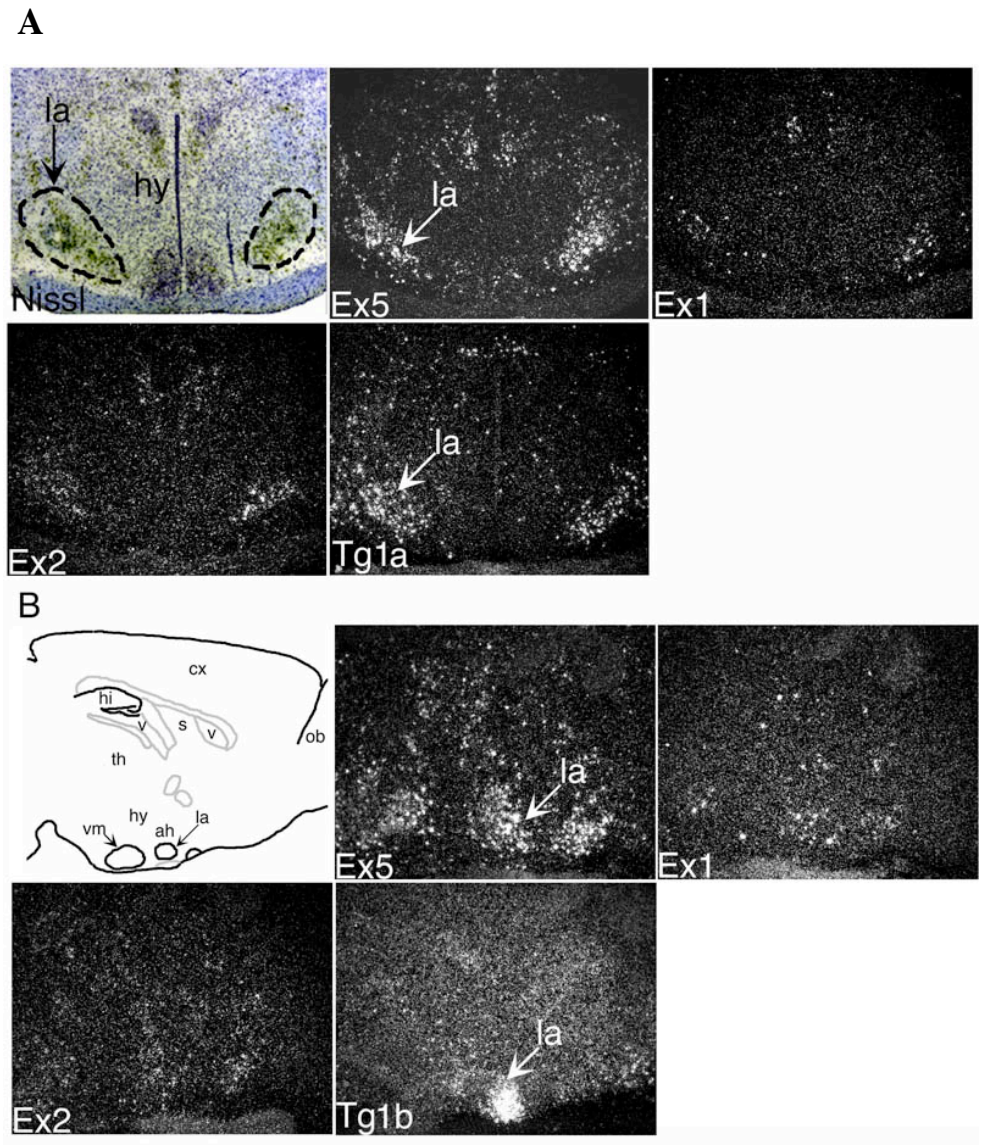

Fig. 16. Coronal (A) and sagital (B) sections of BDNF promoter 1-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the lateroanterior hypothalamic nucleus (la). Tg1a and Tg1b are promoter 1-specific transgenic animals from two distinct transgenic lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 1 (Ex1) or exon 2 (Ex2). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

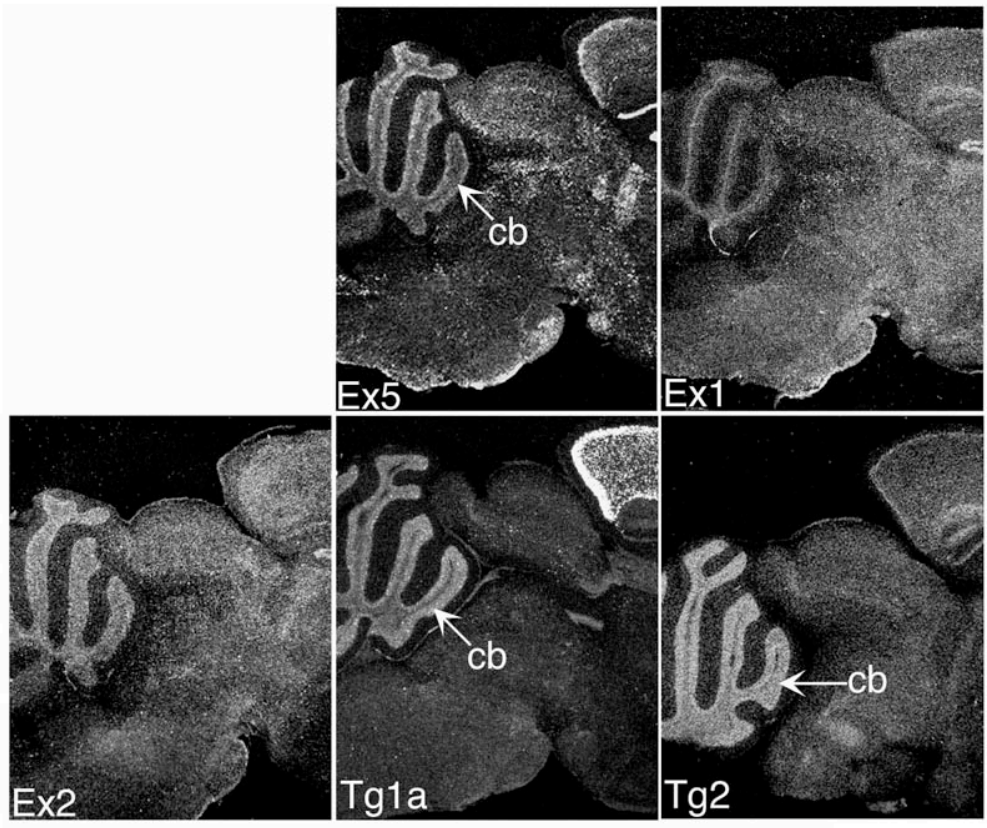

Fig. 17. Sagittal sections of BDNF promoter 1/2-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the granule cell layer of the cerebellum (cb). Tg1a and Tg2 are promoter 1 and promoter 2- specific transgenic animals. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 1 (Ex1) or exon 2 (Ex2). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

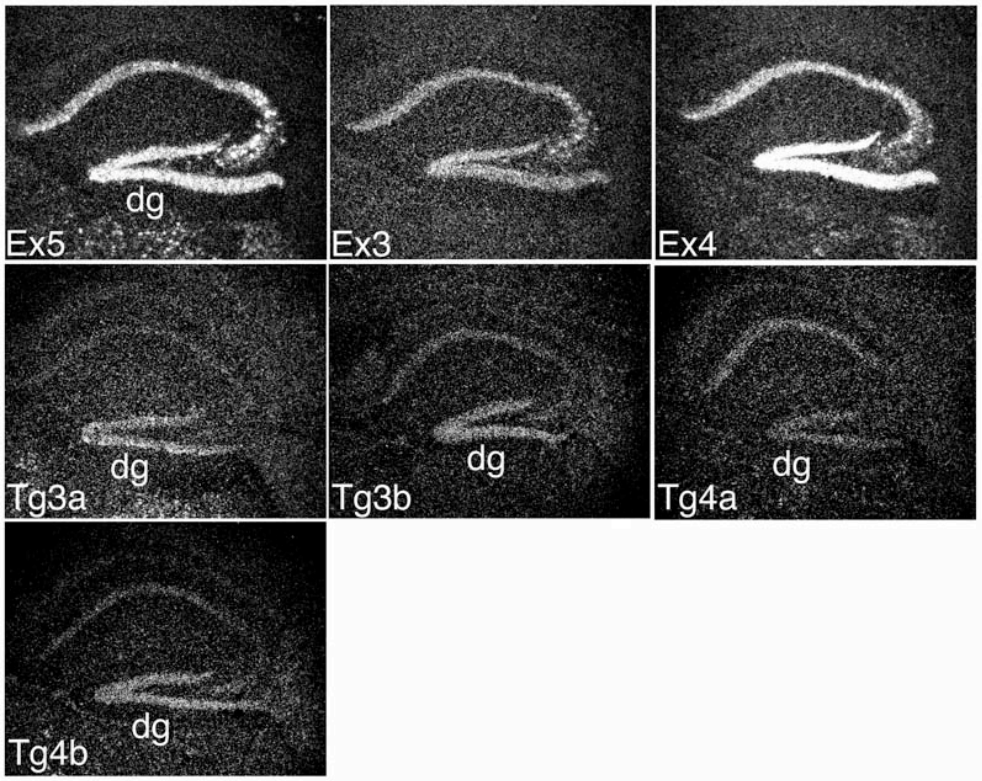

Fig. 18. Sagittal sections of BDNF promoter 3/4-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the hippocampal dentate gyrus (dg). Tg3a and Tg3b are promoter 3-specific transgenic animals from two distinct transgenic lines. Tg4a and Tg4b are promoter 4-specific transgenic animals from two distinct transgenic lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

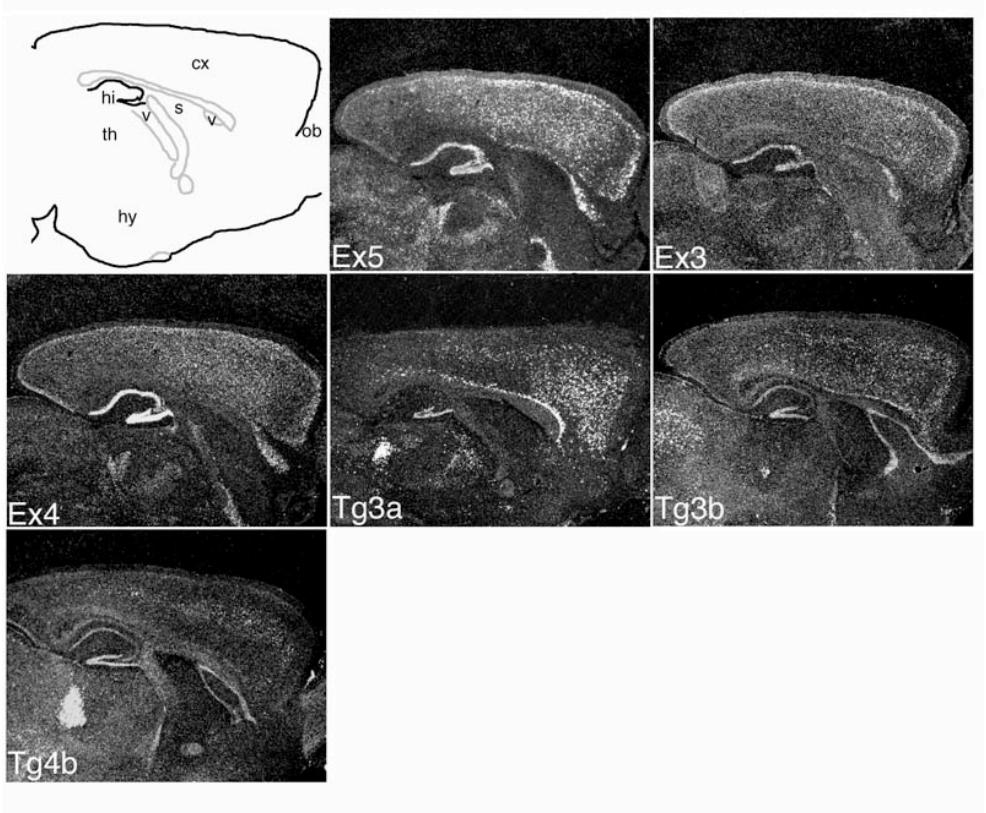

Fig. 19. **Sagittal sections of BDNF promoter 3/4-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the cortex (cx). The striatum (s) serves as a negative control. Tg3a and Tg3b are promoter 3-specific transgenic animals from two distinct lines. Tg4b is a promoter-4 specific transgenic animal. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.**

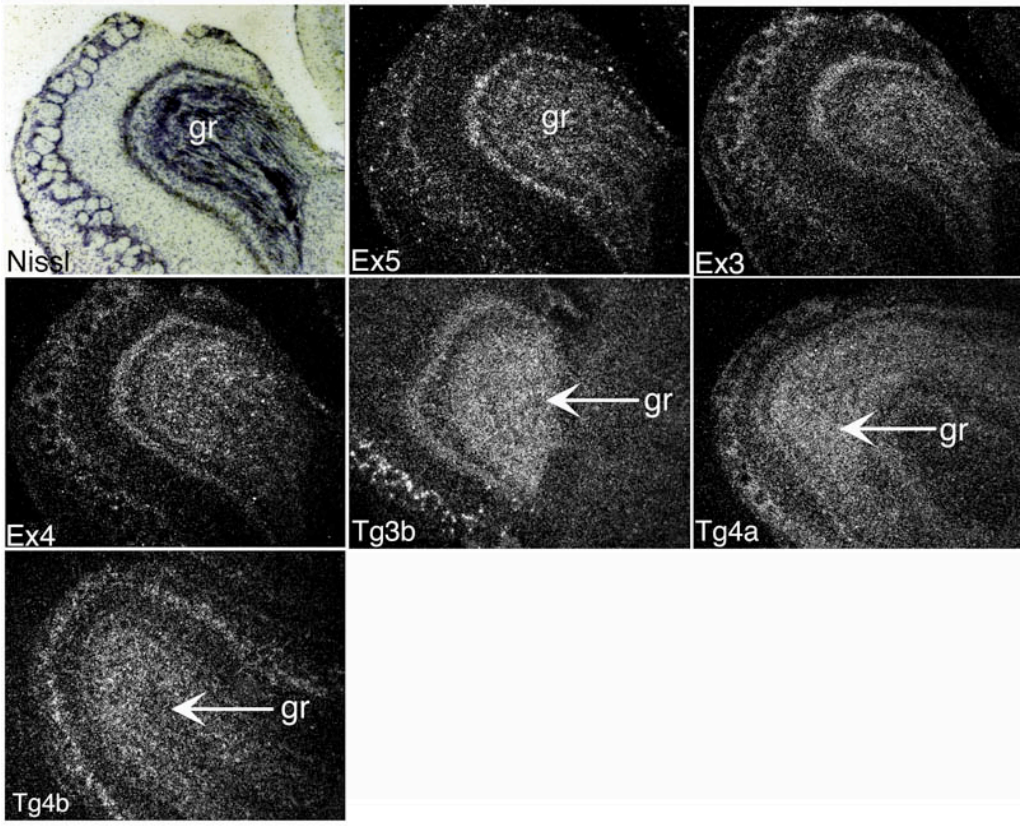

Fig.20. Sagittal sections of BDNF promoter 3/4-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the olfactory bulb granule cell layer (gr). Tg3b is a promoter 3-specific transgenic animal. Tg4a and Tg4b are promoter 4-specific transgenic animals from 2 distinct lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

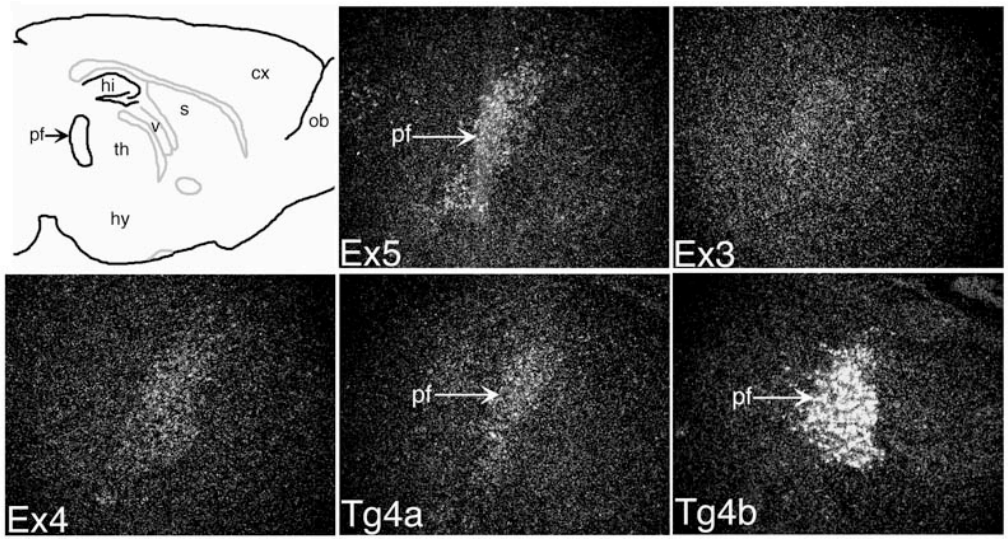

Fig.21. Sagittal sections of BDNF promoter 3/4-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the parafascicular thalamic nucleus (pf). Tg4a and Tg4b are promoter 4-specific transgenic animals from two distinct lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

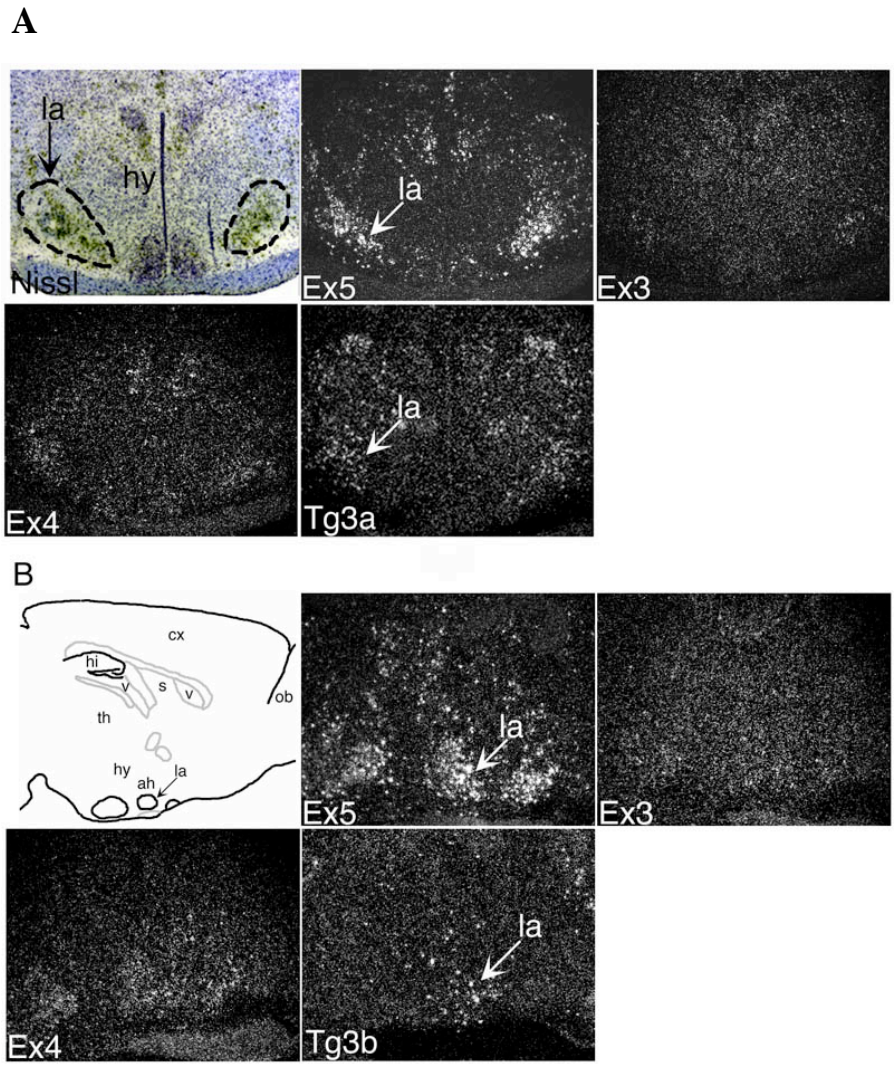

Fig.22. Coronal (A) and sagittal (B) sections of BDNF promoter 3-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the lateroanterior hypothalamic nucleus (la). Tg3a and Tg3b are promoter 3-specific transgenic animals from two distinct lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

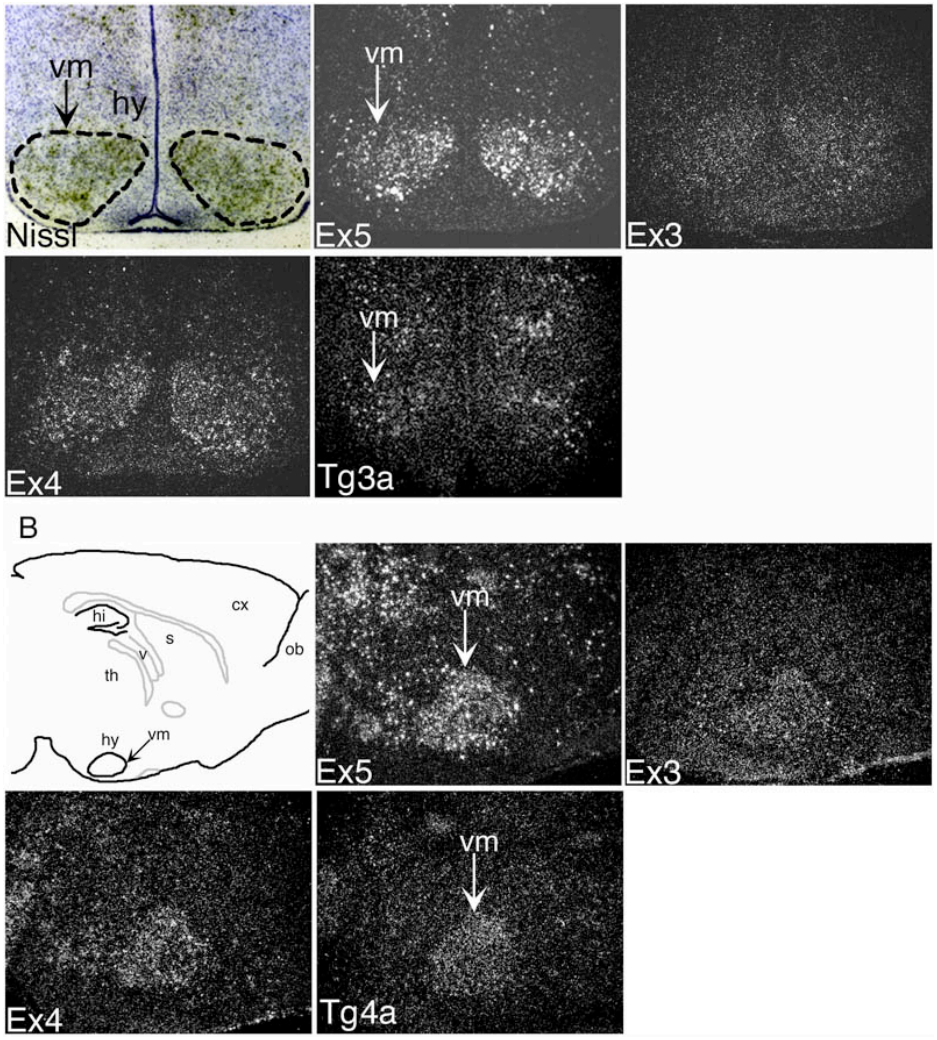

Fig. 23. Coronal (A) and sagittal (B) sections of BDNF promoter 3/4-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the ventromedial hypothalamic nucleus. Tg3a and Tg4a are promoter 3 and promoter 4-specific transgenic animals respectively. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

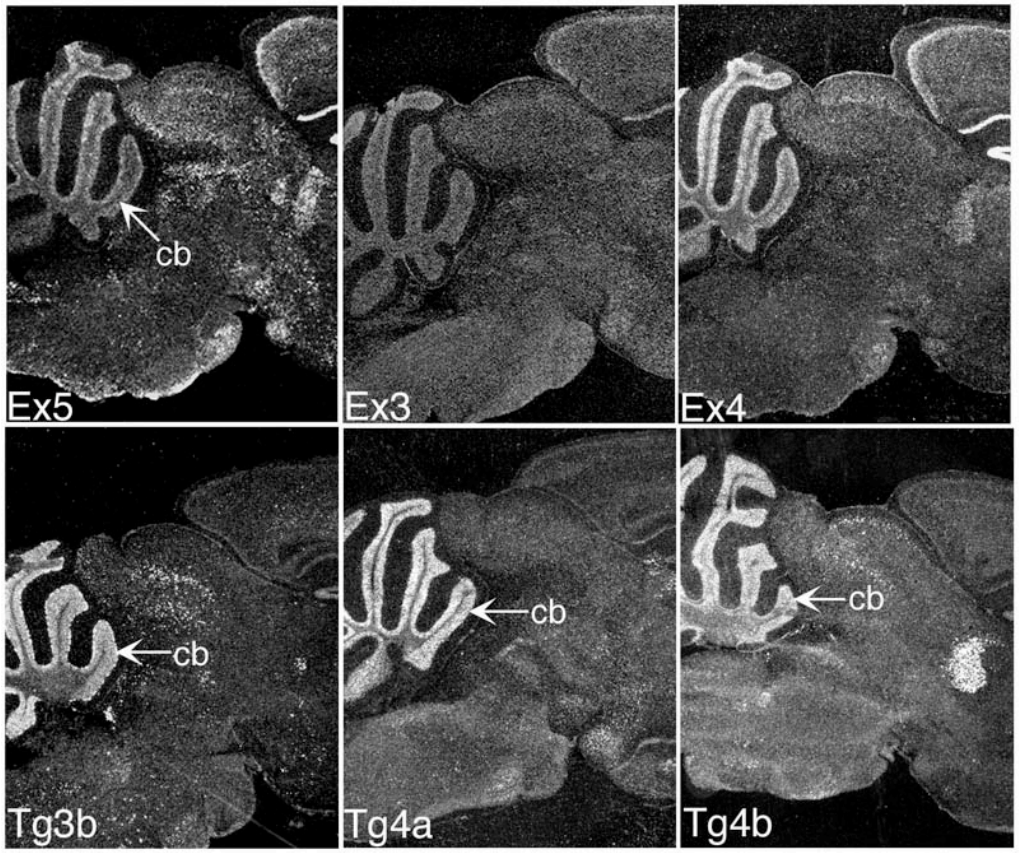

Fig.24. Sagittal sections of BDNF promoter 3/4-specific transgenic lines showing the expression of GFP mRNA by *in situ* hybridization in the cerebellar granule cell layer (cb). Tg3b is a promoter 3-specific transgenic animal. Tg4a and Tg4b are promoter 4-specific transgenic animals from two distinct lines. Transgenic animals express a BDNF-GFP reporter. Transgenic sections were compared to wild-type sections hybridized with either BDNF exon 5 (Ex5), exon 3 (Ex3) or exon 4 (Ex4). Wild-type animals were 7-9 weeks of age. Transgenic animals were 4-8 weeks of age.

Chapter. 5 Comparative genomic analysis applied to the study of brain-derived neurotrophic factor

Introduction

Comparative genomics, also referred to as phylogenetic footprinting is becoming a powerful tool for identifying the elements regulating gene expression in multicellular eukaryotes (Miller et al., 2004). The goal of this study was to identify putative cis-regulatory elements of BDNF using comparative genomic analysis, and discuss them relative to the transgenic studies in chapter 4.

Alignments between mouse, human, chicken and frog were made using zPicture within 100kb flanking regions of the BDNF gene, and the gene itself included. 7 evolutionarily conserved regions (ECRs) between mouse, human and chick were identified. Three of these ECRs (ECR 1, ECR 4 and ECR 7) are partially conserved in the frog. In theory, the higher the conservation of an ECR the higher its likelihood of functionality, making ECRs 1, 4 and 7, the ECRs with highest likelihood of functionality. ECRs 4 and 7 are present within the 3'UTR, which is present in the Timmusk constructs (but not those of the chapter 4 study), and hence these ECRs have the potential to function as key enhancer elements as well as elements important for RNA stability. ECR 1 may be the most interesting candidate for future study due to its high conservation and location within the third intron and in none of the discussed constructs. rVISTA was used to identify transcription factor binding sites within ECRs 1-7, to determine whether this data supports the hypothesis that a given ECR is important in BDNF regulation.

Results

The objective of this study was to identify putative cis-regulatory elements in the mouse BDNF gene and discuss them relative to the transgenic study in chapter 4. For the comparative genomics alignment, the mouse BDNF sequence spanned 100kb on either side of the BDNF gene. Such a large fragment was used for this analysis based partially on the distance of putative neighboring genes

from BDNF and partially on arbitrary criteria (Table 8). Essentially, the objective was to compare conservation close to the BDNF gene to that further away from the BDNF gene.

The mouse sequence was first aligned with the orthologous human sequence using zPicture. The level of conservation obtained was very high, making this alignment in and of itself inappropriate for the identification of putative cis-regulatory elements.

Next, the mouse sequence was aligned with the orthologous chicken sequence. Repeat sequences were eliminated from this alignment by eye, since the zPicture feature Repeatmaster was found to be error prone: it disqualifies sequences with partial repeats that may still constitute potential cis-regulatory elements. In all, 7 ECRs were identified between the mouse, human and chicken (Fig. 26). Of these ECRs, 3 are present within the BDNF gene itself in introns 3 and 5 (ECRs 1, 2 and 3). ECR 1 (426bp) is 7.8kb downstream of the start site of exon 1 and hence just 1.9kb 3' of the chapter 4 transgenic construct 1/2 (Fig. 29). ECR 2 (208bp) is 2.9kb 5' of the start site of exon 3, and ECR 3 (123bp) is 3.4kb 3' of the start site of exon 4, and hence both ECRs are within the chapter 4 transgenic construct 3/4 (Fig. 30). ECR 4 (452bp) is 3.3kb 3' of the start site of the BDNF coding exon which is over 30kb 3' of the chapter 4 transgenic construct 3/4 (Fig. 29). Around 300bp of ECR 4 appear to be within the BDNF 3'UTR. ECR 5 (143bp) is 25kb upstream of BDNF exon 1 start site. ECR 6 (291bp) is 55kb upstream of BDNF exon 1 start site. Hence ECRs 5 and 6 are around 19kb and 50 kb respectively of chapter 4 transgenic construct 1/2. ECR7 (304bp) is special in that it flanks the 3' end of the BDNF coding exon and is within the 3'UTR in its entirety.

Next, the mouse sequence was aligned with the orthologous frog sequence. Only three ECRs were conserved between the mouse, human, chicken, and frog: ECR 1, ECR 4, and ECR 7 . However, ECRs 8 (232bp) and 9 (75bp) are conserved between the mouse, human and frog, but not the chick. They overlap with BDNF exon 1 and BDNF exon 3 respectively.

Based on zPicture, ECRs 1-7 are conserved 91%, 82%, 90%, 94%, 83%, 91%

and 96% respectively between the mouse and the human. ECRs 1-7 are conserved 76%, 75%, 71%, 73%, 72%, 73%, and 92% respectively between the mouse and the chicken. 123bp of ECR 1 are conserved 70% between the mouse and the frog. 332bp of ECR4 are conserved 77% between the mouse and the frog. 261bp of ECR 7 are conserved 83% between the mouse and the frog. Mouse ECR8 (232bp) is conserved 97% with the human and 81% with the frog. Mouse ECR 9 (75bp) is conserved 96% with the human and 70% with the frog.

The program rVISTA was used to identify putative transcription factor binding sites (TFBs) in ECRs 1-7 (Figs. 27-33). Due to the inherent problems with this (and any transcription factor hunting program) these results must be viewed with caution.

In addition, the promoter regions for transgenic constructs 1/2 and 3/4 from chapter 2 were aligned to determine any possible similarity between the regulatory elements. No similarity was found.

Conclusion

In theory, any of the identified ECRs could play a role in any of the many functions of BDNF. The rVISTA data are consistent with the hypothesis that these ECRs could potentially function to regulate BDNF expression in the target tissues of peripheral sensory neurons during development. These ECRs could also potentially function in neural progenitor survival, proliferation, differentiation and neurite outgrowth as well as having a later function in synaptic plasticity, learning and memory.

The fact that ECRs 2 and 3 are located within construct 3/4 which is insufficient for endogenous-like expression speaks to the idea that multiple elements are necessary for BDNF regulation. A similar conclusion can be made based on ECRs 8 and 9, which are conserved between the mouse, human and frog, but not in the chicken, and are located within constructs 1/2 and 3/4 respectively. The fact that ECRs 4 and 7 are highly conserved and present in the Timmusk construct but not in the constructs of this study raises the idea that they may be highly important for BDNF regulation or mRNA stability. The long

distance of ECRs 5 and 6 from the BDNF promoters brings their relevance into question although they are valid candidates for further study. Based on its location (within intron 3), high conservation and absence from all discussed constructs, ECR1 appears to be an excellent if not the best candidate for future study.

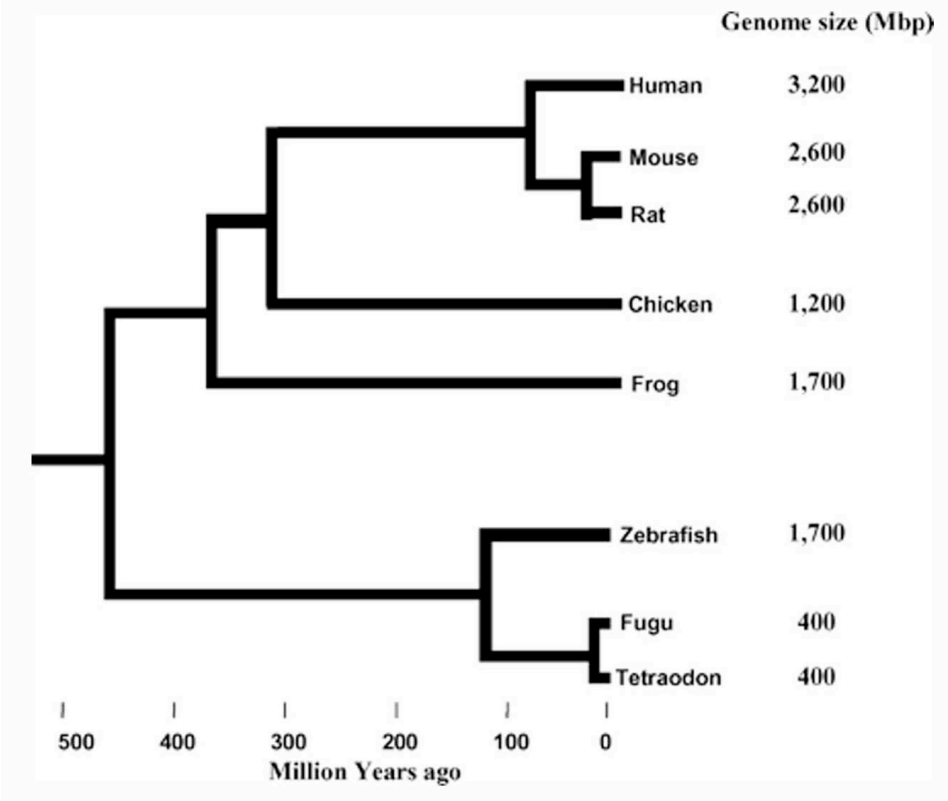

Fig. 25 The approximate divergence time of each of the eight vertebrate species whose genome sequences are currently available is represented. Haploid genome sizes are indicated in million base pairs.

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Chromosome: 2; 11p13; region 109000000-110000000

symbol	Distance from BDNF	Description
0610027B03Rik		Riken cDNA 0610027B03 gene
Kif18a		Kinesin family member 18A
LOC435682	279,376bp	Similar to peptidyl-prolyl cis-trans isomerase A
BDNF		Brain-derived neurotrophic factor
LOC435683	64,919bp	Similar to high mobility group protein-like 10
Lin7c		Lin 7 homolog (c. elegans)
Gpr48		G protein coupled receptor 48

Table 8. BDNF locus on mouse chromosome 2

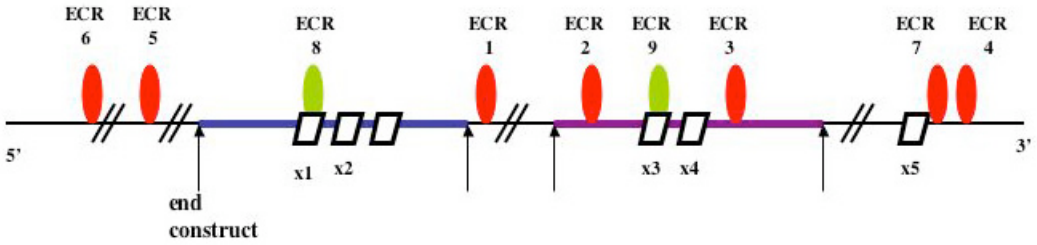

Fig. 26. Diagram of all evolutionarily conserved regions (ECRs) between 3 or more species (species being mouse, human, chicken, and frog) in the mouse brain-derived neurotrophic factor (BDNF) gene, identified by comparative genomics. X1-x5 represent BDNF exons 1-5, with exon 5 encoding the entire BDNF protein. Exons 1-4 are untranslated and associated with a promoter. The unlabelled exon was only recently identified (NCBI entryAY057907). Red ovals represent ECRs conserved between the human, mouse, and chicken and in the case of ECRs 1, 4 and 7, the frog as well. Green ovals represent ECRs conserved only between the mouse, human and frog. Transgenic constructs used in chapter 4 specific to exon 1/2 and exon 3/4 are labeled in blue and purple respectively.

	% ECR homology to mouse and size ECR			
	mouse	human	chicken	frog
ECR1	426bp	91% (430bp)	76% (419bp)	70% (123bp)
ECR2	208bp	82% (197bp)	75% (210bp)	---
ECR3	123bp	90% (123bp)	71% (123bp)	---
ECR4	452bp	94% (464bp)	73% (454bp)	77% (332bp)
ECR5	141bp	83% (141bp)	72% (145bp)	---
ECR6	298bp	91% (297bp)	73% (295bp)	---
ECR7	304bp	96% (307bp)	92% (376bp)	83% (261bp)
ECR8	232bp	97% (232bp)	---	81% (232bp)
ECR9	75bp	96% (75bp)	---	70% (75bp)

Table 9. Table showing 9 evolutionarily conserved regions (ECRs) between 3 or more species (species being mouse, human, chicken, and frog) in the mouse brain-derived neurotrophic factor (BDNF) gene. The table shows the size of each ECR and its percent homology to the mouse.

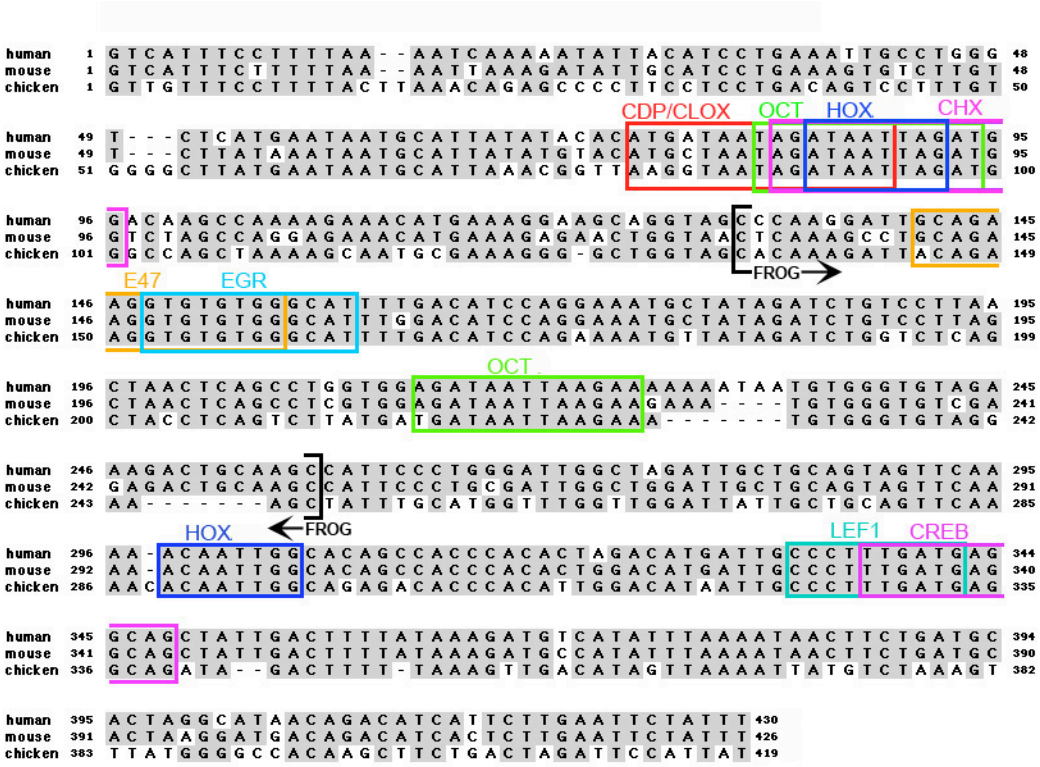

Fig. 27 ECR1: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. Region which aligns with the frog is also shown. rVISTA selected transcription factors are based on the mouse/chicken alignment. Mouse/human homology is 91%. Mouse/chicken homology is 76%. Mouse/frog homology is 70%.

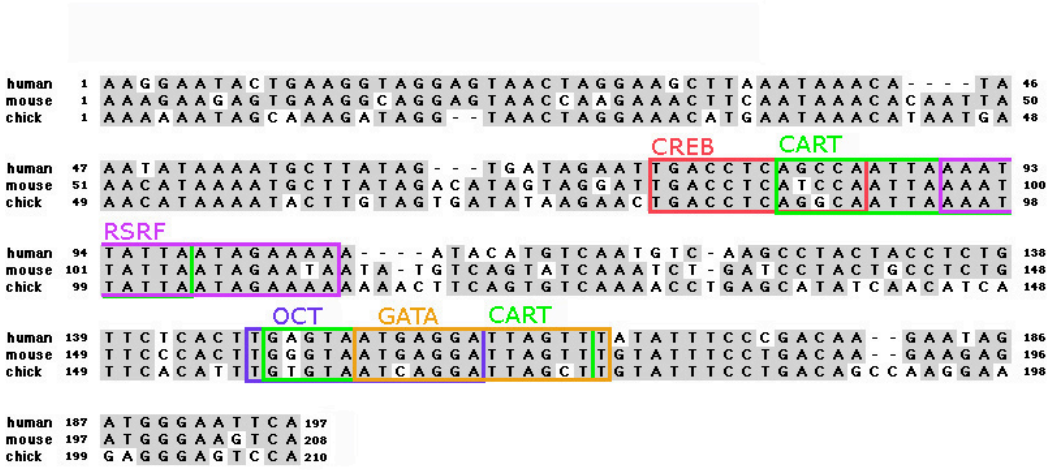

Fig. 28 ECR2: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. rVISTA selected transcription factors are based on the mouse/chicken alignment. Mouse/human homology is 82%. Mouse/chicken homology is 75%.

Fig. 29 ECR3: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. rVISTA selected transcription factors are based on the mouse/chicken alignment. Mouse/human homology is 90%. Mouse/chicken homology is 71%.

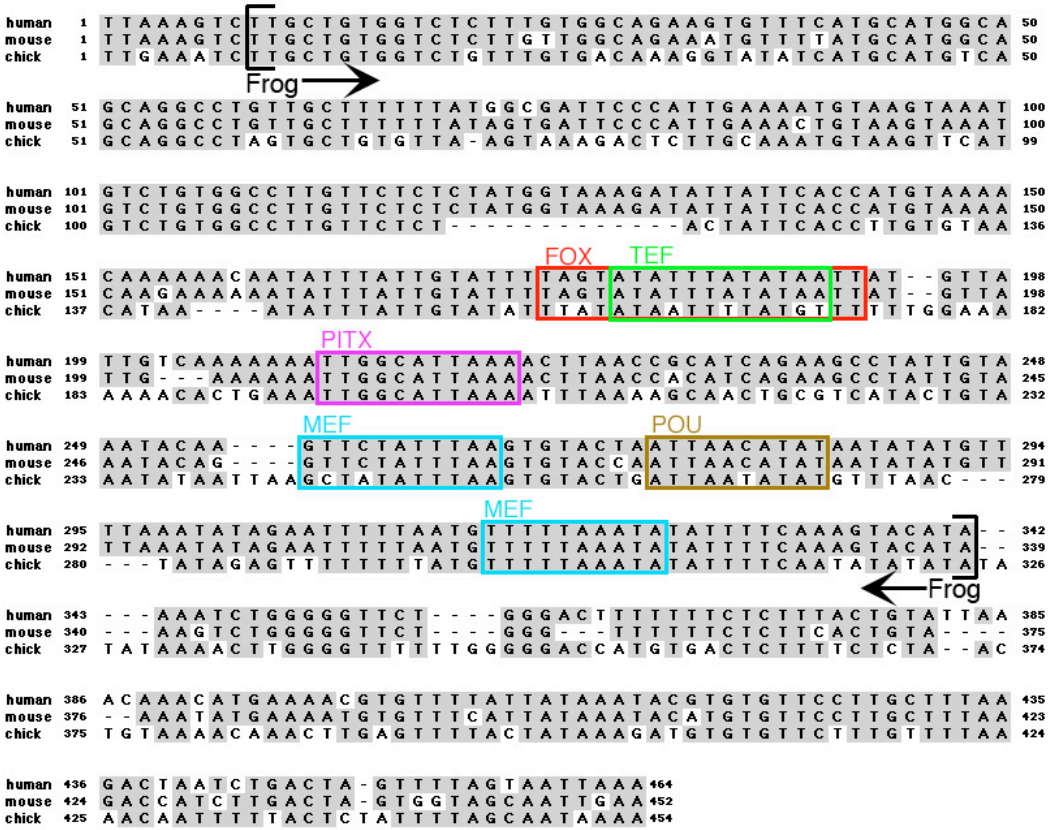

Fig. 30 ECR4: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. Region which aligns with the frog is also shown. rVISTA selected transcription factors are based on the mouse/chicken alignment. Mouse/human homology is 94%. Mouse/chicken homology is 73%. Mouse/frog homology is 77%.

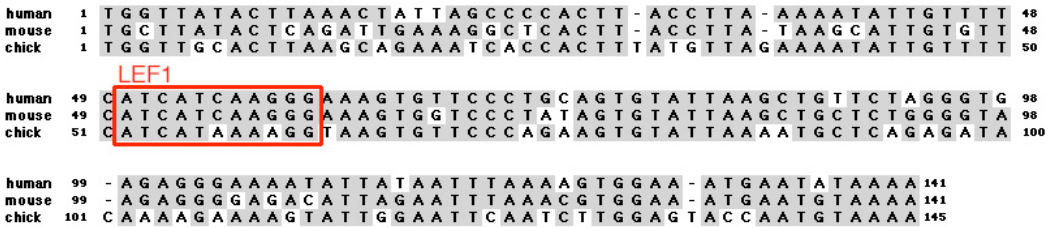

Fig. 31 ECR5: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. rVISTA selected transcription factors based on the mouse/human alignment. (the mouse/chicken alignment yielded no transcription factors). Mouse/human homology is 83%. Mouse/chicken homology is 72%.

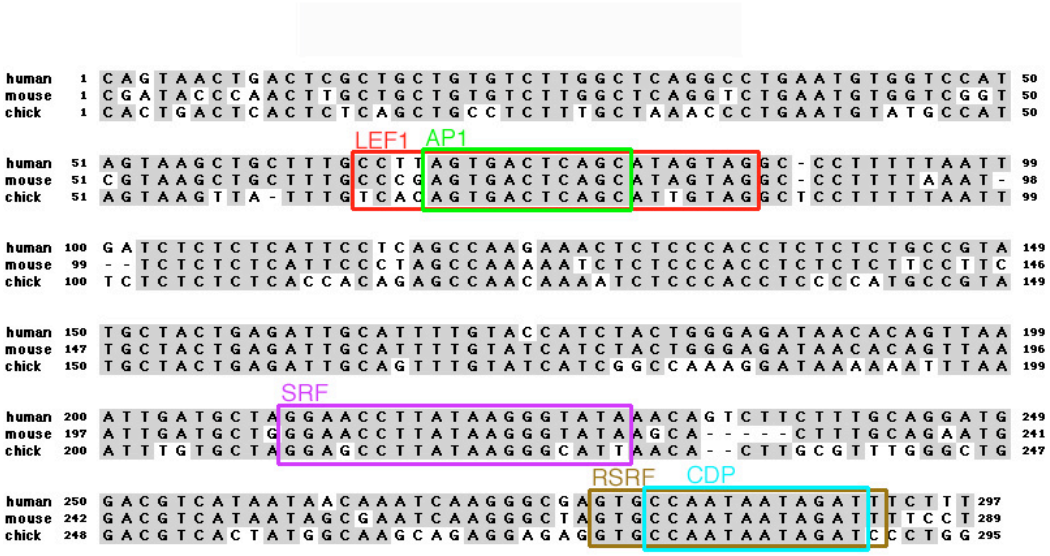

Fig. 32 ECR 6: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. rVISTA selected transcription factors based on the mouse/chicken alignment. Mouse/human homology is 91%. Mouse/chicken homology is 73%.

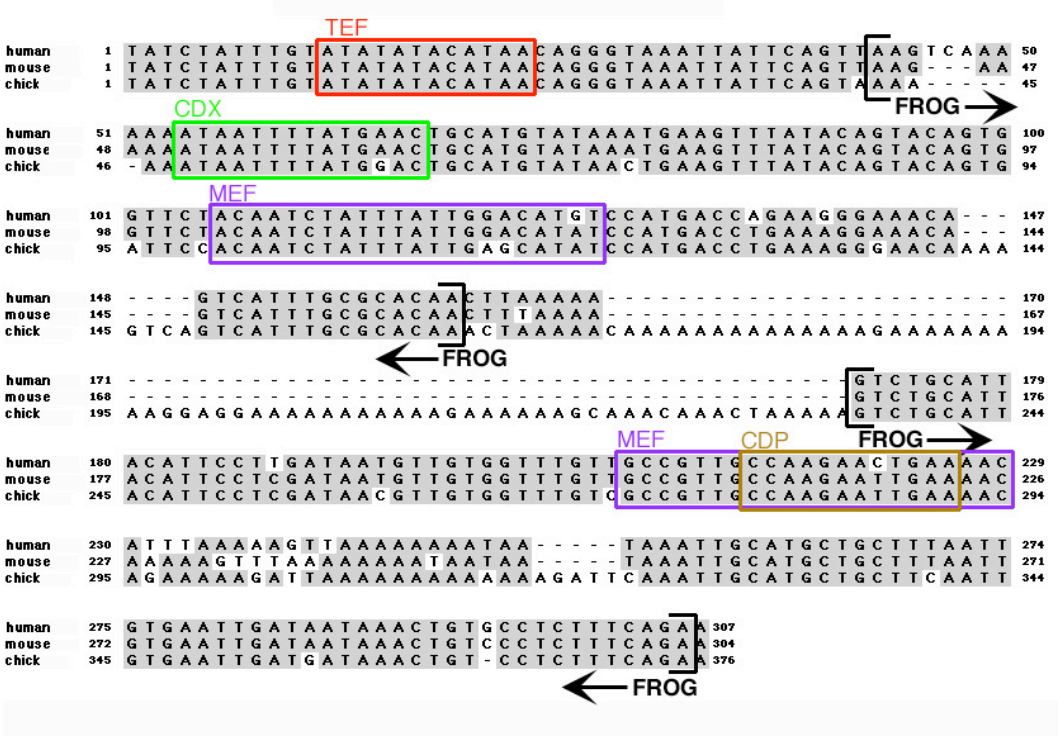

Fig. 33 ECR 7: alignment of human, mouse and chicken BDNF, showing transcription factors selected by rVISTA. rVISTA selected transcription factors based on the mouse/chicken alignment. Mouse/human homology is 96%. Mouse/chicken homology is 92%. Mouse/frog homology is 83%.

Chapter 6. Discussion

As discussed earlier, BDNF is a fascinating neurotrophic factor with highly widespread expression and a wide array of functions. There is a large body of work describing the functions of BDNF, however relatively little is known of its regulation. In this thesis, we attempted to learn more about BDNF regulation by means of: an *in situ* hybridization study of the four distinct exons in the adult mouse brain; use of transgenic animals to define BDNF promoter regions; and use of bioinformatics to identify evolutionarily conserved regions of BDNF.

Differential regulation of the four major BDNF transcripts

It appears likely that the four major BDNF transcripts are differentially regulated not only at the level of transcription, but also at the level of mRNA stability and translatability on account of their differing 5' regions. One hypothesis regarding the differential induction of the four major BDNF transcripts is that the timing, source and concentration of intracellular calcium determines which transcript(s) is expressed (West et al., 2001). In light of the fact that BDNF mRNA was shown to be transported into dendrites, it is also an interesting speculation, that BDNF transcripts tagged by the 4 different exons are differentially trafficked within the neuron (Tongiorgi et al., 1997). Recent evidence suggests that exon 4 is trafficked to the dendrites, while exon 3 remains in the nucleus (Pattabiraman et al., 2005).

In the present *in situ* hybridization study, the goal was to examine the similarities and differences in the expression patterns of BDNF exons 1-4 as a starting point to uncovering potential differential roles for the four BDNF promoters in the adult mouse brain. There are several conclusions that can be drawn from this study.

Firstly, looking at the results in table 1, it appears that the 4 BDNF exons are present in most of the same brain regions. Secondly, the expression pattern of

exon 1 is similar to that of exon 2, and the expression pattern of exon 3 is similar to that of exon 4. Finally, each major BDNF transcript shows differential expression. The section below speculates on the possible functions underlying the most striking examples of differential expression of the four BDNF exons.

Functional speculations based on the location of the 4 BDNF transcripts

Several studies suggest that exon 3 may be highly critical for LTP-type synaptic plasticity (Tabuchi et al., 2000; West et al., 2001). An interesting trend that was observed in exon 3 expression is that it is expressed at relatively high levels in associative areas that are dynamically modified by learning throughout an animals life, while it is expressed at comparatively low levels in relatively hard-wired regions, which are established to a greater degree during development (Kandel et al., 2000). Associative areas include the hippocampus, cortex, amygdala, and cerebellum; while, hard-wired areas include nuclei of the hypothalamus, and the awareness-related nuclei of the thalamus discussed below. Besides LTP-type synaptic plasticity, BDNF has been shown to function in homeostatic plasticity (that is maintaining neural firing rates within certain boundaries) and to have very acute neurotransmitter-like effects at synapses (Kafitz et al., 1999; Leslie et al., 2001). Presumably, these are functions of BDNF that are seen throughout the CNS, and hence one may hypothesize that they are more likely attributed to exon 1,2 and 4 transcripts as opposed to the exon 3 transcript. Furthermore, exon 1 and 3 transcripts as opposed to those of exons 2 and 4, are the dominant species induced by seizures (Kokaia et al., 1994; Metsis et al., 1993; Tetsuya Tsukahara, 1998).

In the hippocampus, there is prominent differential expression in the CA1 and dentate gyrus, where exons 1 and 2 are expressed at markedly lower levels than exons 3 and 4. In the hippocampus, BDNF has been implicated in short term plasticity, LTP, LTD, as well as spatial memory acquisition, consolidation and retrieval (Kijofumi Yamada, 2003; Lu, 2004). In this region, BDNF has also been linked to exercise, stress, depression, and neuronal insults such as seizures and

ischemia (Hashimoto et al., 2004; Kokaia et al., 1994; Russo-Neustadt et al., 2000).

In the cortex, exons 1 and 2 are expressed at significantly lower levels than exons 3 and 4. The cortex is a highly associative and plastic region believed to process sensory and motor stimuli, and function in aspects of learning, memory and emotion (Kandel et al., 2000). The cortex plays a critical role in complex processes such as the planning and execution of actions. In this region, BDNF has been implicated in short term plasticity, LTP, LTD, memory and emotion (Hashimoto et al., 2004; Lu, 2004; Yamada et al., 2002).

Considerable differential expression of the 4 BDNF exons is seen in the central medial, the parafascicular, and the anterodorsal thalamic nuclei. To date there have not been any studies to elucidate BDNF function in the thalamus. The thalamus relays stimuli from all sensory modalities except olfaction to the cerebral cortex for processing (Kandel et al., 2000). The central medial and the parafascicular thalamic nuclei both belong to the family of intralaminar thalamic nuclei, which has been implicated in visceral functions, arousal and awareness (Van der Werf et al., 2002). In the parafascicular nucleus, the exon 4 transcript predominates. The parafascicular nucleus distinguishes itself from the central medial nucleus in that it has predominant projections to the basal ganglia, and relatively sparse projections to the cortex. This fact along with a number of studies implicates this nucleus in the control of motor actions in response to relevant stimuli. In the central medial thalamic nucleus, the transcripts for exons 2 and 4 predominate. In contrast to the parafascicular nucleus, the central medial nucleus has predominant projections to the prefrontal and anterior cingulate cortices, and appears to be more implicated in cognitive awareness. In the anterodorsal thalamic nucleus, the exon 4 transcript predominates. The anterodorsal nucleus, an anterior thalamic nucleus forms a circuit with the anterior and posterior cingulate cortices, the hippocampus, and the mammillary nuclei (Paxionos, 2004). It has been implicated in spatial orientation, spatial memory and attentional functions. The anterodorsal nucleus is unusual among thalamic

nuclei in that it has so called "head direction" (HD) cells which signal the rat's directional heading, as the rat navigates through an environment.

As stated earlier, the hypothalamus is a relatively "hard-wired" region, where expression of exon 3 is markedly lower than that of the other three exons in all examined nuclei. The hypothalamus is believed to play a critical role in homeostasis, which involves the integration of endocrine, autonomic, somatomotor and environmental information. Homeostatic functions include temperature, blood pressure, food-intake, and sleep regulation. Key to hypothalamic function is its control of hormone secretion by the pituitary gland, which is important for its function in mating, maternal, and aggressive behaviors. BDNF has been implicated in feeding and aggressive behavior, both functions in which the hypothalamus is believed to be involved (Kernie et al., 2000; Lyons et al., 1999; Rios et al., 2001). The mammillary body is a hypothalamic region of particularly striking differential expression of the 4 BDNF exons. Exon 3 as well as exon 1 expression is markedly lower than exon 2 and 4 expression in this region. The mammillary body receives dense hippocampal projections, projects predominantly to the anterior thalamic nuclei, and has been implicated in the encoding of spatial information (Vann and Aggleton, 2004). The lateral mammillary nuclei possess head direction neurons (described above) and are important for directional firing of head direction cells of the anterior thalamic nuclei. Medial mammillary neurons were shown to fire in synchrony with hippocampal theta rhythm, which has been linked to spatial processing and LTP.

The cerebellum is a striking region of differential expression as the exon 1 transcript is absent, while all the other exons are expressed at comparable levels. The cerebellum receives somatosensory information from the spinal cord, motor input from the cerebral cortex, and balance input from the vestibular organs (Kandel et al., 2000). It has been implicated in balance, coordination, smooth movement and the learning of motor skills. LTP and LTD of the parallel fiber-Purkinje cell synapse has been implicated in various forms of motor learning, such as associative eyelid conditioning (Hansel et al., 2001). It has been shown that in BDNF null mice, paired-pulse fascilitation (a form of short term plasticity)

at the parallel fiber-Purkinje cell synapse is significantly reduced (Carter et al., 2002). Furthermore, BDNF has been implicated in the stargazer mutant cerebellar phenotype, which consists of ataxia, head tossing and impairment in associative eyelid conditioning (Richardson and Leitch, 2005).

Transgenic study to identify brain-derived neurotrophic factor promoter regions mediating expression in distinct brain areas

The present transgenic study originated from the experiments in our laboratory performed by Kernie et al. (2000). Kernie et al. (2000) discovered that BDNF heterozygous mice had either an obese or a hyperactive phenotype, and these two distinct phenotypes correlated with differential BDNF expression in two hypothalamic nuclei (Kernie et al., 2000). It was hypothesized, that these two different phenotypes resulted from differential silencing of BDNF promoters in the two hypothalamic regions. Hence, the next step was the determination of expression of the four BDNF exons (promoter reporters) in these two hypothalamic regions. The result that all 4 exons were present in both hypothalamic regions, suggested that differential promoter silencing was not responsible for the two distinct phenotypes. However, these results prompted a detailed brain-wide *in situ* hybridization (ISH) study of the expression of the 4 BDNF exons in the adult brain, to determine the extent of differential expression of these exons (chapter3). This ISH study was essential to the present transgenic study on the regulation of BDNF since, noone had previously analysed in detail, the expression of the four endogenous BDNF exons throughout the adult brain at baseline levels.

While many studies show examples of the differential expression of the four major types of BDNF transcripts in response to a variety of stimuli, to date, virtually no information exists regarding the cis-regulatory elements that regulate BDNF expression. The present study, attempted to use transgenic animals to identify promoter regions for BDNF promoters 1-4 that would recapitulate endogenous exon 1-4 expression throughout the brain. Four transgenic constructs specific to each of the four BDNF promoters were designed (Fig. 10, 11). Since

there is a GFP reporter in these transgenic constructs, it is possible to localize transgenic signal by ISH to specific groups of cells. Notably, the reporter used in these constructs is in fact a BDNF-GFP fusion, and it was shown that this BDNF-GFP fusion protein is functional (Table 2, Fig. 12). Using immunohistochemistry to detect GFP was not successful, even though by western blotting, GFP protein was detected in at least one transgenic line (data not shown). In the event that endogenous expression was successfully recapitulated using these constructs, resulting transgenic lines could be used for further studies, such as the identification of cis-regulatory elements and the rescue of BDNF mutant mice. The results of the present study suggest that constructs with promoter regions of size 11.4kb and 16kb for promoters 1/2 and 3/4 respectively are insufficient to drive endogenous-like expression in the brain and nonneural tissues.

Present study versus Timmusk study

It was initially hypothesized that the transgenic constructs of the present study contain all necessary elements to recapitulate endogenous-like expression, based on a previous study (Timmusk et al., 1995), which used similar sized constructs to generate transgenic animals. In the Timmusk study, a CAT reporter was used for transgenic expression and it was shown that high levels of transgenic expression were present in the major brain regions of high endogenous BDNF expression, with minor differences. CAT mRNA and endogenous BDNF mRNA were quantitated for several lines, and it was shown that transgenic mRNA was equivalent or higher than endogenous BDNF mRNA in the hippocampus. For most of the lines, transgenic expression appeared to be highest in the hippocampus of all brain regions, which is true of endogenous BDNF expression. Surprisingly, the Timmusk study showed that most founder lines for each of the constructs described above gave very similar expression patterns, suggesting the presence of an insulator-like element in their constructs. While the CAT enzymatic assay allows for considerably more sensitive detection of signal, in the present study – unlike in the CAT study – it was possible to localize the GFP signal to specific nuclei (as opposed to only entire brain regions) and state

whether expression has an endogenous-like pattern in a given region. The major differences between the Timmusk study and the present study are as follows. Besides the fact that a CAT-BDNF fusion was used instead of a BDNF-GFP fusion, constructs used in the Timmusk study are different in the size of their 3' and 5' regions, (5' regions are longer by 1kb; 3' regions are considerably shorter), and they possess the BDNF 3'UTR and a mini intron (unlike constructs of the present study). As a result of these factors, it is possible that the constructs of the present study lacked cis-regulatory elements present in the Timmusk constructs, or had suboptimal RNA transcription or stability. In summary, the reproducibly high transgenic expression levels seen in most major brain regions in the Timmusk study, were not obtained in the present study. Furthermore, none of the genomic regions used in the present study were sufficient to drive endogenous-like transgenic expression. It is clearly troubling that in the present study only a relatively small fraction of the total number of transgenic animals showed relatively high *in situ* hybridization signal in multiple brain regions. However, by RT-PCR, 20 out of 27 transgenic animals showed expression in the brain, suggesting that the constructs of the present study contain brain-region specific elements. In addition the exon-specific transgenic noneural tissue data is supportive of CNS specificity of exon1-specific transgenics. Also the fact that all four exon-3 and exon-4 specific transgenic lines showed expression in the kidney, suggests that a kidney specific element may be present in these constructs. Nevertheless many key neural and nonneural elements are clearly missing from the constructs of the present study.

Due to the limited number of animals used in this study, particularly for cases where only one animal was used, it is possible that sections showing expression in key regions were missed. Hence, it must not be assumed that nuclei where expression is not reported lack transgenic expression.

Finally, the many differences from endogenous expression and differences in expression between lines generated from the same or similar constructs can be explained by both the fact that key cell-specific regulatory elements are missing in the constructs, and the fact that position effects are at play.

Bioinformatics to elucidate ECRs of BDNF

Although the zPicture parameters were altered to accomodate for ECRs shorter than 100bp and somewhat lower than 70% homology between the mouse and the chicken, it was not possible to find additional ECRs, besides the 7 ECRs discussed below that were sufficiently conserved in the mouse, chicken and human. Two ECRs were also identified that were conserved in the mouse, human and frog, but not the chicken.

The fact that ECRs 2 and 3 are present in the transgenic constructs 3/4 (chapter 2) suggests that important regulatory elements are present in these constructs, even though these elements appear to be insufficient to drive endogenous-like expression throughout the brain. Essentially, these results suggest that multiple elements function in concert for endogenous-like transgenic expression. The same argument can be made for ECRs 8 and 9 which are conserved between the mouse, human and frog, but not the chicken and present in constructs 1/2 and 3/4 respectively. The long distance of ECRs 4, 5, 6, and 7 from the BDNF promoters puts their relevance to BDNF regulation at question. ECR 4 and ECR 7 are present in the 3' UTR and since the 3'UTR of BDNF was present in the constructs of Timmusk et al. 1995 - but not the constructs of the present study- this suggests that ECRs 4 and 7 could have a very important function either as cis-regulatory elements or in mRNA stability. Implying importance for ECR 2, this ECR was also present in the exon 3/4 transgenic construct of Timmusk et. al. 1995 but none of the other 7 ECRs discussed were present in their constructs. In spite of their distance from the BDNF promoters, ECRs 5 and 6 are naturally also valid candidates for cis-regulatory elements of BDNF. The high conservation and proximity of ECR 1 to promoters 1 and 2, makes it a good candidate for a highly important cis-regulatory element of BDNF. All ECRs identified in this study show rVISTA selected binding sites for transcription factors that are potential regulators of BDNF and while as discussed earlier there are many problems with the rVISTA program, this is nevertheless a promising outcome.

Due to the complexity of the BDNF gene, it appears likely, that functional ECRs exist that are conserved only in the human and the mouse, and not all the way down to the chicken; however, it is known that the higher the conservation the higher the chances of functionality. This means that ECR 1 is more likely to be functional than an ECR that is only conserved in the mouse, chicken and human. Due to the high conservation of sequence between the mouse and the human, it would appear to be a guessing game as to which elements are most likely to regulate BDNF, based on this alignment alone. The elements in the exon regions themselves are very highly conserved between the mouse and the human, but these are present in all the constructs and hence did not appear to be sufficient for endogenous-like BDNF expression in the CNS. Finally, it is conceivable that key elements conserved between the human and mouse (but not the chicken) are present in the 1kb 5' regions of the Timmusk constructs not present in those of the present study and this could explain differences between the two studies: mouse-human conservation is clearly high in these regions.

Additionally, the fact that there was no alignment of chapter 2 transgenic construct 1/2 with construct 3/4 suggests that no common cis-regulatory elements function close to each promoter. This may not seem surprising in light of the knowledge of the differential regulation of the 4 promoters.

Correlating BDNF function to that of transcription factors selected to bind ECR 1 by rVISTA

For more in depth analysis of rVISTA results we have chosen ECR1, since as discussed earlier, this appears to be the best candidate for future in vivo studies, although a similar analysis is appropriate for other ECRs. Functional data on transcription factor family members pulled out by rVISTA that bind ECR1 are consistent with the function of BDNF. As discussed previously, in vivo data is necessary to validate any comparative genomics study, nevertheless, many cis-regulatory elements have been discovered using comparative genomics as a starting point. Inspite of being one of the best programs of its kind, there are

many problems with rVISTA and it is sufficient to say that rVISTA will miss relevant transcription factor binding sites and it will identify irrelevant transcription factor binding sites. Below, relevant information regarding the transcription factor families identified by rVISTA for ECR1 will be discussed. Although rVISTA only provides the name of a specific transcription factor as a putative binding candidate for a particular site, other family members of that transcription factor are likely to bind the same site. Hence, the entire family of transcription factors for each specific transcription factor that rVISTA identifies as binding to ECR1 is discussed.

Four different subfamilies of homeodomain genes were pulled out by the rVISTA screen: HOXA4, CDP, CHX10, and OCT-1. The fact that HOXA4 and OCT-1 were pulled out twice is promising in the sense that it increases the likelihood that a transcription factor from these subfamilies indeed binds ECR1.

Hox proteins are part of a larger class of transcription factors that all contain a highly conserved DNA binding domain. This homeodomain (helix-turn-helix) is encoded by a 180 base-pair long conserved DNA sequence the homeobox (Hombria and Lovegrove, 2003). Mammalian hox genes are homologous to drosophila homeotic genes (Hombria and Lovegrove, 2003). They encode a family of transcription factors that have well established functions in specifying regional identity along the antero-posterior axis, and in organogenesis. Hox gene expression in embryonic tissues is consistent with the hypothesis that hox genes may regulate BDNF expression in the peripheral targets of sensory neurons. In the hindbrain, it has been shown that hox genes regulate the expression of other genes in neural progenitors, immature neurons as well as differentiating neural subtypes, and hox genes have also been implicated in forebrain development (Davenne et al., 1999; Gavalas et al., 2003). Certain Hox genes also appear to be present in the adult brain.

CDP/CUX/CUT transcription factors belong to a family that contains a homeodomain and one, two or three CUT repeats which bind DNA (CDP, CDP-2, Cux-1, Cux-2, CLOX) (Nepveu, 2001; Nieto et al., 2004). Cux gene expression in embryonic tissues is consistent with the hypothesis that cux genes may regulate

BDNF expression in the peripheral targets of sensory neurons (Nepveu, 2001; Nieto et al., 2004). Drosophila Cut family genes specify cell identity in a number of tissues. They have been shown to specify neural cell subtype and regulate dendritic morphology in the Drosophila peripheral nervous system. Cux (mammalian) family members have been detected in neurons of the developing and adult brain, and have been hypothesized to function in neural precursor proliferation and neural differentiation. Cux genes were found to generally function as transcriptional repressors (although examples of transcriptional activation exist). The homeobox gene CLOX is a member of the CDP/CUX/CUT family.

CHX10 belongs to the paired class of homeodomain proteins which in addition to a homeodomain, possesses another DNA binding domain known as the paired domain (Haubst et al., 2004; Wong, 1999). Members of this family are candidates for regulating BDNF expression in the target tissues of sensory neurons (Qu et al., 1999). This family has also been implicated in neural patterning, cell proliferation and neural differentiation (Haubst et al., 2004; Wong, 1999). Paired class homeodomain proteins have also been detected in neurons of the adult brain (Ziman et al., 1997).

One member of the POU family of transcription factors was pulled out by the rVISTA screen, OCT-1. The POU domain contains both a diverged homeodomain and a POU-specific DNA binding domain (helix-turn-helix) (Latchman, 1999). Oct-1 was shown to be ubiquitously expressed That is expressed in all tested tissues). Studies indicate that POU family members have critical functions in the developing and adult nervous systems (Latchman, 1999). They have been implicated in neural cell death, neuronal stem cell-specific transcription, neural differentiation and neurite outgrowth. Furthermore, POU family members, specifically Brn-3c have been implicated in regulating BDNF expression in the peripheral targets of sensory neurons (Clough et al., 2004).

E47 is a member of the basic helix-loop-helix family of transcription factors and is characterized by a basic DNA-binding region as well as an HLH domain that mediates homo- or heterodimerisation (Ik Tsen Heng and Tan, 2003;

Massari and Murre, 2000). BHLH factors have been implicated in a wide variety of developmental processes, such as the development of the haemopoietic system, muscle, heart, lung, inner ear and pancreas (Ik Tsen Heng and Tan, 2003; Massari and Murre, 2000). Their presence in embryonic tissues makes them candidates for regulating BDNF expression in the peripheral targets of sensory neurons. They have also been implicated in neural precursor proliferation, neural differentiation, and neural subtype specification. USF bHLH factors are believed to be ubiquitously expressed, and have previously been shown to regulate BDNF (Chen et al., 2003).

The rVISTA screen pulled out LEF1, TCF1 and TCF4 which have virtually identical binding sites and belong to the Lef1/Tcf family of transcription factors (TCF3, TCF4). This family is characterized by a DNA binding domain that consists of 70-80 amino acids that form an L-shaped fold from three alpha-helical segments (Moon et al., 1997; Shimogori et al., 2004). The LEF1/TCF family are nuclear mediators of canonical wnt signaling. Wnt signaling has been implicated in processes that include patterning the anterior-posterior axis, and aspects of organogenesis such as cell proliferation, differentiation, and fate specification. (Moon et al., 1997; Shimogori et al., 2004). In the adult, they have been implicated in carcinogenesis, and degenerative diseases. At least one Wnt family member, Wnt-5b shows unrestricted expression in the embryo. Wnt presence in embryonic tissues makes LEF1/TCF factors good candidates for regulating BDNF expression in the peripheral targets of sensory neurons. To date, wnts have been detected in only select regions of the adult brain.

CREB belongs to the bZIP family of transcription factors (CREM, ATF-1), which possess a basic alpha-helical DNA binding domain with an adjacent leucine zipper dimerization motif (Lonze and Ginty, 2002; Meyer and Habener, 1993). The CREB subfamily members form homo and heterodimers. CREB is activated in response to a wide range of stimuli and is ubiquitously expressed (Lonze and Ginty, 2002; Meyer and Habener, 1993). Hence CREB is a good candidate for regulating BDNF expression in the peripheral targets of sensory neurons during development. Also CREB family transcription factors have been

implicated in embryonic and postnatal neuronal survival, precursor proliferation, and neuronal differentiation as well as in synaptic plasticity, learning and memory.

EGR 1 belongs to the early growth response transcription factor family (EGR 2-4) that is characterized by a highly conserved DNA binding domain composed of three zinc fingers (Knapska and Kaczmarek, 2004). EGR family members are regulated by stimuli such as neural activity, growth factors and hormones as immediate early genes. They have been implicated in hindbrain segmentation, neural proliferation, differentiation, synaptic plasticity, learning and memory (Knapska and Kaczmarek, 2004). At baseline levels, they appear to be expressed throughout the adult brain. Their expression in embryonic tissues suggests that they may also be candidates for regulating BDNF expression in the peripheral targets of sensory neurons.

This study suggests that there is a 426bp ECR in the third intron of BDNF that is highly conserved between the mouse, human, chicken, and frog. Information that was gathered on the transcription factor families identified in an rVISTA search using this ECR is consistent with the function of this ECR in several processes in which BDNF is believed to be involved. These processes include target-derived sensory neuron survival, neural progenitor survival, proliferation, differentiation, neurite outgrowth, synaptic plasticity, learning and memory.

For the validation studies outlined below, based on functionality, conservation and practicality (it does not appear to make sense to pick a member of a very large family with no particular candidate in mind), it appears that three particularly good candidates are OCT-1, CREB, and EGR1. OCT-1 occurs twice in our ECR; both OCT-1 and EGR are conserved all the way down to the frog, and all three transcription factors have the potential to regulate BDNF function in peripheral targets of sensory neurons as well as in the adult brain. It may however be worthwhile to mention that when 50kb chunks of DNA in relatively gene-free regions on 3 separate chromosomes were compared for the number of CREB sites pulled out by rVISTA, on average 1 CREB site was identified per 990bp. Since

in ECR1, 1 CREB site appears in 426bp, these data suggest that the CREB site found in ECR 1 has a strong likelihood of being random.

Future Studies

Based on the *in situ* hybridization study, the transgenic study and the bioinformatics study of this thesis, it appears that the regulation of the BDNF gene is much more complicated than we previously envisioned. For the future, we propose two approaches for elucidating the regulatory elements of BDNF. One approach would involve a minimal enhancer identification/ deletional analysis transgenic strategy using a BDNF bacterial artificial chromosome (bac). This would involve appropriately placing a GFP reporter in a bac construct. Placing GFP behind the exon associated with promoter three may be the optimal initial strategy: to date this is the best characterized promoter, and yet the cis-regulatory elements regulating its endogenous-like expression in the brain have not been identified. Assuming this bac construct contains all relevant BDNF cis-regulatory elements, this could nevertheless be extremely challenging since there may be numerous BDNF regulatory elements that are dispersed or located at long distances from one another. Furthermore, manipulating bacs is challenging in and of itself. An alternative set of experiments could be based on the results of the bioinformatics study of chapter 5. As discussed earlier, ECR 1 appears to be a highly promising candidate for future studies. While ECR 1 may not regulate BDNF or may function in the regulation of a redundant function of BDNF, it is an exciting possibility that ECR1 regulates a nonredundant function of BDNF, and its ablation will yield a dramatic phenotype in the mouse. As a logical starting point, I propose a luciferase assay approach which involves hooking up ECR1 to a basal promoter and a luciferase reporter, and assaying for reporter activity in primary cultures: there is no available cell line expressing BDNF. Since BDNF is known to be regulated by calcium, it would be important to determine, whether KCL depolarization increases luciferase activity. The next step would be to use a transgenic approach which involves hooking up the ECR1 putative enhancer to a

basal promoter and GFP to explore its expression in the CNS and in the peripheral targets of sensory neurons. Depending on the results of this study, it could be interesting to perform the experiments above with another ECR, a construct containing all seven ECRs and depending on these results with combinations of several ECRs. It is possible that the previously discovered calcium response elements in the third promoter of BDNF (discussed earlier) are necessary for high activity-dependent transcription, so if the above studies failed to give positive results, these calcium response elements would be incorporated into the constructs used in the studies above and the experiments above would be repeated. If positive results were obtained with say ECR 1 alone, it would be interesting to mutate specific ECR transcription factor binding sites in a systematic manner to determine if ECR expression is abolished in the transgenic model (see good candidates for mutagenesis above). Other important approaches include mobility shift assays, and competitor DNA assays with putative good candidate binding sites (Latchman, 2004). The Dnase 1 footprinting approach (or variant there of such as the methylation interference assay) can be used to map the transcription factor binding site of an unknown transcription factor. Chromatin IP appears to be the most physiological assay to identify the binding site of a known transcription factor using an antibody to the known factor, although it is labor intensive. To identify a novel transcription factor one can use DNA affinity chromatography (a yeast one hybrid assay can also be used, but has proven more problematic) followed by mass spectrometry. Since the knockout approach is highly risky, costly and time consuming, it is appropriate as a final approach in this type of study.

The regulation of the four major BDNF transcripts is clearly highly complicated, and future studies are necessary to determine the specific functions of the four BDNF promoters. Towards this goal, future studies will need to identify the regulatory pathways upstream of the 4 BDNF promoters. The identification of ECRs in the bioinformatics approach of this study may be highly useful in identifying the cis-regulatory elements of BDNF. To resolve the 4 promoter puzzle, it will also be necessary to address the function of the four major

BDNF transcripts (that have differing 5' UTRs) with respect to stability, translatability and trafficking.

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Vitae

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