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CHRONIC SOCIAL ISOLATION DURING ADOLESCENCE AUGMENTS CATECHOLAMINE RESPONSE TO ACUTE ETHANOL IN THE BASOLATERAL AMYGDALA

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Abstract

Adolescent social isolation (SI) results in numerous behavioral alterations associated with increased risk of alcoholism. Notably, many of these changes involve the basolateral amygdala (BLA), including increased alcohol seeking. The BLA sends a strong glutamatergic projection to the nucleus accumbens and activation of this pathway potentiates reward-seeking behavior. Dopamine (DA) and norepinephrine (NE) exert powerful excitatory and inhibitory effects on BLA activity and chronic stress can disrupt the excitation-inhibition balance maintained by these catecholamines. Notably, the impact of SI on BLA DA and NE neurotransmission is unknown. Thus the aim of this study was to characterize SI-mediated catecholamine alterations in the BLA. Male Long Evans rats were housed in groups of four (GH) or in SI for six weeks during adolescence. DA and NE transporter levels were then measured using Western blot hybridization and baseline and ethanol-stimulated DA and NE levels were quantified using microdialysis. DA transporter levels were increased and baseline DA levels were decreased in SI compared to GH rats. SI also increased DA responses to an acute ethanol (2 g/kg) challenge. While no group differences were noted in NE transporter or baseline NE levels, acute ethanol (2 g/kg) only significantly increased NE levels in SI animals. Collectively, these SI-dependent changes in BLA catecholamine signaling may lead to an increase in BLA excitability and a strengthening of the glutamatergic projection between the BLA and NAc. Such changes may promote the elevated ethanol drinking behavior observed in rats subjected to chronic adolescent stress.

Graphical Abstract

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Keywords

Basolateral Amygdala; Dopamine; Ethanol; Microdialysis; Norepinephrine; Social isolation

INTRODUCTION

Early life adversity in humans is associated with increased risk of developing anxiety disorders and alcohol addiction (Anda et al., 2002). Imaging studies have also shown that excessive childhood stress can disrupt neuronal activity in mesolimbic systems that contribute to anxiety disorders and addiction (Dillon et al., 2009; Oswald et al., 2014). Adolescent social isolation is a model of chronic early-life stress that results in a myriad of behavioral abnormalities. For example, rats that have been socially isolated (SI) in adolescence spend less time on the open arms of an elevated plus-maze compared with rats that were group housed (GH) during this period (Wright et al., 1991; McCool and Chappell, 2009). Social isolation is also associated with increased fear conditioning and decreased fear extinction (Pibiri et al., 2008) and exhibit an overall anxiogenic phenotype on measures of unconditioned anxiety. Furthermore, SI rats display increases in behavioral risk factors associated with alcohol addiction vulnerability, including increased ethanol place preference (Whitaker et al., 2013) and ethanol self-administration (Schenk et al., 1990; Wolffgramm and Heyne, 1991; McCool and Chappell, 2009).

Based on these findings, our group has recently begun to employ a rodent SI model to identify possible neural substrates within the limbic system that may contribute to the increases in addiction-related risk factors engendered by these models. Some of our initial studies have identified profound and enduring SI-dependent alterations in norepinephrine (NE) and dopamine (DA) signaling in the nucleus accumbens (NAc; Karkhanis et al., 2014). However, the behavioral manifestations associated with social isolation, such as increases in anxiety-like behaviors, disruption of fear processing, and increased reward-seeking, are also consistent with an important role of other limbic brain regions, like the basolateral amygdala (BLA). Therefore, in this study we sought to examine the impact of adolescent social isolation on catecholamine signaling in the BLA.

The BLA and the NAc, two regions crucial for mediating drug addiction behaviors, are connected via a glutamatergic projection from the BLA to the NAc (Koob, 2006; Stuber et al., 2011). This robust glutamatergic projection plays a major role in affective motivational

behaviors (Stuber et al., 2011). For example, inhibition of this projection decreases sucrose seeking while facilitation of this pathway increases reward-seeking behavior (Stuber et al., 2011). DA (Rosenkranz and Grace, 1999; Pape, 2005) and NE (Southwick et al., 1999; Buffalari and Grace, 2007; Silberman et al., 2010) play a powerful role in modulating the excitatory efferents of the BLA. The regulation of BLA output is complex as DA (Marowsky et al., 2005; Diaz et al., 2011) and NE (Southwick et al., 1999; Buffalari and Grace, 2007; Silberman et al. 2010) both exert excitatory and inhibitory effects in an intact system.

Behavioral perturbations such as chronic stress, however, can affect the excitation-inhibition balance mediated via DA (Phillips et al., 2002) and NE (Braga et al., 2004; Buffalari and Grace, 2009) in the BLA. Repeated exposure to stress tends to shift the balance in favor of excitation and this shift may contribute to dysregulation of reward-seeking behavior. For example, social isolation during adolescence decreases baseline DA levels (Heidbreder et al., 2000), possibly augmenting the overall excitability of the BLA. Additionally, chronic cold stress increases excitatory effects of NE on neuronal activity in the BLA (Buffalari and Grace, 2009). Furthermore, repeated stress decreases $\alpha 1$ AR-mediated facilitation of GABAergic inhibition in the BLA (Braga et al., 2004), potentially leading to hyperexcitability. As mentioned earlier, increased excitation of the BLA-NAc glutamatergic projection may facilitate reward-seeking behaviors, including increased ethanol-seeking.

Despite the fact that many of the behavioral sequelae associated with SI are consistent with altered BLA function, little is known about the impact of SI on BLA neurotransmission. In addition, no studies to date have examined acute effects of ethanol on DA and NE release in the BLA of awake, behaving animals, despite the fact that acute ethanol has robust effects on the excitability of VTA DA (Brodie et al., 1999; Ding et al., 2009) neurons and LC NE neurons (Pohorecky and Brick, 1977), both of which send strong projections to the BLA. Therefore, here we employed microdialysis in freely moving rats to characterize the effects of adolescent social isolation on ethanol-stimulated DA and NE release in the BLA. Additionally, we used Western blot hybridization to quantify DA and NE transporter protein levels in this brain region. Our findings reveal, for the first time, that social isolation decreases DA transporter protein levels in the BLA and results in a sensitized response of both catecholaminergic systems to acute ethanol administration. These sensitized BLA catecholaminergic responses may contribute to the behavioral phenotype associated with adolescent social isolation, particularly the increased propensity to self-administer ethanol.

MATERIALS AND METHODS

Group and isolation rearing

Male Long-Evans rats were purchased from Harlan at postnatal day (PD) 21. At PD 28, following a week of acclimation in standard housing conditions (four animals per cage, food and water *ad libitum*; 12 h light/dark cycle), rats were randomly assigned to one of two experimental groups: group housed and socially isolated. Rats in the GH condition were housed in groups of four in guinea pig cages (33 cm $\times$ 50.7 cm; Nalgene, Rochester, NY); SI rats were housed individually in rat cages (20.3 cm $\times$ 26.7 cm; Allentown, Allentown, NJ).

Rats were maintained in their respective housing conditions for at least six weeks before western blot hybridization and microdialysis experiments (Fig. 1).

Western blot hybridization

In a separate group of animals, BLA tissue samples were extracted from GH and SI animals at the end of the six week housing paradigm (n = 8 in both groups). DA transporter (DAT) and NE transporter (NET) protein levels in the BLA were quantified using Western blot hybridization. Western blot procedures were similar to those published in previous reports (Christian et al., 2012). Briefly, homogenizing buffer consisting of tissue protein extraction reagent (78510T-PER[®]; Thermo Fisher, Waltham, MA), protease inhibitors for mammalian tissue (P8340; Sigma, St. Louis, MO), and phosphatase inhibitor cocktail 2 and 3 (P5726 and P0044 respectively; Sigma, St. Louis, MO) was added to BLA samples (10 – 20 mg wet weight) dissected from GH and SI animals, disrupted by brief sonication, incubated at 4° C on a rotisserie mixer for 2 hrs, and centrifuged to isolate the supernatant. Protein yield was quantified using a commercially available Pierce BCA assay kit (PI23227; Thermo Fisher, Waltham, MA) and Molecular Devices Spectra Max 384 Plus spectrophotometer (Sunnyvale, CA) utilizing the SoftMax Pro software.

Seven to ten µg of protein was loaded onto 4 – 20% Criterion TGX 18-well precast gels (567-1094; Bio-Rad, Hercules, CA) along with Precision Plus Western C Standards (160-0376; Bio-Rad, Hercules, CA). The membranes were blocked with a solution of Tris Buffered Saline (TBS-T 20× concentrate; J640; Amresco, Solon, OH) and 0.05% Tween-20 (BP337; Fisherbrand, Loughborough, UK) containing 5% Carnation powdered nonfat dry milk (NFM) for 1 hour at room temperature. Subsequently, blots were incubated with agitation overnight at 4°C in TBS-T/1% NFM solution containing a rabbit polyclonal primary antibody that recognized either DATs or NETs. The following antibodies exhibited specificity for the protein targets as indicated by the immunoreactive bands at expected molecular weights: DAT (1:1000; 2231; EMD Millipore, Billerica, MA) and NET (1:500; AMT-002; Alomone Labs, Jerusalem, Israel). Following extensive washing with TBS-T, the blots were exposed to goat anti-rabbit secondary antibody (1:3000; 4914; Sigma, St. Louis, MO) labeled with peroxidase in TBS-T/1% NFM solution for one hour at room temperature with agitation. Detection of bound secondary antibody was performed using enhanced chemiluminescence (34076; Thermo Fischer, Waltham, MA). Band intensity was quantified from digital images using the Bio-Rad Molecular Imager[®] ChemiDoc XRS system (170-8070; Bio-Rad, Hercules, CA) in conjunction with Bio-Rad Quantity One[®] software. Samples from GH and SI animals were always run on the same gel to facilitate direct comparisons.

Microdialysis

Surgery—All rats were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.). A guide cannula with a stylet (MD-2250; BASi Instruments, West Lafayette, IN) was implanted unilaterally over the BLA (anterior, –2.8; lateral, –5.0; ventral, –6.4 with respect to bregma; Fig. 2). The coordinates were determined using a rat atlas (Paxinos and Watson, 2007). The guide cannula was anchored to the skull, and the exposed skull was covered, using fast-drying dental acrylic (Lang Dental Manufacturing Co., Inc., Wheeling,

IL). Ketoprofen (5 mg/ml; 1 ml/kg, s.c.) was administered post-surgery. A microdialysis probe with a membrane length of 2 mm (MD-2200, BASi Instruments, West Lafayette, IN), was inserted through the guide cannula into the BLA immediately after the surgery. The microdialysis probe was connected to a syringe pump (A-99; Razel Scientific Instruments, Inc., Stamford, CT) and perfused with artificial cerebrospinal fluid (aCSF; 148 mM NaCl; 2.7 mM KCl; 1.2 mM CaCl₂; 0.85 mM MgCl₂; pH 7.4) at a flow rate of 1 µl/min for a minimum of one hour before inserting into the guide cannula. Following microdialysis experiments, all rats were perfused transcardially with paraformaldehyde (10%; SF100-4; Fischer Scientific, Fair Lawn, NJ) and the brains were removed for probe placement confirmation (Fig. 2).

Experimental design—Microdialysis experiments were conducted following the adolescent housing paradigm (between PD 70 and 110; GH: n=8; SI: n=11). GH rats that were housed together during adolescence were run at the same time but in separate chambers during microdialysis experiments. The post-surgery recovery time was between 18 and 24 hrs for all rats. Ethanol (1 g/kg and 2 g/kg) was administered intraperitoneally (i.p.) to all rats. The lower dose was always administered 24 hrs prior to the higher dose. Overnight, aCSF flow through the probe was maintained at a rate of 0.4 µl/min. Following the recovery period, the flow rate was increased to 1 µl/min for two hours prior to baseline sample collections. Samples were collected with an interval of 20 min for two hours prior to ethanol administration and are referred to as baseline. Dialysate samples were collected for 100 min following ethanol administration (1 and 2 g/kg). In order to verify whether the elevation in DA and NE levels observed was a result of neurochemical effects of ethanol or a stress response to the injection, a separate group of GH and SI animals were injected with saline after establishing stable baseline levels of DA and NE (n=4 in each group). Samples were collected with 20 min intervals for 100 mins post-injection. Perchloric acid (10 µl) was added to all sample tubes before collection. All samples were frozen at -80° C until analysis.

High Performance Liquid Chromatography—The dialysate samples were analyzed using high performance liquid chromatography (HPLC; ESA/Thermo Scientific, Chelmsford, MA) with electrochemical detection. Samples (10 µl) were injected onto a reverse-phase microbore column (Luna 3µ, C18(2), 100 Å; 100 × 4.60 mm, 3 µm; 00D-4251-E0; Phenomenex, Torrance, CA) for separation. Two separate systems with identical mechanical configurations were used for DA and NE detection. The following mobile phase was used for DA detection: 75 mM monohydrate sodium dihydrogen phosphate, 1.9 mM 1-octanesulfonic acid sodium salt, 100 µl/L triethylamine, 25 µM EDTA, 10% acetonitrile, adjusted to a pH of 3.0 using NaOH. The mobile phase used in the current study was a modified version of the MD-TM mobile phase (70-1332; Thermo Fisher Scientific; Sunnyvale, CA). The mobile phase used for NE detection was modified from a previously published study (Lapiz et al., 2000): 1.65 mM octyl sodium sulfate, 9.2 mM citric acid, 1 ml EDTA, 14% acetonitrile, adjusted to a pH of 5.0 using NaOH.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The western blot data for DAT and NET and the baseline DA and NE data were analyzed using Student's *t*-tests. The microdialysis data were analyzed using a repeated measures two-way analysis of variance (ANOVA) with Bonferroni's *post hoc* analysis. The two independent variables were time and ethanol dose (saline, 1, or 2 g/kg). The dependent variable was either extracellular DA or NE. In a second comparison, the two independent variables were housing and ethanol dose. For this comparison, DA and NE responses were quantified by calculating the area under the curve (AUC) and were analyzed further using a two-way ANOVA followed by Bonferroni's *post hoc* analysis. AUC was calculated individually for each animal and averaged for grouped data. AUC included the entire area on the Y-plane (DA or NE levels) ranging from the baseline (100% mark) to the peak of catecholamine for that particular animal at any given unit in time. This was calculated across the entire X-plane (time), which was held constant for all animals (100 mins). Peaks of DA and NE that did not exceed a 5% increase over baseline were considered noise and were not included in the AUC. All data are reported as mean $\pm$ standard error of the mean. The significance level for all statistical measures was set at $p < 0.05$.

RESULTS

Effects of acute ethanol on extracellular levels of DA

DAT and extracellular DA levels at baseline—DAT protein levels were compared between SI and GH animals using Western blot hybridization. Social isolation resulted in increased DAT protein levels compared to group housing (Fig. 3A; $t_{14} = 3.38$; $p < 0.01$). During the microdialysis experiments, baseline samples were collected for two hours prior to ethanol administration to ensure that the DA level in the BLA was stable and a mean of three samples was used to compare baseline extracellular DA levels in GH and SI rats. Baseline DA levels in SI animals was significantly less than baseline DA levels in GH animals (Fig. 3B; $t_{17} = 2.66$; $p < 0.05$).

Social isolation results in an augmentation of DA responses to a 2 g/kg dose of ethanol in SI rats—A dose comparison within the GH rats showed that 1 and 2 g/kg ethanol increased DA levels in the BLA, however no dose-dependent differences in the increased DA levels were observed (Fig. 4A; $F_{dose(2,15)} = 2.18$; $p > 0.05$). DA levels were also observed to significantly vary with time ($F_{time(7,15)} = 2.75$; $p < 0.05$), but there was no significant interaction between the time and dose variables ($F_{int(14,105)} = 1.12$; $p > 0.05$). In contrast, the dose comparison within the SI rats revealed that 2 g/kg ethanol increased extracellular levels of DA almost three-fold above the 1g/kg dose (Fig. 4B; $F_{dose(2,19)} = 8.67$; $p < 0.01$). The time variable was also observed to be significant ($F_{time(7,19)} = 10.05$; $p < 0.001$) and a significant interaction was observed between the time and dose variables ($F_{int(14,133)} = 3.81$; $p < 0.001$). *Post hoc* analysis revealed that this difference was significantly greater between the 1 and 2 g/kg doses, starting 40 min post ethanol administration and remained elevated until the end of the experiment (100 mins). The difference between saline and 2 g/kg ethanol was significantly different starting 20 min post ethanol administration, which remained elevated until the end of the experiment. A two-way

ANOVA of the AUCs indicated that the augmentation of the DA response was dependent on the housing condition and the ethanol dose (Fig. 4C; $F_{int(2,34)} = 3.37$; $p < 0.05$; $F_{dose(2,34)} = 4.34$; $p < 0.05$; $F_{housing(1,34)} = 1.56$; $p > 0.05$). The *post hoc* analysis confirmed that ethanol (2 g/kg) resulted in enhanced DA responses in the BLA of SI compared to GH rats. Since saline administration did not affect extracellular levels of DA in GH and SI animals, these data show that SI rats have an augmented DA response to ethanol (2 g/kg), primarily due to the neurochemical influence of ethanol on the dopamine system.

Acute ethanol effects on NE in the BLA

NET and extracellular NE levels at baseline—A comparison of NET protein levels between the two groups showed that social isolation did not alter NET protein levels in the BLA (Fig. 5A; $t_{(13)} = 0.71$; $p > 0.05$). In order to confirm that the extracellular NE levels were stable prior to ethanol administration during the microdialysis experiments, three samples preceding ethanol challenge were averaged to obtain baseline NE levels in the BLA of SI and GH rats. A comparison of the two groups showed that isolation rearing did not affect NE levels at baseline (Fig. 5B; $t_{(15)} = 0.47$; $p > 0.05$).

Social isolation results in an augmentation of NE responses following a 2 g/kg dose of ethanol—Acute ethanol did not increase NE levels in GH rats at either dose tested, and no significant difference between the ethanol doses was observed (Fig. 6A; $F_{dose(2,16)} = 2.93$, $p > 0.05$; $F_{time(7,16)} = 0.49$, $p > 0.05$; $F_{int(14,112)} = 0.93$, $p > 0.05$). A dose comparison within SI animals showed that NE levels were significantly elevated following the 2 g/kg dose of ethanol (Fig. 6B; $F_{dose(2,16)} = 10.50$, $p < 0.001$; $F_{time(7,16)} = 5.94$, $p < 0.001$; $F_{int(14,112)} = 3.31$, $p < 0.001$). This effect of ethanol on NE was dose dependent as a 1 g/kg dose of ethanol and saline did not increase NE in the BLA. A two-way ANOVA of the AUC analysis indicated that the robust increase in NE following acute ethanol was dependent on the ethanol dose and the housing condition (Fig. 6C; $F_{dose(2,32)} = 7.77$, $p < 0.001$; $F_{housing(1,32)} = 7.81$, $p < 0.001$; $F_{int(2,32)} = 4.10$, $p < 0.05$). The *post hoc* analysis confirmed that ethanol (2 g/kg) resulted in an augmentation of NE responses in the BLA of SI compared to GH rats. In summary, neither the 1 g/kg nor 2 g/kg dose of ethanol increased NE in the BLA of GH rats, but a significant dose dependent effect was observed in SI rats. Ethanol induced a significant increase in NE in the BLA of SI rats only at the higher dose (2 g/kg) of ethanol. Saline administration did not alter the extracellular levels of NE verifying that the ethanol-induced augmentation of NE observed in SI animals is a result of neurochemical effects of ethanol.

DISCUSSION

The primary goal of the current study was to examine the effects of adolescent social isolation stress on ethanol-induced alterations in DA and NE levels in the BLA. Baseline extracellular levels of DA were significantly decreased in SI compared to GH rats and DAT protein levels were also greater in SI rats. In addition, SI rats exhibited a significantly larger increase in ethanol-stimulated elevation in DA levels in the BLA, relative to GH rats. Social isolation had no significant effect on baseline extracellular NE or NET protein levels. However, ethanol significantly increased BLA NE levels in SI rats but had no effect on NE

levels in GH animals. A mild stressor, injection of the saline vehicle, had no significant effect on DA or NE levels in either GH or SI animals. Together, these data show that isolation rearing during adolescence results in significant alterations in baseline BLA DA levels and significantly augments ethanol-stimulated increases in DA and NE levels in this brain region.

SI-mediated hypodopaminergia and sensitized DA responses to ethanol in the BLA

Social isolation resulted in a significant decrease in baseline DA levels in the BLA. The observation that SI was also associated with an increase in BLA DAT protein content suggests that greater DAT-mediated DA uptake may have contributed to the lower extracellular levels of DA in the BLA of SI animals. Interestingly, several studies have reported that chronic ethanol exposure and withdrawal can also lead to decreased baseline DA levels (Diana et al., 1993; Barak et al., 2011) and increased DAT protein levels (Rothblat et al., 2001; Healey et al., 2008) in the ventral striatum. Moreover, one study reported increased DAT protein levels in the BLA following chronic ethanol exposure in animals with increased anxiety-like behavior (Jiao et al., 2006). Together, these data suggest that the stress associated with either adolescent social isolation or chronic ethanol exposure may lead to a hypodopaminergic state as a result of increased DAT protein levels, thus reducing DA-mediated inhibition of principle neurons in this brain region. Such an effect could promote an increase in BLA excitability, thus contributing to the increase in behavioral risk factors of alcohol addiction observed in SI rats (e.g. increased anxiety-like behavior, increased ethanol self-administration).

A number of prior studies have shown that SI animals exhibit increased ethanol intake and preference compared to GH animals (Wolffgramm and Heyne, 1991; McCool and Chappell 2009, Lopez et al., 2011; Chappell et al., 2013). Therefore, to further examine possible mechanisms that may contribute to increased ethanol drinking behavior, the DA response to an acute ethanol challenge was assessed in the current study. Ethanol (1 g/kg) significantly elevated DA levels in the BLA in both SI and GH animals, although there were no significant differences between the two groups at this dose. In contrast, at a higher dose, ethanol (2 g/kg)-mediated increases in DA levels in the BLA were potentiated in SI compared to GH rats, although the change in absolute DA levels was only slightly higher in SI rats.

Acute ethanol increases the firing rate of VTA DA neurons (Brodie et al., 1999; Ding et al., 2009) which project to the BLA, and SI has recently been shown to increase mGluR and NMDA signaling in VTA DA neurons (Whitaker et al., 2013), possibly explaining the increased ethanol-induced DA release in the BLA of SI animals. It is unlikely that ethanol has its effects directly at the DAT as previous studies show that DA uptake rates do not change following acute ethanol application. Specifically, DAT knockout and wild-type mice have a similar acute ethanol-induced DA increase in the striatum (Mathews et al., 2006) and DA uptake rates are not altered by the application of physiologically relevant doses of ethanol to rat NAc slices (Budygin et al., 2001). In addition, social isolation increases D3 receptor functionality (Phillips et al., 2002) and D3 receptor activation has been shown to increase BLA excitability by inhibiting GABAergic transmission (Diaz et al., 2011).

Therefore, the acute ethanol-associated increase in DA in the BLA may further augment the overall excitability of the BLA leading to increased ethanol-seeking and self-administration.

SI-induced NE responses to acute ethanol in the BLA

The current findings showed that baseline NE levels were not significantly different in SI and GH animals. Moreover, western blot hybridization analysis revealed no differences in NET protein levels in the BLA between SI and GH rats. However, acute ethanol (2 g/kg) challenge induced robust NE responses in SI, but not GH rats, with no group differences observed following an injection of a lower dose of ethanol (1 g/kg).

Stress has profound effects on central nervous system NE signaling, and the nature of these alterations is dependent on the type of stressor used, duration of stress, and the brain region being examined. For example, acute stress increases extracellular NE levels in the hippocampus (Abercrombie et al., 1988), frontal cortex (Rossetti et al., 1990), medial prefrontal cortex (Finlay et al., 1995), hypothalamus (Yokoo et al., 1990), and amygdala (Tanaka et al., 1991). However, chronic stress depletes NE in the hippocampus (Fulford and Marsden, 1997; Spasojevic et al., 2012) and hypothalamus (Fulford and Marsden, 1997). Furthermore, tissue content analysis showed a decrease in NE levels in the NAc (Brenes et al., 2008) and no difference in the cortex, hippocampus, and hypothalamus (Lapiz et al., 2000) following social isolation. Western blot analysis of NET in the BLA in the current study showed no differences in relative NET levels in SI and GH animals. Consistent with the current findings, social isolation also had no effect on NET protein levels in the hippocampus (Spasojevic et al., 2012). Moreover, the present data showed that baseline extracellular NE levels were not significantly different in SI and GH rats. These findings suggest that baseline NE levels are not disrupted by SI in the BLA.

NE has profound effects on BLA excitability via $\alpha 1$ and $\beta 3$ AR-mediated inhibition and $\beta 1/2$ AR-mediated excitation of the BLA (Southwick et al., 1999; Buffalari and Grace, 2007; Silberman et al. 2010; 2012). Specifically, $\beta 3$ ARs enhance lateral paracapsular GABAergic inhibition of BLA pyramidal cells (Silberman et al., 2010), which in turn inhibit the excitability of these neurons (Marowsky et al., 2005). Similarly, $\alpha 1$ AR activation facilitates spontaneous GABA release, further inhibiting the overall excitability of the BLA (Braga et al., 2004). However, chronic stress has been shown to compromise the inhibitory effects of NE, thus shifting the balance in favor of its excitatory effects. For example, chronic restraint stress dramatically reduces $\alpha 1$ AR-mediated facilitation of GABAergic inhibition in the BLA (Braga et al., 2004). Additionally, chronic cold stress exposure increases NE-mediated spontaneous activity of BLA neurons, thus increasing the overall excitability of the BLA (Buffalari and Grace, 2009). This overall increased excitability may facilitate increased reward-seeking behavior and thus the increased ethanol consumption observed in SI animals (Wolfgramm and Heyne, 1991; McCool and Chappell, 2009; Chappell et al., 2013).

In GH animals, the current study found that ethanol (1 and 2 g/kg) had no effect on NE release in the BLA. These data are consistent with others showing no significant changes in NE levels in the BLA following acute ethanol administration in rats housed under standard conditions (Bacopoulos et al., 1978). Following social isolation however, the higher dose of ethanol (2 g/kg) resulted in a robust increase in BLA NE levels that was only

observed in SI animals. Furthermore, NE remained elevated for at least 100 mins. These results suggest that chronic adolescent stress sensitizes the noradrenergic system to ethanol actions within the BLA. This increase in NE transmission in conjunction with the hyperexcitability of the BLA following chronic stress exposure may contribute to increased reward seeking.

BLA and reward-seeking behavior

In an hypodopaminergic state, the activity of the BLA is likely enhanced due to the lack of excitation of local GABAergic interneurons. This increased excitation may further enhance the activity of the glutamatergic projection to the NAc, which is important for increased reward-seeking behavior (Koob, 2006; Stuber et al., 2011). *In vivo* optical inhibition of this projection reduced reward-seeking for sucrose (Stuber et al., 2011). Additionally, silencing the BLA-NAc glutamatergic projection resulted in a lack of cue-induced reinstatement of ethanol-seeking (Sinclair et al., 2012). It is possible that chronic stress, such as periadolescent social isolation, may result in strengthening of this projection as a result of decreased extracellular levels of DA as observed in the current study and others (Heidbreder et al., 2000; Wang et al., 2011). Likewise, behavioral studies show that SI animals self-administer more ethanol and have a greater preference for ethanol over water compared to GH animals (McCool and Chappell, 2009; Chappell et al., 2013). Moreover, as mentioned earlier, D3 receptor functionality is increased following social isolation (Phillips et al., 2002) and D3 receptor activation increases BLA excitability (Diaz et al., 2011). To that effect, systemic administration of D3 antagonists decreases ethanol intake in mice (Leggio et al., 2014) and ethanol preferring rats (Thanos et al., 2005). Specifically, buspirone, an approved drug for anxiety disorders with D3 antagonist properties, decreases ethanol consumption in mice (Leggio et al., 2014). Overall, these data suggest that increasing DA transmission attenuates ethanol seeking, possibly via decreasing BLA excitability.

Previous reports have shown a chronic stress-induced NE-mediated facilitation of BLA excitability (Buffalari and Grace, 2009). Thus the ethanol-induced NE elevation observed only in SI animals in the current study may contribute to the facilitation of reward-seeking behavior in SI animals. Supporting this hypothesis, previous studies report that increased NE transmission via inactivation of $\alpha 2$ ARs potentiates reinstatement of ethanol self-administration, while activating this receptor attenuates ethanol drinking (Le et al., 2005). Additionally, ethanol drinking in alcohol dependent rats is reduced by systemic administration of prazosin, an $\alpha 1$ AR antagonist (Walker et al., 2008). Prazosin (i.p.) also reduces ethanol drinking in stress-induced reinstatement of alcohol seeking in rats (Le et al., 2011). Furthermore, systemic administration of propranolol, a β AR antagonist, reduces operant responding for alcohol (Gilpin and Koob, 2010), as propranolol reduces the overall excitability of BLA, further decreasing reward-seeking behavior.

Sex-dependent differences in stress-induced ethanol intake

We did not examine the effect of SI on catecholamine changes in the BLA of female rats in this study. However, we would expect to observe sex-dependent differences, as there are profound differences between males and females in behavioral responsivity to a wide range of stressors. In contrast to the robust increases in anxiety-like behavior and ethanol intake

induced by this model of early life stress in males, females do not exhibit such changes (Butler et al., 2014; Chappell et al., 2013). However, other kinds of early life stress, such as adolescent social instability, have been shown to increase anxiety-like behaviors in females (McCormick et al., 2013). It would be of interest in future studies to examine ethanol intake and catecholamine dynamics in females under such paradigms.

Conclusions

In conclusion, the current study demonstrated that social isolation results in decreased baseline DA transmission mediated, in part, by an increase in DAT protein levels in the BLA along with a significant increase in ethanol-induced DA and NE levels in this brain region. Although there may be differences in the neurobiological changes associated with contingent vs. non-contingent ethanol exposure, these findings provide important initial evidence that alterations in BLA catecholamine signaling may contribute to the increase in alcohol addiction vulnerability associated with early-life stress. The sensitized ethanol-induced DA and NE responses likely contribute to the increased ethanol intake observed in rodent models of adolescent social isolation, as the imbalance of excitation and inhibition within the BLA results in overall hyperexcitability of BLA glutamatergic efferents, possibly strengthening BLA-NAc connectivity. These findings suggest that medications directed towards reducing NE signaling may prove to be effective treatments of alcoholism, particularly in individuals exposed to early-life stress.

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Figure 1.

A schematic of the experimental paradigm. Male, Long Evans rats arrived at the facility on postnatal day (PD 21) and were maintained in group housing to acclimate for one week. On PD 28, half the rats were housed individually while the other half remained in group housing. Microdialysis and Western blot hybridization experiments were conducted between PD 70 and 110.

Figure 2.
Coronal sections showing microdialysis probe locations measured from bregma.
Microdialysis probes were inserted in the BLA using the Paxinos and Watson (2007) rat atlas.

Figure 3.

DAT and extracellular DA levels at baseline. (A) Western blot analysis revealed increased DAT protein levels in SI compared to GH rats (n=8 in both groups). The inset displays representative immuno blot for DAT protein. (B) SI (n=11) rats had decreased levels of DA at baseline compared to GH (n=8) rats.

Figure 4.

Ethanol-induced DA response in the BLA of group housed (GH) and socially isolated (SI) rats. (A) An ethanol dose comparison within the GH animals showed that the two doses of ethanol have comparable effects on DA release in the BLA. Saline administration did not alter DA levels. GH: 1 g/kg ethanol, $n=7$; 2 g/kg ethanol, $n=7$; saline, $n=4$. (B) An ethanol dose comparison within the SI animals demonstrated that both 1 and 2 g/kg of ethanol increased DA levels in the BLA, but the higher dose (2 g/kg) of acute ethanol resulted in an augmentation of the DA response. Saline administration did not alter the DA levels. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (1 vs. 2 g/kg); , $p < 0.05$; , $p < 0.01$; , $p < 0.001$ (saline vs. 2 g/kg). SI: 1 g/kg ethanol, $n=8$; 2 g/kg ethanol, $n=10$; saline, $n=4$. (C) A two-way ANOVA between housing and dose comparison showed that social isolation results in an augmentation of ethanol-induced DA release only at a higher dose (2 g/kg) of ethanol and only in SI animals, confirming that both housing and dose affect DA responses to ethanol. *, $p < 0.05$.

Figure 5. NET and extracellular NE levels at baseline. (A) Western blot analysis of NET protein revealed no significant differences between SI (n=8) and GH rats (n=7). The inset displays representative immuno blot for NET protein. (B) Extracellular levels of NE at baseline in the BLA of SI (n=9) and GH (n=8) animals. Social isolation did not significantly alter the extracellular levels of NE in the BLA.

Figure 6.

Ethanol-induced NE response in the BLA of group housed (GH) and socially isolated (SI) animals. (A) A dose comparison within the GH rats did not show a significant difference at saline, 1 or 2 g/kg ethanol dose. GH: 1 g/kg ethanol, $n=7$; 2 g/kg ethanol, $n=8$; saline $n=4$. (B) Acute ethanol (2 g/kg but not 1 g/kg or saline) resulted in an augmentation of NE levels in the SI animals. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (1 vs. 2 g/kg); , $p < 0.05$; , $p < 0.01$ (saline vs. 2 g/kg). SI: 1 g/kg ethanol, $n=7$; 2 g/kg ethanol, $n=8$; saline, $n=4$. (C) A two-way ANOVA analysis of dose and housing effects on the ethanol-induced NE response showed that a higher dose of ethanol (2 g/kg) induced and increased NE response in the SI animals. ***, $p < 0.001$.