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Dysregulation of TrkB phosphorylation and proBDNF protein in adenylyl cyclase 1 and 8 knockout mice in a model of fetal alcohol spectrum disorder

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) mediates neuron growth and is regulated by adenylyl cyclases (ACs). Mice lacking AC1/8 (DKO) have a basal reduction in the dendritic complexity of medium spiny neurons in the caudate putamen and demonstrate increased neurotoxicity in the striatum following acute neonatal ethanol exposure compared to wild type (WT) controls, suggesting a compromise in BDNF regulation under varying conditions. Although neonatal ethanol exposure can negatively impact BDNF expression, little is known about the effect on BDNF receptor activation and its downstream signaling, including Akt activation, an established neuroprotective pathway. Therefore, here we determined the effects of AC1/8 deletion and neonatal ethanol administration on BDNF and proBDNF protein expression, and activation of tropomyosin-related kinase B (TrkB), Akt, ERK1/2, and PLCγ. WT and DKO mice were treated with a single dose of 2.5 g/kg ethanol or saline at postnatal days 5-7 to model late-gestational alcohol exposure. Striatal and cortical tissues were analyzed using a BDNF enzyme-linked immunosorbent assay or immunoblotting for proBDNF, phosphorylated and total TrkB, Akt, ERK1/2, and PLCγ1. Neither postnatal ethanol exposure nor AC1/8 deletion affected total BDNF protein expression at any time point in either region examined. Neonatal ethanol increased the expression of proBDNF protein in the striatum of WT mice 6, 24, and 48 h after exposure, with DKO mice demonstrating a reduction in proBDNF expression 6 h after exposure. Six and 24 h after ethanol administration, phosphorylation of full-length TrkB in the striatum was significantly reduced in WT mice, but was significantly increased in DKO mice only at 24 h. Interestingly, 48 h after ethanol, both WT and DKO mice demonstrated a reduction in phosphorylated full-length TrkB. In addition, Akt and PLCγ1 phosphorylation was also decreased in ethanol-treated DKO mice 48 h after injection. These data demonstrate dysregulation of a potential survival pathway in the AC1/8 knockout mice following early-life ethanol exposure.

Introduction

Prenatal ethanol exposure can result in growth retardation, facial dysmorphology, and cognitive and behavioral impairments, a condition known as fetal alcohol spectrum disorders because of the broad range of severity (Bertrand, Floyd, & Weber, 2005; Riley & McGee, 2005; Sokol, Delaney-Black, & Nordstrom, 2003). While there are multiple factors that modulate this severity, including nutritional status, genetics, and amount of alcohol consumed, the timing of exposure to ethanol greatly influences the sensitivity of various regions of the brain to ethanol (Guerri, Bazinet, & Riley, 2009; Hamre & West, 1993; Jones, 2011; Kleiber, Mantha, Stringer, & Singh, 2013; Maier & West, 2001; Thomas, Goodlett, & West, 1998; Tran, Cronise, Marino, Jenkins, & Kelly, 2000; West, Chen, &

Abbreviations: AC, adenylyl cyclase; Akt, protein kinase B; AU, arbitrary units; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; DKO, double knockout; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; G, gestational day; P, postnatal day; proBDNF, pro-brain-derived neurotrophic factor; p-Akt, phosphorylated Akt; p-PLCγ1, phosphorylated PLCγ1; p-TrkB, phosphorylated TrkB; PLCγ1, phospholipase Cγ1; TBST, Tris-buffered saline with Tween 20; TrkB, tropomyosin-related kinase B; WT, wild type.

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Pantazis, 1994). Previous studies have demonstrated profound ethanol-induced neuronal cell loss in various regions of the brain at unique stages during development (Heaton, Mitchell, & Paiva, 1999; Ikonomidou et al., 2000). In line with these observations, expression of the mature form of brain-derived neurotrophic factor (BDNF) protein has been demonstrated to be elevated at time points when specific brain regions are less sensitive to the deleterious effects of ethanol, such as the cortex at postnatal day (P) 21 and the striatum at P14 (Heaton, Paiva, Madorsky, Mayer, & Moore, 2003). BDNF protein levels have also been shown to be increased in the hippocampus after neonatal ethanol exposure (Heaton, Mitchell, Paiva, & Walker, 2000), suggesting a protective role for BDNF (Lindvall, Kokaia, Bengzon, Elmér, & Kokaia, 1994) in addition to its role in both synaptic plasticity (Chapleau, Larimore, Theibert, & Pozzo-Miller, 2009) and dendritic development via its receptor, tropomyosin-related kinase B (TrkB), a member of the receptor tyrosine kinase family (Mooney & Miller, 2011; Yacoubian & Lo, 2000). Interestingly, BDNF mRNA does not appear to be present in the striatum of embryonic or neonatal mice (Baquet, Gorski, & Jones, 2004). The BDNF protein found in the striatum results from anterograde transport primarily from the cortex (Altar et al., 1997; Baquet et al., 2004).

In addition to providing BDNF, the cortex is also one of the main sources of afferent activity for the striatum. During the brain growth spurt period, afferent innervation is particularly important for dendritic growth (Cline, 2001). Loss of neuronal activity during this period can lead to lasting deficiencies in dendritic morphology. Afferent activity leads to an elevation in intracellular calcium, which leads to changes in dendritic morphology (reviewed in Redmond & Ghosh, 2005; Wong & Ghosh, 2002). Activity has also been shown to be required for BDNF-mediated dendritic growth (McAllister, Katz, & Lo, 1996). Taken together with previous studies documenting that the cortex demonstrates neurodegeneration 12-24 h after early-life ethanol exposure, (Heaton, Paiva, Madorsky, & Shaw, 2003; Maas et al., 2005) as well as long-term reductions in dendritic morphology of cortical and medium spiny neurons lasting into adulthood (Hamilton, Whitcher, & Klintsova, 2010; Rice et al., 2012; Susick, Lowing, Provenzano, Hildebrandt, & Conti, 2014), we aimed to determine the effect of neonatal ethanol exposure on the expression of BDNF and activation of TrkB proteins in the striatum.

We have previously demonstrated a reduction in dendritic morphology in mice lacking the calcium/calmodulin stimulated adenylyl cyclases (ACs) 1 and 8 (DKO) compared to wild-type (WT) controls (Susick, Lowing, Provenzano, et al., 2014). In addition, DKO mice have an increased sensitivity to the neurodegeneration caused by neonatal ethanol exposure that is mediated by BDNF-related pathways, including Akt (Conti, Young, Olney, & Muglia, 2009; Maas et al., 2005). AC activity has also been associated with the expression of BDNF in a number of studies. For example, administration of a single dose of NKH477, a forskolin derivative, or forskolin to stimulate AC activity, increased BDNF mRNA expression in the cortex and hippocampus of rats (Morinobu, Fujimaki, Okuyama, Takahashi, & Duman, 1999) and in striatal slices in culture (Goggi, Pullar, Carney, & Bradford, 2003), respectively. Similarly, AC inhibition using SQ22536 decreased the basal expression of BDNF mRNA in rat astrocyte cultures (Caruso et al., 2012). In addition, forskolin can also induce an increase in TrkB mRNA in cortical neuron cultures (Deogracias, Espliguero, Iglesias, & Rodríguez-Peña, 2004). As noted, one pathway through which BDNF/TrkB can regulate neuronal survival and growth is the Akt pathway (Chao, 2003; Minichiello, 2009). Previous work has demonstrated a reduction in the phosphorylation of Akt in the cerebral cortex at P7, P14, and P21 after ethanol exposure throughout gestation (Climent, Pascual, Renau-Piqueras, & Guerri, 2002), 1 h after 3.5 and 4.5 g/kg ethanol (Chandler & Sutton, 2005), and 45 min or 3 h after 5.0 g/kg ethanol from P6-8 (Fattori, Abe, Kobayashi, Costa, & Tsuji, 2008; Tsuji, Fattori, Abe, Costa, & Kobayashi, 2008). Similarly, Akt phosphorylation has been shown to be decreased in the hippocampus 1 h after 3.5 or 4.5 g/kg ethanol at P5 (Chandler & Sutton, 2005), in the cerebellum at P2 after gestational ethanol exposure, or 2 h after ethanol inhalation at P4 and P7 (Heaton, Moore, et al., 2003; Xu et al., 2003), and in the striatum 2 h and 3 h after neonatal ethanol exposure (Young, Straiko, Johnson, Creeley, & Olney, 2008), with genetic deletion of AC1/8 amplifying this acute effect (Conti et al., 2009). However, the long-term impact of neonatal ethanol on this pathway has not been examined. Therefore, we aim to determine the involvement of AC1/8 in the regulation of BDNF protein levels in the striatum and cortex, as well as the activation of the BDNF receptor, TrkB, and the downstream signaling proteins, Akt, extracellular signal-related kinase (ERK) 1/2 and phospholipase C (PLCɣ1) at various time points after ethanol exposure.

Materials and methods

Animals

All mice were backcrossed a minimum of 10 generations to WT C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME). The progeny of both homozygous DKO mutant and WT mice were bred in our colony for the present studies. Mice were maintained on a 12-h light/dark schedule with *ad libitum* access to food and water. All experiments were performed using male mice 5-7 days after birth weighing 2.5-3.0 g (Conti et al., 2009; Maas et al., 2005; Susick, Lowing, Bosse, et al., 2014; Susick, Lowing, Provenzano, et al., 2014; Young & Olney, 2006). Only litters with an even number of male pups in this weight range were used to ensure a littermate was available for use as a saline-treated control. Only 2 pups from a litter, one saline-exposed and one ethanol-exposed, were assigned to a time point, i.e., if 4 males from a single litter were available for treatment, they were used for two different time points. Pups were randomly assigned to either saline- or ethanol-treatment groups and any pups that did not survive to the selected time point were excluded from analysis. Group sizes for the experiments were calculated based on a power analysis using estimates of effect size and variance obtained from preliminary data conducted in the Conti laboratory and pertinent published literature for comparable studies in which desired effect sizes were shown to be significant. A minimum of 3 animals per genotype per treatment for immunoblotting and BDNF ELISA was determined to be required to achieve a sample size for >88% power (minimum power function = 0.88 and $a = 0.05$) with significance set at $p \leq 0.05$. All mouse protocols were in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Wayne State University. Animals were assigned a numerical code at the time of treatment; however, samples were decoded prior to loading samples into the ELISA plates or electrophoresis gels.

Ethanol treatment

WT and DKO pups were injected subcutaneously (Conti et al., 2009; Maas et al., 2005; Susick, Lowing, Bosse, et al., 2014; Susick, Lowing, Provenzano, et al., 2014; Young & Olney, 2006) with a single dose of ethanol (2.5 g/kg) prepared as a 20% solution using 100% ethanol (Decon Laboratories, King of Prussia, PA) in normal saline or corresponding volumes of saline as controls. This single dose of ethanol during P5-P7 represents maternal exposure to alcohol during the third trimester in humans. This treatment results