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Increased BDNF expression in fetal brain in the valproic acid model of autism

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Abstract

Human fetal exposure to valproic acid (VPA), a widely-used anti-epileptic and mood-stabilizing drug, leads to an increased incidence of behavioral and intellectual impairments including autism; VPA administration to pregnant rats and mice at gestational days 12.5 (E12.5) or E13.5 leads to autistic-like symptoms in the offspring and is widely used as an animal model for autism. We report here that this VPA administration protocol transiently increased both BDNF mRNA and BDNF protein levels 5–6-fold in the fetal mouse brain. VPA exposure *in utero* induced smaller increases in the expression of mRNA encoding the other neurotrophins, NT3 (2.5-fold) and NT4 (2-fold). Expression of the neurotrophin receptors, trkA, trkB and trkC were minimally affected, while levels of the low-affinity neurotrophin receptor, p75^{NTR}, doubled. Of the nine 5′-untranslated exons of the mouse BDNF gene, only expression of exons I, IV and VI was stimulated by VPA *in utero*. In light of the well-established role of BDNF in regulating neurogenesis and the laminar fate of postmitotic neurons in the developing cortex, an aberrant increase in BDNF expression in the fetal brain may contribute to VPA-induced cognitive disorders by altering brain development.

Keywords

Autism spectrum disorder; Brain-derived neurotrophic factor; Brain development; Gene promoters; Neurotrophin; VPA

Introduction

Valproic acid (VPA) is a widely used anti-epileptic and mood-stabilizing drug; however, exposure to VPA *in utero* can adversely affect fetal brain development. Children of women

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taking VPA during pregnancy have an increased risk of congenital malformations and impaired cognitive function including reduced IQ and autism (Williams *et al.*, 2001; Bromley *et al.*, 2008; Meador *et al.*, 2009; Ornoy *et al.*, 2009). The critical time window for the induction of autistic symptoms occurs during the first trimester in humans (Surén *et al.*, 2013) and gestational days 11 – 14 (E11 – 14) in rodents (Arndt *et al.*, 2005; Ploeger *et al.*, 2010), during which time the first neurons of the cerebral cortex are generated from proliferating neural progenitors and begin to differentiate into mature neurons. Consequently, a single administration of VPA to pregnant rats or mice during the second week of gestation has been extensively used as an animal model for autism (Rodier *et al.*, 1997; Arndt *et al.*, 2005; Murcia *et al.*, 2005; Schneider and Przewlocki, 2005; Markram *et al.*, 2007; Gogolla *et al.*, 2009; Gandal *et al.*, 2010; Rouillet *et al.*, 2010; Foley *et al.*, 2012; Chomiak and Hu, 2013; and see Patterson, 2011), with the offspring exhibiting increased cortical excitation, learning and memory deficits as well as abnormal fear conditioning and social interactions (reviewed in Markram *et al.*, 2007). Recently, mice exposed to VPA *in utero* were found to exhibit impaired communicative function, delayed auditory evoked potentials, and reduced γ -frequency phase locking factor, all of which are observed in autism (Gandal *et al.*, 2010). The mechanism by which fetal VPA exposure leads to these endophenotypes is not known.

Several lines of evidence suggest that autism is associated with increased expression of the neurotrophin, brain-derived neurotrophic factor (BDNF) (Nelson *et al.*, 2001; Miyazaki *et al.*, 2004; Tsai, 2005; Connolly *et al.*, 2006; Correia *et al.*, 2010). While BDNF is required for normal brain function throughout the lifespan, enhanced BDNF signaling can sometimes be pathogenic. For example, adult transgenic mice overexpressing BDNF are prone to seizures (Binder *et al.*, 2001) and elevation of BDNF enhances pain sensitivity (Merighi *et al.*, 2008). Moreover, during early embryonic brain development, the cell cycle parameters of proliferating neuroblasts and the laminar fate of their progeny are highly sensitive to either increased or decreased BDNF signaling (Fukumitsu *et al.*, 2006, Bartkowska *et al.*, 2007). Thus, BDNF signaling must be tightly regulated to enable normal function in both the developing and adult nervous system.

Based on these considerations, we investigated the possibility that aberrant BDNF signaling mediates the effects of VPA on the developing brain by measuring not only BDNF mRNA and protein but also mRNAs encoding the BDNF receptor, *trkB*, the other neurotrophins, nerve growth factor (NGF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4), as well as their receptors, using a VPA administration protocol that induces autistic behavior in mice and rats. Our results demonstrate that *in utero* VPA exposure increases expression of BDNF, and to a lesser extent, NT3 and NT4, in the fetal brain. Dysregulation of critical steps of prenatal cortical development occurring at the time of VPA exposure, which is predicted to result from increased BDNF signaling (Fukumitsu *et al.*, 2006, Bartkowska *et al.*, 2007), may contribute to postnatal behavioral and cognitive impairments.

Materials and methods

Animals

C57BL/6J mice were paired overnight; the day following the pairing was designated E0.5. Pregnant mice were injected at E12.5 with 400 mg/kg i.p. of VPA (sodium salt; Sigma-Aldrich, St. Louis, MO) in sterile phosphate-buffered saline (PBS) or PBS only. Administration of 600 mg/kg VPA resulted in an approximately 50% reduction in the number of viable fetuses 24 hr later (data not shown); consequently, the dose of VPA was limited to 400 mg/kg in this study. Fetal and maternal brains were removed 3, 6 or 24 hr after the injection.

All procedures using mice were carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the AVMA Guidelines on Euthanasia and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Whole fetal brains or 50 mg samples of maternal frontal cortex were agitated (600 Hz, 1 min) with 1 mm zirconia/silica beads (Biospec, Bartlesville, OK) in 1 ml of QIAzol lysis reagent (Qiagen, Germantown, MD). Total RNA was purified with RNeasy lipid tissue mini kit (Qiagen) including the on-column DNA digestion step. RNA (1 µg) was reverse-transcribed with Transcriptor High Fidelity (Roche, Indianapolis, IN). Primers were designed based on published genome sequences (NCBI, Gene database), except for the BDNF 5'-untranslated exons (I – IXa), which were obtained from Aid *et al.* (2007). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer sequences and qRT-PCR parameters are provided in Appendix, Table A1. Gene expression for each target mRNA was normalized to GAPDH mRNA assayed in the same sample as described by Pfaffl (2001). mRNA levels in VPA-treated brains are reported relative to those in brains from mice that had received a PBS injection (1 = no effect of VPA).

BDNF ELISA

Each sample analyzed consisted of four pooled fetal brains from one pregnancy or 50 mg of the maternal frontal cortex; four different samples (4 pregnancies) were analyzed for BDNF protein expression at 3, 6, and 24 hrs after VPA administration. Tissue was placed in 400 µl or 1 ml of freshly prepared lysis buffer (Szapacs *et al.*, 2004) containing 100 mM Pipes (pH 7.0), 500 mM NaCl, 0.2% Triton X-100, 0.1% NaN₃, 2 mM EDTA-Na₂•2H₂O, 200 µM PMSF, 10 µM leupeptin, 0.3 µM aprotinin and 1 µM pepstatin (all reagents from Sigma-Aldrich), homogenized as described above and stored at –80°C. Total protein was assayed by the BCA method (Pierce, Rockford, IL). BDNF was assayed in triplicate by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Promega, Madison, WI) omitting the optional acid pretreatment; bovine serum albumin (2%) was added to enhance sensitivity. Approximately 0.2 mg of fetal brain protein and 0.25 mg of maternal brain protein was loaded in each sample well.

Cell culture

Six fetal brains from one pregnancy obtained at E12.5 were placed in 1 ml of Liebowitz L15 medium without L-glutamine (Invitrogen, Carlsbad, CA), triturated with a narrow bore Pasteur pipette and suspended in Neurobasal medium supplemented with 2% B27, 1 mM l-glutamine and penicillin/streptomycin (Invitrogen). Cells were seeded at 3×10^5 cells/well in 24-well plates (0.5 ml/well) coated with poly-ornithine (Sigma-Aldrich) and laminin (Invitrogen). Two days after plating, cultures were treated for 7 hrs with VPA and lysed by addition of RNeasy lysis buffer (150 μ l/well). Lysates from three wells in each plate were combined and processed for total RNA using an RNeasy mini kit (Qiagen). Four different cultures, each from a different pregnancy were analyzed for each VPA concentration.

Statistical analysis

In Figures 1, 2, 4, and 5B, each bar represents a different PCR reaction showing the VPA-induced change in mRNA expression relative to the PBS-injected controls $\pm$ sem (1 = no effect of VPA); asterisks indicate expression levels significantly different from control by t-test. In Figures 3 and 5A, BDNF mRNA or protein expression is shown relative to controls $\pm$ sem (1 = no effect of VPA); asterisks indicate expression levels significantly different from control by one-way ANOVA with pairwise multiple comparison by Tukey's method. Unless otherwise stated, statistical analysis was performed using GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA).

Results

In utero VPA exposure stimulates BDNF expression in fetal brain

We investigated whether *in utero* exposure to VPA can modulate neurotrophin expression in the fetal brain. A single i.p. injection of VPA (400 mg/kg) to pregnant females induced a 5.6-fold increase in *BDNF* exon IX mRNA expression in the fetal brain as determined by qRT-PCR (Fig. 1A). Exon IX encodes the proBDNF protein, which is enzymatically processed to the mature, biologically active form subsequent to secretion. The BDNF primer pair used in Figure 1 (Table A1) amplified a sequence entirely within the mature BDNF coding sequence. Virtually identical results were obtained using a primer pair that amplified the N-terminal sequence, which is cleaved from proBDNF in the process of generating mature BDNF (data not shown). *BDNF* exon IX mRNA fell to baseline levels by 24 hr. Smaller (2–2.5-fold) transient increases in NT3 and NT4 expression were also observed in the fetal brain 3 hr after VPA administration, while NGF expression was unaffected at either time. Rapid metabolism of VPA in the mother (serum half-life in adult mouse $\approx$ 1 hr) (Nau and Löscher, 1982) may account for the transient nature of the increase in fetal neurotrophin mRNAs.

We also measured the expression of BDNF mRNA in the frontal cortex of the VPA-injected pregnant females. Surprisingly, BDNF mRNA expression in the maternal brain was not stimulated by VPA at either 3 or 24 hr (Fig 1B); however, expression of NT4, which, like BDNF, activates trkB, was increased 2.8-fold in the maternal brain at 3 hr after VPA administration. Thus, the profile of neurotrophin mRNA responses to administration of VPA at E12.5 is different in fetal brain and maternal frontal cortex (Fig. 1). We did not conduct a

regional study of BDNF mRNA expression in either the fetal or the maternal brain and it remains possible that VPA may induce BDNF expression in areas of the maternal brain other than the frontal cortex.

We also determined the effects of *in utero* VPA exposure on levels of mRNAs encoding the full-length, catalytically-active neurotrophin receptors, trkA, trkB, and trkC, the dominant-negative, truncated trkB isoforms, trkB.T1 and trkB.shc, as well as the low-affinity, non-specific neurotrophin receptor, p75^{NTR}, in the fetal brain (Fig. 2). At 3 hr, VPA exposure had induced only modest increases (<50%; trkA, trkB.FL) or decreases (<25%; trkB.shc; trkC) in the trk receptors; interestingly, p75^{NTR} expression had doubled. As p75^{NTR} mediates neurotrophin signaling independently of the trk receptors (Schor, 2005), enhanced p75^{NTR} signaling may also contribute to the effects of VPA on the fetal brain; however, the role of p75^{NTR} signaling in brain development has not been studied. By 24 hr, increases in neurotrophin receptor mRNA levels had returned to levels at or slightly below baseline (Fig. 2B).

VPA increases expression of BDNF protein in fetal brain

To determine if the up-regulation of BDNF mRNA by *in utero* VPA exposure (Fig. 1A) is accompanied by an increase in BDNF protein expression, we assayed total BDNF protein by ELISA in the fetal and maternal brains. There was no increase in BDNF levels in the fetal brains 3 hr after VPA administration but BDNF protein levels had increased 6-fold at 6 hr after VPA administration and returned to control levels by 24 hr (Fig. 3). Thus, the transient VPA-induced increase in BDNF mRNA precedes the corresponding rise in BDNF protein by about 3 hr in the fetal brain. As expected from the results of BDNF mRNA determination (Fig. 1B), VPA injection did not stimulate expression of BDNF protein in the maternal brain (Fig. 3).

5'-untranslated BDNF exons mediating the effects of VPA

BDNF expression is regulated by alternative splicing of one or more 5'-untranslated exons, each of which has its own unique promoter. In the mouse and rat, there are nine 5'-untranslated exons (I – IXa) plus a single common exon (IX) which encodes proBDNF protein (Aid *et al.*, 2007). To investigate the mechanism of VPA-induced BDNF mRNA expression, we used qRT-PCR to measure expression of the nine 5'-untranslated exons of the mouse *BDNF* gene in the same fetal brain samples used to analyze NGF, NT3 and NT4 expression in Fig. 1A. Whereas all nine exons were detected in adult mouse brain by qRT-PCR (not shown), only *BDNF* exon I, IV and VI expression was detected in the fetal brains. VPA stimulated *BDNF* exons I, IV and VI expression about 4-, 12.5- and 15-fold, respectively (Fig. 4). These data suggest that the VPA-induced increase in BDNF expression probably results from activation of *BDNF* exons I, IV and/or VI.

VPA stimulates BDNF mRNA expression in brain cell cultures

Because VPA is rapidly metabolized *in vivo* and some of its metabolites might be biologically active (Nau and Löscher, 1982), we investigated whether VPA induces BDNF mRNA expression in a cell culture system where there is no drug metabolism by the maternal liver. Cultures were prepared from E12.5 mouse brain; after 2 days *in vitro* (DIV)

cultures were stimulated with VPA (0.3 – 3 mM) for 7 hours. This concentration range was examined because it spans the circulating VPA levels of patients undergoing VPA treatment (Löscher, 1981). RNA was reverse-transcribed and analyzed by qRT-PCR. VPA stimulated expression of mRNA encoding the BDNF protein (exon IX) with a 2-fold increase observed at 3 mM, the highest concentration tested (Fig. 5A). We also assayed the expression of *BDNF* 5'-untranslated exons I – IXA in the cultures. Only exons I, II, IV and VI were detectable. VPA (3 mM) induced a 2 – 4-fold stimulation of *BDNF* exons I, IV and VI, while exon II was not affected (Fig. 5B). Thus, as was observed in the fetal brain *in vivo* (Fig. 4), VPA stimulates *BDNF* gene expression via exons I, IV and VI in fetal brain cultures.

Discussion

Regulation of fetal brain development by BDNF

The development of the fetal cortex is tightly regulated by BDNF signaling. For example, BDNF injected into mouse cerebral ventricles *in utero* led to precocious neurogenesis with a shorter S-phase, premature exit from the cell cycle of the proliferating neuroblasts and altered laminar fate of the postmitotic cortical neurons (Fukumitsu *et al.*, 2006). Abnormal precursor proliferation and cortical development was also observed by Bartkowska *et al.* (2007), who used an *in utero* electroporation approach to either increase or decrease BDNF signaling in the embryonic mouse brain. It should be noted that BDNF expression levels are normally low in the fetal brain and gradually increase to a maximum in the adult (Maisonpierre *et al.*, 1990), however, a rapid, transient increase in BDNF in the fetal brain is clearly abnormal. Thus, a transient VPA-induced increase in BDNF expression in the fetal brain of the magnitude we observed (Figs. 1a and 3) would be expected to both alter the proliferation and differentiation of neural progenitors and modify the laminar fate of their progeny, regardless of the levels achieved in the normal postnatal and adult brain. Such errors in neurogenesis may leave a “signature” that is not corrected during subsequent cortical development (Ben-Ari, 2008), thereby leading to permanent connectivity defects that could impair cognition and behavior in the postnatal animal.

Other modulators of brain development may be regulated by VPA

In this study, we focused on expression of the neurotrophins and their receptors and found that BDNF is the principal neurotrophin target of VPA in the fetal brain. However, BDNF is not the only potential modulator of brain development affected by VPA. For example, as revealed in Fig. 1A, NT3 and NT4 mRNA levels are also elevated, albeit to lesser extents than BDNF. NT3 acts at a different neurotrophin receptor (trkC) than do BDNF and NT4 (trkB), but little is known about its role in regulating fetal brain development. Thus, effects of VPA on the developing fetal brain could be due to the combined actions of elevated BDNF, NT3 and NT4. Further conclusions regarding NT3 and NT4 must await protein measurements as was done for BDNF (Fig. 3).

Factors other than neurotrophins may also mediate the effects of VPA on brain development. For example, mRNA encoding the homeotic gene, *Hoxa1*, is elevated in rat embryos after *in utero* VPA treatment (Stodgell *et al.*, 2006); increased *HoxA1* expression

has the potential to alter multiple developmental processes in the fetal brain, particularly during critical windows of vulnerability. Prenatal VPA exposure in mice has also been reported to reduce expression of the postsynaptic cell-adhesion molecule, neuroligin-3 (NLGN3), mutations in which have been reported in patients with autism (Kolozi *et al.*, 2009). Thus, exposure to VPA alters the expression of multiple genes encoding critical regulators of brain development.

Although the findings reported here and by others (Stodgell *et al.*, 2006; Kolozi *et al.*, 2009) suggest plausible mechanisms for the effects of *in utero* exposure to VPA on brain development, they do not, by themselves, demonstrate that altered expression of a single gene product causes the developmental defects. The degree to which altered expression of any one gene affects cortical neurogenesis and, consequently, cognition and behavior, could be studied using *in utero* viral-mediated siRNA gene silencing strategies (c.f., Bartkowska *et al.*, 2007) or, for BDNF, using transgenic mice with genetically modified trkB, which is sensitive to inhibition by systemically-administered pharmacological blockers (Chen *et al.*, 2005).

Possible mechanism of VPA-induced stimulation of BDNF expression

Because VPA is a histone deacetylase (HDAC) inhibitor, our results raise the possibility that stimulation of *BDNF* transcription may be due to increased acetylation of histones associated with the promoters of 5'-untranslated exons I, IV and/or VI (Fig. 4). Indeed, histone modifications at one or more of these *BDNF* promoters have been reported in adult animal models of epilepsy and depression (Huang *et al.*, 2002; Tsankova *et al.*, 2006) and stimulation of *BDNF* exon IV expression by VPA in neuron cultures has been attributed to HDAC inhibition (Yasuda *et al.*, 2009). Our finding that expression of exons I, IV and VI is stimulated in fetal brain by administration of VPA to the pregnant dam (Fig. 4) and by direct application of VPA to fetal brain cultures (Fig. 5B), raises the possibility that epigenetic modulation of these exons is also important for normal and pathological brain development. Use of fetal brain cultures (Fig. 5), in which responses to VPA were observed to be similar to those in fetal brain *in vivo* (Fig. 4), may facilitate determination of the extent to which the promoters of exons I, IV and VI are modulated histone acetylation.

Genes versus environment

Recent research has linked genetic variants of a number of genes to autism and several of those genes are known to play a role in either brain development or synaptic function (Abrahams and Geschwind, 2008; State and Levitt, 2011; Berg and Geschwind, 2012). Those findings are not incompatible with studies demonstrating that environmental influences such as *in utero* VPA exposure can also lead to autistic behavior. Indeed, as has been previously suggested (Arndt *et al.*, 2005), a combination of genetic and environmental influences may act synergistically to produce autism as has recently been discussed for schizophrenia (Lipina *et al.* 2013; Kannan *et al.* 2013), affective disorders (Renoir *et al.* 2013) and pre-term births (Cha *et al.* 2013). The results of the present study and others (Stodgell *et al.*, 2006; Kolozi *et al.*, 2009) demonstrate that environmental stressors such as VPA can alter the expression of genes that are critical for normal brain development. Such

stressors may be more likely to induce autistic symptoms on a background of inherited genetic abnormalities.

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Abbreviations

BDNF	brain-derived neurotrophic factor
ELISA	enzyme-linked immunosorbent assay
NGF	nerve growth factor
NT3	neurotrophin-3
NT4	neurotrophin-4
trkB	tropomyosin-related kinase B
VPA	valproic acid

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Appendix

Table A.1

List of primers used in qRT-PCR experiments. All primers were designed using the NCBI web site, except for primers amplifying *BDNF* exons, which were obtained from Aid *et al.*, (2007). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The efficiency (Eff) for each primer pair is also indicated.

<i>BDNF</i> Exon IX (coding region, mature protein) (Eff = 2.0)	
Forward	AAAGTCCCGGTATCCAAAGGCCAA
Reverse	TAGTTCGGCATTGCGAGTTCCAGT
<i>BDNF</i> All 5' non-coding exons	

Reverse GAAGTGACAGTCCGCGTCCTTA
BDNF Exon I (Eff = 2.1)
 Forward GTGTGACCTGAGCAGTGGGCAAAGGA
BDNF Exon II (Eff = 2.0)
 Forward GGAAGTGGAAGAAACCGTCTAGAGCA
BDNF Exon III (Eff = 2.0)
 Forward GCTTCTATCATCCCTCCCCGAGAGT
BDNF Exon IV (Eff = 2.0)
 Forward CTCTGCCTAGATCAAATGGAGCTTC
BDNF Exon V (Eff = 1.9)
 Forward CTCTGTGTAGTTTCATTGTGTGTTC
BDNF Exon VI (Eff = 1.8)
 Forward GCTGGCTGTCGCACGGTTCCTT
BDNF Exon VII (Eff = 2.1)
 Forward CCTGAAAGGGTCTGCGGAAGTCCA
BDNF Exon VIII (Eff = 1.9)
 Forward GTGTGTGTCTCTGCGCTCAGTGGA
BDNF Exon IXa (Eff = 2.0)
 Forward CCCAAAGCTGCTAAAGCGGGAGGAAG
NGF (Eff = 2.0)
 Forward: CCAAGGACGCAGCTTTCTATAC
 Reverse: CTGCCTGTACGCCGATCAAAA
NT3 (Eff = 2.0)
 Forward: GGAGTTTGCCGGAAGACTCTC
 Reverse: GGGTGCTCTGGTAATTTTCCTTA
NT4 (Eff = 1.9)
 Forward: TGAGCTGGCAGTATGCGAC
 Reverse: CAGCGCGTCTCGAAGAAGT
trkA (Eff = 2.2)
 Forward CCTTCCGTTTCACCCCTCGGC
 Reverse GGTCAGGTCCTGTAGGGAGAGGC
trkB.FL (Eff = 2.0)
 Forward TTTCTTGCCGAGTGCTACAACCT
 Reverse TGAAAGTCCTTGCCTGCATGTCTG
trkB.T1 (Eff = 1.9)
 Forward ATAAGATCCCACTGGATGGG
 Reverse CGTATAGTCAAACAGCTCGC
trkB.shc (Eff = 2.0)
 Forward GGAATGACCAAGATTCTGTATTGAA
 Reverse GAACCTCTGGGCCATGTGTCT
trkC (Eff = 2.0)
 Forward CTGAGTGCTACAATCTAAGCCC
 Reverse CACACCCCATAGAACTTGACAAT

p75^{NTR} (Eff = 1.9)

Forward CTAGGGGTGTCCTTTGGAGGT

Reverse CAGGGTTCACACACGGTCT

GAPDH (EFF = 1.9)

Forward TGATGACATCAAGAAGGTGGTGAAG

Reverse TCCTTGGAGGCCATGTAGGCCAT

Fig. 1. VPA injection induced a transient increase in BDNF mRNA in the E12.5 fetal brain but not in the maternal brain

Pregnant mice received VPA (400 mg/kg, i.p.) at E12.5. 3 or 24 hrs later, RNA was isolated from both whole fetal brain and maternal frontal cortex and reverse-transcribed for analysis of neurotrophin (and neurotrophin receptor) mRNA levels by qRT-PCR. mRNA levels in VPA-treated brains are reported relative to those in brains from mice that had received a PBS injection (1 = no effect of VPA). **A.** Relative BDNF levels in fetal brain increased 5.6-fold at 3 hrs and returned to control levels by 24 hr. NT3 and NT4 mRNA levels were also transiently increased 2.5- and 2-fold. For BDNF, 7 control (3 pregnancies) and 14 VPA-treated (3) fetal brains were analyzed at 3 hr; 7 control (3) and 10 VPA-treated (3) fetal brains were analyzed at 24 hr. For NT3, NT4 and NGF, 18 control (4) and 17 VPA-treated (4) fetal brains were analyzed at 3 hr; 13 control (3) and 14 VPA-treated (3) fetal brains were analyzed at 24 hr. ***, significantly different from PBS-injected control, $p < .001$. **B.** VPA exposure at E12.5 had no effect on neurotrophin mRNA levels in the maternal brain at either time except for NT4, which was increased about 2.5-fold at 3 hr. For BDNF, 3 control and 3 VPA-treated maternal brains were analyzed at 3 hr and at 24 hr. For NT3, NT4 and NGF, 4 control and 4 VPA-treated maternal brains were analyzed at 3 hr and 3 control and 3 VPA-treated maternal brains were analyzed at 24 hr. ***, significantly different from control, $p < .001$.

Figure 2. Effects of VPA on expression of neurotrophin receptors in E12.5 brain

Data are expressed relative to the mRNA level in control (PBS treated) brains (1 = no effect of VPA). Same fetal brain samples that were analyzed for NGF/NT3/NT4 in Fig. 1. **A.** 3-hr treatment with 400 mg VPA/kg increased the expression of mRNAs encoding trkA ($\approx 50\%$), trkB.FL ($\approx 25\%$) and p75^{NTR} ($\approx 100\%$), while an approximately 20% decrease was observed for trkB.shc and trkC mRNAs; trkB.T1 mRNA expression was unchanged. Significantly different from control: *** = $p < .001$ and * = $p < .05$.

B. Neurotrophin receptor mRNA expression in fetal brain 24 hrs after a single *in utero* VPA exposure (400 mg/kg) at E12.5. Levels of trkA, trkB.FL, and trkB.T1 mRNA were unaffected 24 hrs after VPA exposure, while trkB.shc, trkC and p75^{NTR} mRNA levels remained suppressed by 20 – 25%. Significantly different from control: ** = $p < .01$ and * = $p < .05$.

Fig. 3. VPA injection induced a transient increase in BDNF protein in the E12.5 fetal brain but not in the maternal brain

Pregnant mice received PBS (control) or VPA (400 mg/kg, i.p.) at E12.5. 3, 6 or 24 hrs later, fetal and maternal brains were removed and BDNF protein expression was quantified by ELISA. Each sample analyzed consisted of 4 pooled fetal brains from one pregnancy. Four separate samples (4 pregnancies) plus a sample of each maternal frontal cortex were analyzed $\pm$ VPA at each time point; i.e., a total of 16 fetuses and 4 dams were analyzed $\pm$ VPA at each time point. BDNF protein levels in VPA-treated brains are reported relative to those in brains from mice that had received a PBS injection (1 = no effect of VPA). **Left.** BDNF levels had increased 6-fold in fetal brains by 6 hr after VPA treatment following a delay of at least 3 hr and returned to slightly below unstimulated levels by 24 hr. Note that the level of BDNF mRNA was elevated 3 hr following VPA administration (Fig. 1). **Right.** Consistent with BDNF mRNA levels, there was no increase in BDNF protein expression in maternal brains following VPA injection (right). BDNF protein levels in fetal and maternal brains exposed to PBS were 11.7 ± 3.3 and 24.1 ± 2.8 pg/mg total protein, respectively. Data were analyzed by ANOVA; $F_{(2,9)} = 200$ ($p < .001$) for fetal brain and $F_{(2,9)} = 22$ ($p < .001$) for maternal brain. ***, significantly different from PBS-injected control, $p < .001$. Statistical analysis was performed with SigmaPlot v.12.3 (Systat Software, Chicago, IL).

Fig. 4. VPA injection stimulated expression of *BDNF* 5'-untranslated exons I, IV and VI in fetal brain

Pregnant mice received VPA (400 mg/kg, i.p.) at E12.5. 3 hrs later, RNA was isolated from fetal brains and reverse transcribed for analysis of *BDNF* 5'-untranslated exons I – IXa mRNA expression by qRT-PCR. Only exon I-, IV- and VI-specific mRNAs were detected in fetal brain following VPA administration and expression of those 5'-untranslated exons was stimulated about 4-, 13- and 15-fold, respectively. Same fetal brain samples analyzed for NT3, NT4 and NGF in Fig. 1. ***, significantly different from PBS-injected control, $p < .001$, $n = 14$.

Fig. 5. VPA stimulated expression of BDNF mRNA (coding region, exon IX) (A) and 5'-untranslated exons I, II, IV and VI (B) in cultures of E12.5 fetal mouse brain

Brain cell cultures were prepared from fetal brain at E12.5 as described in Materials and Methods. Two days after plating, fetal mouse brain cultures were treated for 6 hr with 0.3, 1.0 or 3.0 mM (A) or 3 mM VPA (B) and then harvested for determination of BDNF coding region (exon IX) mRNA (A) or 5'-untranslated exon I, II, IV and VI mRNA (B) by qRT-PCR. **A.** Four independent cultures were analyzed for each VPA concentration; all three concentrations of VPA significantly stimulated exon IX mRNA expression. Data were analyzed by ANOVA: $F_{(3,12)} = 50$ ($p < .001$). Statistical analysis was performed using SigmaPlot v.12.3 (Systat Software, Chicago, IL). ***, significantly different from untreated cultures ($p < .001$); **, significantly different from untreated cultures ($p < .02$); #, significantly different from 0.3 μM VPA ($p < .01$). **B.** Only exon I, II, IV and VI mRNAs were detected and of these only exons I, IV and VI mRNAs were stimulated by VPA. Four control and 4 VPA stimulated cultures were analyzed for each *BDNF* 5' untranslated exon. ***, significantly different from untreated cultures.