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Ethanol-induced increases in extracellular dopamine are blunted in brain-derived neurotrophic factor heterozygous mice

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Abstract

Drugs of abuse like ethanol have the ability to stimulate forebrain dopaminergic pathways. Although the positive reinforcing properties of abused substances are largely attributed to their effects on dopamine transmission, alcohol addiction involves complex interactions between numerous molecular mediators. Brain-derived neurotrophic factor (BDNF) is suggested to have a protective role in regulating the reinforcing effects of ethanol. In the present study, we evaluated the effects of an acute, systemic injection of ethanol (2 g/kg) on BDNF protein levels and extracellular dopamine concentrations, measured by *in vivo* microdialysis, in the caudate-putamen of wildtype and heterozygous BDNF mice. In both genotypes, the peak increase in extracellular dopamine following ethanol coincided temporally with a decrease in BDNF protein levels following a similar ethanol treatment. Moreover, the effect of ethanol to increase extracellular dopamine was blunted in heterozygous BDNF mice compared to wildtype mice. While the magnitude of decrease in BDNF protein induced by ethanol was similar between genotypes (two-fold), ethanol treatment induced significantly lower BDNF protein levels in heterozygous BDNF mice overall. These findings suggest the effects of ethanol are influenced by an interaction between BDNF and dopamine transmission, which may relate to the pathway through which BDNF regulates ethanol intake.

Keywords

BDNF; ethanol; dopamine; striatum; caudate-putamen; microdialysis

Introduction

Alcoholism is a prevalent addictive disorder, with approximately 5% of Americans classified as alcohol abusing or dependent [7]. Recently, several susceptibility factors have been identified that are believed to significantly contribute to the development of alcohol addiction [25]. In particular, multiple lines of evidence indicate that brain-derived neurotrophic factor (BDNF) regulates the behavioral effects of ethanol.

BDNF is a soluble protein that is widely distributed throughout the brain and has well established effects on neuronal growth, plasticity, and survival through activation of tyrosine kinase B (TrkB) receptors and its downstream signaling pathways (for review see [4]). Both human and animal studies indicate that BDNF plays a protective role in regulating the

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reinforcing effects of ethanol. In humans, BDNF [20,33,35] and TrkB [36] gene polymorphisms are associated with vulnerability to develop alcohol dependence and undergo relapse following treatment for alcoholism. Further, alcohol-preferring rats have innately lower BDNF levels in the nucleus accumbens (NAc) [37] and inhibition of TrkB receptors increases ethanol consumption in mice [11]. Heterozygote BDNF mice (BDNF^{+/-}) that have a 50% reduction in neuronal BDNF expression [3,32] also exhibit a preference for voluntary ethanol consumption and heightened sensitivity to ethanol in several behavioral procedures [8,21].

The involvement of BDNF in the regulatory pathway underlying the behavioral response to ethanol is further supported by the finding that ethanol modulates BDNF expression. For example, gene microarray analysis in humans indicated BDNF is an ethanol-responsive gene [33] and a rodent study demonstrated that both acute ethanol administration and voluntary consumption increased BDNF mRNA levels in the caudate-putamen (CPu) [21]. Although these results indicate ethanol upregulates BDNF signaling, which in turn inhibits subsequent ethanol drinking; the neuronal substrate mediating this interaction has not been defined. Alcohol exerts its positive reinforcing and stimulating effects primarily through activation of dopaminergic pathways [5,31] and BDNF has been shown to have potent neuromodulatory effects on dopamine transmission [3,6,13,29]. Therefore, the goal of the present study was to measure the functional consequence of acute ethanol administration on BDNF protein levels and extracellular concentrations of dopamine in the CPu of BDNF^{+/-} mice.

Materials and Methods

Levels of BDNF protein and extracellular dopamine were evaluated in separate groups of wildtype and BDNF^{+/-} mice following a single, systemic injection of a moderate dose of ethanol (2 g/kg) or saline (0.9% NaCl). Wildtype and BDNF^{+/-} mice on a C57Bl/6 genetic background were initially obtained from Jackson Laboratories and offspring were raised as a colony in house. Genotype identification was performed essentially as previously described [13] with primers specified by Jackson Laboratories. Mice were housed in groups of 3-4 per cage with food and water *ad libitum* (12-hr light-dark cycle). All animals used in the experiments were males between 8-16 weeks old. All procedures were designed and conducted to minimize pain and discomfort to the animals. Animal care and use was in accordance with the National Institutes of Health Animal Care guidelines and approved by the Wayne State University Institutional Animal Care and Use Committee.

For BDNF protein analysis, mice received an intraperitoneal (i.p.) injection of either saline (0.1 ml) or ethanol (2 g/kg; 15%, w/v) 45 min prior to sacrifice. Brains were sectioned for the CPu which was rapidly frozen in liquid nitrogen. BDNF protein was extracted using the modified procedure described in Szapacs et al. [32]. Briefly, samples were weighed, homogenized in lysis buffer (100 mM PIPES (pH 7), 500 mM NaCl, 0.2% Triton X-100, 0.1% NaN₃, 2% BSA, 2mM EDTA·Na₂·2H₂O, 200 μM PMSF, 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 the Promega BDNF E_{max} ImmunoAssay System. First, a polystyrene 96-well plate was coated overnight at 4°C with anti-BDNF monoclonal antibody (1:1000 in carbonate coating buffer (25 mM NaHCO₃ and 25 mM Na₂CO₃; pH 9.7)). Unabsorbed antibody was removed by washing plates once with TBST (20 mM Tris-HCl (pH 7.6), 150 mM NaCl and 0.05 % (v/v) Tween 20). Plates were then blocked with 1× Block and Sample buffer (Promega assay kit) for 1 h at room temperature, washed once with TBST, and samples or standards were added to the 96-well plate in triplicate. Plates were incubated with the samples and standards for 2 h with shaking (600 rpm) at room temperature and washed five times with TBST. The plates were incubated first with anti-human BDNF

polyclonal antibody (1:500) for 2 h, then anti-horseradish peroxidase conjugate (1:200) for 2 h, also with shaking at room temperature. Plates were washed 5 times with TBST following each step. Finally, plates were developed using TMB One Solution (Promega assay kit) and the reaction stopped with 1 N HCl. Absorbance was measured at 450 nm and BDNF levels were reported in ng/g wet weight of tissue (ng/g ww). Blood ethanol concentration (BEC) was determined from this group of mice using trunk blood harvested after sacrifice. BEC was quantified using the dehydrogenase/NADH-based enzymatic assay (Diagnostic Chemicals Limited) as previously described [2] and values were expressed in mmol/L.

The stereotaxic surgery for microdialysis was performed as previously described [18] with the following modifications. Briefly, a CMA/7 guide cannula was implanted in the CPu of mice under Avertin (20 ml/kg, i.p.) anesthesia. Coordinates (in mm: A +0.8, L -1.3, V -2.5 from Bregma) were determined from the mouse atlas [27] and empirical assessment. Following recovery, a CMA/7 dialysis probe (2 mm membrane length) was inserted and perfused overnight with artificial cerebrospinal fluid (in mM: 147 NaCl, 3.5 KCl, 2 Na₂HPO₄, 1.0 CaCl₂, 1.2 MgCl₂; pH 7.4) at a flow rate of 0.4 µl/min. The next day, the flow rate was increased to 1.1 µl/min for a 1 h equilibration period and dialysate samples were collected from freely moving mice at 20 min intervals. After collection of at least three baseline samples, mice were injected with saline (0.1 ml; i.p.) and samples were collected for 1 h. At the end of the last saline dialysate fraction, mice were injected with ethanol (2 g/kg; 15% w/v; i.p.). Dopamine content from dialysate samples was measured using HPLC with electrochemical detection [18]. Dopamine peak area was integrated and quantified against known standards using LC Solutions Software (Shimadzu Scientific Instruments). Following dialysis, mice were euthanized and brains were sectioned for histological confirmation of probe placement.

Data analysis was performed using GraphPad Prism software. All values were reported as mean ± standard error of the mean (SEM) and the criterion for statistical significance was $p < 0.05$. BEC values were compared by Student's t-test. BDNF protein levels were reported without correction for recovery during tissue extraction. The dopamine response to saline and ethanol treatment was reported as percent of baseline (average of three pre-saline samples). Two-way analysis of variance (ANOVA) with Bonferroni multiple comparison analysis (post-test) was used to test the interaction between genotype (wildtype and BDNF^{+/-} mice) and ethanol treatment on BDNF protein and dialysate dopamine. Additionally, the area under the dopamine concentration curve was calculated from the four dialysate samples following ethanol injection (140 - 200 min) using the trapezoidal method and statistical significance was determined by Student's t-test.

Results

To assess the acute effects of ethanol on both BDNF protein levels and *in vivo* dialysis levels of dopamine, BDNF wildtype and heterozygote mice were injected with a nonhypnotic dose of ethanol (2 g/kg), which resulted in an average BEC of 37.0 ± 6.1 mmol/L ($n = 12$). This did not differ with genotype and was used as an indication that changes in the ethanol-evoked responses across genotype were due to a greater sensitivity to ethanol rather than a difference in the pharmacokinetics of ethanol.

Total tissue levels of BDNF protein were measured from the CPu of wildtype and BDNF^{+/-} mice 45 min after a systemic injection of ethanol or saline (Fig. 1). Two-way ANOVA analysis showed significant main effect for treatment ($F(1,58) = 28.86$, $p < 0.0001$) and genotype ($F(1,58) = 46.82$, $p < 0.0001$). There was also a significant genotype × treatment interaction ($F(1,58) = 4.56$, $p < 0.05$). A post-test indicated BDNF protein levels in the CPu of saline-treated BDNF^{+/-} mice were significantly reduced by ~50% (10.0 ± 1.3 ng/g ww; n

= 5) compared to saline wildtype mice (22.5 ± 1.6 ng/g ww; $n = 6$; $p < 0.001$), consistent with previous reports [3,32]. Acute ethanol administration significantly attenuated BDNF protein levels in wildtype mice (12.0 ± 1.6 ng/g ww; $n = 6$; $p < 0.01$) compared to saline treatment. Interestingly, ethanol treatment reduced BDNF protein levels in wildtype mice to an extent which no longer differed from the BDNF levels observed in saline-treated BDNF^{+/-} mice. In BDNF^{+/-} mice, acute ethanol treatment significantly reduced BDNF protein levels (5.5 ± 1.0 ng/g ww; $n = 6$) compared to both saline-treated BDNF^{+/-} mice ($p < 0.05$) and ethanol-treated wildtype mice ($p < 0.01$).

The mean baseline dialysate concentration of dopamine in the CPu (nM $\pm$ SEM; averaged from three pre-saline samples; Fig. 2), which reflects the sum of exocytotic release and clearance processes, including transporter-mediated uptake and enzymatic metabolism, was 1.2 ± 0.03 nM for wildtype mice ($n = 6$) and 2.2 ± 0.3 nM for BDNF^{+/-} mice ($n = 6$). A two-tailed t-test revealed that the baseline values for dopamine were significantly elevated in the BDNF^{+/-} mice ($p < 0.05$). Systemic injection of saline did not alter dialysate concentrations of dopamine (averaged from three samples) for wildtype (1.1 ± 0.1 nM) or BDNF^{+/-} mice (1.9 ± 0.5 nM). Ethanol administration increased extracellular levels of dopamine in both wildtype and BDNF^{+/-} mice. Data analysis by two-way ANOVA revealed a significant main effect for genotype ($F(1,81) = 4.49$, $p < 0.05$) and time ($F(9,81) = 7.35$, $p < 0.001$), but no interaction ($F(9,81) = 1.56$, $p = 0.14$). Subsequent pair-wise comparisons indicate the peak dopamine response following ethanol injection was significantly attenuated in BDNF^{+/-} mice ($\sim 70\%$ of baseline) compared to wildtype controls ($\sim 190\%$ of baseline) ($p < 0.01$). The area under the curve of the cumulative dialysate dopamine levels of the four samples following the ethanol injection (140 - 200 min) was also calculated (insert for Fig. 2). Wildtype mice had significantly greater ethanol-induced elevations in extracellular dopamine compared to BDNF^{+/-} mice ($p < 0.01$).

Discussion

This study evaluated the effects of a systemic injection of ethanol on BDNF protein levels and extracellular concentrations of dopamine *in vivo* to further understand the striatal neuronal pathway through which BDNF was previously suggested to regulate the reinforcing properties of ethanol [16,21]. Multiple lines of evidence indicate BDNF is neuroprotective by preventing the development of ethanol reinforcing behaviors through a homeostatic mechanism in which ethanol increases BDNF, promoting activation of the TrkB receptor and downstream signaling pathways [10,16]. This mechanism is largely based on changes in BDNF mRNA from brain tissue [21] and protein levels from primary striatal neuronal cultures [15] measured at discrete time points (45 min and 2 h, respectively) following ethanol exposure. In the present study, BDNF protein levels were decreased in the CPu 45 minutes after systemic administration of ethanol. This time point was chosen based on a previous report that 2 g/kg ethanol increased BDNF mRNA expression 45 minutes after administration [21]. In many areas of the brain, the distribution of BDNF protein largely correlates with mRNA expression. However, in the dorsal CPu, BDNF protein is abundant despite low intrinsic BDNF synthesis in this brain region [1]. Rather, striatal BDNF protein is primarily derived from anterograde axonal transport via corticostriatal and nigrostriatal afferents and presynaptic release of BDNF functionally regulates neuronal activity in the CPu [1]. Thus, an effect of ethanol to impair axonal transport, which was demonstrated in primary neuronal preparations of the dorsal root ganglia [22], would lead to a decrease in BDNF protein measured in the CPu. Alternatively, the divergent change in BDNF mRNA reported previously [21] and protein levels in the current study following ethanol administration may result from modulation of local post-translational processing, including conversion of proBDNF to mature BDNF and/or protein degradation. Although the contribution of these factors to the interaction between ethanol and BDNF will become more

apparent as the physiological conditions that regulate neurotrophin expression and trafficking are better understood, our finding highlights the need for further investigation into the temporal and region-specific dynamics of BDNF protein and mRNA changes following ethanol. For instance, it is possible that the decrease in BDNF protein levels after acute ethanol in our study is transient. Thus, the increased expression of BDNF mRNA at this same time point (45 min) reported previously [21] may correspond to elevated levels of BDNF protein at later time points, which was demonstrated 2 h after ethanol administration in primary striatal cultures [15]. In addition, the finding that repeated bouts of ethanol self-administration altered corticostriatal BDNF gene expression [16] suggests that BDNF signaling between the cortex and striatum may be involved in the long-term consequences of ethanol abuse.

An important contribution of this study was to compare the temporal relationship between ethanol-induced changes in BDNF protein and extracellular concentrations of striatal dopamine. Our findings indicate that in the CPu, the peak dopamine response to an acute injection of ethanol corresponds with an attenuation of BDNF protein levels. Although many studies have shown that acute ethanol activation of mesolimbic dopaminergic neurons is essential in the initiation of alcohol reinforcement (for review see [31]), ethanol administration has also been shown to increase the firing rate of nigrostriatal neurons [24] leading to an increase in extracellular dopamine in the CPu [5,19,23]. An upregulation of dopamine transmission in the CPu following ethanol has been implicated in the development of alcohol dependence through the formation of goal-directed responses and behavioral habits associated with compulsive use [34]. A systemic injection of 2 g/kg ethanol significantly increased extracellular levels of dopamine in the CPu of wildtype mice between 40 and 60 min following ethanol administration. These microdialysis results from the CPu are in agreement with several mouse microdialysis studies that have shown an approximate 50 – 170% increase in extracellular dopamine levels in response to a locomotor activating dose of ethanol [17,19,26,28]. One important difference between the present study and those described above is that the majority of mouse microdialysis studies have evaluated ethanol-induced elevations in extracellular dopamine in the NAc (or ventral striatum) [17,26,28] and not the CPu (or dorsal striatum); but see [19]. Importantly, in all the microdialysis studies a robust dopamine response to 2 g/kg of ethanol is observed indicating a conserved dopaminergic mechanism.

BDNF^{+/-} mice were used to study the influence of low endogenous BDNF levels on the effect of ethanol to alter BDNF protein levels and induce dopamine transmission in the CPu. The percent reduction in BDNF protein levels following acute ethanol exposure was similar for wildtype and BDNF^{+/-} mice. However, ethanol treatment induced significantly lower BDNF protein levels in BDNF^{+/-} mice overall. Furthermore, the ethanol-mediated reduction in BDNF levels corresponded with a two-fold decrease in the peak dopamine response following ethanol administration in the CPu of BDNF^{+/-} mice. These findings indicate that ethanol-mediated regulation of endogenous BDNF may, in turn, influence the dopaminergic response to ethanol. Future studies to elucidate the mechanism underlying this interaction will be important to determine how BDNF may modify dopamine transmission following ethanol treatment, since previous studies indicate BDNF can regulate dopamine turnover [1], activity-dependent release [6], and dopamine transporter activity [9]. A future ethanol dose-response study would also provide mechanistic insight in determining if the diminished effect of ethanol to increase extracellular dopamine in BDNF^{+/-} mice is related to a rightward shift in the dose response and/or a decrease in the magnitude of effect.

The ability of BDNF to alter ethanol-induced increases in extracellular levels of dopamine may be a key mediator in the homeostatic pathway through which BDNF is hypothesized to regulate ethanol drinking behavior. Indeed, Ron and colleagues demonstrated that BDNF

decreases ethanol intake by increasing the expression of downstream effectors, particularly dopamine D3 receptors and dynorphin [11,15], both of which regulate dopamine neurotransmission [12,14,30,38]. Additionally, our finding that BDNF^{+/-} mice, which are characterized as “alcohol-preferring” [8,21], have attenuated increases in extracellular dopamine following ethanol in the CPu is consistent with the report of a negative correlation between innate alcohol preference and ethanol-induced dopamine transmission in the ventral striatum [28]. Overall, our study demonstrates that striatal BDNF protein is amenable to acute ethanol regulation and indicates this regulation may influence ethanol-evoked dopaminergic transmission in the CPu, potentially involved in the homeostatic pathway through which BDNF was proposed to modulate ethanol drinking behavior [21].

*Research Highlights

- Ethanol reduced striatal BDNF protein levels in wildtype and BDNF^{+/-} mice.
- BDNF^{+/-} mice had elevated baseline striatal dopamine levels by microdialysis.
- Ethanol-induced increases in extracellular dopamine were decreased in BDNF^{+/-} mice.

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Figure 1.

BDNF protein levels in the CPu of wildtype (WT) and BDNF^{+/-} mice 45 min following a systemic injection (i.p.) of saline or ethanol (EtOH; 2 g/kg). Data are mean $\pm$ SEM of five to six mice per group and expressed as ng/g wet weight of tissue (ng/g ww). *** p < 0.001, ** p < 0.01, compared to the saline-treated wildtype mice and * p < 0.05, compared to saline-treated BDNF mice (two-way ANOVA).

Figure 2.

Extracellular dopamine (DA) concentrations in the CPu of wildtype (WT) and BDNF^{+/-} mice. Saline and ethanol (EtOH) were administered i.p. at the time indicated by the arrow. Data are mean $\pm$ SEM of six mice per group and expressed as percent of uncorrected baseline values. ** $p < 0.01$, compared to wildtype mice (two-way ANOVA). Insert shows the area under the curve for the cumulative increase in extracellular dopamine over the four 20 minute sample collections (140 - 200 min) following the ethanol injection. Data are mean area under the curve (AUC) $\pm$ SEM. ** $p < 0.01$, compared to wildtype mice (Students *t*-test).