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Pharmacology, Biochemistry and Behavior 79 (2004) 599–605

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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In alcohol-treated rats, naloxone decreases extracellular dopamine and increases acetylcholine in the nucleus accumbens: evidence of opioid withdrawal

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Received 25 August 2004; accepted 7 September 2004

Abstract

Withdrawal from ethanol is aversive. The question is why. As with the withdrawal from morphine, nicotine, diazepam and sugar, the ethanol withdrawal state may involve an increase in nucleus accumbens (NAc) acetylcholine (ACh) causing an alteration of the dopamine (DA)–ACh balance in favor of ACh. Therefore the effects of acute and chronic alcohol (1 gm/kg/day i.p.) treatment on extracellular concentrations of NAc ACh and DA were determined before and after naloxone-precipitated withdrawal. Ethanol initially increased DA to 119% of baseline as measured by microdialysis. This was still the case on the 21st day of ethanol injection when DA increased to 126%. There was no effect of ethanol on ACh. However, naloxone (3 mg/kg s.c.) injected the next day decreased extracellular DA to 83% of baseline and caused a significant rise in ACh to 119%. This state of high ACh combined with low DA may contribute to the aversive aspects of alcohol withdrawal.

Keywords: Microdialysis; Ethanol; Rat

1. Introduction

Dopamine (DA) agonists given locally in the nucleus accumbens (NAc) can increase ethanol (ETOH) intake (Samson et al., 1991; Hodge et al., 1992; Samson et al., 1993), suggesting that DA plays a role in alcohol consumption. Conversely, local injection of a dopamine antagonist into the NAc decreases ethanol-seeking behavior (Samson and Chappell, 2004). Ethanol, like most drugs of abuse, increases extracellular DA in the NAc of rats (Di Chiara and Imperato, 1988a,b; Weiss et al., 1993; Bassareo et al., 1996; Lewis, 1996; Yim and Gonzales, 2000). Activation of the dopaminergic system by ETOH seems to be mediated in part by an endogenous opioid system. An opiate antagonist blocks the ethanol-induced increase in accumbens DA release (Acquas et al., 1993; Gonzales and Weiss, 1998), and naltrexone is used in the treatment of alcohol dependence (O'Brien et al., 1996). These findings suggest a major role of endogenous opioid systems in ETOH intake, due in part to interactions with the mesolimbic DA system.

Acetylcholine (ACh) in the NAc works in opposition to DA and can cause an aversive state (Rada and Hoebel, 2001). Extracellular accumbens ACh decreases following systemic or local NAc morphine and is released during naloxone-induced morphine withdrawal. This occurs in both morphine-dependent rats (Rada et al., 1991; 1996) and sugar-dependent rats (Colantuoni et al., 2002). Accumbens ACh is also released during flumazenil-induced withdrawal in diazepam dependence (Rada and Hoebel, 2003) and mecamylamine-induced withdrawal in nicotine dependence (Rada et al., 2001). This led to the hypothesis that ethanol withdrawal might also involve the DA/ACh imbalance. Even though withdrawal behavior is minimal in ethanol-treated rats, the neurochemical signs of the aversive state

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doi:10.1016/j.pbb.2004.09.011

may be present. If naloxone induces opioid withdrawal in ethanol-treated rats, we hypothesize that a decrease in extracellular DA accompanied by release of ACh in the NAc should occur. An abstract of this study has been published (Rada and Hoebel, 2003).

2. Material and methods

2.1. Subjects and surgery

Male Sprague–Dawley rats (Taconic Farms, Germantown, NY) weighing 300–350 g were housed individually on a reversed 12:12-h light/dark schedule with rodent chow pellets and water available ad libitum. For surgeries, subjects were anesthetized with a combination of ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Bilateral 21-gauge stainless-steel guide shafts were stereotaxically implanted for the posterior medial accumbens (shell) as follows: AP +1.2 mm, L 0.8 mm and V 4.0 mm, with reference to bregma, midsagittal sinus, and surface of the level skull, respectively. Rats recovered in the home cage at least 1 week before experiments began. When microdialysis probes were inserted, they extended 5.0 mm beyond the guide shaft to reach a depth of 9.0 mm. All experimental protocols followed National Institutes of Health guidelines for the care and use of laboratory animals and were approved by Princeton University Animal Care and Use Committee.

2.2. Microdialysis procedure

Probes were constructed of silica glass tubing (37 μm i.d., Polymicro Technologies, Phoenix, AZ) inside a 26-gauge stainless-steel tube with a microdialysis tip of cellulose tubing (Spectrum Medical, Los Angeles, CA) sealed at the end with epoxy (6000 MW, 0.2 mm o.d.×2 mm long) (Hernandez et al., 1986; Mark et al., 1991). Probes were inserted and fixed in place with acrylic cement 14–16 h before each experiment to allow neurotransmitter recovery to stabilize. Probes were perfused with buffered Ringer's solution (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.35 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.35) at a flow rate of 0.5 μL/min overnight and 2.0 μL/min starting 2 h before and continuing throughout the experiment. Neostigmine (0.3 μM) was added to the perfusion fluid to improve basal recovery of ACh by hindering its enzymatic degradation. Samples (40 μL) were collected every 20 min during the experiment.

2.3. Dopamine and acetylcholine assays

Dopamine and its metabolites DOPAC and HVA were analyzed by reverse phase, high performance liquid chromatography with electrochemical detection (HPLC-EC). Samples were injected into a 20-μL sample loop leading to a 10-cm column with 3.2-mm bore and 3 μm, C18 packing (Brownlee Model 6213, San Jose, CA). The mobile phase contained 60 mM sodium phosphate, 100 μM EDTA, 1.24 mM heptanosulfonic acid, and 5% vol/vol methanol. DA, DOPAC and HVA were measured with a coulometric detector (ESA Model 5100A, Chelmsford, MA) with the conditioning potential set at +500 mV, and working cell potential at −400 mV.

Acetylcholine was measured by reverse phase HPLC-EC using a 20-μL sample loop with a 10-cm C18 analytical column (Chrompack, Palo Alto, CA). ACh was converted to betaine and hydrogen peroxide by an immobilized enzyme reactor (acetylcholinesterase and choline oxidase from Sigma, St Louis, MO and column from Chrompack) The mobile phase was 200 mM potassium phosphate at pH 8.0. An amperometric detector (EG&G Princeton Applied Research, Lawrenceville, NJ) was used. The hydrogen peroxide was oxidized on a platinum electrode (BAS, West Lafayette, IN) set at 500 mV with respect to a Ag–AgCl reference electrode (EG&G Princeton Applied Research).

2.4. Procedure

2.4.1. Experiment 1: test of acute ethanol on accumbens DA/ACh release

DA and ACh were measured every 20 min until obtaining a stable baseline (±10% in three consecutive samples). Then rats were injected with ETOH (1 g/kg, i.p., N=5) or saline (equal volume, N=5) and four more samples were collected (80 min).

2.4.2. Experiment 2: test of repeated ethanol injection on accumbens DA/ACh release

A separate group of rats was injected with ETOH (1 g/kg, i.p., N=6) or saline (equal volume, N=5) daily for 21 days. They were implanted with microdialysis probes on the evening of Day 20, for sampling on Day 21. After a stable baseline was achieved (±10%), rats were injected for the 21st time with ETOH or saline, and DA and ACh levels were monitored for four samples (80 min).

2.4.3. Experiment 3: test of systemic naloxone on accumbens DA/ACh release in ethanol-treated rats

Separate groups of rats received daily injections of ETOH (1 g/kg i.p., N=8) or saline (equal volume, N=6) for 21 days. Dialysis probes were implanted, and the next day after a stable baseline was observed, rats were injected with naloxone (3 mg/kg s.c.), and DA and ACh extracellular levels were monitored for 80 min.

2.4.4. Experiment 4: test of naloxone on overt withdrawal behavior

Naive rats were injected with 10% (w/v) ETOH (1 g/kg, i.p., N=8) or saline (equal volume, N=8) daily for 21 days.

On Day 22, instead of receiving an injection, each rat was placed in a 43×43 cm open field for 10 min. The enclosure was equipped with an automated location detection system of 16 photobeams per side for horizontal activity and 16 photobeams for vertical activity (Med Associates, St. Albans, VT, USA). An observer, blind to the rat's treatment, stood next to the enclosure and recorded instances of teeth chattering, head weaving, head shakes, wet-dog shakes, and diarrhea. Then the rat received an injection of naloxone (3 mg/kg s.c.) and 5 min later was returned to the open field for an additional 10-min observation period. Next, the rat was immediately placed in the center of an elevated plus maze and videotaped for 5 min. The maze was 60 cm above the floor and had four arms 10 cm wide×48 cm long. Two opposite arms were enclosed by 39-cm-high opaque walls; the other two arms had no walls. An observer blind to the rat's treatment scored the videotape to determine the time spent with all 4 feet in an open arm or a closed arm, or at least 1 foot in the center of the maze.

2.5. Histology and statistics

Histology was performed to verify probe location. Rats received an overdose of sodium pentobarbital and were perfused with 0.9% saline followed by 10% formalin. Brains were removed, and sectioned 40 μm thick from the anterior lobe caudally until probe tracks were identified.

Data were normalized as percent of baseline and analyzed by two-way ANOVA for repeated measures followed when justified by a Newman–Keuls post hoc comparison.

3. Results

3.1. Basal extracellular levels do not change

Basal levels of DA, DOPAC, HVA and ACh are presented in Table 1. The values are not normalized and not corrected for probe recovery. Values did not differ significantly between groups or following chronic injection of ETOH.

Table 1
No change in basal levels of NAc neurochemicals following ethanol injections on Day 1 and Day 21

Neurochemicals	Day 1		Day 21	
	ETOH	SALINE	ETOH	SALINE
ACh (pmol)	0.51±0.05	0.49±0.07	0.56±0.11	0.49±0.06
DA (fmol)	17.7±1.5	14.5±0.9	14.9±1.9	13.3±1.9
DOPAC (pmol)	3.35±0.5	3.46±0.8	3.60±0.4	3.66±0.6
HVA (pmol)	2.82±0.4	2.31±0.4	2.46±0.4	2.33±0.3

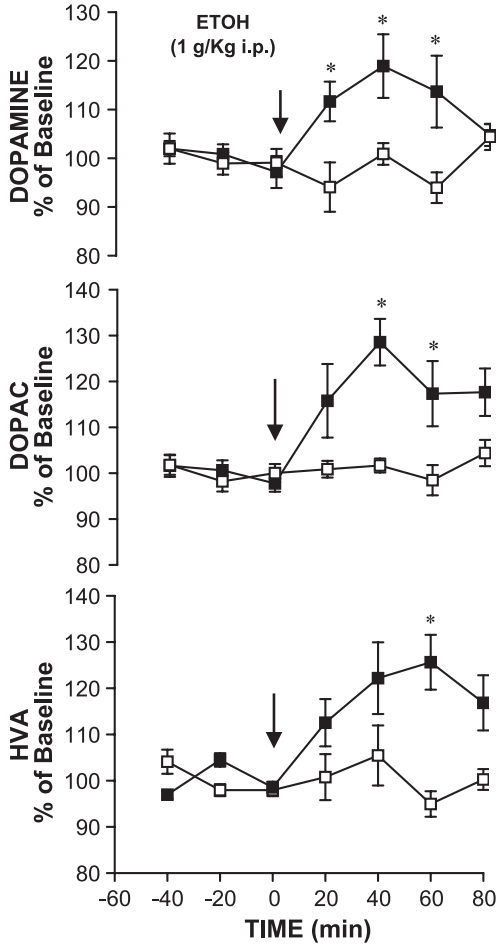

Fig. 1. Ethanol (1 g/kg i.p.) in Experiment 1 releases DA, which is metabolized to DOPAC and HVA, in the NAc. The peak response is 40 min after injection (*p<0.02).

3.2. Experiment 1: acute ETOH significantly increases accumbens DA without affecting ACh

An injection of ETOH significantly increased DA levels to 119±6.5% compared to 100±2% in saline-injected rats (F(6,48)=4.03, p<0.02, Fig. 1). This increase in DA was accompanied by a significant increase in both metabolites DOPAC and HVA (F(6,48)=5.509, p<0.01 and F(6,48)=3.868, p<0.01, respectively; Fig. 1). However, extracellular levels of ACh were unchanged after the ETOH injection (107±6% in ETOH-injected rats vs. 103±4% in saline-injected rats).

3.3. Experiment 2: daily ETOH repeatedly increases accumbens DA

After 21 days of daily injection, no tolerance was seen in ETOH's ability to increase DA levels. DA increased to 126±2.5% compared to 101±2% in saline-treated animals (F(6,48)=6.1, p<0.01, Fig. 2). This increase was similar to that observed following the acute ETOH injection in Experiment 1, with a maximum occurring

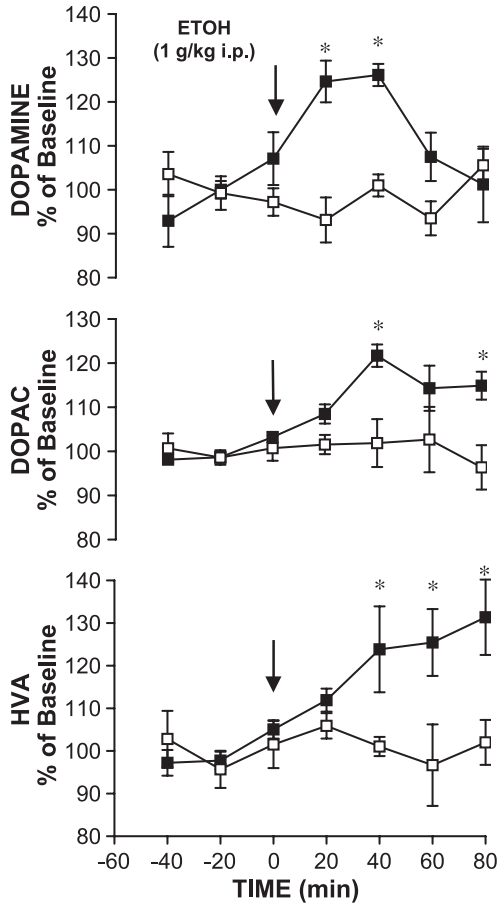

Fig. 2. Ethanol (1 g/kg i.p.) given daily for 21 days in Experiment 2 causes extracellular DA, DOPAC and HVA to increase in the NAc on the last day, as on the first day (*p<0.01).

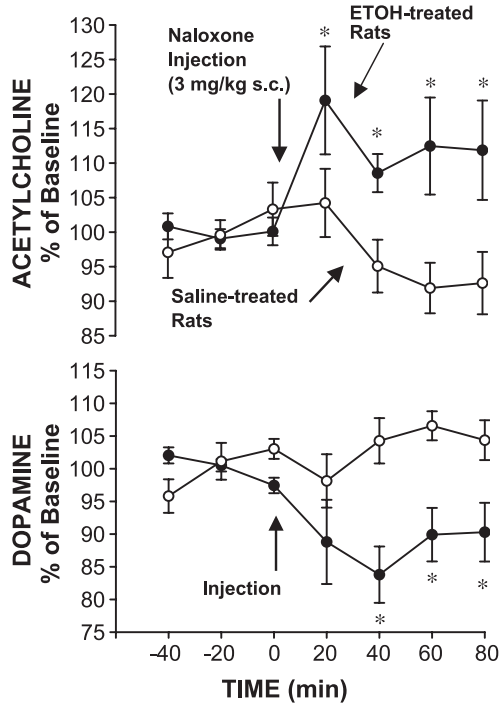

Fig. 3. Rats received ethanol injection (1 g/kg i.p.) daily for 21 days followed by treatment with an opioid antagonist. Naloxone (3 mg/kg s./c.) given on Day 22 caused a decrease in DA (top graph) and release of ACh instead (bottom graph). The ACh effect peaks in about 30 min and both effects last more than an hour. This is a neurochemical sign of opioid withdrawal.

during the 2nd sample. DOPAC and HVA also increased significantly (F(6,48)=5.012, p<0.01 and F(6,48)=4.217, p<0.01 respectively, Fig. 2). Again, ETOH did not change accumbens ACh levels (103±3% in ETOH-treated rats compared to 104±4% in saline-treated animals).

3.4. Experiment 3: naloxone decreases accumbens DA and increases ACh release in ETOH-treated rats

Naloxone (3 mg/kg, s.c.) significantly decreased DA levels to 83±4% of baseline levels (F(6,54)=4.834, p<0.01, Fig. 3), and simultaneously increased extracellular ACh levels to 119±8% (F(6,72)=3.052, p<0.02, Fig. 3). Histology showed that all microdialysis probes were localized mainly in the shell of the NAc (Fig. 4).

3.5. Experiment 4: effect of systemic naloxone on overt withdrawal behavior

There was no difference between the rats treated with naloxone and those treated with saline in any behavioral measure. ETOH-treated rats traveled 3436±614 cm in the open field while saline-treated rats traveled 3168±299 cm. In the plus maze, ETOH-treated rats spent 75±18 s in the center or open arm while saline-treated rats spent 76±11 s.

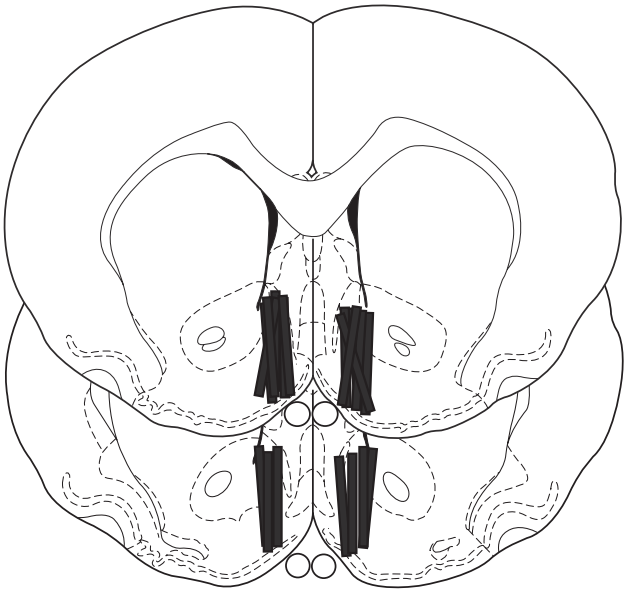

Fig. 4. The tips of the microdialysis probes were mainly localized in the shell of the nucleus accumbens.

4. Discussion

Ethanol produced an increase in DA release on both the 1st day and 21st day of injection, confirming previous findings by others. The lack of tolerance to this important neurochemical effect of ETOH is in agreement with research involving different routes of alcohol administration (Di Chiara and Imperato, 1985; Imperato and Di Chiara, 1986; Fadda et al., 1989; Weiss et al., 1993; Bassareo et al., 1996; Yim and Gonzales, 2000) and in several different lines of alcohol-preferring rats (Kiianmaa et al., 1995; Piepponen et al., 2002). The data are consistent with DA playing a role in ETOH reinforcement of behavior.

Acute and repeated ETOH injections also increased DA metabolites, DOPAC and HVA. This suggests that ETOH releases DA in the NAc and is not acting as a reuptake blocker, confirming a previous study (Yim and Gonzales, 2000). It is known that systemic or local ethanol injections can release DA by acting directly in the NAc (Wozniak et al., 1991; Yoshimoto et al., 1997) or indirectly through activation of dopamine neurons in the VTA (Brodie et al., 1999; Ericson et al., 2003).

Little is known about the effect of ETOH on accumbens ACh. Studies using accumbens tissue slices found that repeated ETOH caused an increase in electrically evoked release of [^{14}C]ACh, and this response was linked to behavioral sensitization (Nestby et al., 1997). In the present studies, neither acute nor repeated ETOH significantly changed ACh release in the NAc. This difference in results can be attributed to the analytical techniques used (tissue slices vs. in vivo microdialysis). Unlike ETOH, which has no effect, or morphine, which decreases accumbens ACh, nicotine, amphetamine and cocaine can increase accumbens ACh levels (Hurd et al., 1990; Lindefors et al., 1992; Mark et al., 1999; Rada et al., 2001), but all these drugs seem to have a larger effect on DA than ACh, judging in terms of percent increase. Thus DA/ACh balance may be a central factor in substance abuse. When a drug increases accumbens DA more than ACh, it is likely to be abused.

In the present study, 21 days of repeated ETOH (1 g/kg, daily) did not change basal levels or the amount of ACh released in the NAc. In the hippocampus and cortex, chronic ETOH decreased basal ACh and acetyltransferase activity (Imperato et al., 1998). This could be due to the different cholinergic systems involved, one being cholinergic interneurons within the accumbens, and the other being a cholinergic projection system from nucleus basalis to the hippocampus.

The accumbens cholinergic system plays an important role in feeding behavior. ACh increases in the NAc at the end of the meal (Mark et al., 1992a,b), and neostigmine infused into the NAc can stop feeding in hungry rats (Mark et al., 1992a, b). A conditioned taste aversion (CTA) also increases ACh (Mark et al., 1995), and injection of neostigmine into the NAc is sufficient to induce a CTA (Taylor et al., 1992). These results suggest that ACh in the NAc is probably involved in signaling normal satiety and taste aversion. It is interesting that the drugs of abuse that increase accumbens ACh, i.e. nicotine, amphetamine and cocaine, are the ones that suppress appetite. Ethanol, on the other hand, releases DA but not ACh. This could be one reason ethanol stimulates feeding rather than suppressing it (Blackburn and Verbalis, 1994; Fujita et al., 2003; Yeomans et al., 2003).

One can speculate as to the underlying neurotransmitter interactions. Neurophysiological observations show that ACh and DA interact in the control of output neurons in the NAc (Galarraga et al., 1999; Calabresi et al., 2000; de Rover et al., 2002; de Rover et al., 2004).The present results are consistent with the hypothesis that ACh in the NAc can counter the effects of DA. This is thought to occur via M1 muscarinic receptors (Chau et al., 2001).

Opioid antagonists given systemically or locally have been shown to block ETOH-induced DA release in the NAc (Acquas et al., 1993; Gonzales and Weiss, 1998). This is thought to be one way that opiate antagonists suppress ETOH intake in rats and humans (Volpicelli et al., 1992; O'Brien et al., 1996; Heyser et al., 1999; Koob et al., 2003). In the present report, systemic naloxone induced a small but significant decrease in DA that was not seen in the control rats. This supports the suggestion that repeated ETOH injections tonically activate endogenous opioid systems that can, in turn, control DA release. One of several ways this may occur is via opioid inhibition of GABA inhibition (i.e. disinhibition) of DA cells in the ventral tegmental area (Di Chiara and Imperato, 1988a; Bergevin et al., 2002; Leite-Morris et al., 2004).

The principle result was that opiate blockade with naloxone in ETOH-treated rats significantly increased extracellular ACh in the NAc. This increase in ACh, and not DA, is similar to the response observed with naloxone-precipitated withdrawal in morphine-dependent animals (Rada et al., 1991; 1996) or sugar-dependent animals (Colantuoni et al., 2002).

This condition, high extracellular ACh relative to DA, may be aversive. Electrical stimulation of the medial hypothalamus that is aversive releases accumbens ACh, and if rats are allowed to bar press to escape from this stimulation they will successfully attenuate the ACh response (Rada and Hoebel, 2001). This suggests that high ACh is part of a neural condition that rats find aversive. Supporting evidence comes from the release of accumbens ACh by a conditioned taste aversion (Mark et al., 1995). Thus ACh released by ETOH withdrawal may create an ACh-mediated aversive condition. Other candidate neurotransmitter systems for aversive withdrawal have been proposed and include corticotrophin-releasing factor (CRF) and the accumbens kappa-opioid receptor system (Spanagel et al., 1992; Thompson et al., 2000; Koob and Le Moal, 2001; Shippenberg et al., 2001; Koob, 2003).

In conclusion, periodic ETOH injections repeatedly release DA in the NAc. At the same time, the animal becomes dependent on ETOH to release opioids. Dependency is shown indirectly by naloxone-induced DA/ACh imbalance indicative of opioid withdrawal. This neurochemical change has been proposed to mediate an aversive state associated with withdrawal (Rada et al., 1991; 1996; 2001; Hoebel et al., 1999; Rada and Hoebel, 2003). In this way, ETOH may be similar to morphine, sugar, diazepam and nicotine, all of which produce a disproportionate rise in accumbens ACh release during withdrawal.

Acknowledgements

Supported by USPHS grants AA-12882, DA-10608 and the Minnie and Bernard Lane Foundation.

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