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Genomic Organization and Identification of Promoter Regions for the *BDNF* Gene in the Pond Turtle *Trachemys scripta elegans*

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Abstract

Brain-derived neurotrophic factor (BDNF) is an important regulator of neuronal development and synaptic function. The *BDNF* gene undergoes significant activity-dependent regulation during learning. Here, we identified the *BDNF* promoter regions, transcription start sites, and potential regulatory sequences for *BDNF* exons I–III that may contribute to activity-dependent gene and protein expression in the pond turtle *Trachemys scripta elegans* (*tBDNF*). By using transfection of *BDNF* promoter/luciferase plasmid constructs into human neuroblastoma SHSY5Y cells and mouse embryonic fibroblast NIH3T3 cells, we identified the basal regulatory activity of promoter sequences located upstream of each *tBDNF* exon, designated as pBDNFI–III. Further, through chromatin immunoprecipitation (ChIP) assays, we detected CREB binding directly to exon I and exon III promoters, while BHLHB2, but not CREB, binds within the exon II promoter. Elucidation of the promoter regions and regulatory protein binding sites in the *tBDNF* gene is essential for understanding the regulatory mechanisms that control *tBDNF* gene expression.

Keywords

BDNF; Promoter; ChIP assay; CREB; Turtle

Introduction

Brain-derived neurotrophic factor (BDNF) is a potent regulator of neuronal development and synaptic function (Reichardt 2006). The structure of the *BDNF* gene is complex and reflects the multiplicity of ways in which it is regulated. For example, the *bdnf* gene in the mouse and rat has eight 5' noncoding exons each of which contains separate upstream promoters and one 3' exon that encodes the BDNF protein (Liu et al. 2006; Aid et al. 2007). Similarly, the human *BDNF* gene has 11 noncoding exons which are spliced independently to the coding exon to produce more than 15 mRNA transcripts (Pruunsild et al. 2007). Multiple promoters determine the timing and tissue-specific expression of the *BDNF* transcripts (Timmusk et al. 1993; Pruunsild et al. 2007). The *BDNF* gene structure described for nonmammalian vertebrates such as chicken (Yu et al. 2009) and turtle (Ambigapathy et

al. 2013) also shares similar organizational features to mammals. The *BDNF* gene supports a complex pattern of regulation by a number of *cis*- and *trans*-acting transcriptional control elements that produce a variety of differentially spliced sense and antisense transcripts. Several transcription factors contributing to the regulation of *BDNF* promoters have been characterized, notably cAMP response element binding protein (CREB), upstream stimulatory factors 1/2 (USF1/2), calcium-responsive transcription factor (CaRF), and BHLHB2 which is a member of the basic helix-loop-helix superfamily of transcription factors (Shieh et al. 1998; Tao et al. 1998, 2002; Tabuchi et al. 2002; Chen et al. 2003; Jiang et al. 2008). CRE is a major *cis*-element and an important regulator of *BDNF* gene expression in neurons. Moreover, activation of CREB facilitates subsequent recruitment of co-activator proteins such as the CREB binding protein (CBP; Chrivia et al. 1993; Finkbeiner et al. 1997) and possibly methyl-CpG binding protein 2 (MeCP2; Chahrour et al. 2008).

Our previous studies showed that the *BDNF* gene in the pond turtle *Trachemys scripta elegans* (*tBDNF*) contains four exons that are alternatively spliced to produce nine different mRNA transcripts (Ambigapathy et al. 2013). Three exons (I–III) code for 5' untranslated regions (UTRs) and the fourth codes for the protein coding sequence along with its 3' UTR. Significantly, *tBDNF* exon expression undergoes differential activity-dependent regulation. This was observed during studies of learning and memory using an in vitro model of eye-blink classical conditioning (reviewed in Keifer and Zheng 2010). In this model, exon I transcripts show no modulation during classical conditioning, exon II transcripts are downregulated, while exon III transcripts are upregulated (Ambigapathy et al. 2013). To further characterize how *tBDNF* transcripts are controlled during learning, in the present study, we identified the *tBDNF* promoter regions, transcription start sites, and potential regulatory sequences for *tBDNF* exons I–III that contribute to activity-dependent gene and protein expression. By using transfection of *BDNF* promoter/luciferase plasmid constructs into human neuroblastoma SHSY5Y cells and mouse embryonic fibroblast NIH3T3 cells, we identified the basal regulatory activity of promoter sequences located upstream of each *tBDNF* exon, designated as pBDNFI–III. Further, through chromatin immunoprecipitation (ChIP) assays, we detected CREB binding directly to a *cis*-regulatory site of exon I and exon III promoters, while BHLHB2, but not CREB, binds within the exon II promoter. Elucidation of the promoter regions and regulatory protein binding sites in the *tBDNF* gene is essential for understanding the regulatory mechanisms that control *tBDNF* gene expression.

Materials and Methods

Experimental Animals

Freshwater pond turtles, *T. scripta elegans*, purchased from commercial suppliers were anesthetized by hypothermia until torpid and decapitated. All experiments involving the use of animals were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of South Dakota Institutional Animal Care and Use Committee.

5' RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE) Analysis of Transcription Start Sites

Only partial sequences of the 5' UTR for each *tBDNF* exon were identified previously (Ambigapathy et al. 2013). To obtain the remaining sequences and determine the *tBDNF* transcription initiation site, 5' RACE was performed using the First Choice RLM-RACE kit (Ambion, Austin, TX). Briefly, total RNA was isolated from the turtle brain and treated with calf intestine alkaline phosphatase (CIP) and tobacco acid pyrophosphatase (TAP) to decap the mRNA. Then, a 5' RACE adapter was ligated and reverse transcription was performed using random decamers and M-MLV reverse transcriptase. Following adapter ligation, nested PCR was carried out with adapter-specific and gene-specific primer pairs. See Table 1 for a list of BDNF primers. The reaction conditions were as follows: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The resulting PCR products were run on a 2.0 % agarose gel, and bands were excised, purified and cloned into pGEM-T Easy Vector (Promega, Madison, WI). The cloned fragments were sequenced by an automated DNA sequence analyzer (ABI model 3730xl) at the DNA core facility in the Department of Biotechnology at Iowa State University.

Genome Walking

To identify and clone 5' flanking sequences of exons I–III of the *tBDNF* gene, genome walking was carried out with the GenomeWalker Universal Kit (Clontech Laboratories Inc., CA) according to the manufacturer's instruction. Briefly, turtle genomic DNA was isolated and the restriction digested with *DraI*, *EcoRV*, *PvuII*, and *StuI*. Then, the DNA preparations were ligated with the genome walker adaptor. Two rounds of PCR were performed with the BD Advantage 2 PCR kit (Clontech) using adapter primers (AP) and gene-specific primers (GSP; Table 2). Amplifications were performed according to the manufacturer's recommendation. The resulting PCR products were subcloned into pGEM-T-Easy Vector (Promega) and sequenced.

In Silico Analysis for Transcription Factor Binding Sites

The 5' flanking region of *BDNF* exons I–III was analyzed to detect the transcription factor binding sites using Transcription Element Search System Software (TESS), MatInspector, and Patch 1.0 that surveyed the TRANSFAC eukaryotic database using default settings. Annotated sequences of the three promoter regions were obtained using a minimum log likelihood ratio (ta) of 6.0 and a maximum likelihood deficit (td) of 8.0 (Schug 2003; Cartharius et al. 2005; Hart et al. 2011).

DNA Constructs

For the promoter analysis (pBDNFI–III), reporter constructs using different portions of the *BDNF* 5' flanking sequence from each exon were amplified by PCR and ligated with the pGL3-Basic firefly luciferase reporter vector. Three sets of constructs were generated. pBDNFI constructs contain 1,176, 882, 640, and 469 bp of the 5' flanking region of exon I and 321 bp of exon I. pBDNFII constructs contain 602, 423, and 286 bp of the 5' flanking region of exon II and 249 bp of exon II. Constructs for pBDNFIII contain 1,004, 707, 567, and 225 bp of the 5' flanking region of exon III and 279 bp of exon III. Primers used to

amplify the constructs are listed in Table 3. PCR primers were designed with *XhoI* and *HindIII* or *XhoI* and *BglII* restriction sites at the 5' end. PCR reactions were carried out by using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PCR products were subcloned into pGEM-T Easy Vector (Promega) and the DNA fragments were confirmed by sequencing. Constructs were generated by digesting the pGEM-T clones with *XhoI*/ *HindIII* (pBDNFI, II) or *XhoI*/*BglII* (pBDNFIII) restriction enzymes (Promega) and resolved on 2.0 % agarose gel. The bands were excised, purified (Purelink Quickgel extraction kit, Invitrogen), and cloned into the pGL3-Basic reporter vector (Promega) digested with the same enzymes. Plasmid DNA from all the constructs was prepared using the ZR Plasmid Mini Prep Kit (Zymo Research, CA). The authenticity of each construct was verified by sequencing.

Cell Culture and Transfection of Luciferase Constructs

SHSY5Y and NIH3T3 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in 5 % CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin in 10 cm plates. After 70 to 80 % confluence, cells were rinsed with 1× PBS. Following trypsinization and mechanical dissociation, cells were subcultured into 24-well plates and incubated at 37 °C for 2 days preceding transfection. Transfection was done after the cells reached 70 to 80 % confluence. pBDNF constructs, pGL3-Basic, or pGL3-SV40 vector was transiently transfected into SHSY5Y and NIH3T3 cells using Lipofectamine 2000 (Invitrogen). Briefly, for each transfection, 2.0 µg of DNA and 5 µl of Lipofectamine were first diluted in OPTI-MEM (GibcoBRL), mixed gently, and incubated at room temperature for 5 min. Cells were rinsed once with PBS and OPTI-MEM was added before incubation with the DNA/Lipofectamine. The pRL-SV40 Renilla luciferase vector (50 ng) was co-transfected in all experiments. Cells were allowed to incubate at 37 °C and the media was replaced after 12 h by fresh DMEM. Cells were harvested 48 h after the start of transfection.

Luciferase Assay

Luciferase activity was measured after transfection using the Dual-Glo Luciferase Assay System (Promega) in a Microplate Scintillation & Luminometer (Packard Topcount NXT) as per instructions. Culture plates were removed from the incubator and equilibrated to room temperature before performing the assay. Cells were washed with PBS and the firefly and Renilla luciferase activities were determined. Firefly luciferase activity was normalized to Renilla luciferase activity to adjust for variations in transfection efficiency among experiments. All experiments were performed in triplicate.

ChIP Assay

ChIP assays were performed as previously described by Weinmann et al. (2001) with minor modifications. Briefly, turtle brainstem was isolated and minced on ice and immediately incubated in 1 % formaldehyde in PBS at 37 °C for 10 min. The tissue was washed with ice-cold PBS solution containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml protease inhibitor mixture) and homogenized with 200 µl SDS lysis buffer. Chromatin

was sheared using a Branson sonifier 250 at three power levels approximately three to four times using 10 s pulses. The sheared chromatin was cleared of insoluble debris by centrifugation at 13,000 rpm for 10 min at 4 °C, and the supernatant was diluted (1:10) with ChIP buffer. Twenty microliters of diluted cell supernatant was set aside to serve as a total chromatin input control. The supernatant containing the sheared chromatin was precleared with salmon sperm-saturated protein A (Millipore) for 1 h at 4 °C on a rotating platform. Then, the agarose was pelleted by centrifugation, and immunoprecipitation was performed with 2 µg of the CREB antibody (Cell Signaling), 4 µg of DEC1 antibody (Santa Cruz Biotechnology), or 2 µg of normal rabbit IgG (Santa Cruz) as a control, or no antibody, at 4 °C overnight with rotation. Immune complexes were collected by incubating with 60 µl of 50 % salmon sperm-saturated protein A for 1 h at 4 °C with rotation. Agarose was pelleted by centrifugation and the immune complexes were washed once with low salt buffer, high-salt buffer, and lithium chloride wash buffer and twice with TE buffer for 5 min. Immunoprecipitated chromatin was eluted twice, each time with 250 µl of freshly prepared elution buffer (1 % SDS, 0.1 M NaHCO₃). The elutes were pooled and 5 M NaCl was added and cross-links were reversed by incubating the samples at 65 °C for 4 h. Samples were then incubated with proteinase K (10 mg/ml) for 1 h at 45 °C. DNA was isolated using phenol/chloroform/isoamyl alcohol, followed by precipitation with ethanol, and then dried and dissolved in water. The same reverse cross-linking and purification procedures were used for the input sample. ChIP and input samples were used for PCR with *tBDNF* primers targeting the region spanning the specific transcription factor binding sites of each exon. See Table 4 for a list of *tBDNF* primers for ChIP. PCR conditions were 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and final elongation at 72 °C for 10 min. PCR products were examined on 2.0 % agarose gels.

Statistical Analysis

Luciferase activity was determined and compared with vector (pGL3-Basic) that did not contain promoters. Measurements were carried out on three independent experiments with each transfection done in triplicate. Data were analyzed using one-way ANOVA followed by post hoc analysis using Fisher's and Bonferroni tests and are presented as means±SEM. *P* values are given in the text.

Results

Identification of *tBDNF* Transcription Start Sites (TSS)

As a first step towards defining the promoter regions of *tBDNF* exons, 5' RACE was performed to identify the gene's TSS, which is designated as the first nucleotide copied at the 5' end of the corresponding mRNA (Sandelin et al. 2007). 5' RACE PCR followed by nested amplification was performed with exon-specific reverse primers and adapter-specific forward primers using total RNA isolated from turtle brain. This yielded the different sized fragments which were cloned into pGEM-T Easy Vector for sequence analysis. The sequencing results revealed that there are two TSS relative to the 3' end of each respective exon located as follows: -728, -589 nt for exon I; -406, -320 nt for exon II; and -326, -213 nt for exon III.

Identification of Regulatory Elements in the Promoter Regions

In silico analysis of the *tBDNF* gene 5' flanking sequence by the TESS, MatInspector, and Patch 1.0 (TRANSFAC Public 6.0) programs (Schug 2003; Cartharius et al. 2005; Hart et al. 2011) detected major eukaryotic promoter elements. Analysis of the flanking regions of each exon I–III found a region containing a cluster of transcription factor binding sites including the following: TATA box, CAAT box, canonical cAMP response element (CRE), AP1, SP1, CCAAT-enhancer binding protein (C/EBP), and E-box (Figs. 1, 2, and 3; see Fig. 1 legend for complete list of abbreviations). Putative C/EBP binding motifs were found throughout the promoter regions. The 5' flanking sequence of exon I contains a CREB half-site (CGTCA) and CCAAT box which were found at –219 and –330 nt from the TSS, respectively. No TATA box was found near the TSS. In addition, CaRE, SP1, AP1, and C/EBP sites were located in the 5' flanking region of exon I. In exon II, the putative TATA box was found at –290 nt from the TSS. The function of this TATA box is not clear at this time as most mammalian genes have TATA boxes at about –30 nt. However, lower eukaryotic genes are reported to have TATA boxes farther away than –30 nt (Struhl 1989). CCAAT boxes were detected at positions –265 and –666 nt. Two E-boxes were found at –96 and –140 nt upstream of the TSS. Two AP1 sites and four C/EBP sites were also observed. Analysis of the 5' flanking region of exon III revealed that there was no TATA box sequence found near the TSS. Four AP1 and SP1 sites, five C/EBP sites, and two E-boxes were found in the upstream sequence. In addition, a CRE half-site was found at –37 nt upstream of the TSS. Analysis of *tBDNF* promoter sequences and transcription factor binding sites suggests the involvement of a variety of transcription factors in the regulation of the *tBDNF* gene. Functional analysis of these transcription factors has yet to be performed to determine their potential role in the regulation of *tBDNF* transcription during classical conditioning.

Identification and Cloning of *tBDNF* Promoter Regions

To determine the promoter activity of *tBDNF* transcription, we constructed a number of 5' flanking regions containing *BDNF* promoter fragments (pBDNF constructs) using specific primers for PCR as described in the "Materials and Methods." The 5' flanking sequences for exon I (from nt –1,176 to +321), exon II (from nt –602 to +249), and exon III (from nt –1,004 to +279) were fused to the pGL3-Basic vector. pRL-Renilla plasmid was co-transfected along with pBDNF constructs into human neuroblastoma (SHSY5Y) or mouse fibroblast (NIH3T3) cell lines. The results from the corresponding luciferase activity are presented in Fig. 4 and reflect the transcriptional activity of the respective portion of the promoter. Luciferase activity of the promoterless control plasmid pGL3-Basic was minimal. pGL3-SV40 acts as a positive control vector and showed maximal activity. As shown in Fig. 4a, luciferase activity of the three constructs derived from exon I, pBDNFIA (–469 to +321), pBDNFIB (–640 to +321), and pBDNFIC (–882 to +321), were significantly increased in both cell lines compared with the promoterless vector ($P<0.0001$). The exception was construct pBDNF1D (–1,176 to +321) which showed reduced luciferase activity compared with other three shorter pBDNF constructs, but this activity was still significant compared to the pGL3 vector in SHSY5Y cells ($P=0.0004$). However, there was

no significant difference in pBDNF1D in NIH3T3 cells ($P=0.10$). Overall, transfection with constructs pBDNFIA and pBDNFIB showed the highest activity in these cultured cell lines.

For exon II, constructs pBDNFIIA (−286 to +249), pBDNFIIIB (−423 to +249), and pBDNFIIIC (−602 to +249) showed luciferase activity that was significantly increased in the SHSY5Y cell lines compared with the promoterless vector (Fig. 4b, $P<0.0001$ except IIC at $P=0.01$). In NIH3T3 cells, pBDNFIIA and IIB constructs showed a significant increase (IIA $P<0.0001$, IIB $P=0.01$), while there was no difference in pBDNFIIIC ($P=0.11$). Constructs pBDNFIIIB and IIC showed relatively less luciferase activity compared with pBDNFIIA in NIH3T3 cells.

In exon III, all four of the constructs showed luciferase activity that was significantly increased in both cell lines compared with the promoterless vector (Fig. 4c, $P<0.05$). Construct pBDNFIIIA (−225 to +279) showed the highest luciferase activity in SHSY5Y cells compared with the other three longer constructs. The longest construct pBDNFIIID (−1,004 to +279) showed significantly less luciferase activity compared with pBDNFIIIA in both cell lines (SHSY5Y $P<0.0001$, NIH3T3 $P<0.05$). Overall, the level of luciferase activity slowly increased in constructs containing progressively shorter fragments of the 5' flanking region of exons I–III. Together, these findings suggest that positive transcriptional regulation is most effectively carried out by elements immediately adjacent to the transcription start site of the exons and that regulatory elements further upstream act to repress the basal transcription of the promoters.

Interactions Between DNA Regulatory Protein Factors and Promoter Elements

A search of transcription factor consensus motifs detected a CREB binding site in the promoter region of exon I and exon III (Figs. 2 and 3). To verify that the CREB transcription factor physically interacted with the CRE sites within the *tBDNF* promoter, a ChIP assay was performed. Primers are illustrated in Fig. 1. Immunoprecipitation of cross-linked chromatin from turtle brainstem with anti-CREB antibody followed by PCR amplification of the region demonstrated that CREB protein selectively binds to this region of the *tBDNF* promoter in both exon I and exon III (Fig. 5a, c). As expected, a CREB binding site on the promoter region of exon II was not detected (Fig. 5b). Interestingly, an E-box, the transcriptional repressor protein binding sequence, was identified by in silico analysis of exon II. To confirm this, a ChIP assay was performed with anti-BHLHB2 antibody (aka DEC1) followed by PCR amplification of the region. The results indicated that the exon II promoter region has the specific binding site CACGTG for BHLHB2 (Fig. 5d). However, BHLHB2 did not bind to the E-box region in exon III (Fig. 5e). These data suggest that promoter regions for *tBDNF* exons I and III possess a canonical CRE binding site, while exon II does not and further indicate that *tBDNF* gene regulation involves CREB- and BHLHB2-mediated signal transduction pathways.

Discussion

Using a combination of in silico analysis and expression assays, we have identified the promoter regions of the *tBDNF* gene and its organization, nucleotide sequence composition, as well as potential transcription factor binding sites that are likely to regulate its expression.

Initially, we analyzed the transcription start sites for each of the *tBDNF* exons I–III. Sequencing of the 5' RACE clones showed that each of the *tBDNF* exons isolated from turtle brainstem samples has two transcription start sites. Multiple start sites have also been identified for rat and human *BDNF* exons (Aid et al. 2007; Pruunsild et al. 2007) as well as for *Xenopus* (Kidane et al. 2009). These results suggest that *tBDNF*, as is the case for other vertebrates, may have alternative transcription start sites possibly related to tissue-specific or activity-dependent expression.

We investigated the transcriptional activity of the *tBDNF* promoters I, II, and III in two different cell lines, SHSY5Y neuroblastoma cells and NIH3T3 fibroblast cells. First, for the exon I promoter region, pBDNFIA and IB constructs showed the most promoter activity although the maximal reporter expression was in SHSY5Y cells. For exon II, pBDNFIIA showed maximal activity for both cell lines, but again, expression in SHSY5Y cells was superior to NIH3T3 cells. Lastly, for the exon III promoter region, the pBDNFIIIA construct showed maximum activity, but both cell lines showed a similar expression pattern. Generally, the SHSY5Y neuronal cell lines showed greater reporter gene expression than the fibroblast cell lines. Additionally, there was a clear pattern of reduced expression with larger fragments of all three promoter regions. This suggests that the reduced activity may be the result of the silencer/repressor elements in the upstream region that have an inhibitory effect on the reporter gene. This effect was observed in both cell lines. Similar effects were also observed in rat *bdnf* exons and mouse *cmklr1* (Hayes et al. 1997; Mårtensson et al. 2004).

A surprising number of potential transcription factor binding sites on the *tBDNF* promoter regions were identified by using in silico analysis that may be important regulatory sites for *tBDNF* gene expression. The most frequent and notable sites found in all three *tBDNF* promoter regions were AP1, C/EBP, and SP1. CREB sites were found in exons I and III, while a BHLHB2 site was found in exon II. The potential AP1 binding sites were identified throughout the promoter region of all three exons. AP1 is a transcriptional regulator composed of members of the fos, jun, and ATF protein families and regulates the basal level of transcription (Sonnenberg et al. 1989; Zenz et al. 2008). The CCAAT box is another important *cis*-element binding site for C/EBP family proteins and was found in promoters I and II. C/EBP and SP1 are potential transcription factors for *bdnf* promoter IV in rat, and overexpression of C/EBP β increased the exon IV *bdnf* promoter activity (Takeuchi et al. 2002). CRE sites and C/EBP binding motifs can control the transcription of various genes regardless of their position in the promoter region (Ptashne 1988). *tBDNF* exon I does not have a TATA box in its promoter region. Similar results were also reported for rat *bdnf* exon I (Hayes et al. 1997). The exon I promoter also does not have any E-box regions. The exon II promoter region contains two E-boxes which may be binding sites for the class B basic helix-loop-helix family proteins USF (at –140 nt) and BHLHB2 (at –96 nt; Rossner et al. 1997; Boyd and Farnham 1997; Wang and Sul 1997). Earlier reports indicated that USF1/2 is required for the transcriptional activation of BDNF-PI and III (Tabuchi et al. 2002; Chen et al. 2003). We confirmed by ChIP analysis that the BHLHB2 transcription factor does indeed bind to the *tBDNF* exon II promoter at the most proximal E-box. BHLHB2 has been shown to act as a transcriptional repressor for its target gene *bdnf* in mice which is discussed further below (Jiang et al. 2008). In silico analysis clearly showed a CREB binding region

located in exons I and III in *tBDNF*. Phosphorylated CREB has been shown to bind to the *BDNF* promoter of several species and functions to upregulate the expression of BDNF protein (Tao et al. 1998; Ou and Gean 2007; Pugazhenthil et al. 2011).

In our model system using the turtle brainstem for studies of learning during classical conditioning, we previously reported that *tBDNF* exon I transcript levels are not modulated, exon II transcripts are downregulated, and exon III transcripts are upregulated (Ambigapathy et al. 2013). As both CREB (Carlezon et al. 2005) and BHLHB2 (Jiang et al. 2008) transcription factors have been shown to be important regulators of *BDNF* expression in learning, we examined the binding of these proteins in the *tBDNF* promoter regions using the ChIP assay. The results confirmed that CREB binds the *tBDNF* promoter at CRE half-site regions of exons I and III, but not exon II. Genome-wide analysis in human revealed that a number of promoters have CRE half-sites (TGACG/CGTC A), and many studies have shown that CRE half-sites drive CREB-dependent transcriptional activation (Mayr and Montminy 2001; Zhang et al. 2005; Lesiak et al. 2013). In exon I, the CRE half-site CGTCA was located relatively far upstream, -219 nt, from the first transcription start site, whereas in exon III, the CRE half-site was located very close, -37 nt, from the first transcription start site. Mayr and Montminy (2001) reported that CRE sites located within 200 nucleotides of the transcription start site are most likely to be optimally functional. ChIP analysis for BHLHB2 shows positive results for binding in the exon II promoter region, whereas exon III shows that there was no specific binding. Jiang et al. (2008) reported that BHLHB2 has a high affinity with CTCGTG (that closely matches the consensus sequence for a class B E-box CACGTG observed here) and a double nucleotide mutation (CTACTG) results in the loss of binding activity. Based on this information, we conclude that the E-box located at -96 to -91 nt in exon II is responsible for binding BHLHB2, while the E-box sequence CATGTG present in the promoter region of exons II and III may be responsible for USF1/2 binding. Taken together, these results suggest that the CREB binding site present in the proximal region of *tBDNF* promoter III may be a positive modulator for *tBDNFIII* transcript expression, while BHLHB2 may act as a negative modulator to suppress *tBDNFII* transcripts during conditioning. The exact mechanism of conditioning-dependent *tBDNF* gene regulation by these transcription factors awaits further study.

In summary, we have characterized the structure of *tBDNF* gene promoters for exons I–III. These findings indicate that the functional promoter regions for all three *tBDNF* exons are immediately upstream of the transcription start sites. Moreover, the promoter activity is likely to be regulated through the coordination of multiple regulatory elements and transcription factors that were identified here. Knowledge of the promoter regions will allow us to investigate conditioning-related regulation of *tBDNF* gene expression as well as the expression of other specific genes involved in learning.

Acknowledgments

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Fig. 1.

Schematic representation of transcription factor binding sites relative to the transcription start site (TSS) in the promoter region of exons I, II, and III of the turtle *BDNF* gene using in silico analysis. The *numbers* indicate the position of nucleotides from the TSS.

Abbreviations: C/EBP CCAAT-enhancer binding protein, SP1 specificity protein 1, AP1 activator protein 1, CREBP1 CREB binding protein 1, CREB cAMP response element binding protein, CaRE calcium response element, USF upstream stimulatory factors 1/2, BHLHB2 basic helix-loop-helix B2. The *arrows* denote the location of primers used for the specific detection of immunoprecipitated DNA fragments containing the targeted transcription factor binding sites for the ChIP assay

[illegible]

Fig. 2.

Nucleotide sequence of the 5' flanking region for *tBDNF* exons I and II. *Numbers on the left* represent the position of nucleotides relative to the transcription start site of exon I. Exon sequences are shown in *bold uppercase letters*. Putative TATA box, CAAT elements, CRE, AP1, SP1, C/EBP sites, and E-boxes are *underlined* and *in bold*. *Asterisk* marks the transcription start sites

tcacaaatgtcttacagtatccctgctaccaccaccacttcagacacagttaatagatgcttcagtgtccctaaccacagattcttgatactgttcat
 gtgcacatgtattgcatttaaggaagggatctcactgtagaaataaaatagatgaattaaattatcactaatataacctttcaat^{AP2}agcctgctgccatt
 -1248 acatttgactggtagatcattcagattagttgttctgaatgtgcaatataaaagaatgggtgtcctgttcaaccattattaaaaataactaagtatgaa
 tgtccctaattgcaggagacaaccatactagtgcagttatatattgtaaatgtgaatgggtccacagaagtgtgatcaaaattcaaaaataaagttagatgt
 gttaactgat^{AP1}gactaatttaacaagtccagtagaagggt^{USF1, 2 C/EBP}cacctgtgcaatcctctgtctgcaaatt^{SP1}ctgccctcagttatacctgagagaaataggt
 C/EBP ^{SP1} ^{SP1}
 -948 ttgtac^{AP1}catgtagaaaataatccttgag^{SP1}gggcagaaatgaagggtagggttgtccaggtgtggctga^{SP1}gggcagtatttggccagtgaatttggtattatt
 ggtaattatacaccagacagaaagaacagagcatgatttttagagccaggtgagtttgcagagctggcagtttgaaaggccatatttttactttagt
^{AP1} ^{C/EBP}
 gct^{AP1}gagttagtctgatctggaaatcattgaacagtaaatatggaacccttttcaatatgctactagacttttag^{C/EBP}gctaaccacatttataaaaaaaagt
 C/EBP
 -648 ccaagaagacttgaatgtggtactttactaagtttttttaaacagtttttaaagccatagcagggaaataaacta^{AP1}agtttaaccacatgtaagccatgct
^{AP1} ^{C/EBP}
 gctcccttaacatatatcaaacattgttttatgtagaaggccttaggtatcctcaaatttcaaaagcctggaatagtc^{AP1}gtgactctt^{C/EBP}ctgatctaataa
^{SP1} ^{AP1}
 atgc^{SP1}aggtgtttcataataaaggcacacaagttgtccaaaggcaaaa^{AP1}tgacttacatacattcattaaagcaaaaccttgcagtgcaggcgaagggcct
 -348 gagcctactgaagtcaatgggacttttgcattgactgcagtgacaacaggttcaggccttaagggttataaaatgtatttgcctcatgggagagatgatg
 atccccctttgaaaaataaactaactaccctgtgctgttttgcgggaaatgggagcaactaaagtgagaacatcataaa^{SREBP1/USF1, 2}catgtgtaaggcactgaaa
 cgaaaccctgttaataaaagatgcatcattctcaatgcatggtattttgattcttgtaatccatgcagtaattgtgtctattagaggcagccagggtatca
^{CREB} *
 -48 tatgacata^{CREB}acacgtcaagaggtgtagagcaagcgctagtgtggttccc^{*}ACCACACTCCCATTCAGAGCGCTAGAGGTCTGATGAAGCTTGAGCTAACCC
^{*}
TAGCGAGCGAGCGAGCATGAAATCTTTCTGTCTTTACCAAGATCAAATGGAGCTACTCGGTGAGGGGATGCCATTATTACCTCCACTATGCAATTTCCAC
TATCAATAATTTAATCTATTTGCTGCATAAACAGAGGGGAAACCAGCAAGACAGATTTTGAAGGGGGAAGAAAAACATCCCCAAATCAGAGCCAATT
 253 TGGGAGTTTCGTGAGAGTTCCACATGCAGCGCTGTTGATATTGACTTATACAAGTAGCCACTAAAAACAAAAGgtgggttttggattttctgtctgtgtgt
 tatgctcagagaaagtctttaggtgtctagattgtgcaaatatgcccctgctgtctccagaaagtgactagcgtaacgttatctggagcgttcatta
 ataaacaatgcttgttattatcttgatt

Fig. 3.

Nucleotide sequence of the 5' flanking region of *tBDNF* exon III. Numbers on the left represent the position of nucleotides relative to the transcription start site of exon III. Exon sequences are shown in *bold uppercase letters*. The locations of putative CRE, AP1, SP1, C/EBP sites, and E-boxes are *underlined and in bold*. Asterisk marks the transcription start site

Fig. 4.

a–c Identification of *tBDNF* promoter regions for exons I–III analyzed by luciferase reporter assay. The size of each of the constructs is illustrated and the numbering is relative to the transcription start site of each exon. Relative luciferase activity is plotted in the histograms and reflects the transcriptional activity of each of the pBDNF constructs for SHSY5Y and NIH3T3 cell lines. The pGL3-control vector, which contains the SV40 promoter and enhancer sequences, was used as a positive control (Control). The pGL3-Basic vector, which lacks promoter and enhancer sequences, was used as a negative control. Values are from three independent experiments and are represented as means $\pm$ SEM. Asterisks indicate significant differences from pGL3. *P* values are given in the text

Fig. 5.

ChIP assays were performed to verify whether CREB and BHLHB2 associates with CRE and E-box sites, respectively, identified by in silico analysis of *tBDNF* promoters. CREB and BHLHB2 antibodies were used to immunoprecipitate cross-linked chromatin from naïve turtle brainstem tissue and PCR performed with primers specific for the *tBDNF* promoter regions. Control samples and inputs were analyzed in parallel. **a–c** PCR products show that CREB binds the promoter regions of exon I and exon III but not exon II. **d–e** BHLHB2 binds to the promoter region of exon II but not to II

Table 1

Primers used for 5' RACE analysis

Primers	Orientation	Sequence (5'→3')
Outer primer (Ambion)	For	GCTGATGGCGATGAATGAACACTG
Inner primer (Ambion)	For	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
RACE Ex1 outer	Rev	CCAACAGATGCTGGAAGGTAA
RACE Ex1 inner	Rev	CTTCTGCTTTGCTCAGTGA
RACE Ex2 outer	Rev	CGTTATACCAACCCCGGAG
RACE Ex2 inner	Rev	CTCTTTTGGCCCCCTATT
RACE Ex3 outer	Rev	TGTTTATGCAGCAAATAGATTA
RACE Ex3 inner	Rev	ATTGCATAGTGGAGGTAATAA

Table 2

Primers used for genome walking

Primers	Orientation	Sequence (5'→3')
AP1	For	GTAATACGACTCACTATAGGGC
AP2	For	ACTATAGGGCACGCGTGGT
EX1-GP1	Rev	GCCAAAAGAGGAATATGTACAGTTG
Ex1-GP2	Rev	AAACATTCTTCCTTGATCTTGAGTG
EX2-GP1	Rev	CTCCTACCTCTGCCTAACTCAGTG
Ex2-GP2	Rev	GGAGGAAAAGGGCTAAAACCTATAC
EX3-GP1	Rev	AAATTATTGATAGTGGAAATTGCATAGT
Ex3-GP2	Rev	ACACATTACTGCATGGATTACAAGA

Table 3

Primers used to construct pGL3-BDNF for promoter analysis

Primers	Orientation	Location from TSS	Sequence (5'→3')
pBDNFIA	For	-469	ACACTCGAGCCACGCTCAGTAACCCTTAT
pBDNFIB	For	-640	ACACTCGAGACCGGTGTATTCATTCAAG
pBDNFIC	For	-882	ACACTCGAGCACTATGCTCTTCTTAGTACT
pBDNFID	For	-1,176	ACACTCGAGTTCTGCTCTGTGTATCCTGAT
pBDNFI	Rev	+321	TCCCAAGCTTAGGAAAGTCTTTCTGGACACT
pBDNFIIA	For	-286	ACACTCGAGATGGCTACAAGGAGCAGCAT
pBDNFIIIB	For	-423	ACACTCGAGTGCTGATGGCTGTGTCAAGT
pBDNFIIIC	For	-602	ACACTCGAGAATCTCATGGCTCAGCTTTCT
pBDNFII	Rev	+249	TCCCAAGCTTTCCAAAGAGGCGACAGAACT
pBDNFIIIA	For	-225	ACACTCGAGACTACCCTGTGCCTGTTTT
pBDNFIIIB	For	-567	ACACTCGAGACCACATGTAAGCCATGCTG
pBDNFIIIC	For	-707	ACACTCGAGTGGAACCCCTTTCAATATGC
pBDNFIIID	For	-1,004	ACACTCGAGTGTGCAATCCTCTTGTCTGC
pBDNFIII	Rev	+279	GGAAGATCTCATGTGGAACCTCTGACGAA

Table 4

Primers used for the ChIP assay

Primers	Orientation	Sequence (5'→3')	Antibody
BDNF-ExI	For	CGCCAATTGGGTTATTTAC	CREB
BDNF-ExI	Rev	CGTCTGGATAAGCGACCTCT	CREB
BDNF-ExII	For	GTCCAGTCAACATGTGATCATCTA	CREB
BDNF-ExII	Rev	GTGAGCTGATGTTCAAGGATAG	CREB
BDNF-ExIII	For	TTAGAGGCAGCCAGGGTATCA	CREB
BDNF-ExIII	Rev	TAATAATGGCATCCCCTGAC	CREB
BDNF-ExIIA	For	TCTACAGATGCTGGGGGAAA	BHLHB2
BDNF-ExIIA	Rev	CTAACTCAGTGCCCCTGTCC	BHLHB2
BDNF-ExIIIA	For	GAAGTCAATGGGACTTTTGTC	BHLHB2
BDNF-ExIIIA	Rev	ACAGGGTTTCGTTTCAGTGC	BHLHB2