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Functional Role of BDNF Production from Unique Promoters in Aggression and Serotonin Signaling

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Brain-derived neurotrophic factor (BDNF) regulates diverse biological functions ranging from neuronal survival and differentiation during development to synaptic plasticity and cognitive behavior in the adult. BDNF disruption in both rodents and humans is associated with neurobehavioral alterations and psychiatric disorders. A unique feature of *Bdnf* transcription is regulation by nine individual promoters, which drive expression of variants that encode an identical protein. It is hypothesized that this unique genomic structure may provide flexibility that allows different factors to regulate BDNF signaling in distinct cell types and circuits. This has led to the suggestion that isoforms may regulate specific BDNF-dependent functions; however, little scientific support for this idea exists. We generated four novel mutant mouse lines in which BDNF production from one of the four major promoters (I, II, IV, or VI) is selectively disrupted (Bdnf-e1, -e2, -e4, and -e6 mice) and used a comprehensive comparator approach to determine whether different *Bdnf* transcripts are associated with specific BDNF-dependent molecular, cellular, and behavioral phenotypes. Bdnf-e1 and -e2 mutant males displayed heightened aggression accompanied by convergent expression changes in specific genes associated with serotonin signaling. In contrast, BDNF-e4 and -e6 mutants were not aggressive but displayed impairments associated with GABAergic gene expression. Moreover, quantifications of BDNF protein in the hypothalamus, prefrontal cortex, and hippocampus revealed that individual *Bdnf* transcripts make differential, region-specific contributions to total BDNF levels. The results highlight the biological significance of alternative *Bdnf* transcripts and provide evidence that individual isoforms serve distinct molecular and behavioral functions.
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INTRODUCTION

A remarkable feature of the genomic structure of the brain-derived neurotrophic factor (*BDNF*) gene is the existence of nine unique promoters that drive expression of *Bdnf* variants that encode the same BDNF protein (Figure 1a; Aid *et al*, 2007; Liu *et al*, 2006; Timmusk *et al*, 1993; West *et al*, 2014). An important but unanswered question is why multiple transcripts encode an identical BDNF protein and whether BDNF produced from individual promoters regulates specific BDNF-dependent functions. As a secreted neurotrophic factor, BDNF regulates diverse biological functions that range from neuronal survival and differentiation during development to synaptic plasticity and cognitive behavior in the adult (Andero *et al*, 2014; Autry and Monteggia, 2012; Castren, 2014; Chao *et al*, 2006; Lu, 2003). Mice engineered to disrupt BDNF signaling display several phenotypes, including hyperphagia-induced obesity, enhanced aggression, changes in cognitive behavior, and blunted response to antidepressant treatments (Autry *et al*, 2011; Chan *et al*, 2006; Ito *et al*, 2011; Lyons *et al*, 1999; Monteggia *et al*, 2004; Monteggia *et al*, 2007; Sakata *et al*, 2013). In line with BDNF's ability to influence multiple pathways, markers of 5-HT signaling as well as GABAergic transmission are altered in these models (Deltheil *et al*, 2008; Guilloux *et al*, 2012; Homberg *et al*, 2014; Hong *et al*, 2008; Huang *et al*, 1999; Luellen *et al*, 2007; Martinowich and Lu, 2008; Rios *et al*, 2006; Sakata *et al*, 2009; Tripp *et al*, 2012). Consistent with data from manipulation of *Bdnf* in rodents, *BDNF* disruption in humans is associated with psychiatric manifestations and neurobehavioral alterations, including obesity and enhanced aggression (Ernst *et al*, 2012; Han *et al*, 2008).

The capacity to mediate such a wide array of behavioral and molecular functions may be afforded by selective expression of distinct *Bdnf* transcripts, which can precisely control cell-specific, temporal and spatial BDNF production.

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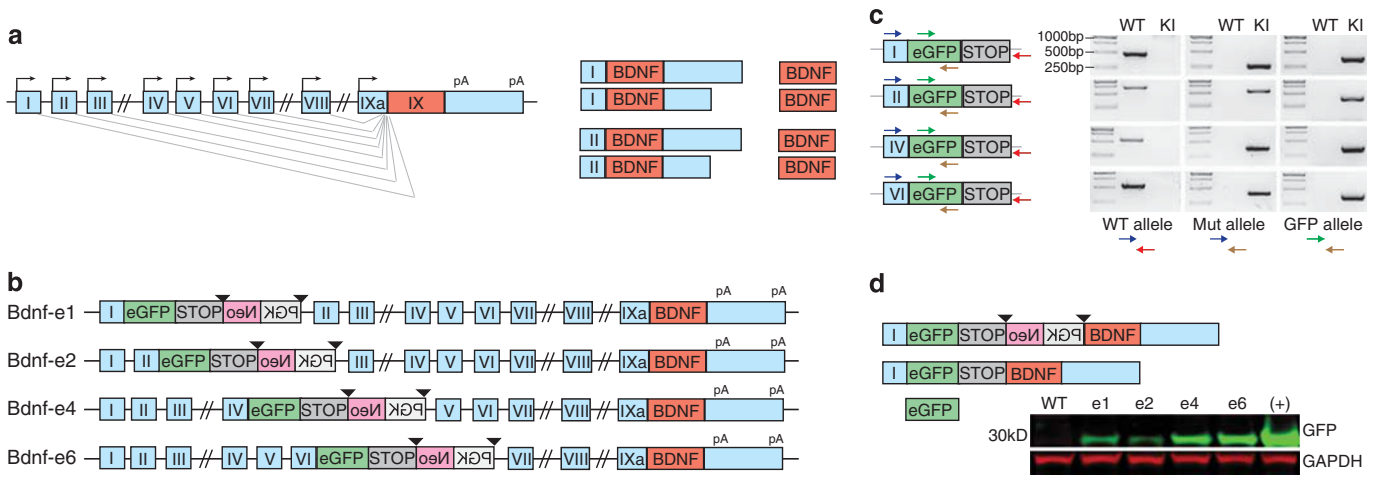

Figure 1 Generation of Bdnf-e1, -e2, -e4, and -e6 mutant mice. (a) Schematic of transcript production from *Bdnf* gene. Transcription is initiated from promoters upstream of individual 5′-untranslated regions (UTRs) and spliced to the common coding exon IX. Each transcript uses one of two polyadenylation sites. (b) Targeting vectors to generate Bdnf-e1, -e2, -e4, and -e6 mice. Vectors were designed to insert an enhanced green fluorescent protein (eGFP)-STOP cassette upstream of the exon's splice donor site with a floxed phosphoglycerate kinase (PGK)-Neomycin (Neo) cassette placed antisense to eGFP. PGK-Neo was later deleted by Cre recombinase expression. (c) PCR analysis of genomic DNA. A 499-bp Ex1 fragment and a 268-bp mutant (Mut) allele fragment were amplified from wild-type (WT) and Bdnf-e1 −/− mice, respectively. A 690-bp Ex2 fragment and a 516-bp Mut allele fragment were amplified from WT and Bdnf-e2 −/− mice, respectively. A 546-bp Ex4 fragment and a 372-bp Mut allele fragment were amplified from WT and Bdnf-e4 −/− mice, respectively. A 566-bp Ex6 fragment and a 367-bp mutant allele fragment were amplified from WT and Bdnf-e6 −/−, respectively. A GFP fragment was amplified from Bdnf-e1, -e2, -e4, and -e6 −/− mice. (d) Upper: example of promoter-I-driven transcription and splicing in Bdnf-e1 mutants. The initial founder line (BDNF-KI) produced a *Bdnf*-I-eGFP-STOP-PGK-Neo-*Bdnf* IX transcript. The floxed PGK-Neo cassette was deleted to produce a second generation of mice (Bdnf-e1) that express a *Bdnf*-I-eGFP-STOP-*Bdnf* IX transcript, leading to GFP production *in lieu* of brain-derived neurotrophic factor (BDNF). Lower: western blotting of GFP in adult HPC of Bdnf-e1, -e2, -e4, and -e6 −/− mice.

Evidence that different *Bdnf* transcripts are directed to distinct subcellular compartments following neural activity supports the view that these transcripts serve unique, or only partially overlapping, functions (An *et al*, 2008; Baj *et al*, 2012; Baj *et al*, 2011; Lau *et al*, 2010; Pattabiraman *et al*, 2005). In rodents, differential production of *Bdnf* splice variants has been documented in a number of models of neurological and neuropsychiatric disease and in response to various pharmacological treatments (Dias *et al*, 2003; Fumagalli *et al*, 2012; Nair *et al*, 2007; Zuccato *et al*, 2001). In humans, misregulation of specific *BDNF* transcripts is associated with several brain disorders, including Huntington's disease, schizophrenia and Alzheimer's disease (Garzon *et al*, 2002; Wong *et al*, 2010; Zuccato *et al*, 2001). Moreover, a selective deletion of *BDNF* exons I–III that spares the remaining portion of the gene is sufficient to cause obesity in humans (Han *et al*, 2008). In mice, selective BDNF disruption from promoter IV, which significantly contributes to activity-dependent BDNF production, leads to impaired GABAergic transmission and behavioral perseverance (Gao *et al*, 2014; Hong *et al*, 2008; Martinowich *et al*, 2011; Sakata *et al*, 2013; Sakata *et al*, 2009).

Since their discovery over two decades ago, it has been hypothesized that *BDNF* isoforms may have discrete, rather than redundant, roles in brain function; however, owing to a lack of tools, there is little scientific proof to substantiate this notion *in vivo*. To determine the biological significance of this 'multiple promoter, same protein' mechanism, we generated four transgenic mouse lines in which BDNF production is selectively disrupted from one of the four major promoters I, II, IV, or VI (Bdnf-e1, -e2, -e4, and -e6 mice). We targeted transcripts containing 5′-untranslated region (5′-UTR) exons I, II, IV, and VI, because they constitute the majority of *BDNF* mRNAs produced in the brain (Aid *et al*, 2007; Pruunsild *et al*, 2007). As BDNF production is only impaired from a single promoter, these models allow us to assess how loss of BDNF from one promoter *vs* another results in different consequences at the molecular, cellular, and behavioral levels. Using a comparator approach, we provide conclusive evidence that *Bdnf* isoforms regulate discrete, partially non-overlapping aspects of BDNF signaling and function *in vivo*. We report that BDNF production from promoters I or II, but not IV or VI, mediates the effects of BDNF on aggressive behavior. This is accompanied by significant decreases in BDNF protein in the hypothalamus (HYP) and selective impairment of 5-HT transporter and 5-HT2A receptor gene expression in Bdnf-e1 and -e2 mutants. In contrast, markers of GABAergic interneurons are significantly downregulated in Bdnf-e4 and -e6 mutants, but not Bdnf-e1 and -e2 mutants. Together, the results allow us to make the unprecedented conclusion that *Bdnf* splice variants have independent functional roles *in vivo*, and that the diverse functions of BDNF signaling can be influenced by selective production from distinct *Bdnf* promoters.

MATERIALS AND METHODS

Mouse Generation

Mice with selective disruption of BDNF production from either promoter I, II, IV, or VI were generated by inserting an enhanced green fluorescent protein (eGFP)-STOP cassette upstream of the respective 5′UTR splice donor site of the targeted exon (Supplementary Figure S1a). A floxed phosphoglycerate kinase (PGK) promoter driving neomycin

(Neo) expression was inserted antisense to eGFP-STOP. For promoter IV, the initial founder line (BDNF-KIV) was generated and described previously (Sakata *et al*, 2009). BDNF-KIV retained a floxed PGK-Neo cassette and showed significant downregulation of remaining *Bdnf* isoforms (Martinowich *et al*, 2011). Therefore, BDNF-KIV and the three additional founder lines (BDNF-KI, -KII, and -KVI) were crossed to a Cre-deleter strain (Jax Stock #006054, Jackson Labs, Bar Harbor, ME), to remove the floxed PGK-Neo cassette in all tissues including germ cells. This cross generated four novel transgenic lines with promoter-specific disruption of BDNF (Bdnf-e1, -e2, -e4, and -e6). In Bdnf-e1, -e2, -e4, and -e6 mutant mice, transcription is initiated from promoter I, II, IV, or VI, producing a 5′-UTR-eGFP-STOP-*Bdnf* IX transcript, which leads to GFP production *in lieu* of BDNF from the targeted promoter. A genotyping strategy was developed to distinguish between Bdnf-e1, -e2, -e4, and -e6 wild-type (WT) and mutant alleles (Supplementary Table S1). In all lines, the original transcript (*Bdnf* I–IX, II–IX, IV–IX or VI–IX, respectively) is not detectable and GFP is inserted correctly (Figure 1c). Male mice were backcrossed to C57Bl/6J > 12 generations before molecular and behavioral experiments. All experiments were conducted in accordance with a protocol approved by the SoBran Biosciences Institutional Animal Care and Use Committee.

Animal Housing Conditions

Animals were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle. Male Bdnf-e1, -e2, and their respective WT control groups were housed in divider caging at weaning due to high levels of fighting-induced death beginning at 5 weeks of age. Male Bdnf-e4, -e6, and their respective WT control groups were group housed at weaning, as there was no observable increase in fighting behavior. For quantitative RT-PCR (qPCR) experiments in adulthood (Figures 3 and 5, and Supplementary Figures S3 and S7), each genotype received a separate WT control group with matched housing conditions. For enzyme-linked immune assay (ELISA) and high-performance liquid chromatography (HPLC) experiments (Figure 3, and Supplementary Figures S3 and S7), Bdnf-e1 and -e2 mice shared a divider-housed WT control group, while Bdnf-e4 and -e6 mice shared a group-housed WT control group. For behavior experiments with Bdnf-e1 and -e2 mutants (Figure 4 and Supplementary Figure S4), WT, Bdnf-e1, and -e2 mice were divider housed before housing with CD1 cagemates. For data analysis, the same WT group was used for Bdnf-e1 and -e2 mutants, and Bdnf-e1 heterozygotes. For behavior experiments with Bdnf-e1 and -e4 mutants, WT, Bdnf-e4, and -e6 mice were group housed by genotype before housing with CD1 cagemates. Bdnf-e4 and Bdnf-e6 mice had separate WT control groups.

Assessment of Developmental Milestones

WT and Bdnf-e1, -e2, -e4, and -e6 animals were tested for developmental weight, eye opening, and selected reflexes from P3 to P14 as described (Heyser, 2004). Briefly, surface righting reflex was analyzed by placing a pup on its back and recording the latency to turn over onto its belly. Auditory startle was measured by operating a clicker 25 cm above each pup and recording jerking, kicking, or squirming in response to acoustic stimuli. Bar holding, a motor development milestone, was assessed as time hanging on a bar (4–7 mm in diameter) using front paws up to a maximum of 10 s.

Cagemate Aggression Assay

For territorial-induced aggression, adult male mice (WT, Bdnf-e1, -e2, -e4, or -e6; $n = 8$–10 per genotype) were placed into customized divider cages (OptiMICE, Animal Care Systems, Centennial, CO) with an adult CD1 male mouse (21–24 g; Harlan Laboratories, Frederick, MD) as a cagemate for 2 weeks. WT and respective experimental groups had identical housing conditions and were not socially isolated before divider caging with CD1s. Dividers physically isolated cagemates, but allowed exchange of bedding, odors, and vocalizations through small holes. The aggression test was conducted by removing the divider and allowing the experimental mice to physically interact with CD1s for 5 min. Interaction sessions were video recorded using CaptureStar software (Clever Systems, Reston, VA). After session completion, the divider was restored and cagemates were separated into the same territories occupied before testing. For each experimental animal, two additional interaction sessions were completed with 48 h between each session. Videos were scored blinded to genotype and offensive aggression was measured by scoring biting attack latency, attack number, mounting latency, and mount number.

RNA Extraction and qPCR

WT and Bdnf-e1, -e2, -e4, and -e6 P28 female mice and adult male mice ($n = 3$–5 per genotype) were killed by cervical dislocation and brain tissues were collected on ice. Total RNA was isolated and extracted from the HYP, prefrontal cortex (PFC), and hippocampus (HPC) using TRIzol (Life Technologies, Carlsbad, CA). RNA was subsequently purified using an RNeasy minicolumn (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrophotomer (Agilent Technologies, Savage, MD). RNA concentration was normalized and reverse transcribed into single-stranded cDNA using Superscript III (Life Technologies). Quantitative PCR was performed using a Realplex thermocycler (Eppendorf, Hamburg, Germany) using GEMM mastermix (Life Technologies) with 40 ng of synthesized cDNA. PCR efficiencies of *Bdnf* primers (Supplementary Table S1) were examined by standard curve of serial-diluted cDNA and melting-curve functionality (Sakata *et al*, 2009). All commercially available Taqman probes (Life Technologies) that were used for qPCR are listed in Supplementary Table S1. Individual mRNA levels were normalized for each well to *Gapdh* mRNA levels.

Western Blotting

WT and Bdnf-e1, -e2, -e4, and -e6 adult male mice were killed by cervical dislocation and hippocampi were dissected and snap frozen in isopentane. Tissue was homogenized and sonicated in lysis buffer containing 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, and proteasome inhibitor cocktail (Sigma-Aldrich, St Louis, MO). SDS was added to tissue homogenate, to a final

concentration of 2% and then homogenates were sonicated again and incubated at 4 °C for 30 min while rotating. Lysates were cleared by centrifugation at 16 000 *g* for 10 min. Total protein concentrations were determined using the BCA Protein Assay Kit (Pierce). Lysates (100 μg) were fractionated via electrophoresis using a 12% Bolt Bis-Tris SDS-PAGE gel (Life Technologies) and transferred onto 0.2 μm nitrocellulose membranes (Life Technologies). Membranes were incubated for 1 h in Odyssey PBS blocking buffer (Li-Cor Biosciences, Lincoln, NE) and probed with anti-GFP (1 : 2000, Roche) and anti-GAPDH (1 : 5000, Millipore) primary antibodies in Odyssey PBS blocking buffer overnight at 4 °C. GFP and GAPDH signals were detected using secondary antibodies conjugated to infrared fluorophores, IR dye 800 donkey anti-rabbit (1 : 20 000, Li-Cor Biosciences) and IR dye 680 donkey anti-mouse (1 : 20 000, Li-Cor Biosciences), respectively. The Li-Cor Odyssey imaging system and software was used for antibody detection.

BDNF Enzyme-Linked Immune Assay

Age-matched WT and Bdnf-e1, -e2, -e4, and -e6 P28 female and adult male mice were killed by cervical dislocation and brain tissues were dissected and snap frozen in isopentane ($n = 3$–5 for each genotype). Whole HYP and unilateral PFC and HPC from each mouse were homogenized by sonication in 3–4 ml of lysis buffer (100 mM PIPES (pH 7), 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 200 μM PMSF, and protease inhibitor cocktail (Sigma-Aldrich), followed by centrifugation for 30 min at 16 000 *g* at 4 °C as previously described (Szapacs *et al*, 2004). Supernatants were then collected and 200 μl aliquots were removed to assess total protein concentration using the BCA Protein Assay Kit (Pierce). BSA was added to the remaining supernatants to a final concentration of 2% and lysates were frozen at − 80 °C until further use. BDNF protein levels were determined by ELISA (BDNF ImmunoAssay System, Promega, Madison, WI). Lysates were loaded directly into 96-well plates without dilution. Absorbances were recorded and analyzed using a Biotek Synergy H1 plate reader (Winooski, VT). BDNF concentration (pg/ml) was normalized to total soluble protein (mg/ml) in each sample and data were expressed as percent change of WT (pg BDNF/mg total protein).

Quantitative Analysis of 5-HT

Age-matched WT and Bdnf-e1, -e2, -e4, and -e6 adult male mice were killed by cervical dislocation and unilateral PFC was dissected and snap frozen in isopentane ($n = 4$–6 per genotype). Concentration of 5-HT was measured by a HPLC system. Briefly, PFC samples were weighed and homogenized in 0.2 M ice-cold perchloric acid. Homogenates were cooled on ice for 30 min to deproteinize and subsequently centrifuged at 20 000 *g* for 15 min at 4 °C. Supernatant samples were filtered through a 0.45-μm filter and 20 μl filtrate was applied to the HPLC system (HTEC-500, Eicom). The system had an Eicompack CAX column (2.0 ID × 200 mm) with a CAX mobile phase (Eicom) and an electrochemical detector set to an applied potential of +400 mV *vs* an Ag/AgCl reference analytical electrode; the flow rate was 0.35 ml/min. Peaks were identified by the standard and the areas were calculated using Envision software. 5-HT content was normalized to tissue weight and data were expressed as percent change of WT (pg 5-HT/mg wet tissue).

Histology

WT and Bdnf-e1, -e2, -e4, and -e6 adult male mice were anesthetized with isofluorane and transcardially perfused with 4% paraformaldehyde. Brains were postfixed overnight, cryoprotected in 30% sucrose, and cut with a frozen microtome in coronal 50 μm sections. Nissl staining was performed using cresyl violet and ethanol washes. Brightfield images were taken at × 2 magnification using an Olympus BX51TF microscope with DP70 color camera. Images were montaged using Neurolucida software (MicroBright Field Bioscience, Williston, VT) to reconstruct complete coronal sections.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism Software (La Jolla, CA). Comparison between two genotypes (WT and *Bdnf* mutant) was made using unpaired Student's *t*-test. Repeated measures of variance analysis (ANOVA) were used for attack latency, attack number, mounting latency, and mounting number across sessions. When applicable, *post hoc* Bonferonni's multiple comparisons were carried out. Data in text and graphical data are presented as means ± SEM. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\&}P < 0.001$, and $^{\#}P < 0.0001$.

RESULTS

Disruption of BDNF Production from Specific Promoters

To address whether BDNF produced from individual promoters serves discrete BDNF-dependent functions, we generated mice with selective disruption of BDNF production from either promoter I, II, IV, or VI. These promoters were targeted because they generate the majority of BDNF in the brain (Aid *et al*, 2007; Pruunsild *et al*, 2007). Targeting was accomplished by genetically engineering the placement of an eGFP-STOP cassette upstream of the respective 5′-UTR splice donor site of the targeted exon (Supplementary Figure S1a) with a floxed PGK-Neo cassette inserted antisense to eGFP-STOP for selection.

The initial founder line to disrupt production from promoter IV (BDNF-KIV) was generated and described previously (Figure 1b; Sakata *et al*, 2009). BDNF-KIV mice were not used for these comparator studies, as they displayed extensive downregulation of alternative *Bdnf* transcripts (Martinowich *et al*, 2011) and expression changes in other chromosome 2 genes (Supplementary Figure S1b). We reasoned that these expression changes were due to PGK-related promoter interference and addressed this issue by removing the existing PGK-Neo cassette to generate a novel second-generation BDNF promoter IV line (Bdnf-e4). To accomplish this, BDNF-KIV and the three unpublished founder lines (BDNF-KI, -KII, and -KVI) were crossed to a Cre-deleter strain, to remove the floxed PGK-Neo cassette in all tissues including germ cells (Figure 1b and Supplementary Figure S1a). PCR from genomic DNA verified PGK-Neo

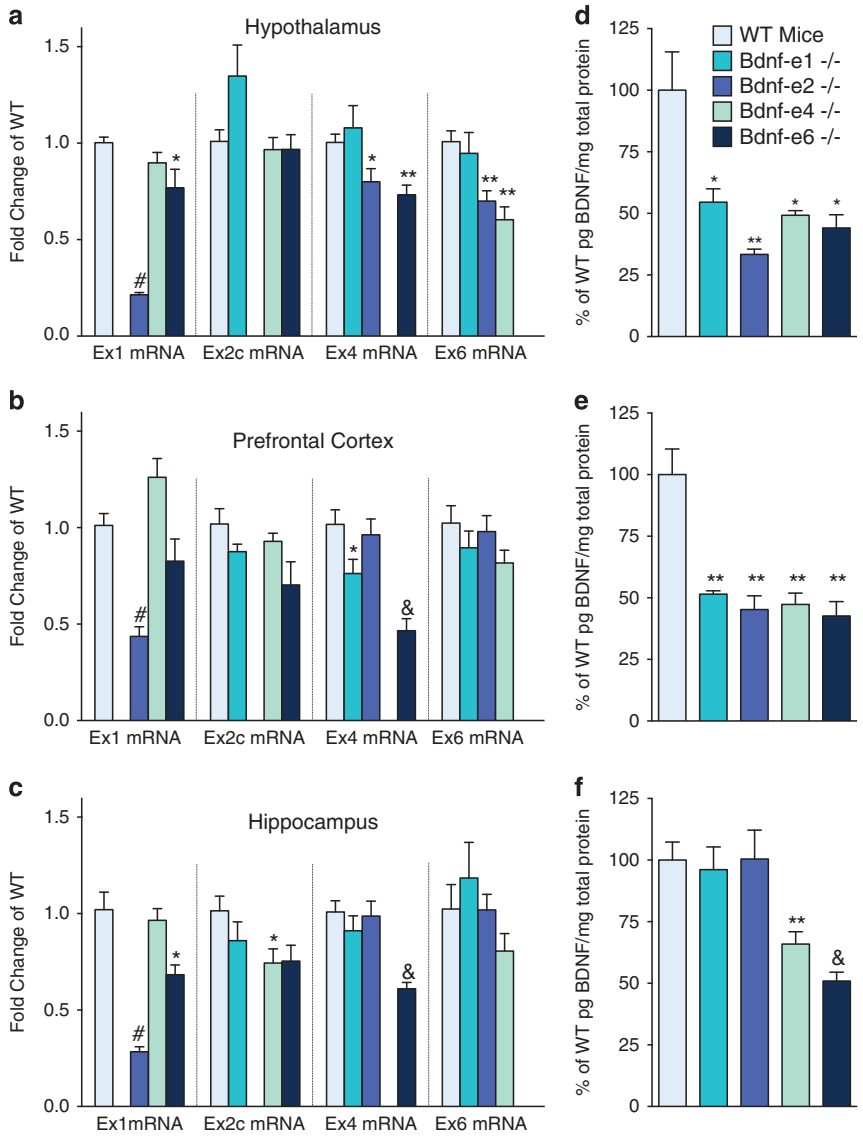

Figure 2 *Bdnf* mRNA and protein expression in postnatal day 28 (P28) Bdnf-e1, -e2, -e4, and -e6 mice. qPCR demonstrating relative expression levels of individual *Bdnf* transcripts (on *x* axis) in P28 wild-type (WT) and Bdnf-e1, -e2, -e4, and -e6 −/− (colored bars) hypothalamus (HYP) (a), prefrontal cortex (PFC) (b), and hippocampus (HPC) (c). In all tissues, disruption from individual promoters blocks transcript production from the targeted promoter. Decreases in transcription from other promoters were observed in specific regions. ELISA quantification demonstrating relative brain-derived neurotrophic factor (BDNF) expression levels in P28 WT and Bdnf-e1, -e2, -e4, and -e6 −/− HYP (d), PFC (e), and HPC (f). Blockade from promoters I, II, IV, or VI causes significant BDNF reductions in HYP and PFC; however, blockade from promoters IV and VI, but not I and II, reduces BDNF in HPC. Data are means ± SEM ($n = 5$ mice; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\&}P < 0.001$, and $^{\#}P < 0.0001$).

removal in the second-generation lines (Supplementary Figure S1c) and qPCR confirmed the absence of changes on chromosome 2 following PGK-Neo deletion (Supplementary Figure S1b). Thus, removal of the PGK-Neo cassette in the novel Bdnf-e4 line corrected the previously observed abnormalities in chromosome 2 gene expression. Bdnf-e1, -e2, -e4, and -e6 mice were used for all future experiments and genotyped by PCR analysis of genomic DNA (Figure 1c and Supplementary Table S1). In Bdnf-e1, -e2, -e4, and -e6 mutant mice, transcription is initiated from promoter I, II, IV, or VI producing a 5′-UTR-eGFP-STOP-*Bdnf* IX transcript, which leads to GFP production *in lieu* of BDNF from the targeted promoter (Figure 1d).

To determine whether our design effectively disrupted expression of the targeted *Bdnf* transcript, we analysed *Bdnf* exon (Ex) 1, 2c, 4, and 6 transcript levels in HYP, PFC, and HPC of postnatal day 28 (P28) Bdnf-e1, -e2, -e4, and -e6 mutant mice (Figure 2a–c). qPCR revealed the absence of transcript expression from the targeted exon (ie, loss of Ex1 transcripts in Bdnf-e1) in all examined regions. Although region-specific regulation of other 5′-UTR-containing transcripts was observed in each line, there was no evidence of widespread promoter suppression at non-targeted *Bdnf* loci as observed in the initial BDNF-KIV founder line (Supplementary Figure S1; Martinowich *et al*, 2011). For example, Bdnf-e1 mice showed decreases in Ex4 transcripts in PFC compared with WT (Fig. 2b; 0.762 ± SEM 0.074,

$p<0.05$), but no changes in other *Bdnf* transcripts in HYP and HPC (Figure 2a and c). These data suggest that alternative regulation of other *Bdnf* transcripts in BDNF-e1, -e2, -e4, and -e6 mutant mice is not a confound of the targeting strategy but rather reflects biological regulation downstream of loss of the targeted transcripts. This notion is strengthened by the fact that the observed misregulations can change across region and development. For example, although Ex4 transcripts are downregulated in Bdnf-e1 mutant PFC at P28 (Figure 2b), they become upregulated in Bdnf-e1 mutant PFC and HPC by adulthood (Figure 3b and c). Interestingly, Bdnf-e2 mutants display region and age-dependent decreases in Ex1 transcripts, suggesting a high degree of cross-talk between promoters I and II (Figure 2 and Supplementary Figure S3).

To assess how loss of BDNF production from individual promoters has an impact on total BDNF, we examined BDNF protein levels in P28 Bdnf-e1, -e2, -e4, and -e6 mutant HYP, PFC, and HPC by ELISA (Figure 2d–f). In HYP and PFC, blockade of *Bdnf* Ex1, 2c, 4, or 6 transcript expression decreased relative BDNF levels ~50% compared with WT. In HPC, blockade of Ex1 and 2 transcripts did not alter total BDNF levels, whereas blockade of Ex4 and 6 transcript expression significantly reduced BDNF production compared with WT ($Ex4 = 65.90\% \pm 5.018$, $p<0.01$; $Ex6 = 50.91\% \pm 3.578$, $p<0.001$). Thus, BDNF derived from promoter IV and VI contributes more substantially to total BDNF expression in P28 HPC than BDNF derived from promoter I or II. Reductions in BDNF caused no gross morphological impairments in brain development (Supplementary Figure S2a–o) and no notable deficits in developmental milestone acquisition, including weight gain, surface righting, bar holding, eye opening, and auditory startle (Supplementary Figure S2p–t). Together, the results demonstrate that the targeting strategy effectively disrupted *Bdnf* transcript and protein production from specific promoters.

To examine the contribution of BDNF derived from individual promoters to total BDNF levels in the mature brain, we analyzed protein levels and *Bdnf* Ex 1, 2c, 4, and 6 expression in HYP, PFC, and HPC of Bdnf-e1, -e2, -e4, and -e6 mice in adulthood (4–5 months). Replicating results from earlier in development at P28, we validated the absence of transcript expression from the targeted locus in all tissues examined (Figure 3a–f and Supplementary Figure S3a–f). Although biological regulation of non-targeted *Bdnf* transcripts was observed in each transgenic line, changes were region specific and in some cases showed different directionality. For example, Bdnf-e1 mice exhibited HYP decreases, but PFC increases in Ex2c and 4 transcripts compared with WT (Figure 3a and b). In Bdnf-e1 and -e2 mutants, BDNF protein was significantly reduced in HYP (Figure 3g and Supplementary Figure S3g) but unaffected in PFC and HPC (Figure 3h and i, and Supplementary Figure S3h and i). On the other hand, in Bdnf-e4 and -e6 mutants BDNF protein was reduced ~50% in HYP, but also >25% in PFC and HPC (Figure 3j–l and Supplementary Figure S3j–l). Together, these results demonstrate that BDNF derived from individual promoters makes differential contributions to total BDNF pools in specific brain regions. Although each promoter is active in all regions, our data suggest that transcription from promoters I and II is more prominent in adult HYP, whereas transcription from promoters IV and VI is more prominent in PFC and HPC.

BDNF Produced from Promoters I and II Regulates Aggression

Mice with BDNF deficiency show diverse phenotypes, including hyperphagia, elevated aggression, and hyperactivity (Chan *et al*, 2006; Ito *et al*, 2011; Lyons *et al*, 1999; Rios *et al*, 2001). Although BDNF reductions are associated with these behaviors, whether disruption from individual promoters is selectively associated with BDNF-dependent behavioral deficits is not established. Using Bdnf-e1, -e2, -e4, and -e6 mice, we investigated how loss of BDNF produced from promoters I, II, IV, and VI has an impact on aggressive behavior. This behavior warranted study due to observations that BDNF-e1 and -e2, but not -e4 and -e6, male mutants displayed striking increases in homecage aggression starting at 5 weeks of age. Indeed, severity of aggression required Bdnf-e1 and -e2 mutant males to be housed in divider caging from weaning to prevent fighting-induced death.

To systematically analyze aggressive behavior, we conducted a cagemate aggression paradigm in which an adult male (WT, Bdnf-e1, -e2, -e4, or -e6 mutant) was divider housed with an age-matched CD1 male. Divider caging physically isolated experimental mice from CD1 cagemates, but a perforated barrier allowed exchange of bedding, odors, and vocalizations. After 2 weeks, the aggression test was conducted in three sessions by removing the divider and allowing experimental animals to physically interact with their CD1 cagemates for 5 min. Across all sessions, Bdnf-e1 mutants were highly aggressive, showing decreased attack latency ($F_{1,19} = 31.90$, $p<0.0001$) and increased attack number compared with WT ($F_{1,19} = 23.96$, $p = 0.0001$; Figure 4a and b). In CD1-WT pairings, CD1s were almost always the aggressors (Figure 4d). Conversely, aggression by Bdnf-e1 mutants was so severe that CD1s rarely attacked them (Figure 4c). In fact, CD1s were four times more likely to attack WT animals than Bdnf-e1 mutants, suggesting that the normally observed CD1 dominance was suppressed in pairings with Bdnf-e1 mutants (Figure 4d). Consistent with convergence of *Bdnf* transcript and protein expression in Bdnf-e1 and -e2 mutants (Figure 3 and Supplementary Figure S3), Bdnf-e2 mutants also displayed elevated aggression characterized by decreased attack latency ($F_{1,19} = 9.592$, $p = 0.0059$) and increased attack number ($F_{1,19} = 4.892$, $p = 0.0394$; Supplementary Figure S4a and b). Although Bdnf-e2 mutants failed to dominate CD1s as strongly as Bdnf-e1 mutants, they neutralized CD1 aggression by demonstrating equivalent attack latency and number as compared with WT animals (Supplementary Figure S4c and d). In sharp contrast, Bdnf-e4 (Figure 4e–h) and -e6 mutants (Supplementary Figure S4e–h) showed no increase in aggression and were significantly dominated by CD1s.

In addition to biting attack behavior, Bdnf-e1 mutants displayed aberrant mounting behavior. Specifically, Bdnf-e1 animals displayed decreased latency to mount CD1s and increased incidences of mounting behavior (Supplementary Figure S5a and b). This trend toward aberrant mounting in BDNF-e1 mutants was almost never observed by CD1s or Bdnf-e2, -e4, or -e6 mutants (Supplementary Figure S5c–h),

suggesting that this pattern of behavior is specifically associated with loss of Ex1 expression. To further investigate the role of Ex1 transcript expression in elevated aggression, we tested Bdnf-e1 heterozygous (+/−) mice in our aggression paradigm. During development, Bdnf-e1+/− mice displayed the expected ~50% downregulation of Ex1 transcript expression in HYP, PFC, and HPC compared with WT, and showed no regulation of non-targeted *Bdnf*

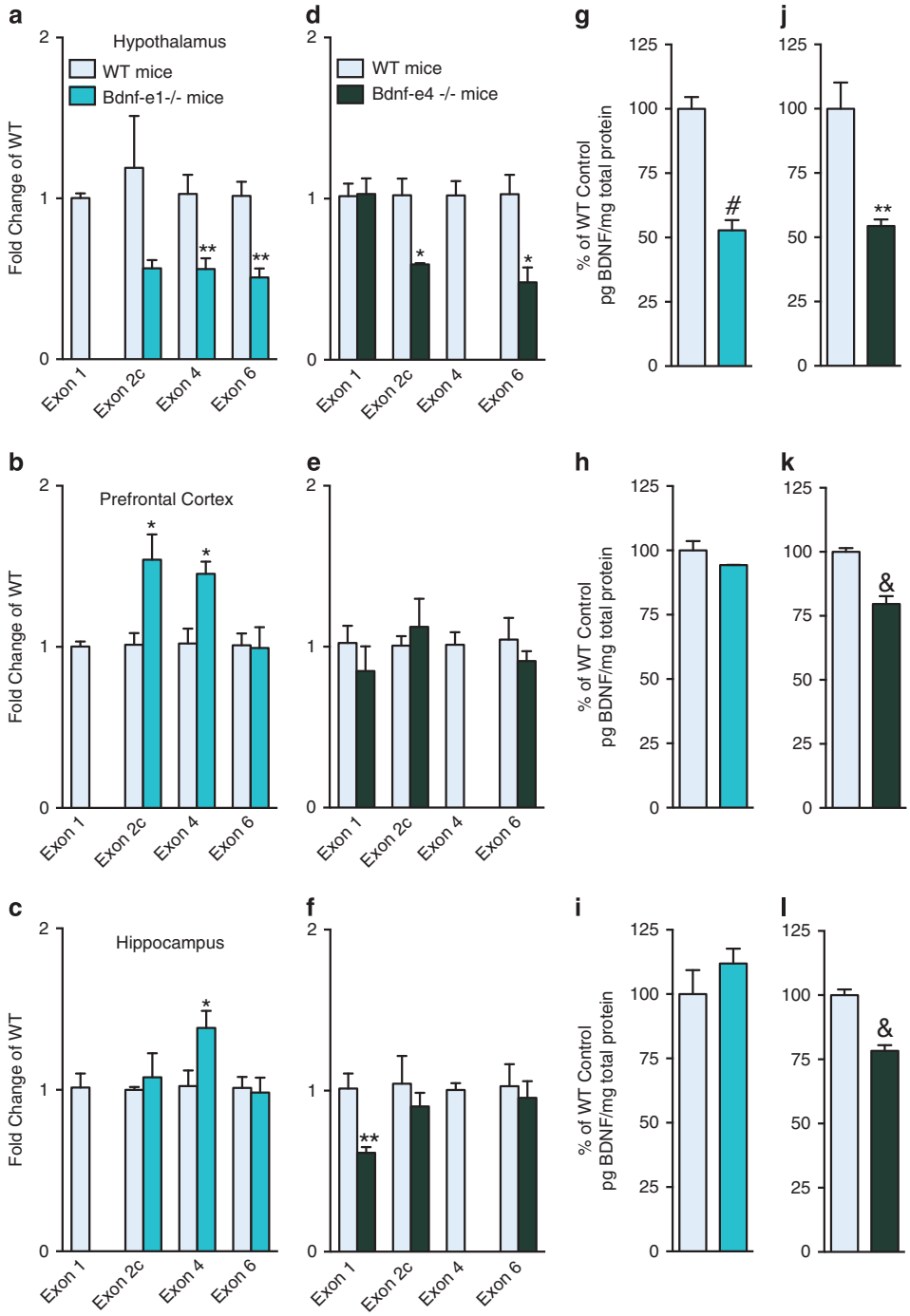

Figure 3 Individual *Bdnf* promoters differentially contribute to brain-derived neurotrophic factor (BDNF) production in adulthood. qPCR demonstrating relative *Bdnf* transcript levels in adult (4–5 month) wild-type (WT) and Bdnf-e1 −/− hypothalamus (HYP) (a), prefrontal cortex (PFC) (b), and hippocampus (HPC) (c). Disruption from promoter I abolishes Ex1 transcripts in all regions, downregulates Ex2c-, 4-, and 6-containing transcripts in HYP, and upregulates Ex2c and 4 transcripts in PFC and HPC. qPCR demonstrating relative expression levels of individual *Bdnf* transcripts in WT and Bdnf-e4 −/− HYP (d), PFC (e), and HPC (f). Disruption from promoter IV causes blockade of Ex4 transcripts in all regions, downregulation of Ex2c- and 6-containing transcripts in HYP, and decreases in Ex1 transcript expression in HPC. ELISA quantification of relative BDNF levels in WT and Bdnf-e1 −/− HYP (g), PFC (h), and HPC (i). Blockade from promoter I causes significant loss of BDNF in HYP, but not in PFC or HPC, of Bdnf-e1 −/−. ELISA quantification of relative BDNF levels in WT and Bdnf-e4 −/− HYP (j), PFC (k), and HPC (l). Blockade from promoter IV causes significant loss of BDNF in HYP, PFC, and HPC. Data are means ± SEM ($n=4$ mice for qPCR; $n=5$ mice for ELISA; $^{*}P<0.05$, $^{**}P<0.01$, $^{\&}P<0.001$, and $^{\#}P<0.0001$).

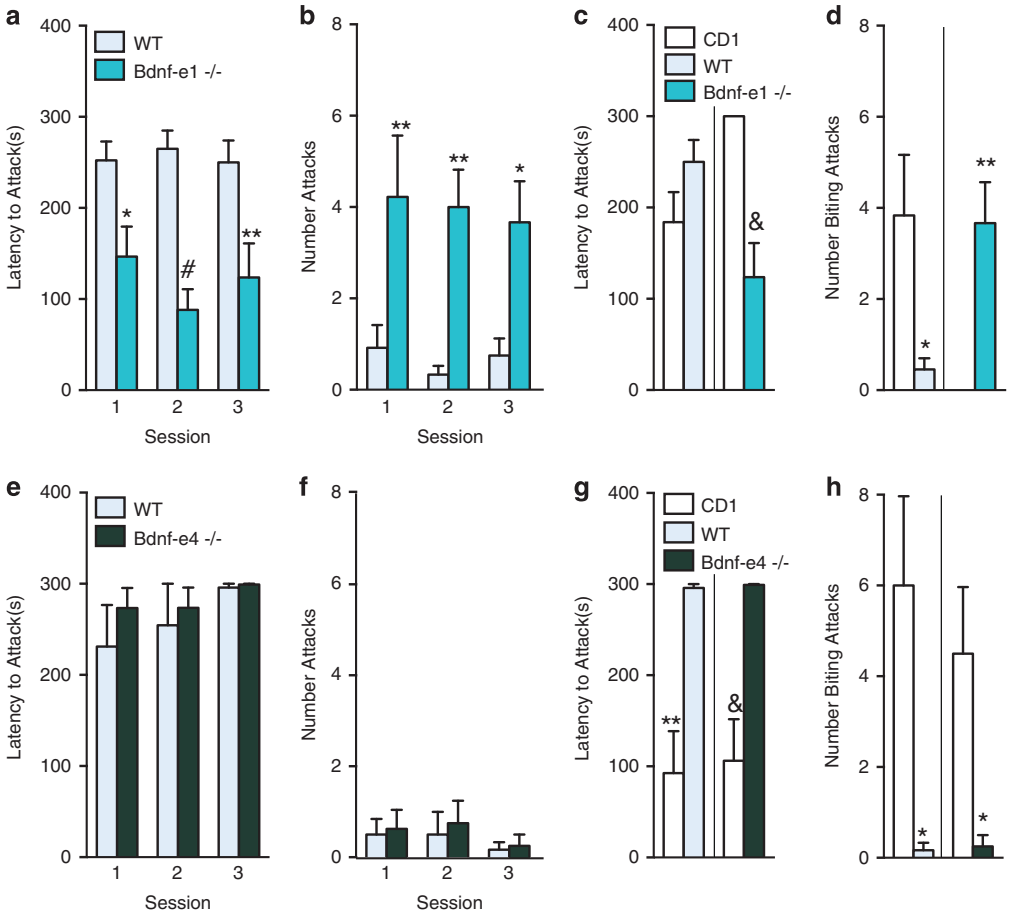

Figure 4 Disruption from promoter I, but not promoter IV, increases aggression in male mice. Biting attack latency (a) and number (b) of Bdnf-e1 −/− across three consecutive sessions. Bdnf-e1 −/− display persistent decreased attack latency and increased attack number over time compared with wild-type (WT). Biting attack latency (c) and number (d) of Bdnf-e1 −/− mice and CD1s from a single session. Unlike WT, Bdnf-e1 −/− attack CD1s significantly faster and more frequently, thereby establishing dominance. Biting attack latency (e) and number (f) of Bdnf-e4 −/− mice across three consecutive sessions. Bdnf-e4 −/− show no change in latency or number of attacks compared with WT. Biting attack latency (g) and number (h) of Bdnf-e4 −/− mice and CD1s from a single session. CD1s attack significantly faster and more frequently than both WT and Bdnf-e4 −/−. Data are means ± SEM ($n = 6$–9 mice per genotype; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\&}P < 0.001$, and $^{\#}P < 0.0001$).

transcripts (Supplementary Figure S6a–c). In adult males, BDNF protein levels were reduced ~50% in HYP, but unchanged in PFC and HPC compared with WT (Supplementary Figure S6d–f). Similar to Bdnf-e1 mutants, Bdnf-e1+/− males showed increased attack number ($F_{1,19} = 6.06$, $p = 0.0236$) and a strong trend toward decreased attack latency compared with WT (Supplementary Figure S6g and h). In addition, Bdnf-e1+/− trended toward dominating CD1s (Supplementary Figure S6j and k) and displaying aberrant mounting behavior (Supplementary Figure S6i and l). Together, the data demonstrate that BDNF disruption from promoters I or II, but not from IV or VI, leads to elevated aggression and provides scientific support for the notion that individual *Bdnf* transcripts are functionally linked to specific behaviors.

Divergent 5-HT and GABA Gene Expression Changes Following BDNF Loss from Promoters I and II *vs* IV and VI

BDNF deficiencies are strongly associated with misregulation of both 5-HT and GABA signaling pathways (Deltheil *et al*, 2008; Guilloux *et al*, 2012; Hong *et al*, 2008; Lyons *et al*, 1999; Rios *et al*, 2006; Sakata *et al*, 2009; Tripp *et al*, 2012). The 5-HT neurochemical signaling system has been consistently associated with control of aggressive behavior (Takahashi *et al*, 2011). PFC circuits are thought to provide an important regulatory role by providing inhibitory control over aggression (Takahashi and Miczek, 2014). In this context, inhibitory circuits mediating GABAergic transmission have been associated with varying levels of aggression. We hypothesized that aggressive Bdnf-e1 and -e2 mutants would show convergent 5-HT and/or GABA gene expression changes distinct from those displayed by Bdnf-e4 and -e6 mutants.

To assess deficits in serotonergic and GABAergic neurotransmission, we compared gene expression levels for markers of 5-HT signaling components and GABA interneurons in HYP, PFC, and HPC of Bdnf-e1, -e2, -e4, and -e6 mice (Figure 5 and Supplementary Figure S7). We also used HPLC to measure 5-HT content in postmortem PFC (Supplementary Figure S7). Disruption of BDNF production from any promoter was sufficient to cause significant alterations in 5-HT and GABA gene expression. However,

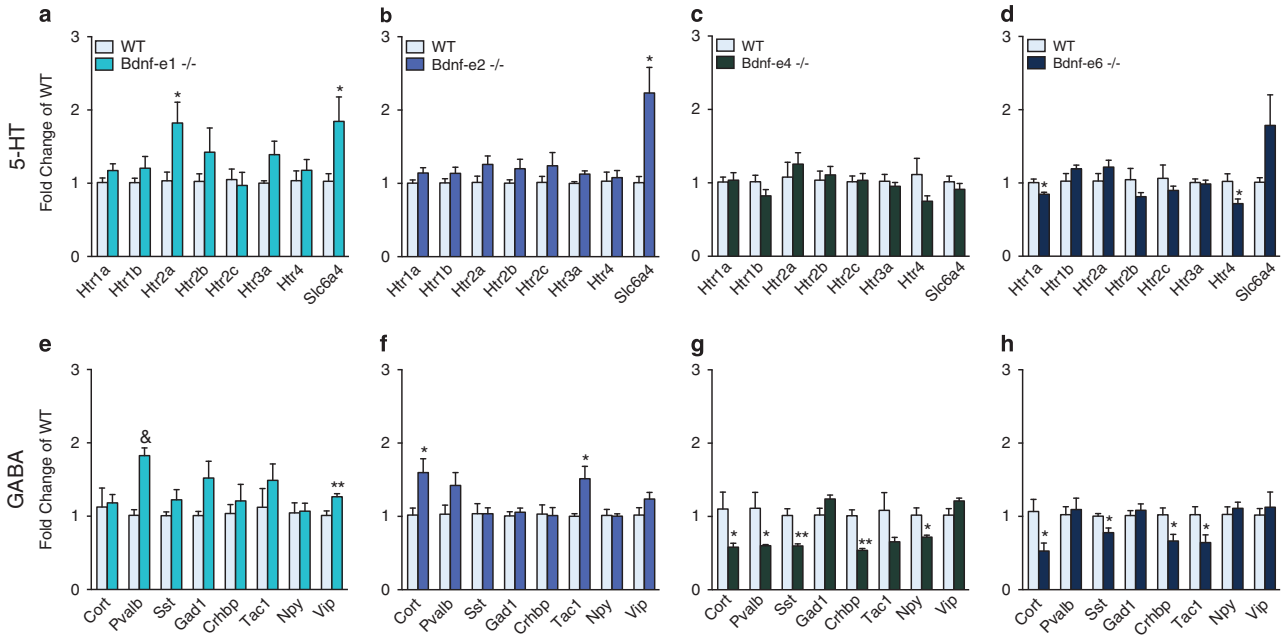

Figure 5 Divergent 5-HT and GABA gene expression changes in Bdnf-e1 and -e2 *vs* -e4 and -e6 mutants. qPCR demonstrating relative prefrontal cortex (PFC) expression levels of 5-HT receptor and 5-HT transporter (*Slc6a4*) transcripts in wild-type (WT) and Bdnf-e1 (a), Bdnf-e2 (b), Bdnf-e4 (c), and Bdnf-e6 (d) mutants. Disruption of individual promoters causes divergent 5-HT gene expression changes, but common *Slc6a4* increases in aggressive Bdnf-e1 and -e2 mutants. qPCR demonstrating relative expression levels of GABA interneuron markers in WT and Bdnf-e1 (e), Bdnf-e2 (f), Bdnf-e4 (g), and Bdnf-e6 (h) mutants. Bdnf-e4 and -e6, but not -e1 and -e2, mutants show widespread downregulation of GABA genes. Data are means ± SEM ($n = 4$ mice; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\&}P < 0.001$).

aggressive BDNF-e1 and -e2 mutants showed convergent 5-HT and GABA gene expression changes that were distinct from 5-HT and GABA deficits in Bdnf-e4 and -e6 mutants. Specifically, Bdnf-e1 and -e2 mutants showed increases in the serotonin transporter 5-HTT (*Slc6a4*) and the serotonin receptor 5-HT2A (*Htr2a*) in the HYP, PFC, and/or HPC compared with WT (Figure 5a and b, and Supplementary Figure S7a, b, e and f). These gene expression changes were accompanied by reduced 5-HT content in the PFC (Supplementary Figure S7i and j). Furthermore, Bdnf-e1 and -e2 mutants showed elevations in GABAergic interneuron markers in the PFC, including parvalbumin (*Pvalb*), vasoactive intestinal polypeptide (*Vip*), cortistatin (*Cort*), and tachykinin (*Tac1*) (Figure 5e and f). On the other hand, Bdnf-e4 and -e6 mutants showed no overlapping deficits in 5-HT receptor and transporter gene expression (Figure 5c and d, and Supplementary Figure S7c, d, g and h) but widespread suppression of GABAergic interneuron markers (Figure 5g and h). Specifically, loss of BDNF from promoters IV and VI caused convergent decreases in *Cort*, somatostatin (*Sst*), corticotropin-releasing factor-binding protein (*Crhbp*), and *Tac1* gene expression in PFC (Figure 5g and h). Unlike Bdnf-e1 and -e2 mutants, Bdnf-e4 and -e6 mutants showed no significant decreases in PFC 5-HT content compared with WT (Supplementary Figure S7k and l). These results demonstrate convergent 5-HT and GABAergic expression changes between Bdnf-e1 and -e2 mutants that diverge from 5-HT and GABA deficits in Bdnf-e4 and -e6 mutants. Together, the data provide evidence that BDNF produced from promoters I and II influences different signaling pathways than BDNF produced from promoters IV and VI.

DISCUSSION

Since the discovery of the four major *BDNF* promoters over two decades ago (Timmusk *et al*, 1993), it has been hypothesized that individual *BDNF* isoforms differentially contribute to BDNF-dependent functions. Support for functional segregation of different *Bdnf* transcripts comes from studies demonstrating that alternative *Bdnf* transcripts mediate precise temporal-, spatial-, and stimulus-specific BDNF production, creating a spatial code for BDNF expression in different brain regions, cell types, and even within distinct subcellular compartments. Although reports demonstrating differential expression and localization of unique *Bdnf* transcripts has fueled interest in their respective potential roles, a lack of evidence has not allowed for the definitive conclusion that BDNF produced from different promoters regulates independent *in vivo* brain functions. Such knowledge is critical given the fact that expression changes in *BDNF* isoforms and epigenetic modifications at individual *BDNF* promoters are frequently reported in both animal and postmortem human studies. Previous efforts to understand the functional significance of individual transcripts focused exclusively on promoter IV because of its established role in activity-dependent transcription (Gao *et al*, 2014; Hong *et al*, 2008; Martinowich *et al*, 2011; Sakata *et al*, 2013; Sakata *et al*, 2009). These studies determined that loss of BDNF expression from promoter IV is sufficient to generate specific behavioral and cellular effects, but whether loss from other *Bdnf* promoters caused similar impairments was not studied (Gao *et al*, 2014; Hong *et al*, 2008; Martinowich *et al*, 2011; Sakata *et al*, 2013; Sakata *et al*, 2009). To address the outstanding question of whether

BDNF produced from different promoters governs discrete molecular, cellular, and behavioral functions, we generated a novel set of transgenic mice in which BDNF production from promoters I, II, IV, or VI is selectively disrupted. Using a comparator approach, these mice allowed us to dissect alternative gene transcription as a key mechanism of BDNF regulation at the functional level. We report that BDNF produced from promoters I and II, but not IV and VI, leads to enhanced aggression and convergent 5-HT deficits. Furthermore, loss of BDNF from promoters IV and VI, but not I and II, causes significant impairments in PFC GABAergic interneuron markers. Our data provide strong evidence that individual *Bdnf* promoters are differentially used *in vivo* and support the hypothesis that BDNF produced from unique promoters regulates distinct molecular and behavioral functions.

To establish the validity of our approach, we quantified *Bdnf* transcript expression in Bdnf-e1, -e2, -e4, and -e6 mice at two different stages, P28 and adulthood (Figure 2 and 3, and Supplementary Figure S3). Although for each line changes in the activity of alternative *Bdnf* promoters was observed, this appears to result from biological cross-talk between promoters and not as a confound of the targeting strategy. This is an important distinction as the initial founder line, BDNF-KIV, exhibited nonbiological interference of other promoters (Martinowich *et al*, 2011). Although BDNF-KIV is a useful tool for examining the impact of impaired activity-dependent BDNF protein production, the updated Bdnf-e4 line addresses the issue of nonspecific promoter interference so that disruption from promoter IV can be selectively evaluated. Biological cross-talk between promoters in our newly generated lines is consistent with results seen after functional loss of expression from *Bdnf* promoter IV; in mice carrying a genetic mutation rendering CREB unable to bind promoter IV, changes in the regulation of other *Bdnf* transcripts are also observed (Hong *et al*, 2008). Although it is possible that expression changes in non-targeted *Bdnf* transcripts may contribute to behavioral phenotypes (ie, downregulation of Ex1 transcripts in Bdnf-e2 mutants), our data suggest that transcription from alternative promoters changes based on age and experience, making it difficult to interpret the functional contribution of biological cross-talk between promoters. For example, Bdnf-e1 mutant mice show no changes in HYP or HPC expression of Ex 2c, 4, and 6 transcripts at P28 (Figure 2a and c); however, in adulthood (~15 weeks), Bdnf-e1 mutants exhibit Ex4 and 6 downregulation in HYP and Ex4 upregulation in HPC (Figure 3a–c). Furthermore, Bdnf-e2 mutants show downregulation of Ex1 transcripts in PFC at P28, but normal Ex1 expression in PFC by adulthood (Figure 2b and Supplementary Figure S3b). Although it cannot be ruled out that alternative regulation may have an impact on molecular and behavior phenotypes, enhanced aggression in Bdnf-e1 heterozygous animals, which do not show any regulation of other *Bdnf* transcripts, (Supplementary Figure S6), strengthen the finding that BDNF produced from promoter I regulates aggressive behavior. Future studies should explore the mechanism underlying this biological cross-talk and how it contributes to BDNF-dependent functions.

Although previous studies confirmed differential spatial and temporal expression of alternative *Bdnf* transcripts in the intact brain (Malkovska *et al*, 2006; Timmusk *et al*, 1994), individual contributions to total BDNF levels were not determined. Important *in vitro* work has shown that individual *Bdnf* exons are differentially translated (Koppel *et al*, 2015; Vaghi *et al*, 2014), but the contribution of individual *Bdnf* transcripts to total levels of BDNF protein *in vivo* and across different brain regions has not been directly tested due to a lack of tools to address this complex question. Here we demonstrate that promoters I, II, IV, and VI are differentially used in HYP, PFC, and HPC. Promoters I and II significantly contribute to total BDNF in adult HYP, but not in PFC and HPC (Figure 3 and Supplementary Figure S3), consistent with previous studies showing relatively higher *BDNF* Ex1 and 2 transcripts in HYP (Han *et al*, 2008). In contrast to promoters I and II, IV and VI contribute more substantially to PFC and HPC BDNF levels. Interestingly, the contribution of each transcript to total protein levels is not the expected one-to-one correlation, suggesting the existence of additional regulatory mechanisms at the level of translation that warrant further investigation. Indeed, *in vitro* studies demonstrate that translation of individual 5′-UTR-containing exons is differentially regulated by specific neurotransmitters and by BDNF itself (Vaghi *et al*, 2014). Furthermore, individual 5′-UTRs have different lengths and regulatory elements that can have an impact on their translatability (Koppel *et al*, 2015; Vaghi *et al*, 2014). Going forward, it will be critical to explore how these translational control mechanisms contribute to BDNF production from individual transcripts in different brain regions.

At the behavioral level, previous studies linked BDNF deficiency to elevated aggression. Mice heterozygous for the *Bdnf*-null allele show decreased attack latency and increased attack number (Lyons *et al*, 1999), and restricted forebrain BDNF knockout causes elevated aggression and heighted social dominance (Ito *et al*, 2011). Although both pre- and postnatal BDNF elimination elevates aggression, fetal loss causes a more severe phenotype (Chan *et al*, 2006). Our results demonstrate enhanced aggression following loss of BDNF from promoters I and II, but not IV and VI. A functional segregation of BDNF produced from promoters I/II and IV/VI is supported by previous studies demonstrating that Ex1/2 and Ex 4/6 transcripts are differentially regulated in the HPC during development (Nair *et al*, 2007; Sathanoori *et al*, 2004). Interestingly, disruption from promoters I and II prominently impaired BDNF expression in HYP, raising the possibility that BDNF produced from these promoters contributes to development of hypothalamic circuits that mediate aggression. This is consistent with previous studies demonstrating that Ex1 and Ex2 transcripts are highly expressed and regulated in the HYP (Han *et al*, 2008; Unger *et al*, 2007). Of all brain areas, HYP is the best-studied in relation to aggression and has been directly implicated in attack behavior (Lin *et al*, 2011; Woodworth, 1971). Although *Bdnf* deletion in ventromedial and dorsomedial HYP of adult mice does not cause elevated aggression (Unger *et al*, 2007), developmental loss of *Bdnf* or disruption in neuronal populations contributing to other HYP circuits may be required for BDNF-dependent aggression. Supporting the notion that individual *Bdnf* transcripts may be used by distinct neuronal populations to mediate specific behaviors is the observation that although Bdnf-e4

and -e6 mutants actually show greater total loss of BDNF protein in the brain, this is not translated to an increase in aggression. Hence, determining whether, and which, specific neuronal populations in HYP require BDNF from promoters I and II to govern social behavior is an important next step. It will also be important to determine the subcellular localization of individual *Bdnf* transcripts in different brain regions, cell types, and subcellular compartments *in vivo*. Previous studies in hippocampal cultures demonstrated spatial segregation of Ex1/4 transcripts in proximal dendrites and Ex2/6 transcripts in distal dendrites (Baj *et al*, 2011). It will be necessary to extend these findings to brain regions containing diverse cell types, such as the HYP, as this may represent another mechanism by which individual *Bdnf* transcripts may execute diverse functions within a single brain region or neuronal population.

At the molecular level, BDNF disruption from any promoter can affect 5-HT signaling components. Low 5-HT levels are associated with increased impulsivity and aggression, and manipulations that increase 5-HT activity are associated with reduced aggression (Takahashi *et al*, 2011). BDNF promotes 5-HT neuron development and function (Eaton and Whittemore, 1996; Mamounas *et al*, 1995), and BDNF deficiency is correlated with enhanced aggression and 5-HT dysfunction, including impaired 5-HT release, altered expression of 5-HT receptors, and deficits in 5-HT2A-mediated excitatory neurotransmission (Chan *et al*, 2006; Lyons *et al*, 1999; Rios *et al*, 2006). The link between BDNF, 5-HT, and aggression is strengthened by studies showing that the selective serotonin reuptake inhibitor fluoxetine ameliorates aggression in both BDNF heterozygotes and mice with deletion of CREB-regulated transcription coactivator 1 (Breuillaud *et al*, 2012; Lyons *et al*, 1999). Indeed, Bdnf-e1 and -e2 mutants show decreased 5-HT content in the PFC compared with WT (Supplementary Figure S7). Based on common dysregulation in aggressive Bdnf-e1 and -e2, but not Bdnf-e4 and -e6 mutants, our results identify 5-HTT and 5-HT2A as important in BDNF-dependent aggression. However, Bdnf-e4 and -e6 mutants also show 5-HT gene expression changes and a trend for reduced PFC 5-HT content independent of an aggression phenotype, suggesting that 5-HT impairments may be necessary but not sufficient for pathological aggression. As 5-HT has been proposed to set the threshold for triggers of aggression (Nelson and Trainor, 2007), one possibility is that increases in 5-HTT and 5-HT2A reduce 5-HT tone, thereby modulating impulsivity. However, 5-HT tone is probably altered in non-aggressive Bdnf-e4 and -e6 mice with 5-HT deficits, raising the possibility of additional mechanisms underlying BDNF-dependent aggression. Interestingly, loss of BDNF production from promoters I and II does not decrease GABA interneuron gene expression in PFC, suggesting that lack of inhibitory control may not be a dominant mechanism mediating BDNF-dependent aggression. Consistent with previous reports implicating activity-dependent promoter IV in GABAergic neurotransmission (Guilloux *et al*, 2012; Hong *et al*, 2008; Sakata *et al*, 2009; Tripp *et al*, 2012), we find suppression of GABA interneuron gene expression in Bdnf-e4 mutants. We also see decreases in GABA interneuron expression in Bdnf-e6 mutant PFC, suggesting that Ex6-containing transcripts may also contribute to BDNF-dependent maturation of cortical inhibition.

Together, the results provide insight into the complex program of *Bdnf* gene transcription and the link between BDNF, 5-HT signaling, and aggression. Importantly, the findings demonstrate functional significance for multiple transcripts encoding an identical BDNF protein and demonstrate the utility of these mice to dissect signaling pathways that have an impact on neural circuits mediating diverse BDNF functions. The findings underscore the importance of alternative *Bdnf* gene transcription and strongly support the notion that BDNF produced from unique promoters differentially has impacts on neurodevelopment, plasticity, and behavior.

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The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)