9-1-2009

Brain Derived Neurotrophic Factor (BDNF) mRNA Expression Levels in Mice Exposed to Learned Helplessness

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Abstract

Brain Derived Neurotrophic Factor (BDNF) is associated with changes in cellular structure that occur during the development of the nervous system, and in the adult brain contributes to neural plasticity. BDNF is found in high concentrations in both the frontal cortex and hippocampus where it has been proposed to play a role in cognition and learning respectively. Low serum BDNF levels can be measured in patients with major depressive disorder, and there is a correlation between decreased BDNF levels and the severity of depression. At this time, research is attempting to solve the question of whether depression results secondary to diminished BDNF levels, or a reduction in BDNF levels occurs as a result of individuals becoming depressed.

Learned Helplessness (LH) is utilized as a model for depression and this is confirmed due to the fact that LH is blocked by antidepressant medications. An advantage of using LH to induce depressive behavior is the fact that the onset of depression in an animal is at a defined time point. The model of Learned Helplessness allows for analysis of depressed patients via LH mice which at this current time is the most accurate model for depression. In this study, our results were consistent with the hypothesis that since BDNF levels are significantly reduced in the medial frontal cortices and hippocampus of depressed patients, as well as in several studies looking at depression in rodents, then BDNF mRNA levels would also be reduced in the brain tissue of LH mice compared to controls after induction of Learned Helplessness. We also predicted that the decrease in the amount of BDNF transcripts would be greater in the medial frontal cortical region (MFC) compared with hippocampal tissue, since deficits in this region have been identified in depressed patients. This was also found to be true in this experiment. The hypothesis was tested by measuring the mRNA message for the five BDNF transcript variants in both the hippocampus and the medial frontal cortex in mice that had experienced inescapable unpredicted foot shocks during learned helplessness training. This data was compared to control mice that did not experience the Learned Helplessness training. Differences in BDNF mRNA levels were then determined thereafter. Levels of mRNA BDNF in the HPC and

MFC of both experimental LH and control animals were quantified using RT-PCR technique.

Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophic factor found originally in the brain as well as the periphery. BDNF is said to be associated with changes in cells and plasticity that occur during the development nervous system, and facilitates the synaptic function in neurons in vitro and in vivo (Lu and Woo, 2005). BDNF also supports the survival of existing neurons. BDNF is active in the hippocampus CA2 and CA3 neurons, cortex, cerebellar cortex, thalamic and hypothalamic nuclei, and the basal forebrain. These include areas important to learning, memory, and higher thought processes. Expression of BDNF may also be seen in the retina, the CNS, motor neurons, the kidneys, and the prostate (Chao, 2003).

BDNF is involved in the expression of many neurotransmitter systems (Mamounas et al., 1995), amyotrophic lateral sclerosis (Apfel, 1997), Alzheimer disease (Connor et al., 1997: Durany et al., 2000), schizophrenia (Takahashi et. al., 2000), and in various forms of major depression (Nibuya et al., 1995). Findings from Karege et al. (2002) indicate that alteration in the expression of neurotrophic factors (primarily BDNF) could be the factor responsible for the interruption of proper neural plasticity. In support of our hypothesis, these findings suggest that antidepressants increase neurotrophin synthesis and enhance neurogenesis, thereby relieving the symptoms of depression (Nibuya et al., 1995; Duman et al., 1997).

Recent studies have given evidence regarding a neurotrophic hypothesis of antidepressant action on depression (Song et al., 2006). This hypothesis explains that decreased BDNF expression could play a role in the atrophy of the hippocampus, which occurs in response to stress in depressed patients. This allows for a role of BDNF upregulation in the treatment of depression (Duman et. al., 1997).

The results given in studies conducted by Song et al. (2006) support this hypothesis in that treatment of antidepressants improve both the changes of

behavior and hippocampus BDNF levels induced by learned helplessness and chronic mild stress. Learned helplessness and chronic mild stress were the damaging factors of cognitive functioning of the mice in this study, but researchers were able to improve these impairments through the use of antidepressant treatment. These cognitive changes were consistent with the alterations of the hippocampal BDNF levels.

BDNF is believed to be involved in several key functions within the brain, which include: regulation of axonal and dendritic growth and guidance, neurotransmitter release, and synaptic structure and plasticity. Mature BDNF also facilitates early phase and late phase long term potentiation in the hippocampus, the region of the brain which has been shown to have the highest neuroanatomical expression of BDNF and its TrkB receptor in the mammalian brain (Lu 2005, Tyler 2002). Patients with major depressive disorder have low serum BDNF levels, and there is a correlation between decreased BDNF levels and the severity of depression. BDNF expression in postmortem hippocampus collected from depressed suicide patients is reduced, whereas it is increased in individuals who were being treated with antidepressants at the time of death (Chen et al 2001; Karege et al 2005). These studies in humans are supported by various studies in rodents (Monteggia et al. 2006). BDNF may even produce an antidepressant-like effect through the antagonism of learned helplessness behavior (Karege 2002). Learned Helplessness is a model for depression based on the hypothesis that depression is induced by uncontrollable stress in individuals with a predisposition.

Exposure to stress induces the stress hormone corticosterone. Corticosterone has been shown to decrease the expression of BDNF in rats, and leads to atrophy of the hippocampus if exposure is chronic (2). This atrophy may also take place in humans who suffer from chronic depression. In order to reduce the expression of BDNF, BDNF heterozygous rats were bred for analysis. These rats exhibited hippocampal atrophy, which suggests a possible link between the development of depression and regulation of BDNF. In contrast, glutamate, exercise, reduction in caloric intake, intellectual stimulation, antidepressants, and electroconvulsive therapy were used to treat depression, and increased the expression of BDNF in the brain, which allows for protection against atrophy.

In mice, BDNF transcripts 1, 2 and 3 are primarily expressed in the central nervous system, whereas variants 4 and 5 are expressed in both the central nervous system and peripheral tissues; transcript 6 shows limited expression in the brain and periphery (Liu et al. 2006). Each of the five BDNF transcripts is present in mouse hippocampus and frontal cortex, in an apparent rank order: BDNF5 > BDNF4 > BDNF1 > BDNF2 > BDNF3 in hippocampus and BDNF5 > BDNF 4 > BNDF3 > BDNF1 > BDNF2 in frontal cortex (Liu et al. 2006). The function(s) of the individual BDNF transcripts is unknown, although differing stimuli regulate the production of unique subsets of transcripts.

Acute, but not chronic, cocaine administration increases BDNF4 transcript in the striatum and frontal cortex (Liu et al. 2006). Chronic (21-day) corticosterone treatment decreased BDNF transcripts containing exons II and V (IV in rat) in the hippocampus and frontal cortex, while chronic (21-day, 1X/day) treatment with desipramine increase transcripts 1 and 4 (3 in rat) in both hippocampus and frontal cortex (Dwivedi et al. 2006). Phenelzine increases transcripts containing exon I in both the frontal cortex and hippocampus, but exon V-containing transcripts only in the hippocampus. Kainate-induced seizures increase the levels of BDNF transcripts 1, 2, and 4 in hippocampus (Timmusk et al. 1993).

In the present study, we predict that since BDNF levels are significantly reduced in the medial frontal cortices and hippocampus of depressed patients, as well as in several studies looking at depression in rodents, then BDNF mRNA levels will also be reduced in the brain tissue of LH mice compared to controls after induction of Learned Helplessness. We further predict that the decrease in BDNF message will be greater in tissue taken from the medial frontal cortical region compared with hippocampal tissue due to the role that the MFC plays in depression; including its involvement in mediating emotional experience, mood, emotional processing, and the impact of these factors on cognition. To test this hypothesis, mRNA message for the BDNF transcript variants (I-V) will be measured in both hippocampus and medial frontal cortex in mice that have previously experience inescapable shocks during learned helplessness training, and will be compared to control mice that do not experience the learned

helplessness training. The mRNA for the BDNF transcripts in the brain will be quantified using RT-PCR technique.

Methods

C57BL/6J adult mice will be used in these studies. All of the procedures involving the animals will be approved by the University of New Mexico Laboratory Animal Care and Use Committee and will be conducted by a trained laboratory technician. The laboratory holds currently approved protocols for these studies. Mice will undergo the learned helplessness (LH) procedure as previously described by (Caldarone et al. 2000). This procedure is well supported as a test for assessing and developing depressive behavior. There are two groups; one undergoes the LH procedure, experiencing 120 inescapable unpredicted footshocks. The other is a control group that experiences the same apparatus, although no foot shocks are presented. There will be a minimum of 8 mice from each of the control and LH groups. One hour following the procedure, the hippocampus and medial frontal cortices of both groups will be analyzed to determine any differences in BDNF mRNA levels.

Learned Helplessness

A Coulbourn™ Habitest© shuttlebox with a stainless steel grid floor for administration of a footshock will be used. The front and back of the box are made of Plexiglas, and the sides, door, and ceiling are made of aluminum. The two-chambered box is housed within a sound-attenuated chamber. A 70% isopropanol solution will be used to clean the apparatus between each mouse and training/testing period. For the experimental group, one mouse will be placed in each chamber of the shuttlebox, and a 0.5 mA shock with a duration of 2 seconds and a frequency of approximately every 10 seconds will be administered through the stainless steel floor to each mouse simultaneously for 1 hour. The shock will be inescapable and set at a random probability of 0.5 shocks every 15 seconds, and the mouse will be removed 30 seconds after the delivery of the last shock. For the control group, mice will be placed in both sides of the shuttlebox for the same amount of time, but no shock will be administered. Following

training, both groups of mice will be euthanized and their brain tissue dissected out and prepared for mRNA analysis.

Assessment of BDNF mRNA Levels

mRNA isolation: mRNA will be isolated using the Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' protocol. Tissue will be placed using a RNA Stabilization Reagent (Qiagen) immediately following dissection. The mRNA concentration will be determined by spectroscopic measurements (OD at 260 nm), using NanoDrop® ND-1000 spectrophotometer (Isogen Life Sciences). Purified mRNA will be stored in 10 µl aliquots at -80^{o}C

cDNA synthesis: 10ng of mRNA from each sample will be reverse transcribed using oligo dT primers and M-MLV reverse transcriptase (Invitrogen). Reverse transcription reactions are carried out in 20 µL following the manufacturer's protocol. Briefly, a 10 µL reaction mixture containing 10 ng mRNA, 1µL oligo dT(500µg/ml), 1µL dNTP mix (10mM each) is heated at 65 ^{0}C for 5 minutes and chilled on ice for 5 minutes, followed by addition of 1 µL (200 units) M-MLV reverse transcriptase, 1 µL RNaseOUT (40 units/µL), 2µL DTT (0.1M) and sterile RNAase free water to 20µL. The whole reaction mixture is incubated at 37 0C for 50 minutes, then 75 ^{0}C for 10 minutes to inactivate the enzyme. The synthesized cDNA is stored at -20 ^{0}C before use.

The suitability of the cDNA for PCR is determined using 2uL of cDNA added to a 40 µL PCR reaction, containing primers specific for mouse BDNG exonVI (see below and Preliminary Results). The primers amplify a 150 bp DNA segment. A control reaction containing 2µl of purified mRNA is run without reverse transcription to confirm the DNA contamination, if any, in the purified mRNA samples. The PCR reaction is analyzed on a 2% (w/v) agarose gel. The amplified 150bp DNA band can be excised, gel purified using a gel extraction kit (Qiagen) and sequenced to confirm the specificity of the primers used.

Primers: Oligonucleotide sequences specific to mouse BDNF Exon I- VI and β Actin were designed using primer Express software (Applied Biosystems) and are given below (5′→3′ sequence):

EXON	SENSE	ANTISENSE
I	CCTGCATCTGTTGGGGAGAC	GCCTTGTCCGTGGACGTTTA
II	CTAGCCACCGGGGTGGTGTAA	AGGATGGTCATCACTCTTCTC
III	GCCGGGCCGGATG	CCGTGGACGTTTACTTCTTTC
IV	CAGAGCAGCTGCCTTGATGTT	GCCTTGTCCGTGGACGTTTA
V	TTGGGGCAGACGAGAAAGCGC	AGGATGGTCATCACTCTTCTC
VI (total)	CAGGTGAGAAGAGTGATGACC	ATTCACGCTCTCCAGAGTCCC

β Actin	Forward	CCATCATGAAGTGTGACGTGG
β Actin	Reverse	GTCCGCCTAGAAGCATTTGCG

Forward primers specific for the upstream exons will be designed in a similar manner and used with the reverse BDNF primer for exonVI, or, if needed, a newly designed reverse primer for exonVI.

The primer concentration is optimized for use in real time PCR to determine the minimum primer concentration giving the lowest Ct (threshold cycle) while minimizing nonspecific amplification. Briefly in a 96 well plate with each amplification in triplicates of 20µL reaction containing 10 µL 2X SYBR green Master mix and a variable concentration of forward and reverse primers is run with thermal cycling parameters as initial step of Ampli Taq gold activation (hold at 95oC for 10 min) followed by 40 cycles of denaturation (hold at 95^{o}C for 10 sec) and annealing (hold at 60^{o}C for 1 min). PCR products are analyzed on 2% (w/v) agarose gels.

Quantitative Real-time PCR assay: Real time PCR will be carried out in a Gene Amp 7700 sequence detection system (Applied Biosystems) using SYBR Green Master Mix Kit (Applied Biosystems). Amplification will be performed in triplicates in a 96-well plate(MicroAmp™ Fast Optical 96-well Reaction plates and MicroAmp™ Optical Adhesive Film, both from Applied Biosystmes) in a total of 20µL reaction containing forward and reverse primers (100nM each final concentration) ,10 µL Power Sybr® Green PCR Master Mix reaction buffer (Applied Biosystems) and 2µL cDNA . The cycling parameters are 950C for 15 Sec, 60 0C for 1 min, 40 cycles after one initial step of 950C for 10 min, which is set to activate AmpliTaq Gold polymerase. The PCR products are monitored by

measuring the increase in fluorescence caused by binding of SYBR Green Dye to the double stranded DNA and Ct values (cycle threshold) are calculated by SDS software v1.9 (Applied Biosystems). The absence of nonspecific amplification is confirmed by analyzing PCR products by 2% (w/v) agarose gel.
The relative quantification of the BDNF gene in different tissue samples is done using a comparative method. β actin is used as endogenous control to standardize the amount of DNA added to the reaction. Each analysis requires 6 reactions (3 for analysis of the target BDNF and 3 for analysis of the internal standard, actin). The ΔCt per FASD and saccharin control sample is calculated and linearized using 2-ΔCt. Finally ΔΔCt between treated and control samples is calculated and linearized using 2-ΔΔCt for overall change. Thus, the amount of target BDNF gene in the FASD mouse normalized to an endogenous reference (β actin) and relative to control saccharin treated BDNF gene is given by an arithmetic formula.

Results

Levels of mRNA BDNF (exons 4, 5, 8) in the HPC and MFC of both experimental LH and control animals were measured and analyzed against a standard curve. The results were analyzed with a T test (Table 1). As described above, ΔΔCt between treated and control samples was calculated and linearized using 2-ΔΔCt for overall change.

Since BDNF levels are significantly reduced in the medial frontal cortices of depressed patients as well as in several studies looking at depression in rodents, we anticipated that mRNA levels would also be reduced in the brain tissue of LH mice compared to controls. This data is consistent with the hypothesis as indicated in figure 1. LH shocked mice displayed significantly higher levels of learned helpless behavior than the control mice. Figure 1 shows that compared to control mice, LH mice had significantly reduced levels of exons V [MFC, $t(6)=10.41$; HPC, $t(6)=4.07$] and VIII [MFC, $t(6)=26.8$; HPC, $t(6)=9.854$]

(* indicates $p<0.01$); ie mRNA BDNF levels were significantly decreased in the medial frontal cortex and hippocampus of LH mice compared to control mice for

exons 5 and 8. The difference seen in exon 4 was an increase in mRNA levels as compared to exons 5 and 8.

Further results indicated a greater decrease in BDNF mRNA in medial frontal cortical brain regions when compared to hippocampal regions also in Figure 1.

Figure 1. Mouse mRNA BDNF. Boxes represent exons and lines connect splice variants. Levels of mRNA BDNF total and specific mRNA transcripts in the medial frontal cortex and hippocampal formation of control and Learned Helplessness mice.

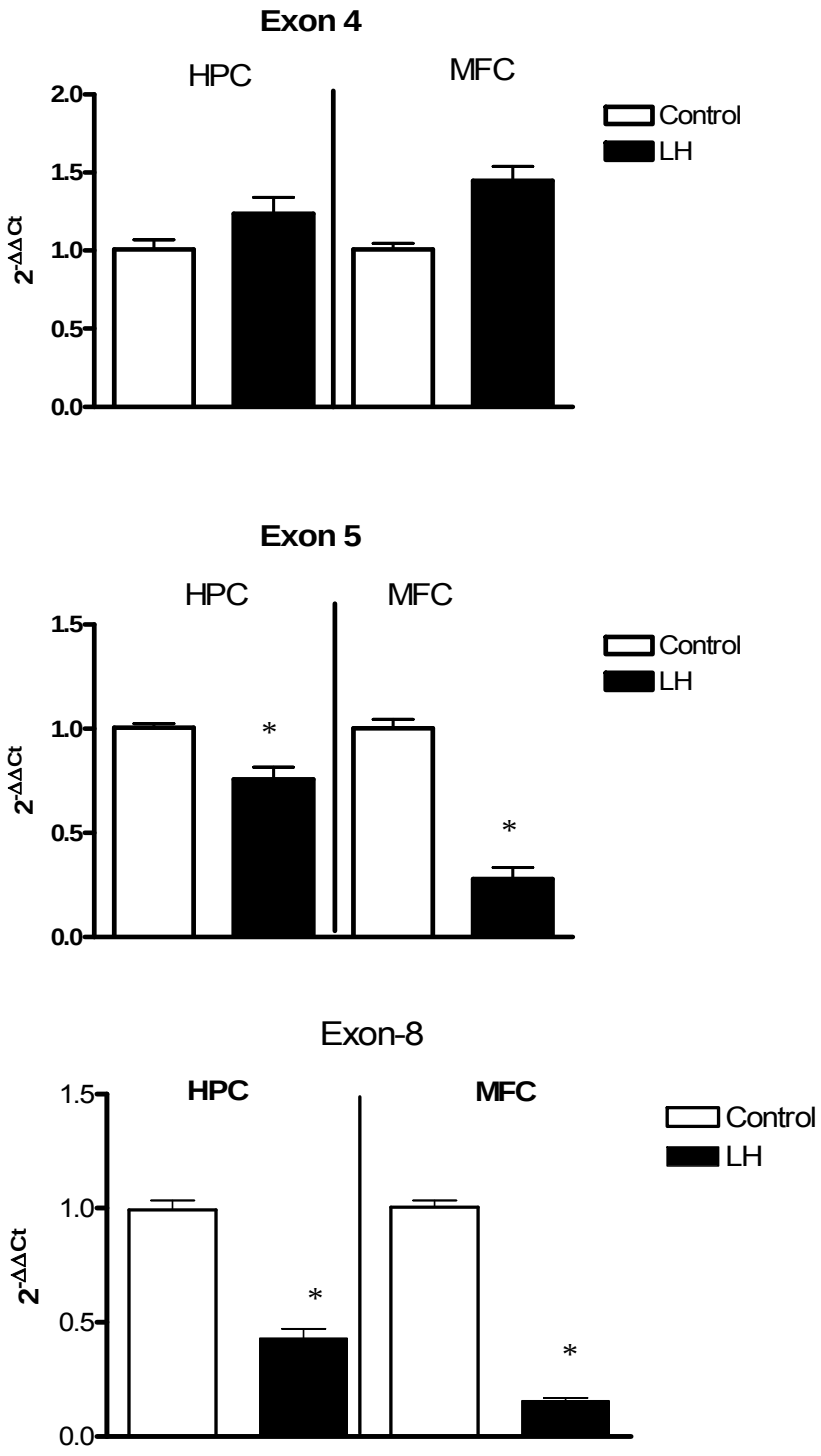

Table 1. T test analysis of BDNF exons.

Exon	Analysis
4	HPC not significant MFC t(6)=4.4 p<0.005
5	HPC t(6)= 4.07 p=0.0066 MFC t(6)= 10.41 p<0.0001
8	HPC t(6) 9.854 p<0.0001 MFC t(6) 26.08 p<0.0001

Exon 8 measures all BDNF transcripts.

Conclusion

In our study, we predicted that induction of depression using Learned Helplessness would result in a decrease in the mRNA levels of one or more of the BDNF transcripts. We further predicted that the decrease in BDNF message will be greater in tissue taken from the medial frontal cortical region compared with hippocampal tissue due to the role that the medial frontal cortex plays in depression. To test this hypothesis, we measured mRNA message for the BDNF transcript variants in both hippocampus and medial frontal cortex in mice that had previously experienced inescapable shocks during Learned Helplessness (LH) training. We then compared them to control mice that did not experience the learned helplessness training.

Our results as shown in Figure 1 indicate that as predicted the LH mice had reduced levels of mRNA in both exon 5 and exon 8. As previously discussed, exon 8 is a measure of the total BDNF mRNA. The fact that there is a reduction in the amount of exon 8 is consistent with prior data that BDNF mRNA is low in concordance with depression and learned helplessness (Karege 2002). In addition, exons 4 and 5 were analyzed because of their precision when utilized as primers and the ability to measure these individual exons more accurately. The amount of BDNF exon 5 in this experiment was decreased as well. However, it is interesting to note that the amount of BDNF exon 4 is increased. Although exon 4 is increased, total BDNF mRNA (ie exon 8) is decreased overall. It is possible that other exons may have yielded similar results to exon 4, however additional exons were not analyzed in this experiment. Further study may be needed to analyze specific exons and the effects of Learned Helplessness on individual components of mRNA. It may be concluded that analyzing the exons as a sum rather than individually is appropriate as individual exons may increase in order to compensate for decreases in alternative exons to yield a total BDNF product. Total BDNF (exon 8) was studied as it yields the most accurate data when analyzing effects of Learned Helplessness on mice.

As predicted, the decrease in BDNF message was more substantial in tissue taken from the medial frontal cortical region compared with hippocampal tissue due to the role that the medial frontal cortex plays in depression. Deficits

in the MFC region have been identified in depressed patients via functional neuroimaging which correlates with depressive symptoms (Drevets 2007). Deficits in patients are appreciated in mediating emotional experience, mood, emotional processing, and the impact of these factors on cognition. The hippocampus, as indicated by our results was affected in addition to the MFC and there is an indicated decrease in BDNF. This is consistent with previous studies in which evidence is given regarding a neurotrophic hypothesis of antidepressant action on depression. Research from Song et al (2006) states that treatment with antidepressants improves both the changes of behavior and hippocampus BDNF levels induced by learned helplessness and chronic mild stress.

Serum BDNF levels can be measured in patients with major depressive disorder (MDD), and there is a correlation between reduced BDNF levels and the severity of depression. More specifically, low BDNF levels correlate with Major Depressive Disorder. The model of Learned Helplessness allows for analysis of depressed patients via mice which at this current time is the most accurate model for depression. In several studies conducted by Song et al. (2006), the hypothesis that treatments with antidepressants improve both the changes of behavior and hippocampus BDNF levels induced by Learned Helplessness was supported. This is consistent with previous data and the data outlined in this experiment. At this time, research is attempting to solve the question of whether depression results secondary to diminished BDNF levels, or a reduction in BDNF levels occurs as a result of individuals becoming depressed. This is important information for patients, as additional knowledge of the function as well as levels of BDNF is useful when analyzing the effects of antidepressant treatment. This in turn will allow for further research in determining effective medications for patients in the future. Further research is needed to analyze the individual BDNF mRNA strands as it is currently unknown whether one specific exon may be more essential in moderating BDNF products than alternative exons. It is possible that there exists a more substantial response to treatment in certain patients because of single nucleotide polymorphisms and specific BDNF transcripts. It is hopeful that the mysteries of BDNF are elucidated in the future, but regardless, BDNF

remains a key component of depression and Learned Helplessness, and is essential to understanding the neurobiology of depression.

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