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Intermittent High Dose Ethanol Exposures Increase Motivation for Operant Ethanol Self-Administration: Possible Neurochemical Mechanism

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Abstract

We investigated the neurochemical mechanism of how high dose ethanol exposure may increase motivation for ethanol consumption. First, we developed an animal model of increased motivation for ethanol using a progressive ratio (PR) schedule. Sprague-Dawley rats were trained to administer 10% ethanol-containing gelatin or plain gelatin (on alternate weeks) in daily 30-min sessions under different fixed ratio (FR) and PR schedules. During FR schedules, rats self administered about 1 g/kg ethanol which was decreased to 0.4 ± 0.03 g/kg under PR10. Rats then received four pairs of either 3 g/kg ethanol or saline injections during the weeks when the reinforcer was plain gelatin. During subsequent ethanol gel sessions, breakpoints and ethanol consumption rose 40% in the high dose ethanol group by the fourth set of injections with no change in plain gel responding. Alterations in amino acids in the ventral striatum (VS) during PR10 responding for 10% ethanol gelatin and plain gelatin was measured using microdialysis sampling coupled with capillary electrophoresis and laser-induced fluorescence detection. There was greater release of taurine, glycine and glutamate in the NAC of the high dose ethanol rats during 10% ethanol-containing gelatin responding, compared to the control rats or during plain gel responding. An increase in the release of glycine in this same brain region has recently been shown to be involved with anticipation of a reward. Thus, it appears that intermittent high dose ethanol exposure not only increases motivation for ethanol responding but may also change neurotransmitter release that mediates anticipation of reinforcement which may play a key role in the development of alcoholism.

Keywords

Ethanol; Operant self-administration; Taurine; Glycine

1. Introduction

Dopamine (DA) release in the nucleus accumbens (NAC) has been proposed as a neurobiological substrate of reward (Koob et al., 1998; Tupala and Tiihonen, 2004). Consistent

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with this hypothesis, ethanol administration increases release of DA (Imperato and Di Chiara, 1986; Yoshimoto et al., 1991, 1992; Diana et al., 1992; Tanda and Di Chiara, 1998; Gonzales et al., 2002; Lominac et al., 2006) in NAC. However, other neurotransmitters in NAC, particularly amino acids, are affected by ethanol and so may play a role in alcohol addiction. For example, taurine levels in NAC are selectively and dose-dependently increased by acute ethanol exposure, and this escalation is enhanced after repeated ethanol treatment (Dahchour et al., 1994, 1996, 2000a; Smith et al., 2004; Li et al., 2008). Similarly, although acute ethanol exposure has small and inconsistent effects on glutamate levels in NAC, repeated ethanol exposure increases glutamate levels (Moghaddam and Bolinao, 1994; Dahchour et al., 1994, 1996, 2000; Selim and Bradberry, 1996; Piepponen et al., 2002; Szumlinski et al., 2005, 2007; Lominac et al., 2006). Finally, increases of glycine in NAC are related to the amount of anticipation of reward (Li et al., 2008) so may be altered if repeated exposure to ethanol changes the reward value of ethanol.

One of the objectives of the present study was to develop a rat model of alcoholism to investigate the neurobiological consequences of forced intermittent ethanol (FIE) exposures that result in loss of consciousness. Recently an ethanol-containing gelatin (10% ethanol, 10% Polycose®, 0.25% gelatin, w/v) has been used to induce reliable and robust self-administration without food or water restriction (Rowland et al., 2005). The ethanol gel produces pharmacologically relevant concentrations of ethanol in the brain of rats (about 8 mM) that are comparable to the concentrations observed after voluntary ethanol drinking (Peris et al., 2006). In the present study, we used this procedure to develop a rat model with increased motivation for alcohol consumption (IMAC) using operant self-administration of ethanol. We chose fixed ratio (FR) schedules to establish baseline responding for ethanol reinforcement and progressive ratio (PR) schedules to assess motivation. We then used the IMAC model to determine whether a history of repeated high dose FIE influences neurotransmitter response to ethanol self-administration or to a 3 g/kg ethanol test injection. We hypothesized that the high dose ethanol group would exhibit sensitized release of glutamate, glycine and taurine in the NAC.

2. Results

Rats were initially trained on FR schedules of reinforcement, increasing from FR1 to FR30 over a 3 month period (Figure 1). Using an FR1 schedule, the average ethanol consumption during the 30-min sessions was 1.3 ± 0.05 g/kg (Figure 2A). During the last 10 days of FR5 responding, average consumption was 1.2 ± 0.05 g/kg (Figure 2B). This decreased during the 10 days of FR30 responding to 0.7 ± 0.05 g/kg ethanol per session (Figure 2C). Blood ethanol concentrations were determined immediately after the 30-min session on Day 11 of the FR1 schedule by sampling tail blood from a subset of these rats (Figure 2D). There was a significant correlation between ethanol dose and blood ethanol concentration.

When rats were moved to daily PR5 sessions (see Figure 1), ethanol consumption was 0.6 ± 0.03 g/kg. There was no difference in breakpoints for ethanol gel (34.5 ± 1.3) versus plain gel (36.2 ± 1.8) on the PR5 schedule. Similar results were found for a PR10 schedule with ethanol consumption of 0.5 ± 0.02 g/kg and breakpoints of 51.8 ± 3.1 for ethanol gel and 52.2 ± 3.8 for plain gel. There was no change in ethanol intake when rats returned to ethanol reinforcement after the 10-day plain gel reinforcement periods for either PR schedule (data not shown).

When the number of responses on the non-reinforced lever were tabulated, there was initially very little responding (2.7 ± 0.5 per FR1 session) and this decreased further over days (FR5 = 2.2 ± 0.3 ; FR30 = 0.7 ± 0.1 ; PR5 = 0.7 ± 0.1 ; PR10 = 0.4 ± 0.1). The average numbers of responses per session on the reinforced lever were: FR1 = 22.5 ± 1.2 ; FR5 = 80.6 ± 3.1 ; FR30 = 263.4 ± 17.1 ; PR5 = 325.9 ± 19.6 ; PR10 = 264.0 ± 21.5 .

In order to determine if high doses of ethanol (similar to what a human might experience when they drink enough ethanol to pass out) could alter motivation for ethanol responding as well as ethanol-induced changes in neurotransmitter levels, it was necessary to give a dose high enough to ensure loss of righting in 100% of subjects. Since Sprague-Dawley rats do not voluntarily consume enough ethanol to cause the loss of righting reflex, we examined whether forced intermittent 3 g/kg ethanol injections (F.I.E.) would affect operant responding and ethanol intake (see Figure 1). Consumption and breakpoint data did not vary across days, therefore we show the average consumption across the 7-day ethanol reinforcement period or the 10-day plain gel reinforcement period in all subsequent figures. Before the F.I.E. injections, the F.I.E. and Control rats consumed the same amount of ethanol (0.46 ± 0.02 g/kg) during the 30-min PR10 sessions. With each pair of high dose ethanol exposures, ethanol consumption rose in the F.I.E. group ($F(4,346) = 3.7$; $P < 0.01$) but not in the Control group (Figure 3A). High dose ethanol exposures did not affect responding for plain gel (Figure 3B). F.I.E. treatment also increased the response breakpoints in the F.I.E. group; although both groups initially had breakpoints of 28 ± 2 , the F.I.E. group increased progressively after high dose ethanol injections to 35 ± 2 , 37 ± 3 , 38 ± 2 and 33 ± 4 for each subsequent ethanol-gel responding period ($F(4,346) = 6.1$; $p < 0.001$).

When the pattern of responses over the 30-minute operant session was examined, over 97% of responses on the reinforced lever occurred during the first 15 minutes of each session regardless of reinforcement type or whether the rats had received saline or F.I.E. injections. There was a significant increase in responding during the first 10 minutes of the third through sixth sessions for rats in the group receiving F.I.E. injections. There was no difference in responding during any of the time periods for saline rats receiving ethanol gel or in any of the rats receiving plain gel reinforcements. These data were supported by a significant Group $\times$ Time interaction ($F(5,350) = 2.9$; $p < 0.05$) for ethanol-reinforced responding but only a significant effect of Time ($F(5,350) = 19.3$; $p < 0.001$) with no effect or interactions involving Group for plain gel-reinforced responding. The Group $\times$ Time interaction that occurred during ethanol-reinforced sessions was due to significantly higher responding during the first 5 and 10 min periods for F.I.E. rats during the later ethanol-reinforced sessions compared to earlier sessions ($F(4,199) = 12.1$ and 13.5 , respectively for 5 and 10 min). In order to determine whether repeated exposure to high dose ethanol caused tolerance in these rats, we measured the duration of the loss of righting reflex after each injection. There was no evidence for tolerance to the loss of righting induced by the eight F.I.E. injections (duration of loss of righting reflex was 61 ± 5 min and 60 ± 7 for the first and eighth injections, respectively).

Intake did not vary across the ethanol-gel operant sessions on the five days before the microdialysis test day (when the animals were tethered to a swivel in the top of the operant chamber), yet both breakpoint and consumption were decreased somewhat in both groups on both days of operant responding during the microdialysis session (Figure 3A and B). Even so, there was still greater consumption of ethanol gel during microdialysis testing in the F.I.E. group compared to the Control group (Figure 3A) with no difference in plain gel responding ($F(1,118) = 5.7$, $P < 0.05$). The breakpoints on the microdialysis days were 21 ± 4 and 32 ± 4 for Control and F.I.E. rats, respectively during ethanol gel responding, and 30 ± 4 and 28 ± 5 for Control and F.I.E. rats, respectively during plain gel responding. ANOVA indicated a significant interaction of Group $\times$ Reinforcer type for breakpoint data ($F(1,118) = 6.2$, $P < 0.05$). As before, over 95% of all reinforcements occurred during the first 15 minutes of the operant sessions regardless of group or reinforcer. All behavioral and microdialysis data are shown only for those animals with accurate probe placement (Figure 4).

Neurotransmitter levels during the operant sessions

On-line detection of the changes in amino acids showed that there was greater release of glycine, taurine and glutamate in the NAC in the F.I.E. rats compared to the Control rats during operant responding for 10% ethanol-containing gelatin (Figure 5). In contrast, there was no difference in the release of these three amino acids in the same animals during responding for plain gel (Figures 6). This observation is supported by an overall significant Group $\times$ Reinforcer $\times$ Time interaction ($F(1028, 7503) = 90.95$, $P < 0.001$ for glycine; $F(1028, 7503) = 26.5$, $P < 0.001$ for glutamate; $F(1028, 7503) = 38.7$, $P < 0.001$ for taurine). There were no significant group differences in baseline concentrations of any neurotransmitters except glutamate which was increased in the F.I.E. group (Table 1). There were no effects of any of the treatments on the levels of aspartate, GABA, glutamine, or serine at any time during testing (data not shown).

During the 30 min operant ethanol gel session, glycine levels increased by 50% in the F.I.E. group and 25% in the Control group (Figure 5A) whereas they were increased to the same degree (about 30%) in both groups during plain gel responding (Figure 6A). During the 30 min operant ethanol gel responding, there was a significant Group $\times$ Time interaction ($F(99, 1485) = 1.65$, $P < 0.05$) which was due to a significant effect of time in the F.I.E. rats ($F(99, 891) = 4.99$, $P < 0.0001$) but not in the Control rats ($F(99, 594) = 1.02$, NS). During the 30 min of operant plain gel responding, there was a significant Time effect ($F(99, 1485) = 1.66$, $P < 0.05$) regardless of group with no interaction or effect of group.

During the first 20 min of operant ethanol gel responding, glutamate levels gradually increased in F.I.E. rats compared with no change in Control rats (Figure 5B). This was supported by a significant Group $\times$ Time interaction ($F(99, 1485) = 1.40$, $P < 0.05$) which was due to a significant effect of time in the F.I.E. rats ($F(99, 891) = 1.48$, $P < 0.05$) but not in the Control rats ($F(99, 594) = 1.19$, NS). There was no effect of responding for plain gel on glutamate levels in either group (Figure 6B).

During the 30 min of operant responding for ethanol gel, taurine levels increased by 15% in both F.I.E. rats and Control rats (Figure 5C). There was a significant effect of Time ($F(99, 1485) = 1.34$, $P < 0.05$) regardless of group with no Group $\times$ Time interaction. During the following 30 min, taurine level was still slightly higher in F.I.E. rats than Control rats. There was a significant effect of Group ($F(1, 15) = 24.34$, $P < 0.05$) with no interaction or effect of Time. There was no effect of responding for plain gel on taurine levels in either group (Figure 6C).

In order to prove that the sensitized release of taurine, glutamate and glycine in F.I.E. rats was due to the F.I.E. treatment and not to the higher level of operant responding that occurred in the F.I.E. group compared to the Control group, we analyzed the data from all rats that received either 2 or 3 reinforcements during the dialysis session. In this way, the F.I.E. group and the Control group had similar breakpoints and ethanol consumption (F.I.E. group: $N=6$, $BP=25 \pm 2.24$, ethanol consumption = 0.41 ± 0.03 ; Control group: $N=6$, $BP=23.3 \pm 2.11$, ethanol consumption = 0.39 ± 0.02). In these subsets of rats, there were still significantly greater ethanol-induced increases in the levels of taurine, glutamate and glycine in the F.I.E. group compared to the Control group ($F(881, 5296) = 28, 31, 18$, and 30 , $p < 0.01$ for taurine, glutamate and glycine, respectively) with no differences between groups during plain gel reinforcement (data not shown).

Since each rat consumed different amounts of ethanol during the microdialysis session, we correlated AUC of the increase of each neurotransmitter with total ethanol consumption during the dialysis session (Figure 7). Increases in glycine, glutamate and taurine were correlated with the amount of ethanol intake for each rat in the F.I.E. group. In contrast, only the increased taurine and glycine release were correlated with ethanol consumption in the control group. In

addition, the slopes of the correlation curves for all three amino acids in the F.I.E. group were significantly higher than that in the control group.

Behavior and neurotransmitter levels during F.I.E. exposure

To determine if the differences in the amino acid responses to operant self-administered ethanol reflected alterations in pharmacological responsiveness to ethanol, all rats received an i.p. test injection of 3 g/kg ethanol one hour after the cessation of ethanol or plain gel responding. The duration of the loss of righting reflex was similar for the FIE (1.17 ± 0.12 hour) and control (1.09 ± 0.26 hour) rats on both days of testing. There was no difference in basal levels of glutamate, glycine or taurine between Day 1 and Day 2 of microdialysis (Table 1). Additionally, there was no difference between days in the magnitude or duration of ethanol-induced changes in any of the amino acids. Therefore, we show only the data from the first injection (Figure 8).

During the first 20 min of sampling, a slight increase of glycine release was observed in the F.I.E. group but not the Control group (Figure 8A) which was supported by a significant effect of time in the F.I.E. rats ($F(80, 720) = 1.58, P < 0.001$) but not in the Control rats ($F(80, 480) = 0.91, NS$). However, during 20 to 45 min after F.I.E. injection, there was a large decrease of glycine release by 40% in the Control rats with a smaller decrease in the F.I.E. rats. This was supported by a significant Group $\times$ Time interaction ($F(99, 1485) = 1.99, P < 0.05$) which was due to a significant effect of Time both in the Control rats ($F(99, 594) = 12.02, P < 0.0001$) and in the F.I.E. rats ($F(99, 891) = 9.83, P < 0.05$).

There was a markedly different effect of 3.0 g/kg ethanol injection on glutamate levels in NAC depending on previous F.I.E. treatment (Fig. 8B). There was an elevation of glutamate levels in F.I.E. rats with no change or a slight decrease in Control rats. This observation was supported by a significant Group $\times$ Time interaction ($F(99, 1485) = 2.34, P < 0.05$) which was due to a significant effect of Time in the F.I.E. rats ($F(99, 891) = 3.29, P < 0.0001$) but not in the Control rats ($F(99, 594) < 1, NS$).

In Figure 8C, the test injection of ethanol elicited a large taurine increase that initially did not differ between the F.I.E. and the Control rats. However, the increase of taurine lasted for at least 2 hours after injection in F.I.E. rats compared to only 25–30 min in the Control group. Thus, during the first 20 min after the injection there was a significant effect of Time regardless of group ($F(99, 1485) = 20.35, P < 0.0001$) with no interaction or effect of group. However, during the period from 20 to 45 min after injection, there was a significant Group $\times$ Time interaction ($F(99, 1485) = 2.35, P < 0.05$) which was due to a significant effect of time in the Control rats ($F(99, 594) = 9.89, P < 0.0001$) but not in the F.I.E. rats ($F(99, 891) = 0.97, NS$). During the last 90 min of collection, there was a main effect of Groups with no effect or interaction of Time.

3. Discussion

Development of IMAC model following F.I.E. exposure

These data confirm that 10% ethanol-containing gel can support robust operant responding in Sprague-Dawley rats resulting in physiologically relevant blood ethanol concentrations after a 30 min FR1 session (Peris et al., 2006). Sprague-Dawley rats are known for poor levels of ethanol self-administration under free access conditions (Mormede et al., 2004; Vengeliene et al., 2005) yet our “jello shot” procedure produces intakes of 1 to 1.5 g/kg that are as least as high as those in other strains using either water deprivation or the sucrose fading procedure. For example, Wistar rats self administer about 0.625g/kg ethanol in a 30 min FR1 operant session (Heyser et al., 1997, 2003); Long-Evans rats consume 0.45g/kg ethanol during a 30-min FR3 session (Arolfo et al., 2004); and, sP rats have a mean ethanol intake in the 0.6 to 1.1

g/kg range over a 30 min FR1 session (Vacca et al., 2002). Thus, the “jello shot” procedure provides a reliable and efficient method for studying an animal model of self-administration of ethanol.

Using PR reinforcement schedules, we found that response breakpoints did not differ for 10% ethanol gel versus plain gel under either PR5 or PR10 schedules. This indicates that the motivation to respond for both kinds of gel is similar in Sprague-Dawley rats. On the other hand, forced intermittent high dose ethanol injections (3 g/kg, i.p.) produced a long-lasting increase in ethanol consumption and in the breakpoints for ethanol responding under PR10 schedules without a corresponding change in responding for plain gel. This occurred in the absence of tolerance to the sedative effects of this high dose (as measured by the duration of the loss of righting reflex). Thus, our IMAC model produces a long-lasting increase in motivation for ethanol self-administration.

Rats completed over 97% of operant responding during the first 15 minutes of each session with no responding occurring during the last 10 minutes of each session regardless of whether the reinforcement was ethanol or plain gel. After F.I.E. exposure, the increase in responding was still primarily during the first 10 minutes of the session. This argues against the development of tolerance to a response slowing effect of intoxication since that would not be expected to occur so quickly in the session. It has been reported there are long term increases in voluntary ethanol consumption in rats after intermittent exposure to alcohol (Rimondini et al., 2002). However, our study is the first to report an effect of intermittent high dose ethanol exposure on motivation for ethanol reinforcement instead of total ethanol consumption.

Imbalance of excitatory and inhibitory neurotransmission in the IMAC model

Traditionally, DA release in NAC is considered to be a neurobiological mediator of the development of alcoholism (Tupala and Tiinonen, 2004). Twenty years of investigation have consistently demonstrated that alcohol administration increases DA levels in NAC, but only a few studies have examined NAC DA during operant self-administration of ethanol (Weiss et al., 1993, 1996; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003). Little attention has been paid to alterations in amino acid neurotransmitters in NAC during ethanol self administration.

Self-administration of ethanol gel elicited greater release of taurine in the NAC of the F.I.E. rats compared to that in the Control rats. It has been suggested that taurine may provide an alternative neuronal mechanism underlying ethanol's effects (Aragon and Amit, 1993; Quertemont et al., 1998). As seen previously, ethanol increased taurine after either operant self-administration (Li et al., 2008) or injection (Smith et al., 2004) in a dose-related fashion. Additionally, our data confirm that of Dahchour and associates that repeated exposure to ethanol further increases taurine levels in NAC (Dahchour et al., 1994; Dahchour et al., 1996; Dahchour & De Witte, 2000a). Behavioral evidence suggests that taurine alters the reinforcing properties of ethanol (Aragon & Amit, 1993; Quertemont et al., 1998b), possibly via modulation of DA transmission. Thus, it is possible that the increase in taurine in the F.I.E. group may be related to the increase in breakpoints in that group.

There is very little evidence that ethanol injections change glutamate levels in ethanol-naïve animals (Dahchour et al., 1994; Dahchour et al., 1996; Quertemont et al., 1998a; Quertemont et al., 2000). Instead, increases in glutamate are generally caused by ethanol withdrawal (Dahchour et al., 1998; Dahchour & De Witte, 1999; Dahchour & De Witte, 2000b; Quertemont et al., 2002; Dahchour & De Witte, 2003) but may also be seen in response to ethanol exposure in ethanol-experienced animals (Roberto et al., 2004; Szumlinski et al., 2005, 2007). We found that F.I.E. treatment resulted in an increase of glutamate levels in NAC either after self-administered or injected ethanol whereas no effect of ethanol exposure on

glutamate levels was seen in control animals. Additionally, the F.I.E. treatment caused an increase in the basal level of accumbal glutamate similar to what has been reported previously (Melendez et al., 2005).

In addition, a sensitized release of glycine was observed in the F.I.E. rats during responding for 10% ethanol gel compared to the increase occurring during responding for plain gel. These results confirm our previous finding that the release of glycine in NAC is involved with alterations in the anticipation of reward (Li et al., 2008) since the F.I.E. rats exhibited higher motivation for ethanol consumption during the operant session. Previously, glycine levels were also increased during the 10 minutes prior to the start of the operant session, relative to glycine levels measured 30 minutes prior to that (Li et al., 2008). Although in the present study, we did not see any difference in basal glycine levels in F.I.E. rats compared to Control rats, we did not collect data earlier than 10 minute prior to the start of the operant session. Therefore, we could not accurately assess whether there was an increase in basal glycine levels prior to the start of the operant session nor whether this differed significantly across groups. Even so, strychnine-sensitive glycine receptors (GlyR) in the NAC regulate both basal and ethanol-induced mesolimbic DA activity (Molander and Söderpalm, 2005a, 2005b) as well as ethanol consumption in ethanol high-preferring male Wistar rats (Molander et. al., 2005). Thus, several recent studies as well as the current findings suggest that glycine may be an important neural mediator of ethanol reinforcement (Li et al., 2008).

Although the neuronal mechanisms mediating the sensitized release of glutamate, glycine and taurine after the F.I.E. treatment are not fully understood, early studies indicate that local perfusion of taurine can increase the level of DA (Ericson et al., 2006) and decrease the levels of glutamate and glycine in NAC (Olive et al., 2000a). In addition, the taurine-induced DA increase, observed after ethanol administration, is completely blocked by perfusion of strychnine in the NAC (Ericson et. al., 2006). ORG 25935, an inhibitor of glycine transport, decreases ethanol intake and ethanol preference in ethanol preferring rats (Molander et. al., 2006). Therefore, GlyRs in the NAC might constitute targets for ethanol in its mesolimbic DA-activating effect particularly via its taurine releasing ability. Taurine has been shown to induce long-lasting enhancement of neuronal firing in striatum and hippocampus implicating taurine as having a role in neuronal plasticity (Chepkova et al., 2002; Sergeeva et al., 2003; Dominy et al., 2004). Very high ethanol doses might cause a profound increase in taurine levels which might be sufficient to result in synaptic plasticity thus changing subsequent responses to ethanol. We hypothesize that alterations in ethanol-induced increases in taurine levels in NAC may be a mechanism for inducing persistent alcohol cravings via an interaction with DA and glutamate efflux. Additionally, reinforcement-induced alterations in DA and glycine may contribute to the effects of ethanol, particularly when motivation for responding has been altered via F.I.E. ethanol treatments. To support these hypotheses, we must determine how these increases in taurine and glycine are associated with alterations in DA and glutamate release.

In summary, we have successfully developed a reliable model of ethanol exposure that can alter motivation for ethanol self administration. This animal model should be useful for studying the early stages of development of alcoholism. The neurochemical changes that underlie this alteration in ethanol responding appear to involve an imbalance of excitatory and inhibitory neurotransmission in NAC which may be the first step for understanding the neuronal adaptations occurring during the development of alcoholism.

4. Materials and Methods

Animals and housing

Female Sprague-Dawley rats were used that were approximately 2.5 mo old and weighed 204 ± 2 g at the beginning of the study. Body weight was recorded once a week. All rats were singly housed in a vivarium with a 12:12 light dark cycle and an ambient temperature of 23 ± 2 °C. Purina 5001 Rodent Chow was available ad libitum at all times except during daily 30-min operant sessions. All procedures were approved by the Institutional Animal Care and Use Committee and were consistent with the NIH Guide for the Care and Use of Laboratory Animals.

Behavioral apparatus

Each chamber consisted of a test cage housed in a sound attenuating isolation cubicle purchased from Coulbourn Instruments (Allentown, PA). Test cages measured approximately 50.8 cm W $\times$ 25.4 cm D $\times$ 30.5 cm H with a grid floor and solid walls. Two levers protruded through one wall of the chamber, placed symmetrically on either side of an optical lickometer. The lickometer was equipped with a photobeam to detect licks. A standard drinking spout was affixed inside the lickometer, such that the spout protruded into the chamber. A 20 ml syringe filled with 10% ethanol containing gelatin or plain gelatin was placed into a syringe pump inside the cubicle, outside of the test cage, before each session. Syringes with gelatin were refrigerated between sessions. Graphic State 3.02 operating software (Coulbourn Instruments, Allentown, PA) was used to control gelatin delivery and collect behavioral data.

Experimental Procedures

Gelatin administration in home cage—Rats received access to 10% ethanol-containing gelatin in jars in their home cage for 24 hrs on the first two days of exposure, then for 6 hrs for two days, then 3 hrs for two days and finally for 1 hr for about 2 weeks (see Peris et al., 2006).

Gelatin administration in the operant chamber

Fixed ratio schedule: After reliably consuming the 10% ethanol-containing gelatin for a 1 hr period of free access, rats were moved to daily 30 minute operant sessions that occurred between 1000 h and 1400 h. Rats were trained to press once for a 0.28 g delivery of 10% ethanol-containing gelatin (FR1). In these sessions, the three cue lights were constantly on over the left lever and a 1s illumination of the house light signaled gelatin delivery. Presses on the right lever had no programmed consequence. After 20 days, rats were moved to an FR5 schedule for another 20 days. To attain FR 30 responding, reinforcement schedules were increased across several days (see Figure 1). Any spillage that occurred was removed from the lickometer and weighed to the nearest .01g and subtracted from the daily total.

Progressive ratio schedule: After 10 days of FR30 training, rats were moved to daily 30 minute PR sessions in which the lever press requirement increased with each successive reinforcement (see Figure 1). Two PR5 and seven PR10 schedules were used; in these schedules, the cost of the Nth reinforcer in a session was 5N or 10N responses, respectively. The last completed ratio was taken as the breakpoint. In keeping with methodology used in the FR schedules, PR sessions were 30 min in length regardless of the rats' performance. However, there was very little responding during the last 15 minutes of any session. Each round of PR5 or PR10 schedule was implemented for 17 days total; the first 7 days responding was reinforced with 10% ethanol-containing gelatin and the next 10 days responding was reinforced with plain (non-alcoholic) gelatin (see Figure 1).

High dose ethanol treatments—Rats were divided into two groups based on their performance after the first two PR10 schedules such that they had equal consumption and breakpoints. Starting with the second 10-day plain gel reinforcement period with PR10 schedules (see Figure 1), the F.I.E. group (N = 7) received injections consisting of 3 g/kg ethanol (20% w/v, I.P.) and the Control group (N = 5) received equivolume saline injections. This dose was chosen since it reliably induces loss of the righting reflex in 100% of animals and the reflex is not regained for about at least one hour (Ye et al., 2009)). All injections occurred at least one hour after the daily operant session on Days 4 and 6 of the second, third, fourth and fifth 10-day period when rats were responding for plain gel reinforcement (Figure 1). Each high dose ethanol injection was divided into three equal volumes given at 5-min intervals. The duration of the loss of righting reflex was measured as difference between the time at which the animals lost consciousness until the time they were able to right themselves three times in one minute.

Blood Ethanol Determinations—The ethanol content of 10 μ l blood samples taken from the tail at the end of an FR1 operant session was determined by the alcohol dehydrogenase assay (Kristoffersen and Smith-Kielland, 2005) adapted for small volume samples (Peris et al., 2006). Briefly each sample was stored in 100 μ l 0.6 M glycine buffer (pH 9.2) on ice until all samples were collected. A standard curve was prepared containing 0, 0.32, 0.63, 1.25, 2.5, 5, 10 or 20 mM ethanol added to 100 μ l glycine buffer. After all samples were collected, 100 μ l of enzyme solution (0.88 mg NAD and 0.29 mg ADH per ml of 0.6 M glycine buffer) was added to each sample and then all samples were incubated at 37°C in a shaking water bath for 20 minutes. Samples were then kept on ice for at least 5 minutes, before transfer to a 96-well plate (Costar, Corning NY) on ice. Absorbance was read at 340 nm using a Synergy HT platereader (Bio-Tek, Winooski VT). Absorbance was converted to mM ethanol by the use of a polynomial equation since at these low volumes and concentrations; the standard curve is not linear. Fits were generally better than $R^2 = 0.98$, with less than 10% error when the absorbance of standard curve values were transformed to mM concentrations.

Surgery and Microdialysis—During the final PR10 training sessions (6PR10 in Figure 1), a time out was introduced before each session which was gradually incremented (by 5 min per day) from 5 min to 120 min. After completion of this sixth 7-day period of PR10 responding for ethanol gel, each rat was anesthetized with isoflurane and placed in a stereotaxic apparatus for surgical implantation of a stainless steel guide cannula. The guide cannula (21 gauge; Plastics One, Roanoke, VA) was positioned to end within 1 mm above the left side of NAC (flat skull coordinates from bregma: +1.8 anteroposterior, +1.3 lateral, -6.2 dorsoventral) and was secured to the skull with dental cement anchored by three stainless steel screws. After surgery, each rat was individually housed and allowed to recover for at least 2 days before daily operant sessions were resumed. During all subsequent operant sessions, rats were tethered to a swivel in the top of the operant chamber by a spring attached to a clip embedded in the dental cement on their skulls. Habituation to the tethering procedure continued for 3 to 7 daily sessions until behavior again stabilized.

Microdialysis probes (o.d. 270 μ m; active length 2 mm; cellulose membrane, 13,000 molecular weight cut-off) were constructed by the method of Pettit and Justice (1989). The probe was perfused with artificial cerebrospinal fluid (aCSF; 145 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.45mM monobasic phosphate, 1.55 dibasic phosphate, pH 7.0–7.4) at 1 μ l/min (CMA 100 microinjection pump; 1 ml Exmire syringe). A calibration curve was generated for each probe by collecting a total of 175 samples after placing the tip of the dialysis probe in seven different concentrations (0.63 - 20 μ M) of a 37°C, well-stirred standard solution containing glutamate, aspartate, GABA, taurine, serine, glutamine and glycine. The mean relative fluorescence of these samples was converted to concentration by plotting peak height versus standard concentration. Thus, extraction fraction in brain dialysate samples was

automatically accounted for, although any differences in diffusion of analytes in solution compared with a complex matrix like brain tissue could create a systematic bias.

At this point, the dialysis probe was inserted into NAC without anesthesia, and rats were placed in the operant chamber 2 hrs before the start of the 30 min session. The levels of amino acids in dialysate were quantified on-line every 15 seconds as described previously (Li et al., 2008) during the 2 hr baseline period; the 30-min operant responding session (with 10% ethanol gel as the reinforcer); for 2 hr after the operant session, and finally for 2 hr following a 3g/kg ethanol i.p. injection. Based on our previous work, 90 min after self administration of 0.7 g/kg ethanol in gel, brain ethanol levels are minimal (Peris et al., 2007). At the end of data collection, probes were removed, dummy cannulas were replaced, and two days later rats were tested again using the same procedure except with plain gel as the reinforcer during the operant session. We have found that this procedure does not interfere with basal levels or with release of amino acids either in response to neuronal stimulation or ethanol (Z. Li, M. King and J. Peris, unpublished observations).

Data Analysis and Histology—The detection of all peaks and the calculation of peak heights and peak areas in these files were performed by “Cutter”, a Lab-View-constructed VI in which electropherograms are batch post-processed (Shackman et al., 2004). The first 10 min of samples collected prior to operant sessions or ethanol injections were used to calculate a baseline. Since there was individual variability in the baseline values of analytes across animals, subsequent data were expressed as a percent of this baseline and calculated across animals as the mean $\pm$ SEM. The area under the curve (AUC) was calculated for each amino acid by summing the increase of each amino acid (as % over baseline) over time during the operant session (see Li, et al., 2008). This value was used to correlate changes in neurotransmitter levels with the amount of ethanol consumption during the microdialysis session.

As an internal standard, we used a peak that is comprised of all the non-charged analytes that are labeled by OPA. This peak (which we will refer to as the OPA peak) is stable relative to other analyte peaks from 1 to 6 hrs after probe implantation (average relative fluorescence = 3.2 ± 0.03 over the course of over 1800 samples or about 6 hrs) since the only factor which might alter its size is the injection efficiency of the flow gate. Thus, it is a good indicator of instrument variability and can be used as a control for the levels of each analyte. In order to reduce inter-sample variability caused by the instrument, we divide the value of each amino acid sample by the value of the OPA peak in that exact same sample.

At the end of the experiment, rats were deeply anesthetized with pentobarbital sodium (120 mg/kg, i.p.). The brains were removed and sectioned to verify the correct position of the probe. Only the data from rats whose probe placement included at least 50% of the active membrane area in NAC were included in the data analyses. There was no difference between the two groups in the number of rats whose probe placements were 70–100 active area in NAC vs only 50–70% active area in NAC. There was also no difference in the neurotransmitter data from probes with 70–100% active area in NAC vs those with 50–70% active area in NAC.

Statistics—Breakpoints and amount of ethanol consumption were the main dependent variables for the PR schedules. Neurotransmitter levels (as a percent of basal) over time were the dependent variable for microdialysis experiments. The data were analyzed using multi-variate ANOVA with JMP-stat software (SAS Institute Inc.). Newman–Keuls post hoc analyses were used to follow up any significant interactions. Additionally basal levels of each analyte were compared for the two groups using T-test. A significance level of $p < 0.05$ was used for all statistical analyses.

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Figure 1.

Summary of fixed and progressive ratio training regimens. The rats were first trained under an FR1 schedule using 10% ethanol-containing gelatin as the reinforcer which was then gradually increased to FR30 over the course of three months. Rats were next tested using PR5 schedules for two cycles of training followed by PR10 schedules for six cycles of training. These cycles each consisted of 7 daily 30-min sessions using 10% ethanol-containing gelatin as the reinforcer followed by 10 daily sessions using plain gelatin as the reinforcer. During the last four cycles of PR10 responding for plain gel, rats received either a 3g/kg ethanol injection (F.I.E. treatment) or saline injection (Control) on Days 4 and 6 after the end of the operant

session. After the last PR10 period of ethanol reinforcement, rats were readied for microdialysis testing.

Figure 2.

Ethanol consumption of rats trained to self-administer 10% ethanol in gelatin under FR1 (Panel A), FR5 (Panel B), and FR30 (Panel C) reinforcement schedules. Operant sessions were 30 minutes long. Shown are means and SEMs for $N = 9$. Panel D: Blood ethanol concentrations determined immediately after the 30-min session on Day 11 by sampling tail blood from 6 of these rats. There was a significant correlation between ethanol dose and blood ethanol concentration.

A. F.I.E increases ethanol consumption

B. F.I.E does not affect plain gel consumption

Figure 3.

F.I.E. treatment increased ethanol consumption (Panel A) but had no effect on plain gel consumption (Panel B). F.I.E. injections (3 g/kg, i.p.) were given on Days 4 and 6 of each of the 10-day plain gel periods occurring between the second and sixth PR10 ethanol sessions. Therefore, by 6 PR10, each rat in the F.I.E. group had received 8 ethanol injections. Since there was no difference in responding on any of the days during the 7-day ethanol cycles or 10 day plain gel cycles, data shown are means and SEMs for the average of all days per cycle for N = 10 and 7 per group. * $p < 0.05$. Ethanol consumption on the day of the microdialysis experiment is indicated by the last set of bars labeled "Exp day".

Figure 4.

Coronal sections showing microdialysis probe placement for all animals. Lines indicate the active dialysis regions. Numbers below the figure represent the position of the slice relative to Bregma. The figure was adapted from Paxinos and Watson (2005). Although some rats had 70–100% active length inside NAC and others had only 50–70% active length in NAC, there was no significant differences in any of the analyte levels between these two sets of rats.

Figure 5.

Neurotransmitter levels in NAC during responding for ethanol gel. There was greater release of glycine (Panel A), glutamate (Panel B), and taurine (Panel C) in the NAC in the F.I.E. rats during operant responding for 10% ethanol-containing gelatin. The data are shown as % baseline for N=10 for the F.I.E. group and N=7 for the Control group. Arrows indicate the start and end of operant responding for 97% of the rats. Data are lined up by the first reinforcement which occurred at time = zero (first arrow) and were averaged across every 10 data points (about 2.5 min) for ease of viewing.

Figure 6.

Neurotransmitter levels in NAC during responding for plain gel. The increase of glycine release that occurred during plain gel reinforcement (Panel A) was not different between groups. There were no changes in glutamate (Panel B) or taurine (Panel C) release in NAC in either group during operant responding for plain gelatin. Arrows indicate the start and end of operant responding for 97% of the rats. The data are shown as % baseline for N=6 for both groups. Data are lined up by the first reinforcement which occurred at time = zero (first arrow) and were averaged across every 10 data points (about 2.5 min) for ease of viewing.

Figure 7.

The correlation between ethanol consumption and neurotransmitter release. Neurotransmitter levels in NAC were well correlated with the amount of ethanol consumed in both groups. The slopes of the regression curves were significant higher in the F.I.E. group than in the Control group for all transmitters. Panel A depicts glycine, Panel B is glutamate and Panel C is taurine.

Figure 8.

Effect of forced high dose ethanol exposure (3g/kg i.p.) on the levels of glycine (Panel A), glutamate (Panel B) and taurine (Panel C) in NAC. There were greater increases in each analyte in the F.I.E. group compared to control after the test injection of 3.0 g/kg ethanol (occurring at time = zero in the figures). The data are shown as % baseline for N=10 for the F.I.E. group and N=7 for the Control group and are averaged across every 10 data points (about 2.5 min) for ease of viewing.

Table 1

Basal concentrations of glutamate, glycine and taurine in NAC.

		Glutamate(μ M)	Glycine(μ M)	Taurine(μ M)
Control (N=7)	Day 1	1.9 ± 0.7	0.71 ± 0.04	3.1 ± 0.13
	Day 2	1.7 ± 0.9	0.69 ± 0.08	3.4 ± 0.32
F.I.E. (N=10)	Day 1	$3.6 \pm 0.5^*$	0.76 ± 0.15	2.9 ± 0.24
	Day 2	$3.9 \pm 0.7^*$	0.81 ± 0.09	3.0 ± 0.18

Basal concentrations were calculated as the average of the first 10 min prior to the start of the operant session which was approximately 2 hours after probe insertion. There was no difference in basal levels between the first and second day of microdialysis testing. There were no differences between groups in the basal levels of GABA, aspartate, glutamine or serine (data not shown).

* indicates $p < 0.05$ compared to Control group using t-test.