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Rescue of glutamate transport in the lateral habenula alleviates depression- and anxiety-like behaviors in ethanol-withdrawn rats

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Abstract

Alcoholism and psychiatric disorders like depression and anxiety are often comorbid. Although the mechanisms underlying this comorbidity are unclear, emerging evidence suggests that maladaptation of the glial glutamate transporter GLT-1 may play a role. Findings from animal and human studies have linked aversive states, including those related to drugs of abuse and depression, to aberrant activity in the lateral habenula (LHb). The relationship between GLT-1 maladaptation, LHb activity, and abnormal behaviors related to alcohol withdrawal, however, remains unknown.

Here we show that dihydrokainic acid (DHK), a GLT-1 blocker, potentiated glutamatergic transmission to LHb neurons in slices from ethanol naïve rats; this potentiation, though, was not observed in slices from rats withdrawn from repeated *in vivo* ethanol administration, suggesting reduced GLT-1 function. Furthermore, GLT-1 protein expression was reduced in the LHb of withdrawn rats. This reduction was restored by systemic administration of ceftriaxone, a β -lactam antibiotic known to increase GLT-1 expression. Systemic ceftriaxone treatment also normalized the hyperactivity of LHb neurons in slices from withdrawn rats, which was reversed by bath-applied DHK. Finally, systemic administration of ceftriaxone alleviated depression- and anxiety-like behaviors, which was fully blocked by intra-LHb administrations of DHK, suggesting that GLT-1's function in the LHb is critical. These findings highlight the significant role of LHb astrocytic GLT-1 in the hyperactivity of LHb neurons, and in depressive- and anxiety-like behaviors during ethanol withdrawal. Thus, GLT-1 in the LHb could serve as a therapeutic target for psychiatric disorders comorbid with ethanol withdrawal.

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Authors contribution

SK conceived and designed all molecular and behavioral experiments. SK and JL performed stereotaxic surgery, behavioral experiments, and western blotting. SK performed electrophysiology. SK and JY performed statistical analysis, prepared figures and wrote the manuscript. All authors contributed to reviewing and editing the manuscript.

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Keywords

Lateral Habenula; alcohol withdrawal; GLT-1; anxiety; depression; glial

1. Introduction

Alcohol abuse disorders (AUDs) and psychiatric disorders such as depression and anxiety are common comorbid conditions (Driessen et al., 2001; Hasin and Grant, 2002; Haver and Dahlgren, 1995; Kessler et al., 1997; Schuckit et al., 1997), yet the mechanisms underlying this comorbidity have not been fully explored. The lateral habenula (LHb), a small epithalamic brain region, reciprocally interacts with several brain regions that are important for motivation and reward, including the serotonergic raphe nuclei, the noradrenergic locus coeruleus, and the dopaminergic ventral tegmental area (Geisler and Trimble, 2008; Hikosaka et al., 2008). The LHb has been considered a key structure in the pathophysiology of depression (Kiening and Sartorius, 2013; Sartorius and Henn, 2007). The evidence from genetic modification further supports this with findings associating depressive-like behaviors with hyperexcitability of LHb neurons (Cui et al., 2014; Li et al., 2011a; Shabel et al., 2014).

Addiction has been linked to the imbalance between synaptic and non-synaptic glutamate, termed glutamate homeostasis (Kalivas, 2009). In the tripartite synaptic architecture, astrocytic processes minimize glutamate overflow by rapidly uptaking glutamate, thereby permitting efficient synaptic signaling (Perea et al., 2009; Rusakov et al., 2011). The glial excitatory amino acid transporter 2 (EAAT2/GLT-1) is particularly important in reducing synaptic glutamate overflow (Rothstein et al., 2005). Besides its well-known function as a scavenger to prevent glutamate spillover into the extrasynaptic space (Danbolt, 2001; Ginsberg et al., 1995; Mitani and Tanaka, 2003), the GLT-1 also acts as an important regulator of synaptic glutamatergic transmission (Ayers-Ringler et al., 2016; Murphy-Royal et al., 2015; Trantham-Davidson et al., 2012; Weng et al., 2007), thereby neuronal activity (Cui et al., 2014). Maladaptation of GLT-1 has been suggested as a contributor to pathological conditions related to alcohol withdrawal. The GLT-1 expression is reduced in the reward-related regions of alcohol-dependent rodents (Abulseoud et al., 2014; Alhaddad et al., 2014). Systemic administration of compounds that potentially increase GLT-1 expression such as ceftriaxone, riluzole, and MS-153 reduces the elevated alcohol consumption seen in alcohol-dependent rodents (Abulseoud et al., 2014; Alhaddad et al., 2014; Besheer et al., 2009; Sari et al., 2011). These studies, together with the finding that GLT-1 maladaptation in the LHb induces depression-like behaviors (Cui et al., 2014), support a potential role for LHb GLT-1 in depressive symptoms occurring during alcohol withdrawal. In the current rat study, we tested the hypotheses that during alcohol withdrawal, astrocyte GLT-1 maladaptation contributes to the hyperexcitability of LHb neurons (Li et al., 2016) and the depressive- and anxiety-like behaviors, using a combination of electrophysiological, pharmacological, molecular, histological and behavioral approaches.

2. Materials and Methods

All procedures were by National Institutes of Health's guidelines and with the approval of the Animal Care and Utilization Committee of Rutgers, the State University of New Jersey, New Jersey Medical School.

2.1. In vivo systemic administration of ethanol and ceftriaxone

Experiments were performed on a total of 112 male Sprague Dawley rats (6–7 weeks old at the start of the experiment, Harlan Laboratories). These rats were group housed in ventilated Plexiglas cages (three per cage) in a climate-controlled room (20–22 °C) with a standard 12-h light/12-h dark cycle (light-off at 19:00) with access to food and water *ad libitum*. Rats were intraperitoneally (i.p.) injected with ethanol (2g/kg in 20% v/v saline), or an equivalent volume of saline twice daily for 10 days. A subgroup of rats from each group also treated with ceftriaxone (200mg/kg/day, i.p.) for the last 5 days during the 10-day ethanol (or saline) administration. Behavioral tests, protein quantifications, and *ex vivo* electrophysiological measurements were performed 24 hours after the last injection.

2.2. Brain slice preparation and electrophysiology

Rats were sacrificed under anesthesia using ketamine/xylazine (80 mg/10 mg/kg, i.p.). Coronal epithalamic slices (300 μ m) were prepared as described (Kang et al., 2017). Briefly, the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in millimolar): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 0.4 l-ascorbate and 11 glucose, and saturated with 95% O₂/5% CO₂ (carbogen). Slices were then incubated >1 hour at 24–25°C in carbogenated aCSF. Electrophysiological events were recorded at ~33°C, with aCSF perfused at 2 ml/min (Kang et al., 2017; Zuo et al., 2017). Patch pipettes (6–8 M Ω) were filled with internal solutions containing (in millimolar): 130 K-methanesulfonate, 10 KCl, 4 NaCl, 10 HEPES, 0.5 EGTA, 2 Mg-ATP and 0.2 Na₂GTP. The spontaneous firing was recorded using the loose-patch cell-attached technique. Miniature EPSCs (mEPSCs) were recorded in the presence of TTX (0.5 μ M). Evoked EPSCs (eEPSCs) were elicited through a bipolar stainless steel electrode placed 200 μ m from the recording site. Access resistance was monitored by a step of –10 mV (0.1 Hz) and experiments were discarded if the access resistance increased >20%. The decay of EPSCs was determined using the “fit exponential” tool in Clampfit (Molecular Devices Union City, CA, USA) based on the average of consecutive EPSCs during 3 minutes for each condition. The kinetics of EPSC decay was best fit by a single exponential (Hendricson et al., 2004; Napier et al., 2012; Takatsuru et al., 2006). Charge transfer was calculated by integrating the area under the EPSC waveform (Li et al., 2015).

We measured neuronal activity in the LHb, particularly in the medial subdivision, where neurons project primarily to the dorsal and median raphe (Proulx et al., 2014; Quina et al., 2015) regions that control serotonin release and have been implicated in generalized anxiety and depression in rats (Sena et al., 2003; Spiacci et al., 2012; Zhao et al., 2015).

2.3. Stereotaxic surgery and postsurgical care

Cannulae implantation was performed using a stereotaxic apparatus (Kopf, Tujunga, CA) under isoflurane (Ohio medical Products, Gurnee, IL) anesthesia as described (Kang et al., 2017). Bilateral guide cannulae (C235G-3.8/Spc 22 gauge; Plastics One, Roanoke, VA) were placed 1 mm above the LHb (−3.7 AP, ±0.75 ML, −4.0 DV). Following a 5 to 7 day recovery period, subjects received an injection of ethanol or equivalent volumes of saline. Before LHb infusion, animals were taken from the colony, brought to the experimental room, and handled for 5 minutes per day until the experimental day during which animals became accustomed to the experimenter, the experimental room, and the manipulation procedure. Histological verification was carried out as described (Zuo et al., 2017). Data obtained at injection sites outside the LHb were excluded from analysis.

2.3.1. Intra-LHb infusions of dihydrokainic acid—A selective GLT-1 inhibitor, dihydrokainic acid (DHK, 12.5 nmol dissolved in aCSF) or vehicle was injected into the LHb (300 nl/side). This amount of DHK has been shown to effectively modulate depression- and anxiety-like behaviors in rats given intra-central-amygdala administrations (John et al., 2015). LHb injections were given 30 minutes before the elevated plus maze, or the 2nd 5-minute forced swim behavioral testing (Li et al., 2011b). Intra-LHb administration of drugs was as described (Zuo et al., 2017): The injector extended 1.0 mm beyond the guide cannulae tip (Fig. 4C), and infusion lasted for 60 seconds. Injectors were left in place for an additional 60 seconds to allow for diffusion.

2.4. Forced swim test

Depression-like behavior was measured with the forced swim test (FST) (Barichello et al., 2016; Slattery and Cryan, 2012) that includes two sessions: an initial 15-minute pretest and the 5-minute test 24 hours later (Kang et al., 2010; Yankelevitch-Yahav et al., 2015). During the pretest, rats are placed in a clear Plexiglas cylinder (51 cm long by 25 cm diameter) filled with water (25±1 °C, 30 cm deep). After the pretest, rats are dried in a warm environment for approximately 30 minutes before being returned to their home cages. The cylinders are rinsed and filled with fresh water between tests. The test sessions are carried out 24 hours later under the same conditions and video-recorded for subsequent scoring. Behavioral activity counts are scored at 5-second intervals throughout the test (Detke et al., 1995). Two independent observers blind to the animal treatments count the amount of time spent immobile, and the latency to immobility. Rats are judged to be immobile whenever they remain floating in the water without struggling and only make movements necessary to remain afloat. Latency to immobility is defined as the time between an introduction to the chamber and the first instance of immobility (Carlezon et al., 2001).

2.5. Elevated plus maze test

Anxiety levels were evaluated with the elevated plus maze (EPM) (Ordookhani et al., 2007). The maze consists of four equally illuminated plastic arms (two open without walls and two enclosed by 30 cm high walls) that radiate at a 90-degree from a central platform 50 cm above the floor. The maze is placed in an isolated, silent, and a dimly lit room with an illumination of 100–140 lux. The animals were released from the central area, and the

behaviors were recorded for 5 minutes with a camera. The maze was cleaned with ethanol and water between each trial. The time spent in open arms, the entries into open arms and the total distance traveled was measured using Smart (ver. 3.0, Pan Lab Harvard Apparatus, Barcelona, Spain).

2.6. Immunofluorescence

Anesthetized rats were transcardially perfused with saline followed by 4% paraformaldehyde (PFA). After post-fixation (overnight, at 4 °C) in 4% PFA, the cryoprotected tissue was sliced (at 30- μ m) using a microtome (Microm HM550, Walldorf, German). Primary antibodies against NeuN (1:1000, Millipore, Billerica, MA), GLT-1 (1:1000, Millipore; 1:1000, Abcam, Cambridge, MA), S100 (1:1000, sigma), and GFAP (1:500, Millipore) were used. Goat anti-rabbit IgG Cy2, and Goat anti-mouse IgG Cy3 antibodies (Jackson Immuno-Research, West Grove, PA) were used at 1:400 dilutions. Control experiments showed no interaction between secondary antibodies, showed species specificity for secondary antibodies, and showed no background staining by secondary antibodies. Immuno-positive cells were visualized with a CCD camera connected to a Nikon Eclipse 80i (Nikon, Melville, NY). The LHb region was identified as described (Aizawa et al., 2012).

2.7. Western blot analysis

To measure the total protein expression of glutamate transporters in the LHb, tissues were punched with 16 gauge syringe ($\varnothing$ 1.194 mm) and homogenized in ice-cold RIPA buffer (Sigma Aldrich, St. Louis, MO) containing 50 mM NaF, 1 mM Na_3VO_4 and a proteinase inhibitor cocktail (Sigma Aldrich). The lysates were separated by SDS-PAGE and probed by immunoblotting with the following primary antibodies: anti-GLT-1 (EAAT2, 1:800, 60 kDa, Abcam), anti-GLAST (EAAT1, 1:800, 60 KDa, Abcam), anti-EAAC1 (EAAT3, 1:800, 57 KDa, Thermo Fisher) or anti- β -actin antibody (1:5000, 42 kDa, BioRad, Hercules, CA), overnight at 4 °C. After washing with TBST, membranes were incubated for 1 hour with Goat anti-rabbit IgG-HRP conjugated antibody (1:2000, Jackson Immuno-Research, West Grove, PA) at room temperature and developed with chemiluminescence reagents (Perkin Elmer, Waltham, MA). Total protein content loaded was then normalized by the GAPDH.

2.8. Drugs

Most reagents, including dihydrokainic acid (DHK), ceftriaxone (CEF), tetrodotoxin (TTX), 6,7-dinitroquinoxaline-2, 3-dione (DNQX), and DL-2-amino-5-phosphono-valeric acid (AP-5) were from Sigma (St. Luis, MO, USA); DI-threo-benzyloxy aspartate (TBOA) from Tocris (Bristol, United Kingdom), ethanol (95%, stored in glass bottles) from Pharmacop Products (Brookfield, CT, USA).

2.9. Data analysis and statistics

All data are expressed as mean $\pm$ s.e.m. To calculate the percent change in excitatory post-synaptic current (EPSC) or firing frequencies for a given cell, baseline recordings during a 3-minute period before drug application were averaged and normalized to 100%. Statistical significance was assessed using an unpaired or paired two-tailed Student's *t*-test, a one-way

ANOVA with *post hoc* test for multiple group comparisons, or a Kolmogorov-Smirnov (K-S) test, where appropriate. Values of $p < 0.05$ were considered significant.

3. Results

3.1. The function of glial excitatory amino acid transporter 2 (GLT-1) is reduced in the LHb of ethanol-withdrawn rats

To determine whether the function of GLT-1 has been changed following repeated cycles of ethanol administration and withdrawal, we compared the effects of dihydrokainic acid (DHK), a selective GLT-1 inhibitor in the LHb neurons from rats of ethanol-naïve (CTRL) and of ethanol withdrawal (Post-EtOH). In general agreement with a previous study showing that inhibition of GLT-1 increases the decay time of spontaneous EPSCs (sEPSCs) (Murphy-Royal et al., 2015), bath-applied DHK (100 μ M) significantly increased the decay time and charge transfer of the sEPSCs (Fig. 1A, B), the miniature EPSCs (mEPSCs) (Fig. 1C, D), as well as the evoked EPSCs (eEPSCs) of LHb neurons of CTRL rats (Decay time CTRL: paired *t*-test, $p = 0.0018$ for sEPSCs, $p = 0.0386$ for mEPSCs, $p = 0.0313$ for eEPSCs; Charge transfer: paired *t*-test, $p = 0.023$ for sEPSCs, $p = 0.019$ for mEPSCs, $p = 0.016$ for eEPSCs, Fig. 1). Bath-applied TBOA, a non-specific inhibitor of glutamate transporters also increased the decay time and charge transfer of sEPSCs in the LHb neurons of CTRL rats (Decay time: paired *t*-test, $p = 0.0227$; Charge transfer: paired *t*-test, $p = 0.0335$, Fig. 1B). In contrast, DHK did not alter the decay time and charge transfer of sEPSCs in LHb neurons of Post-EtOH rats (Post-EtOH, Decay time: paired *t*-test, $p = 0.5760$, Charge transfer: paired *t*-test, $p = 0.067$; Fig. 1A, B). DHK did not alter sEPSC amplitude of LHb neurons of both CTRL and Post-EtOH rats (paired *t*-test, CTRL: before $[23.0 \pm 1.5]$ versus DHK $[23.7 \pm 1.5]$ pA, $p = 0.763$, Post-EtOH: before $[25.5 \pm 3.8]$ versus DHK $[23.4 \pm 2.6]$ pA, $p = 0.647$; Fig. 1B, D, F). DHK or TBOA increased sEPSC frequency (DHK: paired *t*-test, $p = 0.005$; CTRL versus Post-EtOH, Unpaired *t*-test, $p = 0.018$; TBOA: paired *t*-test, $p = 0.0309$, Fig. 1A, B). However, DHK did not alter mEPSC frequency (Paired *t*-test, $p = 0.3648$, Fig. 1C, D).

3.2. Systemic ceftriaxone treatment rescues GLT-1 protein expression in the LHb of ethanol-withdrawn rats

GLT-1 is predominantly expressed in astrocytes, and at lower levels in the excitatory terminals of neurons in some brain regions (Petr et al., 2015). As shown in the immunofluorescent staining data in figure 2B–D, GLT-1 is primarily expressed in astrocytes in the LHb, and to a greater extent than those in the Medial Habenula (MHb) (Fig. 2A–E) (Cui et al., 2014). To determine whether the GLT-1 expression in the LHb was altered during ethanol withdrawal, we quantified GLT-1 expression by western blotting. Quantification analysis indicates no difference between GLT-1 expression in the LHb of CTRL rats with and without saline injection (Unpaired *t*-test, No injection versus CTRL, $p = 0.7411$, Fig. 2F), indicating that the procedure of systemic administration did not change LHb GLT-1 expression. However, the GLT-1 expression was significantly lower in the LHb of Post-EtOH rats relative to CTRL rats (One-way ANOVA, $F_{(2,9)} = 10.73$, $P = 0.0041$; *post-hoc* $p = 0.0046$, Fig. 2G).

Ceftriaxone, a β -lactam antibiotic, has been shown to upregulate GLT-1 expression in several brain areas, including those related to reward (Abulseoud et al., 2014; LaCrosse et al., 2016; Miller et al., 2008; Rothstein et al., 2005; Sari et al., 2010; Trantham-Davidson et al., 2012). To determine whether ceftriaxone can rescue the reduction of LHb GLT-1, we treated rats with ceftriaxone (200 mg/kg/day for 5 days, i.p.). Ceftriaxone treatment significantly increased GLT-1 expression in the LHb of Post-EtOH rats (*post-hoc* $p=0.0191$, Fig. 2G). Expression of GLAST (EAAT1, one way ANOVA, $F_{(2,9)}=0.12$, $P=0.8884$, Fig. 2H) and EAAC1 (EAAT3, one way ANOVA, $F_{(2,9)}=0.1775$, $P=0.8403$, Fig. 2I) in the LHb was not altered in either Post-EtOH or Post-EtOH+CEF rats.

3.3. Systemic administration of ceftriaxone normalizes firing of LHb neurons in ethanol-withdrawn rats

Reduced glutamate uptake due to down-regulation of GLT-1 may contribute to the hyperexcitability of LHb neurons (Cui et al., 2014). We thus examined the effects of DHK on the activity of LHb neurons. Bath-applied DHK (100 μ M) substantially accelerated spontaneous firing of LHb neurons in slices of CTRL rats (by $72.8 \pm 17.3\%$ of baseline, One-way ANOVA, $F_{(2,27)}=6.207$, $P=0.0061$; *post-hoc* $p=0.0077$, Fig. 3A–C). This effect of DHK is probably mediated by glutamate transmission, as it was substantially attenuated by DNQX (20 μ M) and AP5 (50 μ M) (Unpaired t -test, $p=0.0011$, Fig. 3D–F). Notably, bath-applied DHK had no significant effect on the firing rate of LHb neurons in slices of Post-EtOH rats (by $7.9 \pm 4.8\%$ of baseline; *post-hoc* $p=0.9634$, Fig. 3A–C), but it accelerated that of LHb neurons of the Post-EtOH rats that received systemic ceftriaxone (by $67.7 \pm 13.5\%$ of baseline, *post-hoc* $p=0.0228$, Fig. 3A–C).

To determine whether other glutamate transporters, in addition to GLT-1, also play a role in the increased excitability of LHb neurons during ethanol withdrawal, in the presence of DHK, we added TBOA (100 μ M), a nonspecific inhibitor of glutamate transporters to the bath. The addition of TBOA significantly increased LHb firing rate of both CTRL and Post-EtOH rats (CTRL, by $46.5 \pm 11.6\%$ of baseline, paired t -test, $p=0.0280$; Post-EtOH, by $40.7 \pm 11.6\%$ of baseline, paired t -test, $p=0.0395$; CTRL versus Post-EtOH, Unpaired t -test, $p=0.7357$, Fig. 3G). These results suggest specific down-regulation of GLT-1 during ethanol withdrawal.

3.4. In ethanol-withdrawn rats, systemic administration of ceftriaxone prevents depression-like behaviors, which are restored by intra-LHb administration of dihydrokainic acid

Having found that during ethanol withdrawal LHb GLT-1 protein expression and function are reduced and that systemic administration of ceftriaxone can rescue this reduction, we tested the hypothesis that GLT-1 down-regulation contributes to the depressive-like behaviors. As expected, in the forced swim test, the total immobility time was higher (Fig. 4A) and the latency to immobility was shorter (Fig. 4B) in Post-EtOH rats than in CTRL rats, and these changes were alleviated after systemic ceftriaxone treatment (Fig. 4A, B).

Because ceftriaxone has biological actions beyond increasing GLT-1 synthesis and expression, to determine whether GLT-1 in LHb mediates the effects of ceftriaxone, we

compared the effects of ceftriaxone in the absence and presence of intra-LHb DHK. Intra-LHb DHK largely reduced ceftriaxone's effects (One-way ANOVA, immobility time: $F_{(3,29)}=11.91$, $P<0.0001$ and latency to immobility: $F_{(3,29)}=3.062$, $p=0.0437$, Fig. 4A, B), indicating that GLT-1 mediates most of the ceftriaxone's effects in the LHb.

3.5. In ethanol-withdrawn rats, systemic ceftriaxone administration prevents anxiety-like behaviors, which are restored by intra-LHb administration of dihydrokainic acid

We also examined the role of LHb GLT-1 in anxiety-like behaviors. As shown in Figure 5, the time spent in open arms and entries into open arms of the elevated plus maze (EPM) was significantly reduced for Post-EtOH rats, suggesting increased anxiety levels (Fig. 5A, B). These anxiety-like behaviors were rescued by systemic ceftriaxone treatments but were restored by intra-LHb infusion of DHK (One-way ANOVA, time in open arms: $F_{(3,31)}=5.612$, $p=0.0034$, open arm entries: $F_{(3,31)}=7.987$, $p=0.0004$, Fig. 5A, B). The total distance traveled was similar among groups (One-way ANOVA, $F_{(3,31)}=0.2019$, $p=0.8943$, Fig. 5C).

These results suggest that the GLT-1 reduction in the LHb contributes to the depression- and anxiety-like behaviors in Post-EtOH rats without altering locomotion.

4. Discussion

We report here the data for the effects of ceftriaxone, a beta-lactam antibiotic known to increase GLT-1 expression, to induce normalization of the hyperactivity of LHb neurons in slices of ethanol-withdrawn rats, and to alleviate depression- and anxiety-like behaviors in withdrawn rats. Moreover, these effects could be abolished by dihydrokainic acid (DHK), a selective GLT-1 inhibitor, administered directly into the LHb. These results suggest that LHb hyperactivity during alcohol withdrawal is a result of reduced GLT-1, an effect that manifests phenotypically as anxiety and depression.

In the current study, we first showed that DHK increases the decay time of sEPSCs, mEPSCs and eEPSCs in LHb neurons of CTRL rats, this is consistent with recent evidence for a physiological role of GLT-1 in shaping synaptic transmission (Murphy-Royal et al., 2015). Interestingly, DHK had no significant effect on LHb sEPSCs of Post-EtOH rats, suggesting a compromised functioning of GLT-1. Our western blot data showing that GLT-1 protein expression was reduced and that systemic administration of ceftriaxone rescued this reduction supported this possibility. Also, DHK significantly increased firing in LHb neurons of CTRL rats but not in LHb neurons of Post-EtOH rats. However, in Post-EtOH rats treated with ceftriaxone, DHK increased the firing. The effect of DHK on LHb firing is largely mediated by the elevated glutamate transmission since the antagonists of AMPA and NMDA receptors substantially reduced it. However, other contributors, such as reduced GABAergic transmission, or a shifted of co-release of GABA and glutamate (Meye et al., 2016) should also be considered.

Counteracting LHb hyperexcitability has been considered a promising approach in the development of antidepressants (Grandy et al., 2013; Lecca et al., 2014; Li et al., 2011a; Proulx et al., 2014; Sartorius and Henn, 2007; Sartorius et al., 2010). Our data here suggest

that GLT-1 downregulation-induced LHb hyperactivity in alcohol-withdrawn rats may contribute to the depressive-like behaviors. This is consistent with previous preclinical evidence showing that GLT-1 contributes to the pathophysiology of mood disorders, including those related to AUDs. For example, GLT-1 expression in the nucleus accumbens of rodent is reduced after ethanol withdrawal (Abulseoud et al., 2014; Alhaddad et al., 2014). LHb GLT-1 knockout induces depressive-like behaviors (Cui et al., 2014); and pharmacological inhibition of GLT-1 in the central amygdala induces both anxiety- and depressive-like symptoms (John et al., 2015). Through a combination of electrophysiological, biochemical, pharmacological and behavioral approaches, our study provides strong evidence for the antidepressant and anxiolytic effects of GLT-1 up-regulation. GLT-1 is crucial in uptaking synaptic glutamate; so increased GLT-1 expression in the LHb of Post-EtOH rats could counteract the hyperexcitability of LHb neurons. The implications of this finding are significant, as they strengthen the idea that LHb hyperexcitability contributes to the pathophysiology of ethanol withdrawal, and they introduce the role of astrocytic GLT-1 in this process.

However, the ceftriaxone-induced increase in GLT-1 expression may not manifest uniformly throughout the brain (Abulseoud et al., 2014; Alhaddad et al., 2014), although GLT-1 could be crucial in modulating glutamate levels (Mitani and Tanaka, 2003; Tanaka et al., 1997) and neuronal excitability (Cui et al., 2014). Increased glutamate signals in the LHb can induce symptoms of depression such as learned helplessness, social avoidance, disrupted circadian rhythms, and sleep disturbance in rats (Cui et al., 2014; Grandy et al., 2013). This finding not only adds to growing evidence that increased glutamate levels in the LHb may induce some such symptoms but also highlight the LHb as a target area for clinical implications of the glutamate homeostasis theory in addiction and affective disorders. It would be worthwhile, however, to examine additional mechanisms for reductions in glia function of alcoholics, as these are potential pathophysiological features associated with the symptoms of both depression and anxiety (Di Benedetto and Rupprecht, 2013; Miguel-Hidalgo et al., 2010).

Another major finding of the present study is that disruption of astrocytic glutamate uptake in the LHb engenders profound anxiogenic effects. Previous studies have suggested ethanol withdrawal-induced anxiogenic symptoms (Gibula-Bruzda et al., 2015; Gilpin et al., 2015; Kliethermes, 2005; McBride, 2002). Our findings that ceftriaxone rescues depression- and anxiety-like behaviors in Post-EtOH rats, and that intra-LHb DHK administration attenuates this rescue suggest that the down-regulation of astrocytic GLT-1 activity in LHb neurons is a key factor in the observed withdrawal symptoms. This GLT-1 dysfunction mimics an astrocyte pathology observed in patients with depression (Rajkowska and Stockmeier, 2013). A major implication of these findings is that restoration or enhancement of astrocytic function may represent an effective therapy for patients experiencing anxiety comorbid with depression, or depression and anxiety comorbid with ethanol withdrawal.

Although DHK selectively targets GLT-1, site-specific DHK infusion can have effects besides increasing glutamate. For example, intra-hippocampus DHK in high dose affects extracellular phosphoethanolamine levels (Lehmann and Hamberger, 1983). Since alterations in phospholipid metabolism are known to be involved in the pathophysiology of

depression and anxiety (Modica-Napolitano and Renshaw, 2004; Muller et al., 2015), we are unable to entirely rule out the other potential effects of DHK, and their effect on the observed behavioral consequences. This probability, however, is minuscule because of the low dose used, which is over 100 fold smaller than that known to increase phospholipid levels. Therefore, it is more likely that DHK infusions increased LHb activation solely by its inhibition of GLT-1. Altogether, our findings are consistent with the phenomenon of tonic hyperactivation (Morris et al., 1999; Roiser et al., 2009) and glial deficits (Cui et al., 2014) observed in the LHb of patients with major depression disorder and depression-like rodent models.

Converging lines of evidence indicate that ceftriaxone that could penetrate the blood-brain barrier increases GLT-1 expression and function in rodents (Knackstedt et al., 2010; Miller et al., 2008; Rothstein et al., 2005) as well as in primary human astrocytes (Lee et al., 2008). Evidence suggests that a plausible mechanism by which ceftriaxone-induces GLT-1 upregulation is via activation of the transcription factor Nuclear Factor- κ B (NF κ B) (Lee et al., 2008). NF κ B signaling activates a GLT-1 promoter and induces p65 translocation through I κ B proteasomal degradation. Additionally, modulation of astrocytic GLT-1 expression by a mammalian-target-of-rapamycin (mTOR)-Akt-NF κ B signaling cascade has also been suggested (Arami et al., 2013; Wu et al., 2010). This cascade may be implicated in ceftriaxone's antidepressant-like effects since the mTOR pathway has been suggested as a target in the pathophysiology of depression (Ignacio et al., 2015). Thus, the NF κ B pathway may have a critical role in GLT-1 up-regulation after ceftriaxone treatments.

Interestingly, DHK increased sEPSC frequency but not the mEPSC frequency of LHb neurons in slices of CTRL rats. These results suggest that DHK acts on the action potential-driven events due to intrinsic properties of presynaptic cells and network activity. Indeed, a previous rat study found that DHK induced a persistent N-ethyl-D-aspartate receptor-mediated inward current in the hypothalamic neurons (Potapenko et al., 2012). The lateral hypothalamus, a brain area, normally cut together in the same coronal sections with the LHb, sends robust glutamatergic signals to the LHb (Stamatakis et al., 2016). Notably, DHK did not increase sEPSC frequency in the LHb neurons of Post-EtOH rats. Therefore, it will be interesting to determine whether the GLT-1 in the efferent brain areas of the LHb is also involved in the adaptation occurring during ethanol withdrawal.

One limitation of our study is that ethanol was involuntary experimenter administered, although this administration method has been used widely in alcohol research field (Okamoto et al., 2006; Wang et al., 2012; Wang et al., 2010). It will be interesting to assess if similar changes in the glutamate-associated regulation, as well as the effects of ceftriaxone and DHK observed in the current study, will also be seen in the LHb of rats withdrawn from voluntary ethanol administration. Another limitation of the current study is that we did not study the effects of DHK/ceftriaxone on ethanol drinking.

In conclusion, here we show novel findings regarding the impact of GLT-1 modulation in the LHb in depression- and anxiety-like behaviors during ethanol withdrawal. During withdrawal, GLT-1 expression and function in the LHb are decreased, contributing to enhanced LHb activity. Systemic ceftriaxone treatment rescues not only the expression and

function of LHb GLT-1 but also the depression and anxiety phenotypes. Intra-LHb infusion of a GLT-1 specific inhibitor, DHK, restores the expression of depression and anxiety phenotypes otherwise rescued by ceftriaxone. Collectively, these findings provide crucial insight into the potential clinical strategies that make use of GLT-1 activators, as these may present novel targets for the treatment of alcoholism and its comorbid psychiatric disorders.

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Highlights

- GLT-1 function and expression are reduced in the LHb of ethanol-withdrawn rats
- Ceftriaxone normalizes GLT-1 function and LHb activity of withdrawn rats
- Ceftriaxone prevents depression- and anxiety-like behaviors of withdrawn rats
- Intra-LHb dihydrokainic acid attenuates ceftriaxone's effects on behaviors of rats

Figure 1.

Withdrawal from repeated ethanol exposure attenuates the activity of the glial glutamate transporter GLT-1 in the lateral habenula. (A) Representative sEPSC traces showing the GLT-1 inhibitor, dihydrokainic acid (DHK, 100 μ M) increases the decay time in LHB neurons from ethanol-naïve (CTRL), but not those from withdrawn (Post-EtOH) rats. Scale bar: 500 ms, 20 pA for sEPSC trains; 5 ms for individual sEPSCs. (B1) Bar graphs showing sEPSC decay time, charge transfer, and frequency in CTRL, and in Post-EtOH, in the absence (before) and presence of 100 μ M DHK. Paired *t*-test, **p*<0.05, ***p*<0.01. Numbers of cells are indicated. (B2) Bar graphs showing the percent of changes induced by 100 μ M

DHK in sEPSC decay time, charge transfer, and frequency in CTRL and Post-EtOH. Unpaired *t*-test, **p*<0.05, ***p*<0.01. (B3-F) are data from CTRL rats. (B3) Bar graphs showing TBOA (100 μ M) increases sEPSC decay time, charge transfer, and frequency of LHb neurons. Paired *t*-test, **p*<0.05. *n*_{cells}=6. (C) Representative mEPSC traces in LHb neurons showing the increase in decay time in DHK. Scale bar: 5 ms, 5 pA. (D) mEPSC decay time, charge transfer, and frequency in LHb neurons in the absence and presence of DHK (100 μ M). Paired *t*-test, **p*<0.05, ***p*<0.01. *n*_{cells}=6. (E) Representative traces of evoked EPSCs (eEPSCs) showing DHK (100 μ M) increases decay time in LHb neurons. DNQX completely blocked this current. Scale bar: 5 ms, 50 pA (F) DHK (100 μ M) increases eEPSC decay time and amplitude in LHb neurons. Paired *t*-test, **p*<0.05. *n*_{cells}=7.

Fig. 2.

The reduction of GLT-1 expression in the LHb of Post-EtOH rats is rescued by systemic administration of ceftriaxone (200 mg/kg per day for 5 days, i.p.). (A) Schematic location of LHb and medial habenula (MHb). (B–D) Co-immunofluorescence of GLT-1 and NeuN (Neuronal marker) (B), GLT-1 and S-100 (astrocyte marker) (C), and GLT-1 and GFAP (astrocyte marker) (D) in the Hb. Scale bar: 200 μ m. (E) Schematic location of glutamate transporters in an LHb synapse. (F–I) Western blots (upper) and quantification (lower) showing the expression of GLT-1 (EAAT2, F, G), GLAST (EAAT1, H), and EAAC1 (EAAT3, I) in the LHb among the groups. Numbers inside columns indicate the number of rats. F, Unpaired *t*-test, $p=0.7411$; G–I, One-way ANOVA, $F_{(2,9)}=10.73$, $P=0.0041$; *post-hoc* $*p<0.05$, $**p<0.01$.

Fig. 3.

Systemic administration of ceftriaxone normalizes the activity of LHB neurons during ethanol withdrawal, which is blocked by bath application of DHP. Representative traces (A) and bar graphs (B) showing the increased frequency of spontaneous firing recorded with the cell-attached technique from LHB neurons in brain slices of ethanol-withdrawn rats (Post-EtOH), compared to that of ethanol-naïve (CTRL) rats. Systemic ceftriaxone treatments (200 mg/kg per day for 5 days, i.p.) attenuate the increased firing rate (Post-EtOH+CEF). Bath perfusion of DHP blocks the effects of systemic ceftriaxone treatment on firing. Paired *t*-test, ****p*<0.001. (C) Bar graphs showing percent potentiation by bath application of DHP (100 μ M) in firing rate among the groups. One-way ANOVA, $F_{(2,24)}=5.832$, $P=0.009$; *post-hoc* **p*<0.05, ***p*<0.01. (D–F) in ethanol-naïve rats. (D) Representative traces showing DHP (100 μ M) increases spontaneous firing rate in LHB neuron in the absence or presence of glutamate receptor inhibitors, DNQX (20 μ M) and AP5 (50 μ M). (E) Bar graphs showing the reduction in the firing rate of LHB neurons by DNQX and AP5. $n_{\text{cells}}=5$. Paired *t*-test, **p*<0.05. (F) Bar graphs showing bath application of DHP (100 μ M) increases the spontaneous firing rate in the absence or presence of the DNQX (20 μ M) and AP5 (50 μ M) in LHB neurons. Bath application of DNQX and AP5 blocks the acceleration on firing induced by DHP. Unpaired *t*-test, ***p*<0.01. $n_{\text{cells}}=4/\text{group}$. (G) Bar graphs showing in the presence of both TBOA and DHP, no difference was found in percent changes in firing rates between the LHB neurons of the CTRL and Post-EtOH rats. $n_{\text{cells}}=4/\text{group}$. Paired *t*-test, **p*<0.05 versus basal firing; CTRL versus Post-EtOH, Unpaired *t*-test, *p*=0.7357. n. s. means not significant.

Fig. 4.

Attenuation of depression-like behaviors induced by systemic ceftriaxone is reduced by intra-LHb DHK. (A, B) Data in the Forced swim test. Immobility time was increased (A) and latency to immobility was reduced (B) during withdrawal, which was attenuated by systemic administration of ceftriaxone (200 mg/kg per day for 5 days, i.p.), and intra-LHb infusion of DHK (12.5 nmol, 300 nl/side) reduced the effects of ceftriaxone. Numbers inside columns indicate the number of rats. One-way ANOVA, immobility time: $F_{(3,29)}=11.91$, $P<0.0001$ and latency to immobility: $F_{(3,29)}=3.062$, $P<0.05$; *post-hoc* ** $p<0.01$, *** $p<0.001$. (C) A representative figure showing cannulae implantation above the LHb. Scale bar, 1 mm. (D) Schematic of injection sites. Triangles and circles represent the locations of injector tips in the rats for FST test and EPM test, respectively. Numbers at the right indicate the distance from bregma (mm).

Fig. 5.

Intra-LHb DHK reduces attenuation of anxiety-like behaviors induced by systemic ceftriaxone. (A–D) The measurement of anxiety-like behaviors in elevated plus maze (EPM) test. (A) Representative traces on EPM. Systemic ceftriaxone treatment (200 mg/kg per day for 5 days) ameliorated the reduction of time in open arms (B) and open arm entries (C) during withdrawal. Intra-LHb infusion of DHK (12.5 nmol, 300 nl/side) reduced the effects of ceftriaxone. (D) No changes were observed in total distance traveled. Numbers inside columns indicate the number of rats. One-way ANOVA, time in open arms: $F_{(3,31)}=5.612$, $P=0.0034$, open arm entries: $F_{(3,31)}=7.987$, $P=0.0004$, total distance traveled: $F_{(3,31)}=0.2019$, $P=0.8943$; *post-hoc* * $p<0.05$, ** $p<0.01$, *** $p<0.001$.