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Acetylcholine in the accumbens is decreased by diazepam and increased by benzodiazepine withdrawal: a possible mechanism for dependency

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Abstract

Diazepam is a benzodiazepine used in the treatment of anxiety, insomnia and seizures, but with the potential for abuse. Like the other benzodiazepine anxiolytics, diazepam does not increase dopamine in the nucleus accumbens. This raises the question as to which other neurotransmitter systems are involved in diazepam dependence. The goal was to monitor dopamine and acetylcholine simultaneously following acute and chronic diazepam treatment and after flumazenil-induced withdrawal. Rats were prepared with microdialysis probes in the nucleus accumbens and given diazepam (2, 5 and 7.5 mg/kg) acutely and again after chronic treatment. Accumbens dopamine and acetylcholine decreased, with signs of tolerance to the dopamine effect. When these animals were put into the withdrawal state with flumazenil, there was a significant rise in acetylcholine (145%, $P<0.001$) with a smaller significant rise in dopamine (124%, $P<0.01$). It is suggested that the increase in acetylcholine release, relative to dopamine, is a neural component of the withdrawal state that is aversive.
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1. Introduction

Diazepam (Valium®) is a benzodiazepine that binds to a specific site on the γ-aminobutyric acid (GABA) receptor (Stephenson, 1995). It is frequently used as an anxiolytic; however, prolonged treatment may result in the development of dependence in animals and humans (Martin et al., 1993; Woods et al., 1992). Dependence is revealed by withdrawal signs and unpleasant symptoms following either abrupt suspension of drug intake or injection of a specific benzodiazepine antagonist such as flumazenil (Martin et al., 1993; Woods et al., 1992). Withdrawal symptoms, including anxiety, discomfort, extreme dysphoria, paresthesias and psychotic reactions, have been documented (Ashton, 1991; Saxon et al., 1997). Several authors suggest that patients maintain the intake of benzodiazepines to avoid the discomfort of withdrawal symptoms (Griffiths and Weerts, 1997; Lucki et al., 1991). It is generally agreed that benzodiazepines can be addictive (Martin et al., 1993; Woods et al., 1992).

The mesolimbic dopamine pathway plays a key role in addictive behavior (Berridge and Robinson, 1998; DiChiara and Imperato, 1988; Hernandez and Hoebel, 1988; Hoebel et al., 1999; Koob et al., 1996; Petit and Justice, 1989; Wise, 1989). Rats self-administer dopamine agonists directly into the nucleus accumbens (Hoebel et al., 1983; Ikemoto et al., 1997), and almost all drugs of abuse increase extracellular dopamine levels in the nucleus accumbens (DiChiara and Imperato, 1988), even when delivered locally (DiChiara and Imperato, 1988; Hernandez and Hoebel, 1988; Rada et al., 1996). Conversely, dopamine antagonists block self-administration of dopaminergic drugs (Caine et al., 1995; Caine and Koob, 1994). Since benzodiazepines are an exception to the rule that drugs of abuse potentiate the mesolimbic dopamine system (DiChiara and Imperato, 1988), we hypothesized that another neurotransmitter system is probably involved.

A major aspect of drug addiction is the alleviation of aversive withdrawal symptoms (Koob, 2001). The accumbens is involved in the aversive aspects of drug withdrawal.

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In morphine-dependent rats, the accumbens was found to be one of the most sensitive brain sites to produce a conditioned place aversion when methylnaloxonium was locally injected (Stinus et al., 1990). Several experiments suggest that acetylcholine from interneurons in the accumbens could be involved in aversive aspects of drug withdrawal. For example, naloxone-induced withdrawal in morphine-dependent rats produces a significant increase in accumbens acetylcholine levels (Fiserova et al., 1999; Rada et al., 1991, 1996). Similarly, mecamylamine-induced withdrawal in nicotine-dependent animals also releases acetylcholine in the nucleus accumbens (Rada et al., 2001). A direct causal effect is shown with local injection of neostigmine to increase acetylcholine in the nucleus accumbens, which induces a conditioned taste aversion (Taylor et al., 1992). Furthermore, a conditioned taste aversion significantly increases extracellular levels of acetylcholine in the nucleus accumbens (Mark et al., 1995). All these results point to a possible role of the accumbens cholinergic system in aversion. Previous studies show that acute diazepam decreases acetylcholine significantly in the cortex and in the hippocampus (Dazzi et al., 1995a,b; Imperato et al., 1994a,b; Zsilla et al., 1976), but the effect of diazepam on acetylcholine in the nucleus accumbens has not been previously investigated.

The present study monitored dopamine and acetylcholine simultaneously following acute and chronic diazepam injection and after flumazenil-induced withdrawal. The prediction was that diazepam would decrease basal acetylcholine release and withdrawal would increase it.

2. Materials and methods

2.1. Subjects and surgery

Forty-one Sprague–Dawley male rats weighing 300–320 g were housed in individual wire cages in a climate controlled room with a 12/12 reverse light/dark cycle and with food and water ad libitum. For surgery, they were anesthetized with ketamine hydrochloride (50 mg/kg i.p.) supplemented with xylazine (10 mg/kg i.p.). Bilateral guide shafts made of 21-gauge stainless steel tubing were stereotaxically implanted above the nucleus accumbens at coordinates: AP: 1.2 mm, L: 1.0 mm and V: 4.0 mm. A week later, microdialysis probes were inserted and cemented in place to extend another 5 mm to reach the shell of the nucleus accumbens. The experiments were conducted with approval of the Institutional Animal Care and Use Committee.

2.2. Microdialysis procedure

Concentric microdialysis probes used in this study were made in the laboratory (Hernandez et al., 1986). A cellulose hollow fiber (6000 MW cutoff) was attached at one end of a 26-gauge stainless steel tube and plugged with epoxy at the tip, leaving 2-mm cellulose exposed. The microdialysis probe was cemented in place 16 h before the experiment.

The probe was perfused with a modified Ringer's solution (142 mM NaCl, 3.9 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.35 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.4) at a flow rate of 0.5 μl/min overnight and 2 μl/min starting 2–3 h before the experiment. A small amount of neostigmine (0.3 μM) was added to the perfusion fluid to retard enzymatic degradation of acetylcholine.

2.3. Neurochemical assays

Acetylcholine and dopamine were measured simultaneously by splitting the sample into two equal volumes that were injected in separate high performance liquid chromatographs with electrochemical detectors (HPLC-EC). Acetylcholine was measured by reverse-phase, HPLC-EC using an ESA model 580 pump and mobile phase of 200 mM potassium phosphate (pH 8) at a flow rate of 0.6 ml/min. Brain dialysates were injected into a 20-μl sample loop leading to a 10-cm C18 analytical column to separate acetylcholine, which was then converted to betaine and hydrogen peroxide by an immobilized enzyme reactor (columns from Varians). Pure acetylcholine (Sigma Chem.) was used as the standard. Detection was accomplished with an amperometric detector (model 400, EG&G Princeton Applied Res.) that oxidized the hydrogen peroxide with a platinum electrode (BAS) set at 0.5 V with respect to a Ag-AgCl reference electrode (EG&G Princeton Applied Res.). The enzyme reactor was prepared with acetylcholinesterase and choline oxidase (Sigma Chem.).

Dopamine and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed by reverse-phase HPLC-EC (Coulochem model 5100; ESA). A phase II column with 3.2-mm bore and 3-μm C18 packing was used. The mobile phase was 60 mM sodium phosphate monobasic, 100 μM EDTA, 1 μM heptanosulfonic acid and a 4.5% v/v methanol (pH 3.6) at a flow rate of 1 ml/min.

2.4. Drugs and chemical reagents

Diazepam (7.5, 5.0 or 2.5 mg; Sigma Chem.) was used for acute and chronic administration and prepared in 100 μl Tween 80+400 μl water. Flumazenil (Roche Pharmaceutical) in a 1 mg/5 ml solution was used as a benzodiazepine receptor antagonist to cause withdrawal.

2.5. Treatment 1: test of acute diazepam on accumbens acetylcholine and dopamine

Samples were collected at the beginning of the dark phase of the circadian cycle every 20-min starting 60 min before the drug injection. Three samples were used as baseline control, and then subjects were injected with diazepam (7.5, 5.0 or 2.5 mg/kg i.p., n=7, n=6 and n=5,

Table 1
Basal levels of acetylcholine, dopamine and metabolites following chronic diazepam treatment

Neurotransmitter	Before diazepam treatment	After chronic diazepam
Acetylcholine	130±10 fmol/20 μl	80±10 fmol/20 μl[a]
Dopamine	7.6±1.4 fmol/20 μl	6.6±0.05 fmol/20 μl
DOPAC	7±1.1 pmol/20 μl	5.5±0.47 pmol/20 μl
HVA	6±0.87 pmol/20 μl	5±0.94 pmol/20 μl

[a] Indicates $P<0.05$.

respectively), or vehicle (equal volume, $n=6$) followed by collecting five more samples post-injection.

2.6. Treatment 2: test of chronic diazepam on accumbens acetylcholine and dopamine

After the animals were treated with acute diazepam (7.5 mg/kg dose) or vehicle in treatment 1 (above), then using light anesthesia (metaphane), two 7-cm by 1.47-mm I.D. sylastic tubes each containing 90 mg of recrystallized diazepam were implanted under the skin. A third tube of diazepam was implanted 7 days later in accord with a procedure for chronic treatment previously reported (Martin et al., 1993; Wala et al., 1997). These authors report that this procedure provides sustained release of diazepam for over 28 days, so in our experiment it is expected that diazepam was still being released on day 14. Control animals had empty tubes implanted. On day 13, new dialysis probes were set in place on the opposite side of the brain (in counterbalanced order), and day 14, 20-min samples were collected for 1 h before, and 1 h 40 min after, an injection of diazepam (7.5 mg/kg i.p., $n=7$) or vehicle ($n=5$). Thus, in total, these animals received an acute injection followed by chronic infusion and then another injection.

2.7. Treatment 3: test of flumazenil-induced withdrawal on accumbens acetylcholine and dopamine

The rats from treatment 2 (above) were left in the dialysis cage with the microdialysis probes in place, and on day 15 at the beginning of the dark cycle flumazenil (1.0 mg/kg i.p.) was injected. Samples were collected for 80 min to assess the effects of flumazenil.

2.8. Behavioral tests

Separate groups of rats were implanted with intracerebral guide shafts aimed at the accumbens. One group was given subcutaneous diazepam capsules (same doses as those used in treatment 2) and another group was given empty capsules. On day 13, both groups of rats ($n=6$/ea) were tested in an open-field for 10 min (Med Associates, St. Albans, VT, USA) to make sure that diazepam did not produce a locomotor deficit. The next day rats were injected with flumazenil (1.0 mg/kg i.p.) and 15 min later placed in the elevated plus-maze for 5 min. This test was video taped, and an observer blind to treatment measured the time spent in the open arm, closed arm and in the intersection.

2.9. Histology and statistics

Histology was performed to verify injector and probe location. Subjects received an overdose of sodium pentobarbital and were intracardiacally perfused with 0.9% saline followed by 10% formalin. Brains were removed and frozen for sectioning. Sections, 40 m thick, were taken from the anterior lobe caudally until probe tracks were identified.

Microdialysis data was normalized as percent of baseline and analyzed by multifactorial analysis of variance (ANOVA) for repeated measures followed by post-hoc analysis (Newman–Keuls). Basal levels and behavioral tests were analyzed using Student's t-test. Results are presented as the mean±standard error of the mean (S.E.M.).

3. Results

3.1. Acetylcholine, dopamine and metabolite basal levels before and following repeated injections of diazepam

Initial acetylcholine levels were 130±10 fmol/20 μl and decreased significantly following repeated injections of diazepam to 80±10 fmol/20 μl ($T(6)=2.72$, $P<0.04$, Table 1). Dopamine and metabolite basal levels did not change significantly before or after repeated injections of diazepam (Table 1).

3.2. Treatment 1: an injection of diazepam lowers extracellular acetylcholine and dopamine

Both doses of diazepam (7.5 mg/kg and 5.0 mg/kg) decreased accumbens acetylcholine levels to 63±6% and 64±5% of basal levels, respectively, which was significantly different than vehicle ($F(7,63)=9.37$, $P<0.001$, Fig. 1). A lower dose (2.5 mg/kg) produced a milder decrease in acetylcholine to 77±4% of baseline levels. The effect

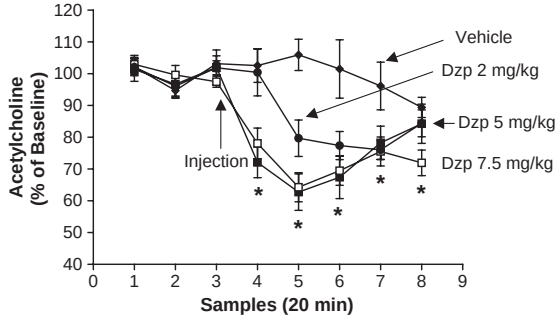

Fig. 1. Diazepam (Valium®) injected i.p. inhibited the release of acetylcholine in the nucleus accumbens (DZP=diazepam; *$P<0.01$ relative to vehicle).

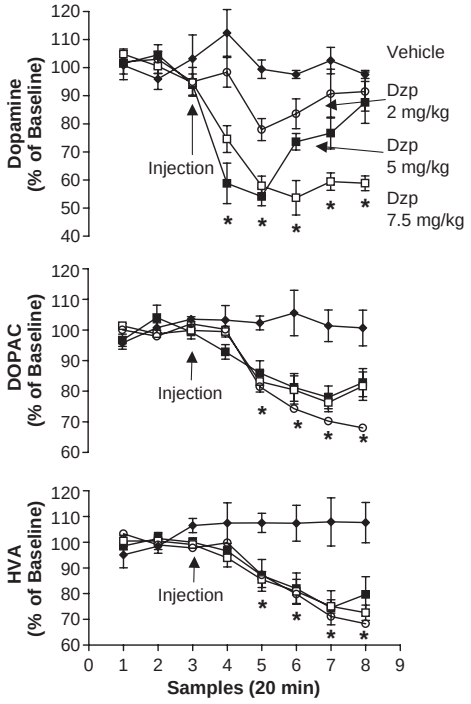

Fig. 2. Diazepam injection decreased extracellular dopamine and its metabolites in the nucleus accumbens (DZP=diazepam; *P<0.01 relative to vehicle).

on acetylcholine was correlated with the dose (y=−8.6x+104.17, R^2=0.9787).

Both doses of diazepam (7.5 and 5.0 mg/kg i.p.) also produced a significant decrease in dopamine, to 54±3% and 54±6%, respectively. The lower dose (2.5 mg/kg) produced a milder effect with a decrease to 78±5% of baseline. This was statistically significant when compared to the minimal effects of the vehicle (F(7,70)=13.67, P<0.001, Fig. 2 top).

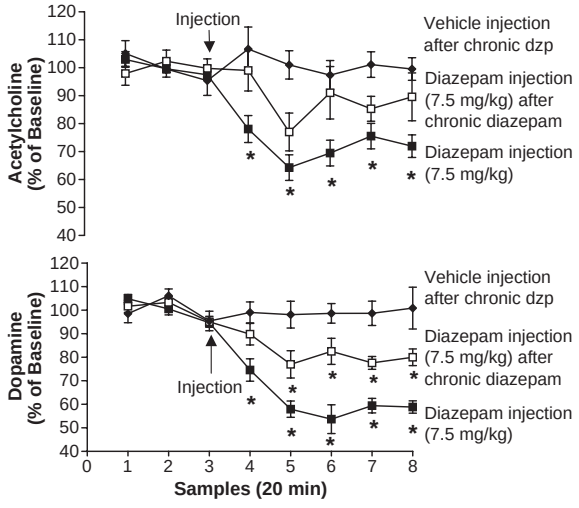

Fig. 3. Diazepam injected after chronic subcutaneous infusion for 14 days is still effective in lowering DA (bottom graph; *P<0.05 compared to vehicle or first diazepam injection). It also lowered ACh but this was not statistically significant when compared to vehicle (P=0.08). Curves from Figs. 1 and 2, for the first diazepam injection, are shown for comparison.

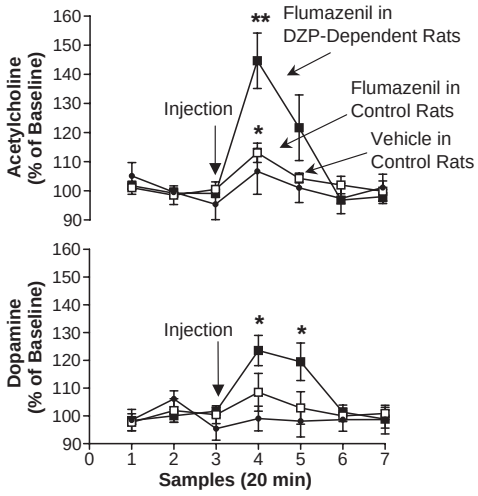

Fig. 4. The benzodiazepine receptor antagonist, flumazenil, 1 mg/kg i.p., caused the release of ACh (top graph) and DA (bottom graph) (DZP=diazepam; *P<0.01 for ACh and P<0.05 for DA, relative to control groups).

The effect on dopamine showed dose-dependency when comparing 0, 2.5 and 5 mg/kg (y=−9.1x+99.917, R^2=0.999). All doses also significantly decreased DOPAC and HVA (DOPAC F(12,90)=5.08, P<0.001, Fig. 2 middle; HVA F(12,90)=4.7, P<0.001, Fig. 2 bottom).

3.3. Treatment 2: an injection of diazepam following chronic infusion lowers acetylcholine and dopamine

Diazepam injection following 14 days of continuous infusion produced a non-significant trend to decrease acetylcholine levels to 77±7% compared to vehicle (F(7,70)=1.9, P=0.08, Fig. 3 top) and an equal, statistically significant, decrease in extracellular levels of dopamine to 77±6% (F(7,70)=3.33, P<0.005, Fig. 3 bottom). Both of these decreases in acetylcholine and dopamine were significantly milder than the effect observed for the same dose on day 1 for acetylcholine (F(7,77)=2.63, P<0.05 and for dopamine F(7,84)=5.709, P<0.01). However, basal extracellular levels of acetylcholine were significantly lower on day 14 than day 1

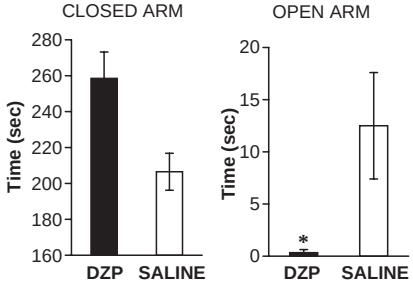

Fig. 5. In diazepam-treated rats (solid bar), flumazenil, 1 mg/kg i.p., decreased time spent in the open arm of the plus-maze and increased time spent on the closed arm when compared to control rats also injected flumazenil (DZP=diazepam; * indicates P<0.05).

(130±10 fmol/20 μl day 1 and 80±10 fmol/20 μl day 14; $t(6)=2.92$, $P<0.05$), while basal dopamine levels did not change significantly (7.6±1.1 fmol/20 μl day 1 and 6.6±0.05 fmol/20 μl day 14). Basal levels for neurotransmitters and metabolites are presented in Table 1.

3.4. Treatment 3: flumazenil increases acetylcholine and dopamine

A low dose of flumazenil (1.0 mg/kg i.p.) given to the chronic diazepam-treated rats caused a large increase to 145±9% in accumbens acetylcholine levels ($F(12,72)=4.59$, $P<0.001$, Fig. 4 top). There was little or no effect on untreated control animals (103±3% of basal levels). Vehicle injection did not affect diazepam-treated rats (107±8%).

Simultaneously, flumazenil produced a mild, although significant, increase in accumbens dopamine levels to 124±5% ($F(12,72)=2.90$, $P<0.01$, Fig. 4 bottom) compared to 108±7% in untreated control rats or 101±9% in treated rats injected with vehicle.

3.5. Behavioral tests

Open-field: on day 13, there was no significant difference between diazepam or saline-treated rats in distance traveled (2904±236 cm vs. 2847±221 cm) demonstrating that, at least on day 13, there was no motor deficit that could confound the results in the plus-maze.

In the plus-maze on day 14, rats chronically treated with diazepam and then given flumazenil did not venture into the open arm (0.3±0.3 s), staying most of the time in the closed arm (259±15 s) or in the intersection (40±15 s). These results were significantly different when compared to saline-treated rats injected with flumazenil (12.5±5 s in the open arm; $t(10)=2.38$, $P<0.04$, 207±10 s in the closed arm; $t(10)=2.89$, $P<0.02$ and 82±6 s in the intersection; Fig. 5). Thus, flumazenil caused anxiety in this behavioral test.

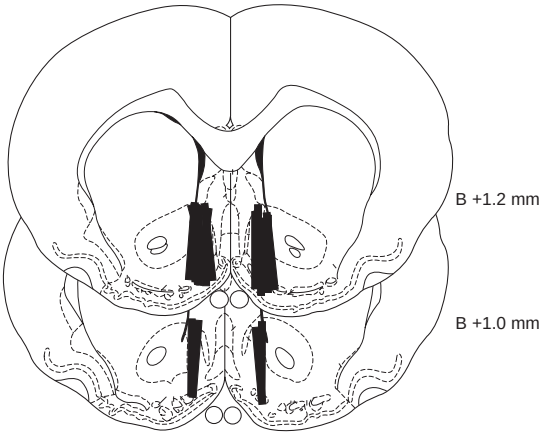

Fig. 6. Frontal sections showing microdialysis probe tracks in the nucleus accumbens, primarily the shell region (*B*=distance from bregma).

3.6. Histology

The tips of the dialysis probes were localized in the nucleus accumbens shell and core, largely within the shell, as shown in Fig. 6.

4. Discussion

It is well known that most addictive drugs increase extracellular dopamine in the nucleus accumbens, however; diazepam is also addictive and, contrary to other drugs, it decreases dopamine levels. So there must be either a different site that is important in diazepam's addictive power or another neurotransmitter system that mediates the addiction. Acetylcholine interneurons in the accumbens are believed to be involved in the addictive properties of several drugs (Hoebel et al., 1999). For example, morphine significantly decreases acetylcholine levels in the nucleus accumbens (Fiserova et al., 1999; Rada et al., 1991, 1996) and, during naloxone precipitated withdrawal, acetylcholine increases (Fiserova et al., 1999; Rada et al., 1991, 1996). The same pattern during mecamylamine-induced withdrawal in nicotine-dependent rats (Rada et al., 2001) was observed indicating that it applies to both opiates and psychostimulants.

To summarize the new results, the present study now shows that an acute systemic injection of diazepam can significantly decrease not only extracellular dopamine in the accumbens as reported by others (Finlay et al., 1992; Invernizzi et al., 1991), but also acetylcholine (Fig. 1). Rats treated continuously for 14 days and then injected with diazepam showed a milder, but still significant, decrease in dopamine with a decrease in acetylcholine as well (Fig. 2). Injection of flumazenil did the opposite by inducing a significant increase in both accumbens acetylcholine and dopamine release, with acetylcholine high relative to dopamine (Fig. 4).

Studies show that dopamine levels in the nucleus accumbens follow a circadian rhythm, with high levels during the rats' active cycle (dark period) and lower levels during the resting cycle (light period) (Castañeda et al., 2004; Paulson and Robinson, 1994). Our experiments were performed at the beginning of the dark period to make sure the observed decrease was due to the drug and not to a circadian response.

Acute diazepam dose-dependently decreased dopamine levels in the accumbens; however, both of the high doses of diazepam used in this study produced similar responses. This effect is probably due to a "floor-effect" suggesting that 5 mg/kg maximally inhibits dopamine output in the nucleus accumbens; although note that the 7.5 mg dose had a longer lasting effect. The decrease in dopamine release is probably mediated in part through GABA receptors in the ventral tegmental area. Local GABA receptor agonists injected in the ventral tegmental area decrease extracellular dopamine

levels in the nucleus accumbens, while an increase is obtained when a GABA receptor antagonist is used (Xi and Stein, 1998). A GABA receptor antagonist injected locally in the nucleus accumbens also increases dopamine release (Yan, 1999). These reports suggest that dopamine cells, in the ventral tegmental area and terminals in the nucleus accumbens, are under tonic inhibition by GABA, specifically through $GABA_A$ receptors.

Acute diazepam decreases extracellular levels of acetylcholine in the accumbens. This decrease may also be mediated directly through a GABAergic system. Previous research has suggested that GABA tonically inhibits cholinergic interneurons in the nucleus accumbens and striatum via $GABA_A$ receptors (DeBoer and Westerink, 1994; Rada et al., 1993).

To be relevant to human drug abuse, chronic studies are needed. In earlier studies, repeated diazepam injections did not cause tolerance to the decrease in dopamine release in the nucleus accumbens (Finlay et al., 1992; Motzo et al., 1997). However, the results presented here suggest that there can be tolerance to the decrease in dopamine. This difference can be attributable to the difference in drug administration, three daily injections in earlier studies vs. the subcutaneous capsules used here.

Basal dopamine levels in the nucleus accumbens were not significantly different between microdialysis on one side the first day and microdialysis 2 weeks later on the opposite side. Therefore, the observed tolerance to the acute diazepam-induced dopamine decrease was probably not due to a change in basal levels, but instead was a change in neural responsivity to diazepam.

Diazepam-treated animals showed a significant decrease in basal acetylcholine levels after two weeks of treatment. Studies of neurotransmitter basal levels could be confounded by probe recovery issues as well as a different site being probed; however, the significantly lower basal acetylcholine accompanied by no modification in basal dopamine levels suggests that the decrease in acetylcholine basal levels is real and probably caused by persistent inhibition by diazepam.

Injection of the specific benzodiazepine antagonist, flumazenil, after chronic diazepam infusion, induced a significant increase in both accumbens dopamine and acetylcholine release. The effect of flumazenil was almost twice as strong for acetylcholine, with a 45% increase for acetylcholine compared to 25% for dopamine. Signs of anxiety as recorded in the plus-maze accompanied these neurotransmitter changes. Flumazenil, at a high dose, can increase acetylcholine levels in the hippocampus and cortex of naïve rats (Imperato et al., 1994a; Dazzi et al., 1995a), and at lower doses, in diazepam-dependent rats (Dazzi et al., 1995b). The present study shows that a low dose of flumazenil increases acetylcholine in the nucleus accumbens of diazepam-dependent animals. This new finding suggests that accumbens acetylcholine may play a role in diazepam-withdrawal.

Flumazenil is well known to induce withdrawal in rats and humans when they become dependent on diazepam (Martin et al., 1993; Pratt et al., 1997; Woods et al., 1992). In naïve rats, flumazenil is known to have no effect on accumbens dopamine release; however, in dependent rats, a significant dopamine increase occurs during withdrawal (Motzo et al., 1997). The present results replicate that finding, with a mild dopamine increase in diazepam-dependent animals during withdrawal. This small increase in accumbens dopamine was probably due to the lower dose of flumazenil used in the present study.

Considering that acetylcholine basal levels were significantly lower in chronically treated animals, an increase in acetylcholine release in the nucleus accumbens could be viewed as a normalization of original values. However, it is not known how the cholinergic system compensated for this persistent decrease in acetylcholine levels. Receptor up-regulation is usually reported, so in this case an increase in accumbens acetylcholine levels could be interpreted as a significant increase in the responsivity of the system. Further research will be needed to clarify this.

The nucleus accumbens seems to be involved in aversive states as well as positive and negative reinforcement (Hoebel et al., 1999; Rada and Hoebel, 2001; Rada et al., 1998). Local injection of methylnaloxonium directly into the nucleus accumbens of morphine-dependent animals is sufficient to induce a conditioned place aversion (Stinus et al., 1990). Recently, it has been shown that injection of a $GABA_A$ agonist in the caudal part of the nucleus accumbens shell elicits a robust conditioned place avoidance and negative aversive facial reactions to sucrose (Reynolds and Berridge, 2002). Precipitated withdrawal from diazepam caused fos-like immunoreactivity (Dunworth et al., 2000) and increased glucose utilization in the accumbens (Pratt et al., 1997). One of the neurotransmitter systems in the nucleus accumbens involved in this aversive state may include the acetylcholine interneurons (Hoebel et al., 1999).

Acetylcholine in the nucleus accumbens appears to be involved in the inhibition of appetitive behaviors and the aversive aspects of drug withdrawal. Previous studies from this laboratory show that, during free-feeding, accumbens acetylcholine reaches a maximum at the beginning of the satiation process during a meal (Mark et al., 1992). A conditioned stimulus in a conditioned taste aversion paradigm also increases accumbens acetylcholine (Mark et al., 1995), and experimentally increasing acetylcholine levels in the nucleus accumbens with neostigmine is sufficient to induce a conditioned taste aversion (Taylor et al., 1992). Aversive handling stimulation also increases accumbens acetylcholine levels (Pfister et al., 1994; Thiel et al., 1998). Increases in accumbens acetylcholine release were observed following naloxone-induced withdrawal in morphine-dependent animals (Fiserova et al., 1999; Rada et al., 1991, 1996), also during mecamylamine-induced with-

drawal in nicotine-dependent rats (Rada et al., 2001) and now in diazepam-dependent rats following a flumazenil injection. These results suggest that the increase in accumbens acetylcholine in diazepam-dependent rats is responsible, at least partly, for the aversive aspects of flumazenil-induced withdrawal. If this is true, then it follows logically that part of diazepam's abuse potential is its ability to lower accumbens acetylcholine as a form of self-medication for withdrawal.

A decrease in extracellular levels of dopamine in the nucleus accumbens has been suggested to signal aversion during morphine-induced and nicotine-induced withdrawal (Acquas and DiChiara, 1992; Diana et al., 1995; Hildebrand et al., 1998; Pothos et al., 1991; Rada et al., 2001). However, in diazepam-dependent animals, flumazenil elicited the opposite response, an increase in dopamine. This implies that it is probably the relationship between dopamine and acetylcholine that determines aversiveness, not either neurotransmitter alone.

The increase in accumbens dopamine following an injection of flumazenil in diazepam-treated rats could be related to an increase in locomotor activity. Most behaviors that increase dopamine (feeding, drinking, psychostimulants) also activate the animal (Hernandez and Hoebel, 1988; Hernandez et al., 1987; Mark et al., 1992). Flumazenil may have acted as a locomotor arousing agent thus increasing dopamine in the accumbens.

In conclusion, diazepam decreased extracellular levels of dopamine and acetylcholine in the nucleus accumbens, and tolerance to this effect was clear for dopamine but not for acetylcholine. Flumazenil-induced withdrawal, on the other hand, produced an increase in both acetylcholine and dopamine, with the effect being relatively mild for dopamine and stronger for acetylcholine. These results confirm the initial prediction that diazepam inhibits accumbens acetylcholine release and withdrawal disinhibits it. This may be relevant in the appearance of the negative state during benzodiazepine withdrawal that contributes to benzodiazepine addiction.

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