Ethanol increases glutamate neurotransmission in the posterior ventral tegmental area of female Wistar rats

Zheng-Ming Ding, Ph.D, **Eric A. Engleman, PhD**, **Zachary A. Rodd, PhD**, and **William J. McBride, PhD**

Institute of Psychiatric Research, Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN 46202

Abstract

Background—The posterior ventral tegmental area (pVTA) mediates the reinforcing and stimulating effects of ethanol (EtOH). Electrophysiological studies indicated that exposure to EtOH increased glutamate synaptic function in the VTA. The current study determined the neurochemical effects of both acute and repeated EtOH exposure on glutamate neurotransmission in the pVTA.

Methods—Adult female Wistar rats were implanted with microdialysis probes in the pVTA. During microdialysis, rats received acute i.p. injection of saline or EtOH (0.5, 1.0 or 2.0 g/kg) and extracellular glutamate levels were measured in the pVTA. The effects of repeated daily injections of EtOH (0.5, 1.0, or 2.0 g/kg) on basal extracellular glutamate concentrations in the pVTA and on glutamate response to a subsequent EtOH challenge were also examined.

Results—The injection of 0.5 g/kg EtOH significantly increased (120–125 % of baseline), whereas injection of 2.0 g/kg EtOH significantly decreased (80% of baseline) extracellular glutamate levels in the pVTA. The dose of 1.0 g/kg EtOH did not alter extracellular glutamate levels. Seven repeated daily injections of each dose of EtOH increased basal extracellular glutamate concentrations (from 4.1 ± 0.5 to 9.2 ± 0.5 μM) and reduced glutamate clearance in the pVTA (from $30 \pm 2\%$ to $17 \pm 2\%$), but failed to alter glutamate response to a 2.0 g/kg EtOH challenge.

Conclusion—The results suggest that the low dose of EtOH can stimulate the release of glutamate in the pVTA, and repeated EtOH administration increased basal glutamate transmission in the pVTA, as a result of reduced glutamate clearance.

Keywords

ethanol; glutamate; microdialysis; ventral tegmental area

Address correspondence to: Dr. Zheng-Ming Ding, Indiana University School of Medicine, Institute of Psychiatric Research, 791 Union Drive, Indianapolis, IN 46202-4887 USA, Phone: 317-274-2336; Fax: 317-274-1365; zding@iupui.edu.

Introduction

The mesolimbic system projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAC) is involved in the reinforcing and rewarding effects of ethanol (EtOH) (Koob and Volkow, 2010; McBride et al., 1999). Systemic administration of EtOH increased dopamine neuronal activity and dopamine levels in the VTA (Campbell et al., 1996; Gessa et al., 1985). Inhibition of VTA dopamine neuronal activity reduced EtOH self-administration (Hodge et al., 1993; Nowak et al., 2000). EtOH can be self-infused into the posterior VTA (pVTA) but not the anterior VTA (Rodd-Henricks et al., 2000; Rodd et al., 2004). Similarly, EtOH stimulates mesolimbic dopamine neurons within the pVTA but not the anterior VTA (Ding et al., 2009b). Furthermore, the pVTA appeared to be a neuroanatomic site supporting EtOH seeking behavior (Hauser et al., 2011).

The VTA receives glutamatergic inputs from various brain regions and glutamate regulates the function of local dopamine neurons (Kalivas, 1993; White, 1996). A number of electrophysiological studies indicate that EtOH can alter VTA glutamate functions. For example, acute administration of EtOH, both in vivo and in vitro, inhibited NMDA receptor activity in the VTA (Allgaier et al., 1999; Steffensen et al., 2000). Both passive administration or chronic voluntary consumption of EtOH increased glutamate synaptic function onto VTA dopamine neurons, indicated by an increased ratio of evoked AMPAR to NMDAR currents (Saal et al., 2003; Stuber et al., 2008). Furthermore, chronic voluntary EtOH drinking enhanced the spontaneous glutamate release in VTA slices (Stuber et al., 2008). Withdrawal from repeated injections of EtOH increased the probability of NMDA receptor-induced burst firing in VTA dopamine neurons (Hopf et al., 2007). At the molecular level, long-term EtOH exposure up-regulated the expression of the NMDA receptor NR1 subunit and the AMPA receptor GluR1 subunit in the VTA (Ortiz et al., 1995). These findings indicate that VTA glutamate transmission plays an important role in the reinforcing effects of EtOH and EtOH self-administration.

Using microdialysis techniques, research has demonstrated the neurochemical effects of EtOH on the mesolimbic glutamate system. In the NAC, acute administration of EtOH produced biphasic changes of extracellular glutamate levels. In general, low to moderate doses of EtOH appeared to increase, whereas high doses of EtOH appeared to decrease extracellular glutamate levels in the NAC (Moghaddam and Bolinao, 1994; Piepponen et al., 2002; Quertemont et al., 2002; Selim and Bradberry, 1996). In addition, chronic EtOH exposure enhanced basal extracellular glutamate levels in the NAC, as revealed with a conventional microdialysis technique (Dahchour and De Witte, 2000). Two studies utilizing quantitative no-net-flux (NNF) microdialysis also indicated increased basal extracellular glutamate concentrations in the NAC after repeated daily i.p. injections of EtOH in Sprague Dawley rats and C57BL/6J mice (Kapasova and Szumlinski, 2008; Melendez et al., 2005) These studies suggested an up-regulation of basal glutamate neurotransmission following a prior EtOH exposure. However, not all studies have observed such an increase (Goulding et al., 2011; Szumlinski et al., 2008). Furthermore, a history of EtOH exposure appears to alter the glutamate response to EtOH. A challenge injection of ethanol (1.5 – 2.0 g/kg) increased extracellular glutamate levels in the NAC in mice repeatedly treated with EtOH but had no effect in naïve mice (Kapasova and Szumlinski, 2008; Szumlinski et al., 2007).

In contrast to the NAC, the neurochemical effects of EtOH on the glutamate system in the VTA have not been as well characterized. Given the important role of the pVTA in mediating the reinforcing and stimulating effects of EtOH (Ding et al., 2009b; Hauser et al., 2011; Rodd-Henricks et al., 2000), the current study examined the effects of acute and repeated EtOH exposure on extracellular glutamate levels in the pVTA using microdialysis techniques. The hypothesis to be tested was that acute administration of ethanol would produce a dose-dependent increase on extracellular glutamate levels in the pVTA, and repeated daily i.p. injections of EtOH would increase basal extracellular glutamate concentrations in the pVTA and enhance glutamate response to a subsequent challenge injection of EtOH.

Methods and Materials

Animals

Adult female Wistar rats (weight 250 to 300 g, Harlan, Indianapolis IN, USA) were housed in pairs in temperature- and humidity-controlled rooms maintained on a reversed 12-hr light-dark cycle (light on at 10:00 p.m.) with food and water available *ad libitum*. Female rats appear to maintain their head size better than male rats for more accurate stereotaxic placements and have been used in several studies requiring accurate placements (Ding et al., 2009b, c; Rodd-Henricks et al., 2000). The estrous cycle was not monitored in the present study. However, counterbalanced experiments were conducted on different days so that any effect of a given phase of the estrous cycle was distributed across experimental conditions. All experimental procedures were conducted during the dark phase. Protocols were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. All experiments were conducted in accordance with principles outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Chemical agents

EtOH (190 proof) was obtained from McCormick Distilling, Weston, MO. O-phthalaldehyde powder and diluents for glutamate derivatization were purchased from Pickering Laboratory, Inc, CA. The salts used in the aCSF and mobile phase were all HPLC grade and obtained from Sigma (St. Louis, MO, USA).

Cannula implantation and probe insertion

Rats were stereotaxically implanted with one 18-gauge guide cannula (Plastics One, Inc., Roanoke, VA, USA) aiming at the pVTA (AP −5.6 mm, ML +2.1 mm, DV −9.0 mm) (Paxinos and Watson, 1998). The cannula was implanted at a 10° angle to the vertical. Rats were individually housed following surgery, and were allowed to recover from surgery for at least 5 days and received daily habituation and handling in the microdialysis chambers. Following the recovery, microdialysis probes (active membrane length 1.5 mm, inner diameter 200 μm, molecular weight cut-off: 13,000, Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA) were constructed (Benveniste and Huttemeier, 1990) and inserted into the pVTA approximately 16–18 hr before the microdialysis, as previously described (Ding et al., 2009a).

Acute effects of i.p. injection of EtOH—General microdialysis followed the procedure described previously (Ding et al., 2009a). Briefly, rats were placed into microdialysis chambers and connected to a Harvard pump with PE20 tubing (inner diameter 0.38 mm, Becton Dickinson & Co., MD, USA). Microdialysis started with a 90-min wash-out period during which artificial cerebrospinal fluid (aCSF: 140.0 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 2.0 mM $Na_2HPO_4 \cdot 7H_2O$, 1.0 mM $MgCl_2$, pH 7.2–7.4) was perfused through probes, followed by the collection of baseline samples. Then, rats were divided into four groups (n = 6–8 / group) and each group received an i.p. injection of saline or one dose of EtOH (0.5, 1.0 or 2.0 g/kg, 15% EtOH in saline). Six samples were collected thereafter. Samples were collected at 20-min intervals at a flow rate of 1.0 μl/min. Samples were frozen immediately on dry ice and were stored at −70°C before analysis.

A separate experiment examined the effects of Ca++ free aCSF on the 0.5 g/kg EtOH-induced alteration of extracellular glutamate levels in the pVTA. Microdialysis started with the perfusion of Ca++ free aCSF (140.0 mM NaCl, 3.0 mM KCl, 2.0 mM $Na_2HPO_4 \cdot 7H_2O$, 2.2 mM $MgCl_2$, 1 mM EGTA, pH 7.2–7.4); baseline levels stabilized approximately 80 min after the perfusion of Ca++ free aCSF (see results on page 11). After the collection of three baseline samples, rats (n = 7) received i.p. injection of EtOH (0.5 g/kg) and six samples were collected following the EtOH injection.

Effects of repeated i.p. injections of EtOH—One experiment utilized the quantitative no-net-flux microdialysis technique to examine the effects of repeated i.p. injections of saline or EtOH (0.5, 1.0 or 2.0 g/kg) on basal extracellular glutamate concentrations in the pVTA. Approximately three to four days after surgery, rats (n = 6 – 8 /group) received daily i.p. injection of saline or EtOH for seven consecutive days. Microdialysis was conducted on the 8th day. During microdialysis, baseline samples were collected following the 90-min washout period. Then, four different concentrations of glutamate (1, 5, 10 or 20 μM in aCSF) were perfused through the pVTA in a random order. The glutamate concentrations in each perfusate were verified to be the stated concentrations on HPLC instruments before the perfusion by comparing the peak area of each concentration to the calibrated standard curve. Liquid swivels were employed to facilitate line switches between different concentrations of glutamate. Both the inlet and outlet of the collection assembly were constructed to allow a 10-min lag time. The perfusion period for each concentration was 25 min after correcting for the lag time of the collection assembly. After the perfusion of the last glutamate concentration, the perfusion medium was changed to aCSF for 20 min. Samples were collected every five minutes at a flow rate of 2.0 μl/min.

Glutamate analysis

Glutamate was analyzed with a reversed-phase high performance liquid chromatography system with electrochemical detection, as described previously (Donzanti and Yamamoto, 1988). Briefly, precolumn derivatization of glutamate with *o*-phthalaldehyde was performed using an ESA Model 542 autosampler. The mobile phase consisted of 25% methanol (v/v), 100 mM $Na_2HPO_4 \cdot 7H_2O$, pH 6.75 and was delivered by an ESA 582 solvent delivery system. Dialysis samples were injected onto a reverse-phase column (BDS Hypersil C18 Pioneer, 150×2 mm) and glutamate was separated and detected with a BAS LC-4C

amperometric detector with the oxidation potential set at +550 mV and the sensitivity set at 0.2 μA. The outputs from the detector were sent to a ChromPerfect chromatography data analysis system. An external standard curve was constructed by calibrating with four concentrations of glutamate standards (1, 5, 10 and 20 μM). Briefly, each concentration of glutamate standard was injected into HPLC system three times and corresponding peak areas were recorded from resulting chromatograms. A linear regression curve plotted the peak areas against the concentrations of standards. The concentrations of glutamate in either samples or perfusates were quantified by comparing peak areas with the calibrated standard curve.

Histology

At the end of each experiment, rats were euthanized with an overdose of CO_2 inhalation and 1% bromophenol blue was then perfused through probes in the pVTA. Brains were removed quickly and frozen immediately on dry ice and stored at −20 °C. Sections (40 μm) were sliced on a cryostat microtome and stained with cresyl violet for verification of the placements of probes with reference to the rat brain atlas of Paxinos & Watson (1998).

Statistical analysis

For conventional microdialysis, the last three baseline samples were averaged and extracellular glutamate levels were normalized and expressed as percent of the baseline. ANOVAs with repeated measures on time were employed to analyze the normalized data demonstrating the time-course effects. If significant differences were detected with ANOVAs ($p < 0.05$), post-hoc tukey's b tests were performed to determine the individual differences.

For no-net-flux microdialysis, data were analyzed with multiple linear regressions, as described previously (Parsons and Justice, 1994; Thielen et al., 2004). The glutamate concentrations (0, 1, 5, 10 and 20 μM) perfused through the probes were defined as $[GLU]_{in}$, and the glutamate concentrations obtained from microdialysis samples were defined as $[GLU]_{out}$. The net gain or loss of glutamate was defined as $[Glu]_{in}-[Glu]_{out}$ and was calculated for each sample. These values of $[GLU]_{in} - [GLU]_{out}$ were plotted as 'y' axis against the values of $[GLU]_{in}$ as 'x' axis. The slope (the extraction fraction, Ed) and the x-intercept (the extracellular glutamate concentrations) for each group were determined by multiple linear regression modeling using the SAS System for Windows, version 8.02. Individual replicate data from all the animals in all groups were analyzed by ANOVA to compare the effect of EtOH on the slope and x-intercept.

Results

Histology

Fig. 1 shows the representative placements of probes in the pVTA. The pVTA is defined as the level of the interpeduncular nucleus from 5.3 mm to 6.0 mm posterior to bregma (Ding et al., 2009a; Rodd-Henricks et al., 2000). The correct placement for probes had to have at least 75% of the active membrane within the pVTA. Some probes also covered a small portion of the red nucleus to the dorsal side and a small portion of the interpeduncular

nucleus to the ventral side. Approximately 85% of animals fulfilled the criteria and were included in the analysis.

Effects of acute i.p. injection of EtOH

Extracellular glutamate levels in each group were: 3.3 ± 0.8 μM for the 'saline' group (n = 8), 4.0 ± 1.6 μM for the '0.5 g/kg EtOH' group (n = 8), 3.5 ± 0.7 μM for the '1.0 g/kg EtOH' group (n = 8), and 3.5 ± 1.2 μM for the '2.0 g/kg EtOH' group (n = 6). There were no significant differences in basal extracellular glutamate levels among these groups $(F_{(3, 26)} = 9.24, p = 0.97)$.

Figure 2 shows the time-course effects of i.p. injection of EtOH on extracellular glutamate levels. An overall "time" × "treatment" two-way ANOVA indicates that there are significant effects of time, treatment and time × treatment interaction (all F values > 1.79, all p values < 0.05). The injection of either saline or 1.0 g/kg EtOH did not significantly alter extracellular glutamate levels (Fig 2A & 2C, p > 0.05). However, the injection of 0.5 g/kg EtOH significantly increased extracellular glutamate levels during the 20–60 min period to approximately 120–125% of the baseline levels (Fig 2B, p < 0.05). On the other hand, the injection of 2.0 g/kg EtOH significantly decreased extracellular glutamate levels during the 80–120 min period to approximately 80% of the baseline levels (Fig 2D, p < 0.05). In addition, perfusion of the pVTA with the Ca++ free aCSF prevented the 0.5 g/kg EtOH-induced increase of extracellular glutamate levels (Fig 2B, $F_{(1,13)} = 11.6, p < 0.01$)

A separate experiment (n = 10) examined the effects of perfusion with the Ca++ free aCSF on basal extracellular glutamate levels in the pVTA (data not shown). After the perfusion medium was changed to Ca++ free aCSF, extracellular glutamate levels were reduced and significant reduction occurred approximately 80 min after the change of the perfusion medium and remained reduced throughout the end of the perfusion (180 min, $F_{(14, 126)} = 2.22, p < 0.05$). The average extracellular glutamate levels during this period (80 – 180 min) were $80 \pm 5\%$ of baseline levels. Extracellular glutamate levels returned to baseline levels following the change of the perfusion medium back to Ca++ containing aCSF.

Effects of repeated daily injections of EtOH

Figure 3 shows the results from the no-net-flux experiments. Multiple linear regression lines plotting $[GLU]_{in} - [GLU]_{out}$ as 'y' axis and $[GLU]_{in}$ as 'x' axis were shown in Figure 3A. Extracellular glutamate concentrations, as represented by the x-intercept of each line, were: 4.1 ± 0.5 μM for the 'saline' group, 6.6 ± 0.4 μM for the '0.5 g/kg EtOH' group, 6.7 ± 0.4 μM for the '1.0 g/kg EtOH' group, and 9.2 ± 0.5 μM for the '2.0 g/kg EtOH' group (Figure 3B). ANOVA revealed a significant effect of treatment $(F_{(3, 390)} = 6.59, p < 0.001)$. Repeated i.p. injections of each dose of EtOH significantly increased extracellular glutamate concentrations in the pVTA compared to repeated saline injections (p < 0.05). In addition, the extracellular glutamate concentrations in the '2.0 g/kg EtOH' group were significantly higher than those in all other groups (p < 0.05).

The extraction fractions (Eds) as represented by the slope of each line in each group were: $30 \pm 2\%$ for the 'saline' group, $22 \pm 2\%$ for the '0.5 g/kg EtOH' group, $17 \pm 2\%$ for the '1.0 g/kg EtOH' group, and $20 \pm 2\%$ for the '2.0 g/kg EtOH' group (Figure 3C). ANOVA

revealed a significant effect of treatment ($F_{(3, 387)} = 9.20$, $p < 0.001$). Further analysis indicated that repeated i.p. injections of each dose of EtOH significantly reduced Ed values in the pVTA compared to those in the 'saline' group ($p < 0.05$). The Ed values in three EtOH-treated groups were not significantly different from one another ($p > 0.05$).

The experiment examining pVTA glutamate response to a challenge dose of EtOH following 7-day repeated EtOH injections used the dose of 2.0 g/kg EtOH for both pretreatment and challenge, as suggested by the greatest effect of this dose of EtOH in the NNF study (Fig. 3). Basal extracellular glutamate levels were 3.0 ± 0.9 μM for saline-pretreated rats (n=6) and 3.8 ± 1.4 μM for EtOH-pretreated rats (n = 5). During the microdialysis, rats in both pretreatment groups received a challenge injection of saline followed by an injection of 2.0 g/kg EtOH. Since the effects of the challenge injections were similar for both pretreatment groups, the data were combined and presented in Fig. 4. The repeated measures ANOVA revealed a significant effect of time ($F_{(18, 180)} = 4.26$, $p < 0.001$). The injection of saline did not significantly alter extracellular glutamate levels. In contrast, the injection of 2.0 g/kg EtOH reduced extracellular glutamate ($p < 0.05$) to levels previously observed following i.p. injection of 2.0 g/kg EtOH in animals that had not received any pretreatment (Fig. 2D).

Discussion

The results of the current study indicate that both acute and chronic EtOH exposure altered extracellular glutamate transmission in the pVTA. Acute systemic administration of EtOH produced a biphasic effect on extracellular glutamate levels in the pVTA. Ca++ free aCSF prevented the increase of extracellular glutamate levels in the pVTA induced by the low dose of EtOH, suggesting that the low dose of EtOH may act, at least in part, through increasing Ca++ dependent release of glutamate. Furthermore, repeated systemic administrations of EtOH increased basal extracellular glutamate concentrations and reduced Ed values in the pVTA, suggesting that repeated EtOH exposure produced adaptive changes in the pVTA glutamate system, resulting in decreased glutamate clearance and increased extracellular glutamate concentrations. However, repeated injections of EtOH did not appear to alter subsequent glutamate response to a challenge dose of EtOH.

It has been shown that EtOH produced a biphasic effect on extracellular glutamate levels in the NAC of rats (Moghaddam and Bolinao, 1994). Similarly, the current study demonstrated that systemic administration of EtOH also produced biphasic alteration of extracellular glutamate levels in the pVTA (Fig. 2). In the Moghadddam & Bolinao (1994) study, 0.5 g/kg EtOH increased extracellular glutamate levels in the NAC to over 250% of the baseline, whereas the current study indicated only a moderate increase in the pVTA, i.e., to approximately 120–125% of the baseline (Fig. 2B). These results suggest that NAC may be more responsive to the excitatory effects of EtOH on extracellular glutamate than the pVTA. In both the NAC and pVTA, 2.0 g/kg EtOH appeared to induce a similar delayed reduction of extracellular glutamate levels (Moghaddam and Bolinao, 1994, Fig. 2D), suggesting that extracellular glutamate in both regions may be similarly responsive to the inhibitory effect of EtOH. The injection of 1.0 g/kg EtOH did not significantly alter extracellular glutamate levels in the pVTA, which is consistent with one recent study (Kemppainen et al., 2010).

However, the Kemppainen et al. study (2010) also did not find any effect with the i.p. injection of 2.0 g/kg EtOH. The different findings may be due to different rat strains used between these two studies. Indeed, several studies indicated that different strains of rats responded differently to EtOH with regard to changes in the extracellular glutamate levels in the NAC (Piepponen et al., 2002; Quertemont et al., 2002; Selim and Bradberry, 1996; Yan et al., 1998). Furthermore, the lack of significant changes with 2.0 g/kg EtOH in the Kemppainen et al. study (2010) may be due to the observed response of extracellular glutamate to saline. In that study, saline injection produced an approximately 30% reduction of VTA extracellular glutamate levels, which may have prevented observing any decrease in extracellular glutamate levels with the injection of 2.0 g/kg EtOH.

In addition, the current study indicated that perfusion with the Ca++ free aCSF through the pVTA significantly reduced basal extracellular glutamate levels to approximately 80% of the baselines, suggesting that approximately 20% of basal extracellular glutamate levels sampled with microdialysis may be coming from Ca++ dependent release. The results are consistent with a previous study indicating that blockade of the N-type Ca++ channel in the NAC significantly reduced local extracellular glutamate levels by 30% (Baker et al., 2002). Similar findings were also reported in the striatum (Morari et al., 1993). However, recent evidence suggests that astrocytes can release glutamate in a calcium-dependent manner (Reyes and Parpura, 2009). Therefore, it is possible that Ca++ depletion in the current study may also reduce glutamate released from astrocytes.

Low to moderate doses of EtOH have been shown to increase extracellular glutamate levels in various brain regions, including the NAC and hippocampus (Moghaddam and Bolinao, 1994; Selim and Bradberry, 1996). The present findings with acute low dose EtOH are in agreement with the results obtained in the NAC and hippocampus. *In vitro* electrophysiological studies performed on VTA slices and isolated VTA neurons also indicated that acute exposure to low concentrations of EtOH increased glutamate release in the VTA, involving a dopamine D1 receptor mechanism (Deng et al., 2009; Xiao et al., 2009). Further studies will be needed to determine whether systemic administration of EtOH is acting on glutamate terminals within the pVTA and/or brain regions projecting to the pVTA, e.g. the laterodorsal and pedunculopontine tegmental nuclei.

The mechanisms underlying the reduction of extracellular glutamate levels by 2.0 g/kg EtOH were not explored in the current study. One previous study (Yan et al., 1998) demonstrated that acute injection of 2.0 g/kg EtOH attenuated high K+ induced synaptic release of glutamate in the NAC, suggesting that 2.0 g/kg EtOH may reduce synaptic release of glutamate in the NAC. Similar mechanisms may also underlie the effect of this dose of EtOH in the pVTA. EtOH may exert its effects through areas projecting to the pVTA and/or locally within the pVTA. For example, ethanol has been shown to inhibit persistent activity in the medial prefrontal cortex, a region that sends glutamate projections to both the VTA and NAC (Tu et al., 2007).

Repeated EtOH exposure increased basal extracellular glutamate concentrations and reduced Ed values in the pVTA compared to the saline treatment (Figure 3B). The results are consistent with previous no-net-flux microdialysis studies in the NAC of both rats (1.0 g/kg)

and mice (2.0 g/kg) using similar repeated injection paradigms (Kapasova and Szumlinski, 2008; Melendez et al., 2005). These results suggest that repeated EtOH exposure reduced extracellular glutamate clearance, leading to increased basal extracellular glutamate concentrations. However, the current findings do not exclude a possible increase of basal glutamate release in the VTA following repeated EtOH treatment. Chronic EtOH exposure has been shown to increase spontaneous glutamate release in the VTA (Stuber et al., 2008), which could contribute to enhanced basal extracellular glutamate concentrations observed in the current study. Furthermore, 2.0 g/kg EtOH induced a greater increase in the extracellular glutamate concentrations compared to the two lower doses, but there was no difference in glutamate clearance among the 3 doses. These results suggest that an additional increase of glutamate release may contribute to the effects of the highest dose of EtOH.

Following 7 daily repeated injections of EtOH, a challenge dose of 2.0 g/kg EtOH produced an approximately 20% of reduction in extracellular glutamate levels in the pVTA, similar to the effects examined in the acute injection study (Fig. 2D). This finding is different from the predication based on findings from the NAC (Kapasova and Szumlinski, 2008; Szumlinski et al., 2007), which indicated significantly greater increases of NAC glutamate levels induced by a challenge injection of EtOH following a period of EtOH exposure. Different brain regions examined may account for this difference. These findings suggest that the NAC may be more responsive to EtOH than the pVTA in rats with a history of EtOH exposure.

Extracellular glutamate is mainly cleared by re-uptake mechanisms maintained by membrane glutamate transporters (Danbolt, 2001). Therefore, it is possible that repeated EtOH treatment may down-regulate glutamate transporter function by either decreasing the density of the transporters and/or by modifying transporter activities. The study of Melendez et al (2005) indicated that seven daily injections of 1.0 g/kg EtOH reduced Na+ dependent glutamate uptake in an in vitro assay, but did not alter protein levels of two major glutamate transporters, suggesting that repeated EtOH may alter the post-translational modifications of glutamate transporters in the NAC, e.g., phosphorylation. Reduced glutamate transporter function was also reported in other studies with different EtOH exposure paradigms. Glutamate uptake activity was reduced in the cerebral cortex of alcohol-preferring cAA rats following voluntary drinking of EtOH for 20 months compared to that in alcohol naïve cAA rats (Schreiber and Freund, 2000). Incubation of rat cortical cells with 50–100 mM EtOH for 6 hr produced significant a 10–15% reduction of glutamate uptake and led to approximately a 70% increase of glutamate in the incubation medium (Singh et al., 1994).

The increased glutamate transmission following either acute or repeated injections of low to moderate doses of EtOH may have significant functional implications. For example, the increased extracellular glutamate levels in the pVTA following acute injection of the low dose of EtOH may contribute to EtOH induced behavioral stimulation and increase of extracellular dopamine in the NAC (Imperato and Di Chiara, 1986). In addition, the increased basal extracellular glutamate concentrations in the pVTA following repeated injections of EtOH may contribute to increased basal extracellular dopamine concentrations in the NAC (Smith and Weiss, 1999). Given the importance of the mesolimbic dopamine transmission in the positive reinforcement of EtOH, the increased glutamate

neurotransmission in the pVTA, therefore, may contribute to the reinforcing effects of EtOH.

In summary, the current study demonstrated that acute injection of EtOH produces a biphasic effect on extracellular glutamate levels in the pVTA. Repeated EtOH exposure reduced clearance of extracellular glutamate, leading to increased basal extracellular glutamate concentrations in the pVTA. These changes may be involved in mediating the reinforcing and rewarding effects of EtOH.

Acknowledgements

References

Allgaier C, Scheibler P, Muller D, Feuerstein TJ, Illes P. NMDA receptor characterization and subunit expression in rat cultured mesencephallic neurons. Br J Pharmacol. 1999; 126:121–130. [PubMed: 10051128]

Baker DA, Xi ZX, Shen H, Swanson CJ, Kalivas PW. The origin and neuronal function of in vivo nonsynaptic glutamate. J Neurosci. 2002; 22:9134–9141. [PubMed: 12388621]

Benveniste H, Huttemeier PC. Microdialysis-theory and application. Prog Neurobiol. 1990; 35:195–215. [PubMed: 2236577]

Campbell AD, Kohl RR, McBride WJ. Serotonin-3 receptor and ethanol-stimulated somatodendritic dopamine release. Alcohol. 1996; 13:569–574. [PubMed: 8949951]

Dahchour A, De Witte P. Taurine blocks the glutamate increases in the nucleus accumbens microdialysate of ethanol-dependent rats. Pharmacol Biochem Behav. 2000; 65:345–350. [PubMed: 10672989]

Danbolt NC. Glutamate uptake. Prog Neurobiol. 2001; 65:1–105. [PubMed: 11369436]

Deng C, Li KY, Zhou C, Ye JH. Ethanol enhances glutamate transmission by retrograde dopamine signaling in a postsynaptic neuron/synaptic bouton preparation from the ventral tegmental area. Neuropsychopharmacology. 2009; 34:1233–1244. [PubMed: 18784647]

Ding ZM, Liu W, Engleman EA, Rodd ZA, McBride WJ. Differential effects of dopamine D_2 and $GABA_A$ receptor antagonists on dopamine neurons between the anterior and posterior ventral tegmental area of female Wistar rats. Pharmacol Biochem Behav. 2009a; 92:404–412. [PubMed: 19480073]

Ding ZM, Rodd ZA, Engleman EA, McBride WJ. Sensitization of ventral tegmental area dopamine neurons to the stimulating effects of ethanol. Alcohol Clin Exp Res. 2009b; 33:1571–1581. [PubMed: 19485970]

Ding ZM, Toalston JE, Oster SM, McBride WJ, Rodd ZA. Involvement of local serotonin-2A but not serotonin-1B receptors in the reinforcing effects of ethanol within the posterior ventral tegmental area of female Wistar rats. Psychopharmacology. 2009c; 204:381–390. [PubMed: 19165471]

Donzanti BA, Yamamoto BK. An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. Life Sci. 1988; 43:913–922. [PubMed: 2901021]

Gessa G, Muntoni AL, Collu M, Vargiu L, Mereu G. Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. Brain Res. 1985; 348:201–203. [PubMed: 2998561]

Goulding SP, Obara I, Lominac KD, Gould AT, Miller BW, Klugmann M, Szumlinski KK. Accumbens Homer2-mediated signaling: a factor contributing to mouse strain differences in alcohol drinking? Genes Brain Behav. 2011; 10:111–126. [PubMed: 20807241]

Hauser SR, Ding ZM, Getachew B, Toalston JE, Oster SM, McBride WJ, Rodd ZA. The posterior ventral tegmental area mediates alcohol-seeking behavior in alcohol-preferring rats. J Pharmacol Exp Ther. 2011; 336:857–865. [PubMed: 21148248]

Hodge CW, Haraguchi M, Erickson H, Samson HH. Vental tegmental microinjections of quinpirole decrease ethanol and sucrose-reinforced responding. Alcohol Clin Exp Res. 1993; 17:370–375. [PubMed: 8098187]

Hopf FW, Martin M, Chen BT, Bowers MS, Mohamedi MM, Bonci A. Withdrawal from intermittent ethanol exposure increases probability of burst firing in VTA neurons in vitro. J Neurophysiol. 2007; 98:2297–2310. [PubMed: 17699688]

Imperato A, Di Chiara G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther. 1986; 239:219–239. [PubMed: 3761194]

Kalivas PW. Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. Brain Res Rev. 1993; 18:75–113. [PubMed: 8096779]

Kapasova Z, Szumlinski KK. Strain differences in alcohol-induced neurochemical plasticity: a role for accumbens glutamate in alcohol intake. Alcohol Clin Exp Res. 2008; 32:617–631. [PubMed: 18341649]

Kemppainen H, Raivio N, Nurmi H, Kiianmaa K. GABA and glutamate overflow in the VTA and ventral pallidum of alcohol-preferring AA and alcohol-avoiding ANA rats after ethanol. Alcohol Alcohol. 2010; 45:111–118. [PubMed: 20075026]

Koob GF, Volkow ND. Neurocircuitry of addiction. Neuropsychopharmacology. 2010; 35:217–238. [PubMed: 19710631]

McBride WJ, Murphy JM, Ikemoto S. Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. Behav Brain Res. 1999; 101:129–152. [PubMed: 10372570]

Melendez RI, Hicks MP, Cagle SS, Kalivas PW. Ethanol exposure decreases glutamate uptake in the nucleus accumbens. Alcohol Clin Exp Res. 2005; 29:326–333. [PubMed: 15770106]

Moghaddam B, Bolinao ML. Biphasic effect of ethanol on extracellular accumulation of glutamate in the hippocampus and the nucleus accumbens. Neurosci Lett. 1994; 178:99–102. [PubMed: 7816349]

Morari M, O'Connor WT, Ungerstedt U, Fuxe K. N-methyl-D-aspartic acid differentially regulates extracellular dopamine, GABA, and glutamate levels in the dorsolateral neostriatum of the halothane-anesthetized rat: an in vivo microdialysis study. J Neurochem. 1993; 60:1884–1893. [PubMed: 8097237]

National Research Council. Guide for the care and use of laboratory animals. Washington, D.C: National Academy Press; 1996.

Nowak KL, McBride WJ, Lumeng L, Li T-K, Murphy JM. Involvement of dopamine D_2 autoreceptors in the ventral tegmental area on alcohol and saccharin intake of the alcohol-preferring P rat. Alcohol Clin Exp Res. 2000; 24:476–483. [PubMed: 10798583]

Ortiz J, Fitzgerald LW, Charlton M, Lane S, Trevisan L, Guitart X, Shoemaker W, Duman RS, Nestler EJ. Biochemical actions of chronic ethanol exposure in the mesolimbic dopamine system. Synapse. 1995; 21:289–298. [PubMed: 8869159]

Parsons LH, Justice JB. Quantitative approaches to in vivo brain microdialysis. Crit Rev Neurobiol. 1994; 8:189–220. [PubMed: 7923396]

Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 4th ed.. New York: Academic Press; 1998.

Piepponen TP, Kiianmaa K, Ahtee L. Effects of ethanol on the accumbal output of dopamine, GABA and glutamate in alcohol-tolerant and alcohol-nontolerant rats. Pharmacol Biochem Behav. 2002; 74:21–30. [PubMed: 12376149]

Quertemont E, Linotte S, De Witte P. Differential taurine responsiveness to ethanol in high- and low-alcohol sensitive rats: a brain microdialysis study. Eur J Pharmacol. 2002; 444:143–150. [PubMed: 12063074]

Reyes RC, Parpura V. The trinity of Ca2+ sources for the exocytotic glutamate release from astrocytes. Neurochem Int. 2009; 55:2–8. [PubMed: 19171170]

Rodd-Henricks ZA, McKinzie DL, Crile RS, Murphy JM, McBride WJ. Regional heterogeneity for the intracranial self-administration of ethanol within the ventral tegmental area of female Wistar rats. Psychopharmacology. 2000; 149:217–224. [PubMed: 10823401]

Rodd ZA, Melendez RI, Bell RL, Kuc KA, Zhang Y, Murphy JM, McBride WJ. Intracranial self-administration of ethanol within the ventral tegmental area of male Wistar rats: evidence for involvement of dopamine neurons. J Neurosci. 2004; 24:1050–1057. [PubMed: 14762123]

Saal D, Dong Y, Bonci A, Malenka RC. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. Neuron. 2003; 37:577–582. [PubMed: 12597856]

Schreiber R, Freund WD. Glutamate transport is downregulated in the cerebral cortex of alcohol-preferring rats. Med Sci Monit. 2000; 6:649–652. [PubMed: 11208385]

Selim M, Bradberry CW. Effect of ethanol on extracellular 5-HT and glutamate in the nucleus accumbens and prefrontal cortex between the Lewis and Fischer 344 rat strains. Brain Res. 1996; 716:157–164. [PubMed: 8738232]

Singh SP, Ehmann S, Snyder AK. Ethanol and glucose-deprivation neurotoxicity in cortical cell cultures. Metabolism. 1994; 43:1108–1113. [PubMed: 7916117]

Smith AD, Weiss F. Ethanol exposure differentially alters central monoamine neurotransmission in alcohol-preferring versus -nonpreferring rats. J Pharmacol Exp Ther. 1999; 288:1223–1228. [PubMed: 10027862]

Steffensen SC, Nie Z, Criado JR, Siggins GR. Ethanol inhibition of N-methyl-D-aspartate responses involves presynaptic r-aminobutyric acid B receptors. J Pharmacol Exp Ther. 2000; 294:637–647. [PubMed: 10900243]

Stuber GD, Hopf FW, Hahn J, Cho SL, Guillory A, Bonci A. Voluntary ethanol intake enhances excitatory synaptic strength in the ventral tegmental area. Alcohol Clin Exp Res. 2008; 32:1714–1720. [PubMed: 18627359]

Szumlinski KK, Ary AW, Lominac KD, Klugmann M, Kippin TE. Accumbens Homer2 overexpression facilitates alcohol-induced neuroplasticity in C57BL/6J mice. Neuropsychopharmacology. 2008; 33:1365–1378. [PubMed: 17568396]

Szumlinski KK, Diab ME, Friedman R, Henze LM, Lominac KD, Bowers MS. Accumbens neurochemical adaptations produced by binge-like alcohol consumption. Psychopharmacology. 2007; 190:415–431. [PubMed: 17225170]

Thielen RJ, Engleman EA, Rodd ZA, Murphy JM, Lumeng L, Li T-K, McBride WJ. Ethanol drinking and deprivation alter dopaminergic and serotonergic function in the nucleus accumbens of alcohol-preferring rats. J Pharmacol Exp Ther. 2004; 309:216–225. [PubMed: 14718606]

Tu Y, Kroener S, Abernathy K, Lapish C, Seamans J, Chandler LJ, Woodward JJ. Ethanol inhibits persistent activity in prefrontal cortical neurons. J Neurosci. 2007; 27:4765–4775. [PubMed: 17460089]

White FJ. Synaptic regulation of mesocorticolimbic dopamine neurons. Annu Rev Neurosci. 1996; 19:405–436. [PubMed: 8833449]

Xiao C, Shao XM, Olive MF, Griffin WC 3rd, Li KY, Krnjevic K, Zhou C, Ye JH. Ethanol facilitates glutamatergic transmission to dopamine neurons in the ventral tegmental area. Neuropsychopharmacology. 2009; 34:307–318. [PubMed: 18596684]

Yan QS, Reith ME, Yan SG, Jobe PC. Effect of systemic ethanol on basal and stimulated glutamate releases in the nucleus accumbens of freely moving Sprague-Dawley rats: a microdialysis study. Neurosci Lett. 1998; 258:29–32. [PubMed: 9876044]
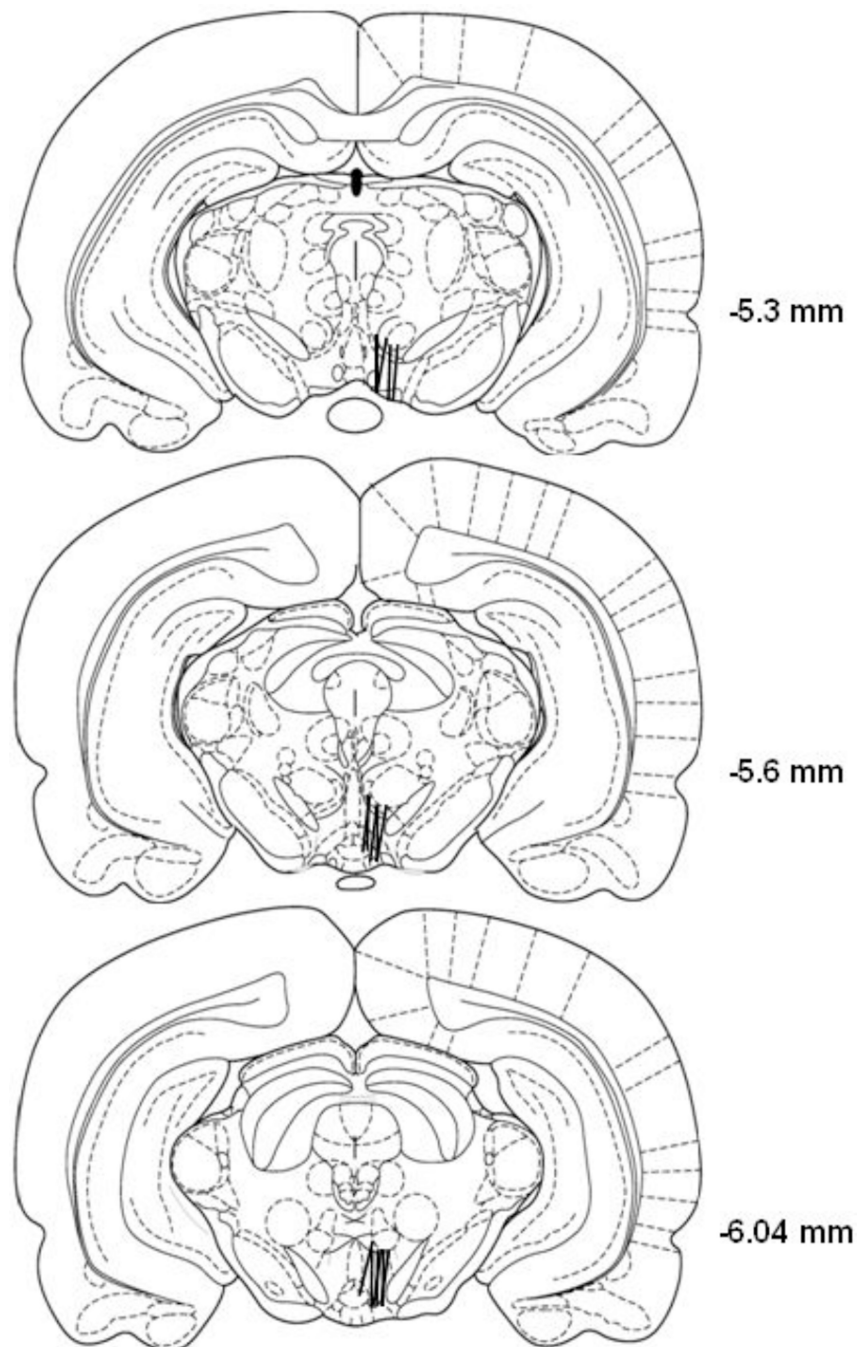
Figure 1.
Representative placements of microdialysis probes in the posterior ventral tegmental area
(pVTA). The pVTA was determined based on previous findings (Ding et al., 2009b; Rodd-
Henricks et al., 2000). Overlapping probes are not shown for clarity purposes. The lines
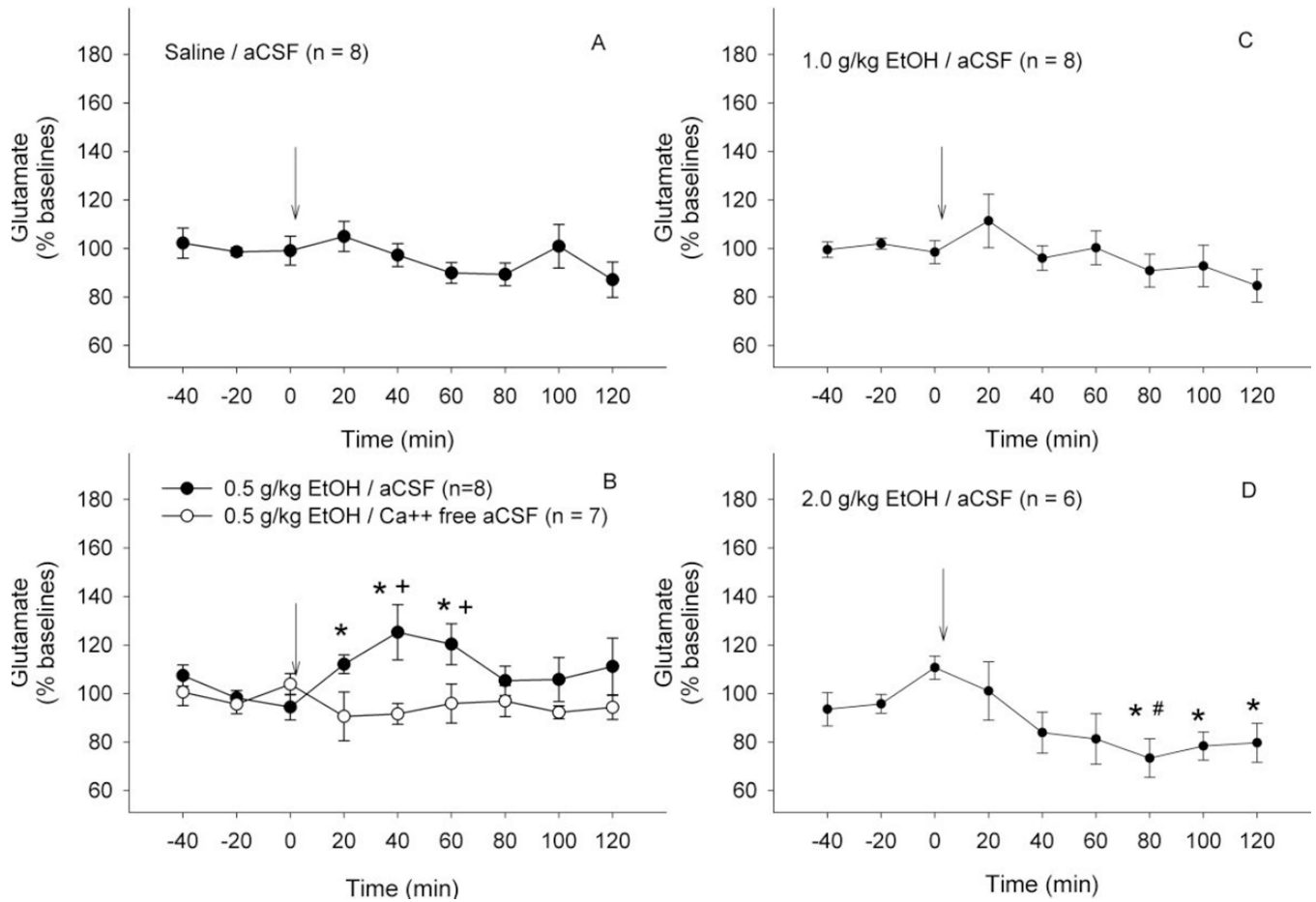represent the 1.5-mm active length of the microdialysis membrane.

Figure 2.
Time-course effects of intraperitoneal injection of saline or EtOH (0.5, 1.0 or 2.0 g/kg) on extracellular glutamate levels in the posterior ventral tegmental area during perfusion with normal aCSF, or in the case of 0.5 g/kg EtOH, during perfusion with aCSF or Ca^{++} free aCSF. *, $p < 0.05$, significantly different from the baseline level; #, $p < 0.05$, significantly different from the 'saline' group; +, $p < 0.05$, significantly different from the 'saline' and '0.5 g/kg EtOH/Ca^{++} free aCSF' groups.
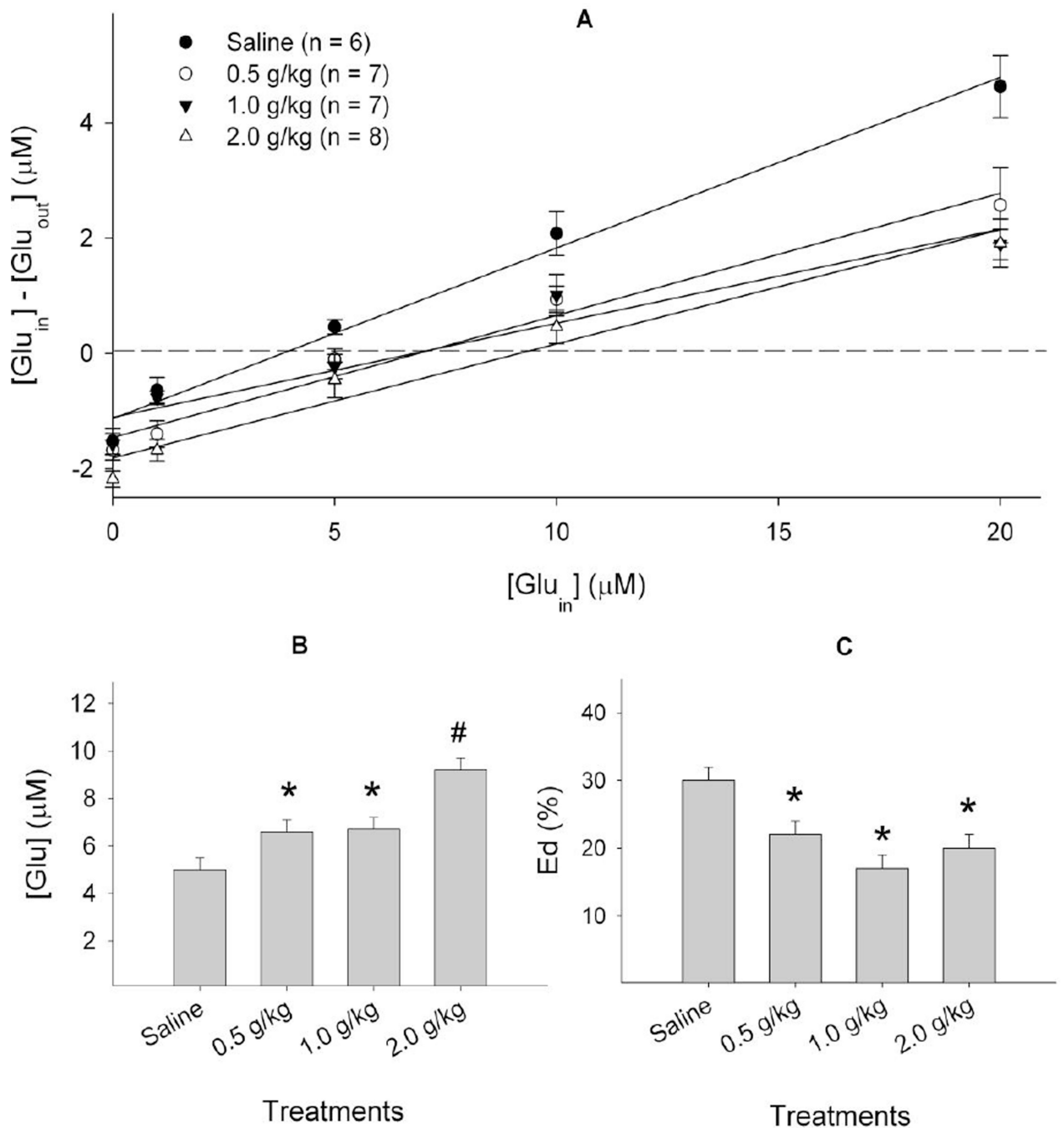
Figure 3.
Effects of repeated daily i.p. injections of saline or EtOH (0.5, 1.0 or 2.0 g/kg) for 7 consecutive days on extracellular glutamate concentrations and extraction fractions (Eds) in the posterior ventral tegmental area. A) linear regression plots of glutamate for the saline, 0.5, 1.0 and 2.0 g/kg EtOH groups; B) extracellular glutamate concentrations determined from points of no-net-flux; C) Ed values of glutamate determined from the slope of the plots for the linear regression analysis. *, $p < 0.05$, significantly higher than the 'saline' group. #, $p < 0.05$, significantly higher than all other groups.
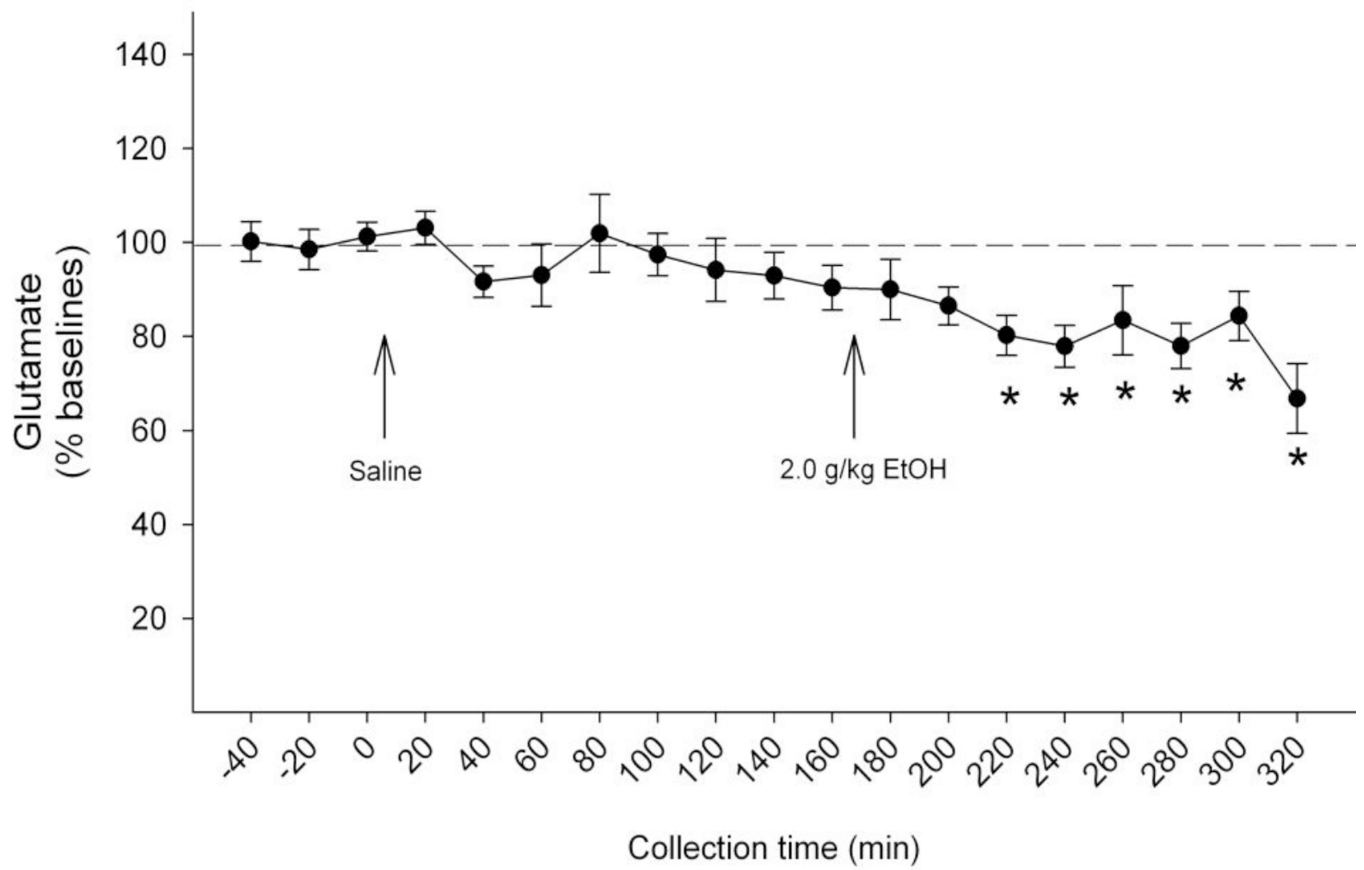
Figure 4.
Effects of i.p. injection of saline and 2.0 g/kg EtOH in rats pretreated for 7 days with daily injections of either saline (n = 6) or 2.0 g/kg EtOH (n = 5). Since the challenge injections produced similar effects in both groups, the data from two groups were combined. * p < 0.05, significantly lower than baseline levels.