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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.

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### Keywords

Fetal alcohol spectrum disorder; Brain-derived neurotrophic factor; Tropomyosin-related kinase B; Akt; Striatum

## 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, & 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

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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 (PLCγ1) 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

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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 $p$ 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 in blood alcohol concentrations 15 min after injection of 276 ± 5 mg/dL and 262 ± 9 mg/dL in WT and DKO pups, respectively (Susick et al., 2014b). All experimental pups were placed on a heating pad at 31 °C (Conti et al., 2009; Maas et al., 2005; Susick, Lowing, Bosse, et al., 2014; Susick, Lowing, Provenzano, et al., 2014; Young & Olney, 2006) away from dams until the ethanol pups regained consciousness, at which time all pups were returned to their dams. Time away from dams was limited to less than 3 h to minimize maternal separation effects. Pups were euthanized 2, 4, 6, 24, or 48 h after injection or at P14, P21, or P30, and brains were dissected.

### BDNF ELISA

Striatal and cortical tissues were rapidly dissected and snapfrozen in liquid nitrogen for analysis by enzyme-linked immunosorbent assay (ELISA). Samples were homogenized in lysis buffer (100 mM PIPES [pH 7], 500 mM NaCl, 0.2% Triton X-100, 0.1% $NaN_3$, 2% BSA, 2 mM EDTA $Na_2$-$2H_2O$, 200 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 0.3 μM aprotinin [pH 8] and 1 μM pepstatin) and centrifuged (16,000 × *g*) for 30 min at 4 °C. Following centrifugation, the supernatant was isolated and stored at −80 °C. BDNF protein was quantified using an ELISA (Promega $E_{max}$ Immunoassay; Madison, WI) according to kit instructions and as detailed in (Bosse & Mathews, 2011; Szapacs et al., 2004). Samples and standards were run in triplicate in Greiner Bio-One High Binding Flat Bottom 96-well microplates. Plates were read at 450 nm on a Synergy HT plate reader (BioTek, Winooski, VT). Replicates were averaged together and normalized to total protein measured using the

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Pierce 660 nm protein assay (Thermo Fisher; Rockford, IL) with bovine serum albumin (BSA) as the standard. Pups from at least 4 separate litters were treated, with a total of 4–8 pups per genotype per condition. Normalized values (pg BDNF/mg protein) were used for statistical analysis.

## Immunoblotting

Whole-cell extracts from striatal tissues were prepared by homogenization in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol with protease and phosphatase inhibitors) and centrifuged (10,000 × *g*) for 10 min at 4 °C. Protein (10 μg) was separated on a 4–12% Bis-Tris gel using MOPS running buffer (Invitrogen, Grand Island, NY). Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus with NUPAGE transfer buffer at 15 V for 40 min (Invitrogen, Grand Island, NY) and exposed to antibodies against proBDNF (OSB00016G; Pierce, Rockford, IL), phosphorylated TrkB (p-TrkB; p-Tyr 705; SAB4300256; Sigma-Aldrich St. Louis, MO), TrkB (GT15080; Neuromics, Edina, MN), phosphorylated Akt (p-Akt; p-Ser 473; 9271; Cell Signaling, Danvers, MA), Akt (9272; Cell Signaling, Danvers, MA), phosphorylated ERK1/2 (p-ERK1/2 p-Thr202/Tyr204; 4377; Cell Signaling, Danvers, MA), ERK1/2 (4698; Cell Signaling, Danvers, MA), phosphorylated PLCγ1 (p-Tyr 783; 14008; Cell Signaling, Danvers, MA), PLCγ1 (2822; Cell Signaling, Danvers, MA) or Actin (A5060; Sigma-Aldrich) at 1:1000 (p-TrkB, p-Akt, and p-PLCγ1), 1:2000 (p-ERK1/2 and PLCγ1), 1:4000 (TrkB, Akt, and ERK1/2), 1:5000 (proBDNF and Actin) in blocking buffer (5% BSA [p-TrkB, p-Akt, Akt] or 5% dry milk [proBDNF, TrkB, p-PLCγ1, PLCγ1, and Actin]) in Tris-buffered saline with 0.1% Tween 20 [TBST] overnight at 4 °C. Membranes were incubated in secondary antibodies against rabbit (1:1000; p-TrkB and p-PLCγ1, 1:4000; p-Akt and Actin, 1:5000; proBDNF, Akt, p-ERK1/2, ERK1/2, and PLCγ1) or goat (1:4000; TrkB) for 1 h following washing in TBST. Immunoblot signals of p-TrkB, full-length TrkB, truncated TrkB, p-Akt, Akt, p-ERK1/2, ERK1/2, PLCγ1, and Actin were detected using SuperSignal West Dura Chemiluminescent substrate (ThermoScientific, Rockford, IL), p-PLCγ1 was detected using SuperSignal West Femto Chemiluminescent substrate (ThermoScientific, Rockford, IL), and all signals were quantified using densiometric analysis with Image J software (NIH; (Conti et al., 2007)) and are presented as a ratio of proBDNF to Actin, phosphorylated full-length TrkB to total full-length TrkB, p-Akt to total Akt, p-ERK1 to total ERK1, p-ERK2 to total ERK2, and p-PLCγ1 to total PLCγ1. Three to four animals from at least 3 different litters were treated with ethanol for each genotype, with littermates used for saline controls.

## Statistical analysis

SigmaPlot 12 was used to perform independent two-way ANOVAs for each time point with Genotype and Treatment as the between-subjects factors for immunoblot data of proBDNF/Actin, p-FL-TrkB/FL-TrkB, p-FL-TrkB/Actin, FL-TrkB/Actin, p-T-TrkB/T-TrkB, TTrkB/Actin, p-Akt/Akt, p-Akt/Actin, Akt/Actin, p-ERK1/ERK1, p-ERK1/Actin, ERK1/Actin, p-ERK2/ERK2, p-ERK2/Actin, ERK2/Actin, p-PLCγ1/PLCγ1, p-PLCγ1/Actin, and PLCγ1/Actin. Statistica 6.0 was used to perform three-way ANOVAs for striatal and cortical BDNF measurements with Time, Genotype, and Treatment as the between-subjects factors. Tukey *post hoc* tests were performed where appropriate.

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# Results

## Brain-derived neurotrophic factor

The established involvement of BDNF in the regulation of dendritic development (Baker, Stanford, Brown, & Hagg, 2005; Chapleau et al., 2009; McAllister et al., 1996; Numakawa et al., 2010) suggests that the previously observed effects of ethanol on dendritic morphology at P30 (Susick, Lowing, Provenzano, et al., 2014) may act via dysregulation of the BDNF pathway. However, a three-way ANOVA failed to demonstrate a significant effect of Genotype, Treatment, an interaction of Genotype × Treatment, or any interactions with Time for BDNF protein expression in the striatum (Fig. 1A) of ethanol-treated WT or DKO mice compared to respective saline controls as measured using a BDNF ELISA. Likewise, postnatal ethanol exposure or AC1/8 deletion at P5–P7 did not alter levels of BDNF protein expression in the cortex (Fig. 1B) at any time point in either genotype. Effects of Time were demonstrated for both brain regions with *post hoc* analyses revealing a significant increase in BDNF at P14, P21, and P30 compared to 2, 4, 6, 24, and 48 h in both brain regions (effect of Time; striatum [$F(7,138) = 93.49$, $p < 0.001$], cortex [$F(7,170) = 193.46$, $p < 0.001$]).

The mature BDNF protein is transcribed as proBDNF which, when cleaved by a convertase into the mature form, activates TrkB. The BDNF ELISA used here is not capable of differentiating between the pro- and mature forms of BDNF; therefore, immunoblottingwas used to determine the expression patterns of proBDNF using whole-cell extracts from the striatum of WT and DKO mice. ProBDNF protein expression following neonatal ethanol exposure was normalized to Actin and was analyzed using two-way ANOVAs for each time point. No significant effects of either genotype or ethanol exposure were found at the 2- or 4-h time points (Table 1). However, significant main effects of Genotype and Treatment were found at 24 h and significant interactions between Genotype and Treatment were found for proBDNF protein expression 6 and 48 h after ethanol exposure (24-h Genotype [$F(1,11) = 12.95$, $p = 0.007$], Treatment [$F(1,11) = 11.84$, $p = 0.009$]; 6-h interaction [$F(1,11) = 13.07$, $p = 0.007$]; 48-h interaction [$F(1,11) = 10.58$, $p = 0.012$]). *Post hoc* analysis revealed a significantly higher level of proBDNF in saline-treated DKO mice compared to saline-treated WT mice only at the 6- and 24-h time points (Fig. 2), as well as an increase in proBDNF expression in ethanol-exposed WT mice at 24 and 48 h. Interestingly, ethanol-exposed DKO mice demonstrate a significant decrease in proBDNF expression 6 h after injection (Fig. 2). The expression of proBDNF was also significantly lower in ethanol-treated DKO mice compared to ethanol-exposed WT mice at 6 and 48 h. No significant effects of Genotype, Treatment, or interaction between Genotype and Treatment on proBDNF protein levels were found for any other time points (Table 1).

## Phosphorylation of TrkB

Currently, little is known about the influence of ethanol on the activation of TrkB, the BDNF receptor. Therefore, using immunoblot analysis, phosphorylated and total full-length (FL-TrkB) and truncated (T-TrkB) TrkB protein expressions were quantified using whole-cell extracts from striatum of the WT and DKO mice used for the BDNF ELISAs. No significant effects of Genotype or Treatment were demonstrated at 2 or 4 h after neonatal ethanol exposure (Table 1). However, a significant main effect of Treatment was found at 6 and 48 h,

while a significant main effect of Genotype was found at 24 and 48 h (6-h Treatment [$F(1,12) = 7.40$, $p = 0.026$]; 48-h Treatment [$F(1,12) = 6.72$, $p = 0.029$]; 24-h Genotype [$F(1,11) = 40.88$, $p < 0.001$]; 48-h Genotype [$F(1,12) = 10.63$, $p = 0.010$]). A Genotype × Treatment interaction was also found at the 24-h time point [$F(1,11) = 29.45$, $p < 0.001$]. *Post hoc* analysis revealed a significant decrease in phosphorylated TrkB in ethanol-treated WT pups compared to saline-treated controls at all 3 time points (6, 24, and 48 h). In DKO mice, no significant effect of ethanol was found 6 h after exposure; however, a significant increase was shown at the 24-h time point followed by a non-significant decrease in ethanol-exposed pups by 48 h, compared to saline-treated DKO mice (Fig. 3). Interestingly, saline-treated DKO pups exhibited a reduction in phosphorylated TrkB compared to saline-treated WT pups at the 48-h time point, which was not found at 6 or 24 h. The ratio of phosphorylated full-length TrkB to total TrkB was also analyzed at P14, P21, and P30, with no significant effects demonstrated at any time point (Table 1). No significant effects of ethanol exposure or genetic deletion of AC1/8 were found for FL-TrkB normalized to Actin (Table 1).

Mature BDNF can also bind to the truncated TrkB receptor, but this binding does not result in activation of the downstream pathways, including Akt, as occurs with activation of the FL-TrkB; therefore, phosphorylation of the truncated TrkB protein relative to total truncated TrkB protein was also analyzed. No significant effects of ethanol or genetic deletion of AC1/8 were found at the 2 or 4-h time points (Table 1). A significant main effect of Genotype was found for the 24-h time point as well as significant interactions of Genotype and Treatment at both 6 and 48 h after ethanol exposure (24-h Genotype [$F(1,11) = 9.07$, $p = 0.017$]; 6-h interaction [$F(1,12) = 5.50$, $p = 0.044$]; 48-h interaction [$F(1,12) = 6.09$, $p = 0.036$]). *Post hoc* analyses revealed a significant increase in phosphorylation of T-TrkB in saline-treated DKO mice compared to saline-treated WT mice at the 6 and 24-h time points, a significant increase in p-T-TrkB following ethanol exposure in WT mice at 24 h compared to saline-treated WT controls, and a significant decrease in p-T-TrKB following ethanol exposure in DKO mice compared to ethanol-exposed WT mice at 48 h. A significant interaction of Genotype and Treatment was also found at P14 with *post hoc* analysis revealing a significant reduction in p-T-TrkB in ethanol-exposed DKO mice compared to both saline-treated DKO mice and ethanol-exposed WT mice (interaction [$F(1,11) = 10.75$, $p = 0.011$]). A significant main effect of Genotype was identified at P21, with DKO mice demonstrating a reduction in phosphorylated TrkB compared to WT, when both treatment groups are combined (P21 [$F(1,13) = 7.49$, $p = 0.021$]). No significant effects were found at P30 (Table 1). Analysis of T-TrkB normalized to Actin demonstrated significant main effects of Genotype at 6 and 24 h and P14 and Treatment at 6 and 48 h and P14 (6-h Genotype [$F(1,12) = 12.30$, $p = 0.007$]; 24-h Genotype [$F(1,11) = 8.27$, $p = 0.021$]; P14 Genotype [$F(1,11) = 104.79$, $p < 0.001$]; 6-h Treatment [$F(1,12) = 8.44$, $p = 0.017$]; 48-h Treatment [$F(1,12) = 5.32$, $p = 0.047$]; P14 Treatment [$F(1,11) = 8.13$, $p = 0.021$]). An interaction of Genotype and Treatment was identified only at P14 with *post hoc* analysis revealing a significant reduction in T-TrkB in saline-treated DKO mice compared to saline-treated WT mice, a significant reduction following neonatal ethanol exposure in WT mice compared to saline-treated WT controls, and a significant increase in DKO mice following ethanol



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exposure compared to saline-treated DKO mice (Table 1; P14 interaction [$F(1,11) = 17.47$, $p = 0.003$]).

### Phosphorylation of downstream TrkB targets

To examine the functional effects of the observed changes in TrkB phosphorylation, the phosphorylation of the downstream proteins, Akt, ERK1/2, and PLCγ1, were also analyzed by immuno-blotting. Two-way ANOVAs revealed no significant effects of Genotype, Treatment, or interaction of Genotype and Treatment at 6 or 24 h after neonatal ethanol exposure for p-Akt (Fig. 4). However, at the 48-h time point, a significant interaction of Genotype × Treatment was found (48-h interaction [$F(1,11) = 9.54$, $p = 0.015$]). *Post hoc* analysis revealed that the phosphorylation of Akt was significantly higher in saline-treated DKO mice compared to saline-treated WT mice and significantly lower in ethanol-exposed DKO mice compared to saline-exposed DKO mice (Fig. 4). No significant effects were found for the later time points for p-Akt (Table 1).

No significant effects of either neonatal ethanol exposure or genetic deletion of AC1/8 on the phosphorylation of ERK1 or ERK2 were demonstrated at the 6, 24, or 48-h time points (Fig. 5). A significant effect of Genotype was found at P14, with the DKO animals having significantly lower p-ERK2 compared to WT mice when both treatment groups were combined (Table 1; [$F(1,12) = 6.399$, $p = 0.032$]). No other significant effects were found for p-ERK1 or p-ERK2 (Table 1).

Immunoblot analysis of phosphorylated PLCγ1 revealed a significant main effect of Genotype at the 4-h time point, with the DKO mice having a significant reduction in p-PLCγ1 compared to WT mice when both treatment groups were combined (Table 1; [$F(1,11) = 12.161$, $p = 0.008$]). *Post hoc* analyses also revealed significant reductions in p-PLCγ1 in saline- and ethanol-treated DKO mice compared to respective WT groups (Table 1). No significant effects of Genotype, Treatment, or interaction of Genotype and Treatment were found at the 2, 6, or 24-h time points (Table 1 and Fig. 6). At the 48-h time point, there was a significant effect of treatment, with the ethanol-exposed mice demonstrating a significant reduction in p-PLCγ1 compared to saline-treated mice, regardless of genotype (Fig. 6; [$F(1,11) = 7.37$, $p = 0.023$]). Further analysis also revealed a significant reduction in p-PLCγ1 in ethanol-treated DKO mice compared to saline-treated DKO mice (Fig. 6). No significant effects were found for P14 or P21. At P30, there was a significant effect of Genotype with DKO mice demonstrating a significant increase in p-PLCγ1 compared to WT mice [$F(1,11) = 8.626$, $p = 0.019$] with *post hoc* analysis revealing a significant increase in p-PLCγ1 in ethanol-treated DKO mice compared to ethanol-exposed WT mice (Table 1).

## Discussion

Previous work has demonstrated reductions in the dendritic morphology of medium spiny neurons after neonatal ethanol exposure in the caudate (Susick, Lowing, Provenzano, et al., 2014) and nucleus accumbens shell (Rice et al., 2012). Due to the ability of BDNF to regulate dendritic development, in the current study we analyzed the expression of BDNF in the striatum and cortex at various time points after neonatal ethanol exposure. Here, we failed to demonstrate significant changes in total BDNF protein levels after neonatal ethanol

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exposure in either the striatum or the cortex at any of the time points examined. Previous work has yielded conflicting reports on the effect of neonatal ethanol exposure on BDNF levels. Similar to that observed in the present study, work by Maas et al. found no significant effect of subcutaneous injection of 2.2 g/kg ethanol at P7 in the brains of WT or DKO mice, 1 or 8 h after exposure (Maas et al., 2005). Heaton et al. also failed to show a change in BDNF protein expression at P1 in the cortex/striatum following gestational ethanol exposure (Heaton et al., 2000). Additionally, no change in BDNF expression was found in the cortex or striatum of P7–P8 rats exposed to 1 g/kg ethanol from gestational day (G) 5-G20; however, treatment with 3 g/kg ethanol resulted in a decrease in BDNF protein in the hippocampus and cortex (Feng, Yan, & Yan, 2005). Similarly, a study by Climent et al. exposed rats to ethanol throughout gestation and lactation (ethanol intake ~14.3 g/kg/day), and demonstrated that BDNF levels in the cerebral cortex were decreased following ethanol exposure (P5, P14, and P21), but these levels return to baseline levels by P35 (Climent et al., 2002). Caldwell et al. also demonstrated a decrease in BDNF protein in the medial frontal cortex following ~9.6 g/kg/day ethanol exposure throughout gestation (Caldwell et al., 2008).

In contrast, other studies have demonstrated increases in BDNF expression following neonatal ethanol exposure. For example, Heaton et al. demonstrated that BDNF levels in the cortex/striatum rise acutely after ethanol exposure from P4–P10 and return to baseline by P21 (Heaton et al., 2000). This group also found an increase in striatal BDNF 24 h after ethanol inhalation at P3 (Heaton, Paiva, Madorsky, Mayer, et al., 2003), although no differences in BDNF expression were found 0, 2, or 12 h after ethanol exposure. Another study demonstrated an increase in BDNF + cells in the central extended amygdala 2 and 24 h following a “binge-like” paradigm of 2 doses of 2.5 g/kg ethanol 2 h apart at P7 with no effect on BDNF + cells in the pyriform cortex 2, 12, or 24 h after injection (Balaszczuk, Bender, Pereno, & Beltramino, 2013). Another study by Heaton et al. found an increase in BDNF protein expression in the cortex immediately after ethanol inhalation at P7, followed by a decrease 2 h after exposure, and then another increase 12 h after ethanol treatment (Heaton, Paiva, Madorsky, & Shaw, 2003). These differences in outcomes are speculated to arise from the differences in dosing paradigms and variances in the temporal vulnerability of the brain regions examined. The time of sampling could also be critical as changes in neurotrophic support could lead to apoptotic, protective, or adaptive pathways (Heaton, Paiva, Madorsky, & Shaw, 2003). One possible confounding factor to BDNF analysis is that immature or proBDNF has been shown to increase after repeated prenatal stress with a concomitant decrease in mature BDNF (Yeh, Huang, & Hsu, 2012). Since the BDNF ELISA used in the current study is not capable of differentiating between these two types of BDNF, we examined the expression of proBDNF using immuno-blotting and found an increase in proBDNF 24 and 48 h after ethanol exposure in WT mice (Fig. 2). This finding, along with no change in total BDNF as determined by ELISA, could indicate a decrease in the mature form of BDNF.

Although we have previously demonstrated a reduction in basal dendritic morphology in AC1/8 knockout mice (Susick, Lowing, Provenzano, et al., 2014) and AC activity has been shown to play a role in BDNF mRNA expression (Caruso et al., 2012; Goggi et al., 2003; Morinobu et al., 1999), we found no differences in basal total BDNF protein expression

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between the saline-treated WT and DKO mice at any time point examined. However, as shown in Fig. 2, the levels of proBDNF are reduced in ethanol-treated DKO mice compared to ethanol-exposed WT mice at 6 and 48 h after exposure. These data, combined with the lack of a difference in total BDNF protein, suggest a higher level of mature BDNF in the DKO mice compared to WT following neonatal ethanol exposure, which could be an overcompensation in the AC1/8 knockout mice.

AC activity has also been implicated in the expression and regulation of TrkB (Deogracias et al., 2004; Komaki, Ishikawa, & Arakawa, 2012). Therefore, to further explore the effects of AC1 and 8 deletion and neonatal ethanol exposure on the regulation of the BDNF signaling pathway, we next examined the activation of the BDNF receptor, TrkB. There are two important TrkB isoforms, the full-length TrkB receptor and a truncated TrkB receptor. The truncated TrkB receptor lacks the kinase domain and is reported to act as a competitive antagonist of the full-length receptor (Middlemas, Lindberg, & Hunter, 1991). A significant difference in phosphorylation of FL-TrkB between saline-treated WT and DKO mice was demonstrated only at the 48-h time point. Previous work has demonstrated that the expression of AC1/8 increases during development in the cortex and hippocampus of mice. TrkB expression can be stimulated via a PKA/cyclic AMP-responsive element-binding protein-(CREB) dependent mechanism and has been shown to be increased following forskolin administration in cortical cultures (Deogracias et al., 2004). Therefore, this shift in AC expression could contribute to an increase in FL-TrkB phosphorylation in WT mice which is absent in DKO animals, resulting in the observed reduction in baseline phosphorylation of FL-TrkB in DKO mice compared to WT mice at 48 h and not at the earlier time points (Conti et al., 2007). Interestingly, the phosphorylation of tyrosine 705 of the full-length receptor is decreased 6 and 24 h after neonatal ethanol exposure in WT mice. However, in DKO mice, no effect of ethanol exposure is found at 6 h while the phosphorylation of FL-TrkB is significantly increased 24 h after ethanol exposure (Fig. 2), suggesting dysregulation or overcompensation of this pathway. Interestingly, by 48 h after ethanol exposure, both WT and DKO mice demonstrate a significant decrease in phosphorylation of FL-TrkB compared to saline-treated controls. This decrease in phosphorylated FL-TrkB was not a result of a reduction in total full-length TrkB, as no significant decreases in the ratio of full-length TrkB to Actin were found (Table 1). A previous study failed to demonstrate a significant effect of ethanol exposure on the phosphorylation of TrkB in the cerebellum, cortex, or striatum (Feng et al., 2005). However, this study used a gestational exposure paradigm, while the window of vulnerability for the striatum is during the third-trimester equivalent (P5–P7). In addition, the study by Feng et al. quantified phosphorylation of tyrosine 515, which is the site for Shc interaction (Huang & Reichardt, 2001), while we measured tyrosine 705, which is located in the activation loop of TrkB. When tyrosine 705 is phosphorylated, it allows the binding of ATP (Segal et al., 1996).

Mature BDNF can also bind to the truncated TrkB receptor, which would then limit the availability of BDNF to bind to and activate the full-length receptor. Phosphorylation of T-TrkB was only significantly increased following ethanol exposure at the 24-h time point in WT mice, although this coincides with a reduction in phosphorylation of p-FL-TrkB at this time point. A reduction in p-FL-TrkB was also found at 6 and 48 h, while no effect of

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ethanol on p-T-TrkB was seen at these time points. In addition, a significant reduction in p-T-TrkB was demonstrated following neonatal ethanol exposure in DKO mice at the 48-h time point and no concurrent increase in p-FL-TrkB was found.

What is most surprising from these data is that no changes in TrkB activation are observed at 2 or 4 h after ethanol administration, when an increase in cell death is observed in the striatum of both WT and DKO mice (Conti et al., 2009; Olney et al., 2002; Young & Olney, 2006). This suggests that in our model, activation of TrkB is not involved in the acute survival of neurons after ethanol exposure at P5–P7. The reduction in TrkB phosphorylation in WT mice at 24 h may be related to the increase in neurodegeneration in the cortex found 12–24 h after neonatal ethanol exposure (Heaton, Paiva, Madorsky, & Shaw, 2003; Maas et al., 2005), which could lead to a decrease in afferent activity. However, the increase in TrkB activation observed at 24 h in the DKO mice may be indicative of a disruption in the delicate balance of neurotrophic factor signaling in the striatum and ultimately result in the long-term effects of ethanol we have previously demonstrated (Susick, Lowing, Provenzano, et al., 2014). Surprisingly, these changes in TrkB activation did not correspond to changes in Akt phosphorylation. Although Akt can be activated via phosphoinositide-3 kinase following activation of TrkB, the disconnect between alterations in Akt at 2 and 3 h following ethanol exposure demonstrated previously (Conti et al., 2009) and TrkB phosphorylation demonstrated here, suggest that regulation of Akt by other pathways involving the ACs, such as protein kinase A, plays a more dominant role than TrkB following neonatal ethanol exposure in this model.

As shown in Fig. 4, the phosphorylation of Akt was increased in saline-treated DKO mice compared to saline-treated WT controls only at the 48-h time point. Previous work has demonstrated an increase in Akt activity in cells treated with five compounds, including forskolin, that stimulate an increase in cAMP levels (Sable, Filippa, Hemmings, & Van Obberghen, 1997). Therefore, as suggested for FL-TrkB, baseline changes in Akt phosphorylation between WT and DKO mice at 48 h and not at the earlier time points could be attributed to the AC1/8 expression changes that occur during development (Conti et al., 2007), revealing a new pattern of regulation for Akt activation.

In addition to Akt, TrkB activation can also lead to the phosphorylation and activation of ERK1/2. Previous work has demonstrated that phosphorylation of ERK1 and 2 are reduced 2 and 3 h after ethanol exposure in DKO mice, while only ERK2 is significantly decreased in WT mice 2 h after ethanol treatment (Conti et al., 2009). Here we demonstrate no further changes in ERK1/2 phosphorylation following neonatal ethanol exposure at any time point examined. TrkB can directly phosphorylate another downstream protein, PLCγ1 on Tyr 783. Here, we found a significant reduction of PLCγ1 phosphorylation after neonatal ethanol exposure, regardless of genotype, only at the 48-h time point. These data indicate that either T-TrkB is affecting the downstream signaling of the BDNF receptor or other mechanisms of Akt, ERK, and PLCγ1 regulation, such as insulin, nerve growth factor, or other growth factors play a more prominent role than TrkB in our model. Another possible mechanism for changes in the phosphorylation of these signaling proteins is regulation by NMDA and/or GABA receptors. A previous study has demonstrated that ethanol, MK-801 (to block NMDA receptors), or flurazepam (to activate GABA receptors) all decreased the

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phosphorylation of ERK in the cortex of adult mice (Kalluri & Ticku, 2002). Finally, a lack of changes in downstream effectors found here could be a result of the time course used here not being suitable for detecting changes in Akt, ERK, and PLCγ1.

Although we were unable to detect changes in total BDNF protein levels in either the striatum or the cortex following neonatal exposure to 2.5 g/kg ethanol, a result that was independent of AC1/8 activity, we have demonstrated AC1/8-dependent changes in proBDNF expression, activation of TrkB, the major BDNF receptor, and Akt. Future studies will be aimed at determining the effects on other pathways regulated by TrkB, such as SHP2, as well as downstream pathways affected by these alterations in TrkB signaling, the role of AC1/8 in regulating TrkB activation, and the pathway(s) involved in regulation of Akt activation.

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## 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 |

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| | |
|---|---|
| **PLCγ1** | phospholipase Cγ1 |
| **TBST** | Tris-buffered saline with Tween 20 |
| **TrkB** | tropomyosin-related kinase B |
| **WT** | wild type |

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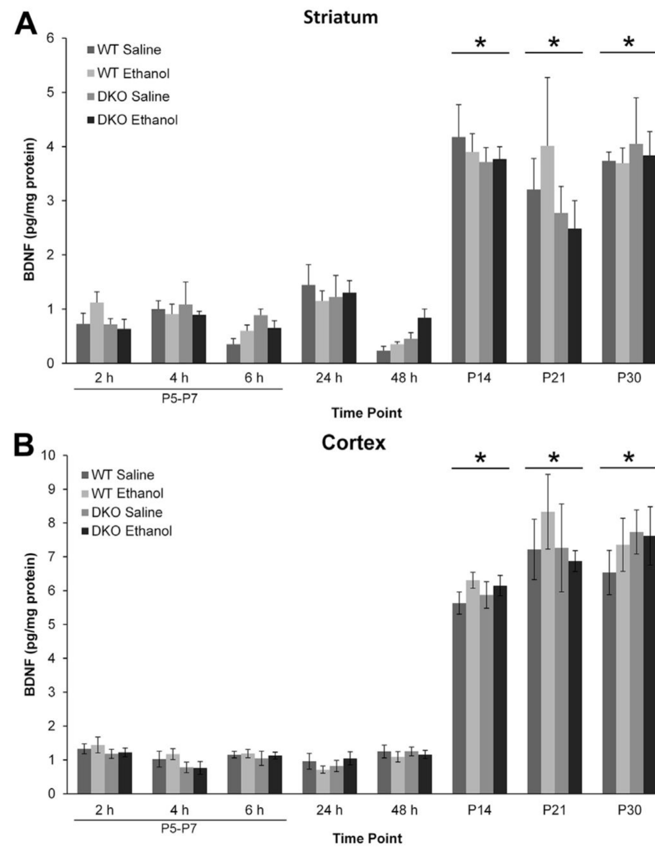


**Fig. 1.**

Postnatal ethanol exposure does not affect BDNF levels in the striatum or cortex. BDNF protein levels in (A) striatal or (B) cortical tissue from WT or DKO mice at various time points following subcutaneous injection of 2.5 g/kg ethanol or saline at P5–P7. BDNF levels are presented as pg of BDNF per mg of total protein. Mean ± SEM, $n = 4–8$ per group. * Different from 2, 4, 6, 24, and 48 h (main effect of time), $p < 0.001$. BDNF, brain-derived neurotrophic factor; DKO, double knock out; WT, wild type.

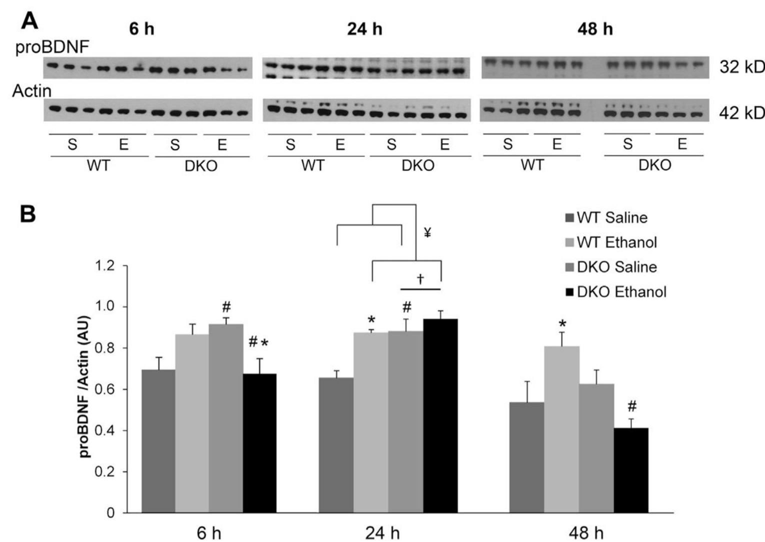


**Fig. 2.**

Effect of neonatal ethanol exposure on proBDNF protein expression in the developing striatum. (A) A single immunoreactive band at ~32 kD, representing proBDNF, was detected in all samples. Equal loading conditions were verified by immunodetection of Actin. (B) Densitometric analysis of proBDNF/Actin demonstrates that saline-treated DKO mice have an increase in the expression of proBDNF at the 6- and 24-h time points compared to saline-treated WT mice. Neonatal ethanol exposure significantly increased the expression of proBDNF in WT mice at the 24- and 48-h time points while decreasing this expression in DKO mice at the 6- and 48-h time points, compared to respective saline-treated controls. ProBDNF expression was also significantly different between ethanol-exposed WT and DKO mice at 6 and 48 h. Mean ± SEM, $n = 3$ per group, † main effect of Genotype, ¥ main effect of Treatment, * different from respective saline-treated control, # different from respective WT group. AU, arbitrary units; DKO, double knock out; proBDNF, pro form of brain-derived neurotrophic factor; WT, wild type.



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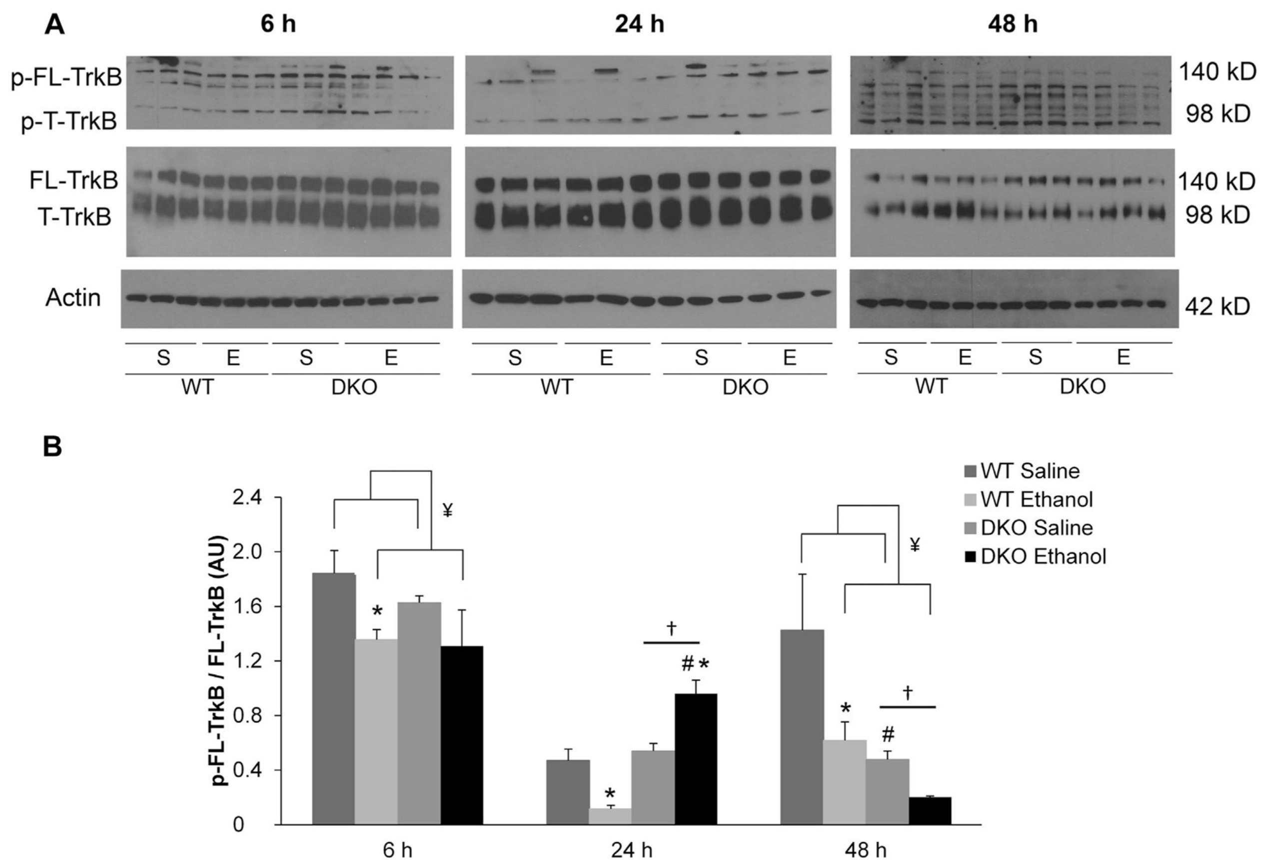


**Fig. 3.**

Acute effects of ethanol exposure on the phosphorylation of TrkB in the developing striatum of WT and DKO pups. (A) A single immunoreactive band at ~145 kD, representing the phosphorylated or total full-length TrkB protein and a single immunoreactive band at ~95 kD, representing the phosphorylated or total truncated TrkB protein were detected in all samples. Equal loading conditions were verified by immunodetection of Actin. (B) Immunoblot analysis of phosphorylated and total full-length TrkB and Actin demonstrates that exposure to 2.5 g/kg ethanol at P5–P7 significantly decreases phosphorylation of full-length TrkB in striatal protein extracts obtained 6 and 24 h after ethanol injection compared to WT saline-treated controls. In contrast, phosphorylated TrkB is increased in DKO pups 24 h after ethanol exposure compared to saline-treated controls. By 48 h after ethanol exposure, phosphorylation of full-length TrkB was significantly decreased in striatal protein extracts from both genotypes compared to respective saline controls. Densitometric analysis of phosphorylated full-length TrkB/total full-length TrkB expressed in arbitrary units, mean ± SEM, $n = 3–4$ per group, † main effect of Genotype, ¥ main effect of Treatment, * different from respective saline-treated control, # different from respective WT group. AU, arbitrary units; DKO, double knock out; FL-TrkB, full-length tropomyosin-related kinase B; T-TrkB, truncated tropomyosin-related kinase B; WT, wild type.



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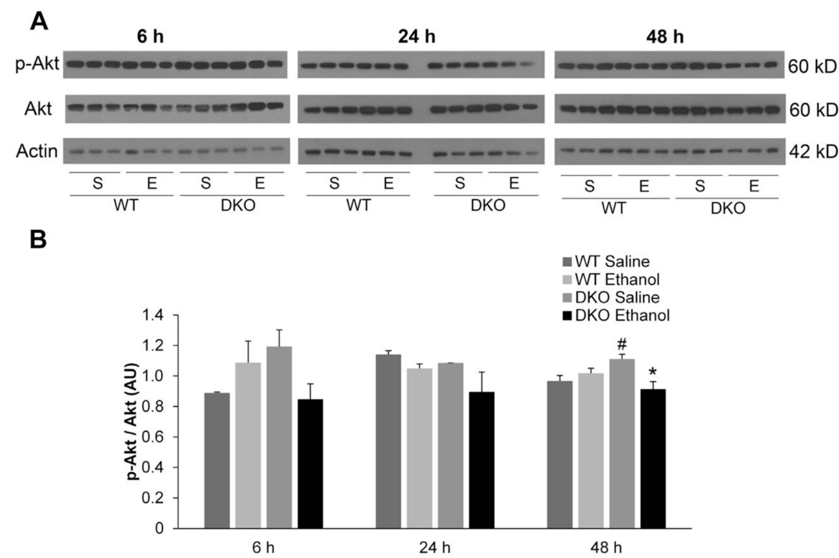


**Fig. 4.**

Phosphorylation of Akt in the developing striatum of WT and DKO mice 6, 24, and 48 h after neonatal ethanol exposure. (A) A single immunoreactive band at ~60 kD, representing the phosphorylated or total full-length Akt protein was detected in all samples. Equal loading conditions were verified by immunodetection of Actin. (B) Immunoblot analysis of phosphorylated and total Akt demonstrates that exposure to 2.5 g/kg ethanol at P5–P7 decreases phosphorylation of Akt in striatal protein extracts from DKO mice obtained 48 h after ethanol injection compared to saline-treated DKO mice. Phosphorylation of Akt is also increased in saline-treated DKO pups compared to saline-treated WT controls. No significant effects of ethanol or genetic deletion of AC1/8 were found at 6 or 24 h. Densitometric analysis of phosphorylated Akt/total Akt expressed in arbitrary units, mean ± SEM, $n = 3$ per group, * different from respective saline-treated control, # different from respective WT group. AU, arbitrary units; DKO, double knock out; WT, wild type.

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**Fig. 5.**

ERK phosphorylation is unchanged in the striatum of WT and DKO mice 6, 24, and 48 h after neonatal ethanol exposure. (A) Two immunoreactive bands at ~44 and 42 kD, representing the phosphorylated or total full-length ERK1 and ERK2 proteins were detected in all samples. Equal loading conditions were verified by immunodetection of Actin. (B) Immunoblot analysis of phosphorylated and total ERK1/2 demonstrates that neither exposure to 2.5 g/kg ethanol at P5–P7 or genetic deletion of AC1/8 had any effect on the phosphorylation of ERK1/2 at the 6-, 24-, or 48-h time points. Densitometric analysis of phosphorylated ERK1/2/total ERK1/2 expressed in arbitrary units, mean ± SEM, $n = 3$ per group. AU, arbitrary units; DKO, double knock out; ERK, extracellular signal-related kinase; WT, wild type.

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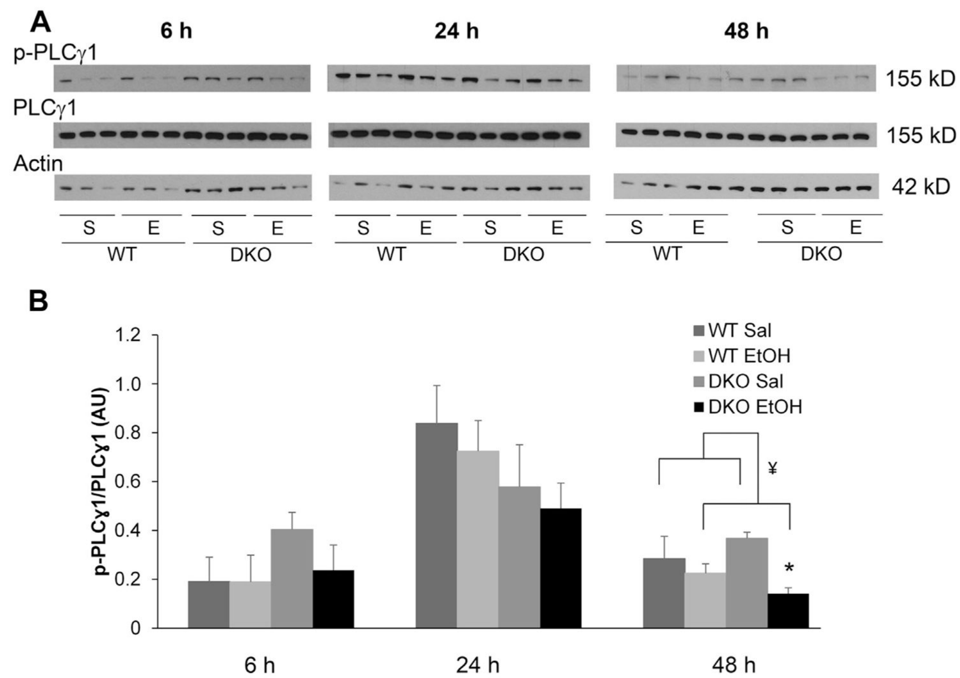


**Fig. 6.**

Phosphorylation of PLCγ1 is decreased 48 h after neonatal ethanol exposure in the developing striatum of WT and DKO mice. (A) A single immunoreactive band at ~140 kD, representing the phosphorylated or total full-length PLCγ1 protein was detected in all samples. Equal loading conditions were verified by immunodetection of Actin. (B) Immunoblot analysis of phosphorylated and total PLCγ1 demonstrates that exposure to 2.5 g/kg ethanol at P5–P7 decreases phosphorylation of Akt in striatal protein extracts obtained 48 h after ethanol injection compared to saline-treated controls. Phosphorylation of PLCγ1 is also decreased in ethanol-treated DKO pups compared to saline-treated DKO controls. No significant effects of ethanol or genetic deletion of AC1/8 were found at 6 or 24 h. Densitometric analysis of phosphorylated PLCγ1/total PLCγ1 expressed in arbitrary units, mean ± SEM, $n = 3$ per group, ¥ main effect of treatment, * different from DKO saline control. AU, arbitrary units; DKO, double knock out; PLCγ1, phospholipase Cγ1;WT, wild type.

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## Table 1

Immunoblotting quantification (arbitrary units ± SEM).

| Protein | Time point | WT saline (mean ± SEM) | WT ethanol (mean ± SEM) | DKO saline (mean ± SEM) | DKO ethanol (mean ± SEM) |
|---|---|---|---|---|---|
| Pro-BDNF/Actin | 2 h | 1.21 ± 0.02 | 0.94 ± 0.18 | 0.89 ± 0.07 | 0.86 ± 0.16 |
| | 4 h | 0.84 ± 0.07 | 0.83 ± 0.14 | 0.71 ± 0.11 | 0.85 ± 0.22 |
| | P14 | 0.38 ± 0.16 | 0.42 ± 0.16 | 0.52 ± 0.17 | 0.73 ± 0.03 |
| | P21 | 1.07 ± 0.41 | 1.36 ± 0.15 | 1.32 ± 0.08 | 1.16 ± 0.14 |
| | P30 | 0.74 ± 0.09 | 0.62 ± 0.09 | 0.34 ± 0.09 | 0.65 ± 0.09 |
| p-FL-TrkB/FL-TrkB | 2 h | 0.38 ± 0.12 | 0.34 ± 0.14 | 0.45 ± 0.10 | 0.53 ± 0.09 |
| | 4 h | 0.79 ± 0.12 | 0.77 ± 0.06 | 0.83 ± 0.03 | 0.85 ± 0.04 |
| | P14 | 1.14 ± 0.14 | 1.39 ± 0.15 | 1.30 ± 0.04 | 1.28 ± 0.06 |
| | P21 | 1.20 ± 0.19 | 0.97 ± 0.05 | 0.79 ± 0.05 | 0.78 ± 0.05 |
| | P30 | 0.55 ± 0.15 | 0.61 ± 0.06 | 0.80 ± 0.05 | 0.54 ± 0.13 |
| FL-TrkB/Actin | 2 h | 0.86 ± 0.11 | 1.02 ± 0.04 | 1.08 ± 0.04 | 1.07 ± 0.07 |
| | 4 h | 1.56 ± 0.39 | 1.20 ± 0.13 | 1.19 ± 0.12 | 0.98 ± 0.13 |
| | 6 h | 0.43 ± 0.05 | 0.53 ± 0.01 | 0.51 ± 0.01 | 0.51 ± 0.01 |
| | 24 h | 0.86 ± 0.04 | 1.03 ± 0.04 | 1.18 ± 0.11 | 1.34 ± 0.17 |
| | 48 h | 0.53 ± 0.09 | 0.51 ± 0.13 | 0.55 ± 0.11 | 0.86 ± 0.10 |
| | P14 | 1.32 ± 0.19 | 1.03 ± 0.10 | 1.26 ± 0.22 | 1.82 ± 0.52 |
| | P21 | 0.93 ± 0.21 | 1.11 ± 0.03 | 1.14 ± 0.04 | 0.92 ± 0.10 |
| | P30 | 1.18 ± 0.01 | 1.53 ± 0.07 | 1.32 ± 0.06 | 1.49 ± 0.06 |
| p-T-TrkB/T-TrkB | 2 h | 0.65 ± 0.20 | 0.55 ± 0.18 | 0.53 ± 0.14 | 0.64 ± 0.16 |
| | 4 h | 1.00 ± 0.16 | 0.94 ± 0.11 | 0.98 ± 0.13 | 1.29 ± 0.25 |
| | **6 h** | 0.25 ± 0.05 | 0.46 ± 0.08 | **0.80 ± 0.10**[d] | 0.41 ± 0.18 |
| | **24 h**[a] | 0.22 ± 0.07 | **0.44 ± 0.03**[c] | **0.53 ± 0.06**[d] | 0.49 ± 0.08 |
| | **48 h** | 0.73 ± 0.16 | 0.87 ± 0.22 | 0.97 ± 0.02 | **0.31 ± 0.04**[c,d] |
| | **P14** | 0.60 ± 0.08 | 0.77 ± 0.06 | 0.66 ± 0.14 | **0.29 ± 0.07**[c,d] |
| | **P21**[a] | 1.30 ± 0.25 | 1.08 ± 0.01 | 0.90 ± 0.13 | 0.83 ± 0.06 |
| | P30 | 0.69 ± 0.20 | 0.57 ± 0.16 | 0.57 ± 0.04 | 0.64 ± 0.22 |
| T-TrkB/Actin | 2 h | 0.93 ± 0.06 | 1.13 ± 0.09 | 1.24 ± 0.07 | 1.01 ± 0.12 |
| | 4 h | 0.94 ± 0.14 | 1.10 ± 0.20 | 1.04 ± 0.15 | 0.90 ± 0.19 |

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| Protein | Time point | WT saline (mean ± SEM) | WT ethanol (mean ± SEM) | DKO saline (mean ± SEM) | DKO ethanol (mean ± SEM) |
|---|---|---|---|---|---|
| | **6 h**[a,b] | 0.72 ± 0.03 | 0.64 ± 0.01 | 0.63 ± 0.01 | 0.60 ± 0.02 |
| | **24 h**[a] | 1.00 ± 0.04 | 1.13 ± 0.08 | 1.29 ± 0.08 | 1.58 ± 0.21 |
| | **48 h**[b] | 0.81 ± 0.20 | 0.93 ± 0.14 | 0.70 ± 0.14 | 1.30 ± 0.14 |
| | **P14**[a,b] | 1.52 ± 0.10 | **1.14 ± 0.06**[c] | **0.66 ± 0.08**[d] | **0.74 ± 0.06**[d] |
| | P21 | 0.75 ± 0.23 | 1.08 ± 0.03 | 1.08 ± 0.03 | 0.94 ± 0.05 |
| | P30 | 1.14 ± 0.02 | 1.33 ± 0.07 | 1.15 ± 0.07 | 1.21 ± 0.03 |
| p-Akt/Akt | P14 | 2.36 ± 0.34 | 1.36 ± 0.20 | 1.53 ± 0.25 | 2.64 ± 0.48 |
| | P21 | 0.71 ± 0.07 | 1.07 ± 0.09 | 1.01 ± 0.08 | 1.67 ± 0.33 |
| | P30 | 0.53 ± 0.07 | 0.52 ± 0.06 | 0.44 ± 0.08 | 0.55 ± 0.04 |
| p-ERK1/ERK1 | **P14**[a] | 0.60 ± 0.28 | 0.81 ± 0.17 | 0.77 ± 0.19 | 0.33 ± 0.10 |
| | P21 | 0.62 ± 0.17 | 0.61 ± 0.09 | 0.53 ± 0.12 | 0.65 ± 0.15 |
| | P30 | 0.97 ± 0.18 | 1.11 ± 0.17 | 0.90 ± 0.14 | 0.37 ± 0.18 |
| p-ERK2/ERK2 | P14 | 1.27 ± 0.20 | 1.47 ± 0.10 | 1.14 ± 0.09 | 0.73 ± 0.27 |
| | P21 | 1.02 ± 0.12 | 0.93 ± 0.06 | 0.92 ± 0.07 | 0.94 ± 0.10 |
| | P30 | 1.24 ± 0.05 | 1.14 ± 0.09 | 0.94 ± 0.14 | 0.79 ± 0.18 |
| p-PLCγ1/PLCγ1 | 2 h | 0.58 ± 0.04 | 0.60 ± 0.04 | 0.79 ± 0.10 | 0.61 ± 0.07 |
| | **4 h**[a] | 0.81 ± 0.03 | 0.78 ± 0.12 | **0.51 ± 0.10**[d] | **0.52 ± 0.01**[d] |
| | P14 | 0.87 ± 0.05 | 0.79 ± 0.11 | 0.59 ± 0.11 | 0.70 ± 0.07 |
| | P21 | 0.75 ± 0.23 | 1.08 ± 0.04 | 1.08 ± 0.19 | 0.94 ± 0.21 |
| | **P30**[a] | 0.57 ± 0.08 | 0.43 ± 0.12 | 0.70 ± 0.40 | **0.89 ± 0.06**[d] |

Bold indicates significant differences were found.

[a]Main effect of genotype.

[b]Main effect of treatment.

[c]Different from respective saline.

[d]Different from respective WT.