

No Role of the Dopamine Transporter in Acute Ethanol Effects on Striatal Dopamine Dynamics

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KEY WORDS microdialysis; voltammetry; DA transporter knock-out mice

ABSTRACT The acute effects of ethanol on dopamine (DA) release and clearance in the caudate–putamen were evaluated in wild-type and dopamine transporter (DAT) knockout (DAT-KO) mice, using microdialysis and voltammetry. Dialysate DA levels were elevated, ~80% above baseline levels, after administration of 2 g/kg ethanol in both wild-type and DAT-KO mice. In brain slices containing the caudate–putamen, a low (20 mM) concentration of ethanol produced no change in electrically stimulated DA release in either wild-type or DAT-KO mice. A high concentration (200 mM) of ethanol caused a similar decrease in DA release in slices from both types of mice. DA clearance was unaltered across the genotypes at low and high concentrations of ethanol. The fact that ethanol had similar effects in wild-type and DAT-KO mice, measured by *in vivo* microdialysis or brain slice voltammetry, supports the idea that acute ethanol does not interact with the DAT to produce its effects on the DA system. **Synapse 60:288–294, 2006.** © 2006 Wiley-Liss, Inc.

INTRODUCTION

The rewarding, stimulating, and addictive properties of drugs of abuse are often associated with an elevation in dopamine (DA) levels in brain areas, such as the caudate–putamen and nucleus accumbens. Ethanol shares this action with many other psychostimulants, including cocaine, amphetamine, and methylphenidate (Ritalin). Termination of the DA signal is accomplished by diffusion of DA molecules away from receptors, metabolism, and uptake by the DA transporter (DAT). DA uptake through the DAT is the major regulator of DA levels (Jones et al., 1998). Many psychostimulants interact directly with the DAT at the level of presynaptic terminals (Giros and Caron, 1993; Schenk, 2002). In contrast, ethanol increases the firing rate of DA cell bodies in the ventral tegmental area and substantia nigra (Brodie et al., 1990, 1999; Mereu and Gessa, 1985), leading to enhanced DA signaling in terminal fields (Di Chiara and Imperato, 1985; Tang et al., 2003; Yan, 1999). There is currently a debate as to whether ethanol also has the ability to directly alter DAT function (Budygin et al., 2001a; Mayfield et al., 2001; Robinson et al., 2005; Sabeti et al., 2003; Yim and Gonzales, 2000).

Acute and chronic ethanol studies have shown a potential link between DAT and ethanol, but the exact mechanism is unclear. Specifically, studies involving

nonhuman primates have shown an increase in caudate–putamen DA uptake rates measured by voltammetry, after 18 months of ethanol exposure (Budygin et al., 2003), and rats treated for a year with ethanol show increased caudate–putamen DAT levels measured by western blotting (Rothblat et al., 2001). However, in humans suffering from alcoholism, the changes in DAT are not as clear. Laine et al. (1999) reported that alcoholics had decreased DAT numbers, while Volkow et al. (1996) showed no changes in DAT numbers during withdrawal. In addition, Tiihonen et al. (1995) and colleagues found divergences in DAT activity, depending on the presence or absence of violent behavior in subjects. Obviously, there is much debate on the long-term effects of ethanol on the DAT. However, to fully comprehend the effects of ethanol on DA uptake, potential acute interactions need to be thoroughly evaluated. To date, acute ethanol administra-

Contract grant sponsor: NIAAA; Contract grant numbers: AA0765, AA11997; Contract grant sponsor: NRSA; Contract grant number: DA016498; Contract grant sponsor: ABMRF; Contract grant numbers: AA014091 and AA013900.

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Received 4 January 2006; Accepted 5 April 2006

DOI 10.1002/syn.20301

Published online in Wiley InterScience (www.interscience.wiley.com).

tion in rodent models has produced conflicting results. A single dose of ethanol has been reported to increase (Sabeti et al., 2003; Wang et al., 1997), decrease (Lin and Chai, 1995; Robinson et al., 2005), or to have no effect (Budygin et al., 2001a; Gonzales et al., 2004; Yavich and Tiihonen, 2000; Yim and Gonzales, 2000) on DA uptake.

DAT knock-out (DAT-KO) mice have proven to be a valuable tool in understanding the mechanism of action of drugs of abuse (Giros et al., 1996; Jones et al., 1998). DAT-KO mice provide an opportunity to directly evaluate whether ethanol is altering DA dynamics via the DAT or by another mechanism (Budygin et al., 2001a,b; Mayfield et al., 2001; Robinson et al., 2005; Sabeti et al., 2003; Yim and Gonzales, 2000). This is an important area of research, because understanding how ethanol modulates DA will aid in understanding ethanol-mediated reinforcement and, ultimately, alcoholism.

In the present study, we utilized *in vivo* microdialysis to compare changes in extracellular DA levels after systemic administration of 2 g/kg ethanol in freely moving wild-type and DAT-KO mice. A stimulating 2 g/kg ethanol dose was chosen, since previous studies using C57/BL6 mice used this dose to evaluate extracellular DA changes (Tang et al., 2003; Zapata et al., 2005). Cyclic voltammetry was also employed to determine local effects of ethanol on DA dynamics, specifically DA clearance, in caudate–putamen slices. In sum, the *in vivo* microdialysis and *in vitro* voltammetry data demonstrate that the acute effects of ethanol on DA neurotransmission are not DAT-dependent.

MATERIALS AND METHODS

Animals

Littermate mice, expressing zero (DAT-KO) or two (wild-type) intact copies of the DAT gene (*Slc6a3*), were generated as previously described on a mixed 129SvJ/C57BL background (Giros et al., 1996). For all experiments, male wild-type or DAT-KO mice were used that were 2–3 months old. Animals were housed in groups of three or four per cage, with food and water *ad libitum* on a 12-h light–dark cycle. Experimental protocols adhered to National Institutes of Health Animal Care Guidelines and were approved by the Wake Forest University Institutional Animal Care and Use Committee.

Fast-scan cyclic voltammetry in brain slices

Mice were killed by decapitation, and the brains were rapidly removed and cooled in ice-cold, preoxygenated (95% O₂/5% CO₂), modified in Krebs buffer (containing (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl, 25 NaHCO₃, 11 glucose, 20 HEPES, 0.4 L-ascorbic acid), and the pH was adjusted to 7.4. The tissue was then sectioned into 400- μ m thick coronal

slices containing the caudate–putamen, with a vibrating tissue slicer (Leica VT1000S, Vashaw Scientific, Norcross, GA). Slices were kept in a reservoir of oxygenated aCSF at room temperature until required. Thirty minutes before each experiment, a brain slice was transferred to a submersion recording chamber, perfused at 1 ml/min at 25°C with oxygenated Krebs, and allowed to equilibrate. DA release was evoked by single, rectangular, electrical pulses (300 μ A, 2 ms/phase, biphasic). DA was detected using voltammetry as described earlier (Budygin et al., 2002; Phillips et al., 2003). Electrodes were calibrated with 1- μ M DA to convert the signal from current to concentration. Measured time courses of DA before and after ethanol (20 or 200 mM) were analyzed with a Michaelis–Menten based set of kinetic equations (Phillips et al., 2003), to determine the maximal concentration of DA released by stimulation and the rate of DA uptake for wild-type mice. Since DAT-KO mice lack the DAT, DA clearance curves obtained from these animals were fit to a pseudo-first-order rate constant (k). To compare kinetics between genotypes, a rate constant k was calculated for wild-type mice by dividing V_{\max} by K_m , as described before (Budygin et al., 2002; Jones et al., 1998; Mateo et al., 2004a).

Surgery for *in vivo* microdialysis

Briefly, mice were anesthetized with 65 mg/kg ketamine and 4.4 mg/kg xylazine, administered in a volume of 20 μ l/g. A guide cannula for a CMA/7 microdialysis probe (CMA/Microdialysis, Chelmsford, MA) was implanted into the caudate–putamen, using coordinates determined from mouse atlases (Franklin and Paxinos, 1997; Slotnick and Leonard, 1975), and refined by empirical determination (anterior, +0.6; lateral, –1.8; ventral, –2.5 from bregma). The guide cannula was anchored, and the exposed skull was sealed with a fast-drying two-part epoxy (Loctite, Fastneal, State College, PA). Immediately after dialysis, mice were killed by cervical dislocation, and the brains were removed for histological confirmation of probe placement.

Microdialysis

The microdialysis procedure is essentially as described by Mateo et al. (2004b), with the following modifications. Briefly, as mice were recovering from anesthesia, microdialysis probes (2 mm membrane length, 0.24 mm o.d.; Cuprophane, 6 kDa cut-off; CMA-7, CMA/Microdialysis, Chelmsford, MA) were connected to a syringe pump and perfused with artificial cerebral spinal fluid (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 0.85 mM MgCl₂; pH 7.4) at a flow rate of 0.2 μ l/min over night. Approximately 12 h later, the flow rate was increased to 0.6 μ l/min and allowed to adjust for 1 h before the four baseline samples

were collected at 20-min intervals, and analyzed immediately by high-performance liquid chromatography with electrochemical detection. Dialysate samples (10 μ l) were injected onto a reverse-phase microbore column (150 \times 1 mm², C-18 UniJet, Bioanalytical Systems, West Lafayette, IN) for separation, followed by detection at a glassy carbon electrode (+0.65 V, Bioanalytical Systems). The mobile phase consisted of 25 mM sodium acetate, 1 mM sodium octanesulfonate, 2 mM EDTA, and 10% acetonitrile (pH 5.8). Ethanol was injected i.p. (i.p.), and the samples were collected for 3 h.

Statistics

Statistical analyses using one-way ANOVA, two-way ANOVA, and paired Student's *t*-tests were performed with GraphPad Prism (Graph Pad Software, San Diego, CA). The data are presented as mean \pm SEM. The criterion of significance was set at $P < 0.05$.

RESULTS

Ethanol-stimulated DA release in vivo

Ethanol (2 g/kg, i.p.) was administered at the start of the last baseline sample. DA was measured subsequently in 20-min intervals. DA levels in three samples prior to ethanol were averaged to obtain mean baseline values, to determine the effect of ethanol on extracellular DA levels in striatum by calculating the percent change from baseline. Baseline dialysate DA levels before the injection of ethanol were 2.1 ± 0.4 nM ($n = 7$) for wild-type and 9.4 ± 3.0 nM ($n = 6$) for DAT-KO mice ($t = 2.6$, $P < 0.05$). After administration of 2 g/kg ethanol, DA dialysate levels were measured in the caudate–putamen (Fig. 1). An increase of DA level by ethanol was observed in caudate–putamen [$F_{9,80} = 3.13$, $P < 0.01$], with a maximal elevation for wild-type and DAT-KO mice to $186 \pm 25\%$ at 60 min ($n = 7$) and $166 \pm 24\%$ at 80 min ($n = 6$) of baseline, respectively (Fig. 1). There was no difference between the wild-type and DAT-KO response to ethanol [$F_{1,80} = 0.29$; $P = 0.59$]. Thus, the increase in extracellular DA levels in the caudate–putamen after an i.p. dose of ethanol is not dependent on the DAT, and is not significantly altered by its absence.

DA signals are changed by acute ethanol identically in caudate–putamen slices from wild-type and DAT-KO mice

Two studies suggest that ethanol changes the rate of DA uptake (Mayfield et al., 2001; Robinson et al., 2005). Using DAT-KO mice, we can specifically address the contribution of DAT to the effects of ethanol on DA levels. Voltammetry was used to evaluate the effects of ethanol on DA dynamics in mice with normal transporter levels and mice lacking the transporter. Figure 2

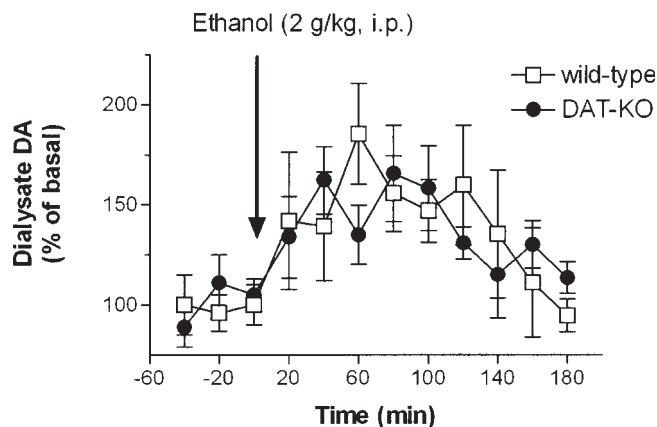


Fig. 1. Ethanol (2 g/kg) increased extracellular DA levels, in the caudate–putamen of wild-type and DAT-KO mice, as measured by microdialysis. Three baseline samples were collected and, then, ethanol was administered i.p. during the start of the last baseline sample (arrow). Ethanol increased DA levels ~ 90 and $\sim 70\%$ in wild-type and DAT-KO mice, respectively. Data are mean \pm SEM values from five or six mice and are expressed as percent of baseline values.

depicts the voltammetry data obtained in brain slices from a representative wild-type and a knockout mouse, before and after ethanol perfusion. The ascending phase of the DA signal primarily represents release during the stimulus pulse (1 pulse). The descending portion of the overflow curves represents the rate of DA clearance, which is clearly longer in the knockout compared to the wild-type mice (100 vs. 1 s, respectively).

In caudate–putamen slices, 20 or 200 mM ethanol had no effect on DA clearance in wild-type ($k = 25.1 \pm 0.2$, 25.1 ± 0.3 , 25.1 ± 0.2 s⁻¹ for 0, 20, and 200 mM ethanol, respectively) or knockout mice ($k = 0.02 \pm 0.002$, 0.02 ± 0.001 , 0.02 ± 0.002 s⁻¹ for 0, 20, and 200 mM ethanol, respectively) (Fig. 3). Only 200 mM ethanol caused a decrease in the amplitude of the DA signal in slices from both wild-type (2.7 ± 0.03 and 1.9 ± 0.02 μ M, for 0 and 200 mM ethanol ($P < 0.05$)) and knockout (0.7 ± 0.04 and 0.5 ± 0.05 μ M, for 0 and 200 mM ethanol ($P < 0.05$)) mice and the percent decrease in signal ($\sim 25\%$) was the same in wild-type and DAT-KO mice (Fig. 4).

DISCUSSION

The precise mechanism through which acute ethanol modifies dopaminergic neurotransmission remains unclear, since there is a controversy over the interaction of ethanol with DAT (Budygin et al., 2000, 2001b; Gonzales et al., 1998; Maiya et al., 2002; Mayfield et al., 2001; Robinson et al., 2005). The primary goal of this article was to clarify the involvement of DAT in the effects of ethanol on the DA system. We report here that ethanol concentrations of 20 and 200 mM had identical effects on DA dynamics in the caudate–putamen of wild-type and DAT-KO mice. Since the absence of the DAT did not alter the DA effects of etha-

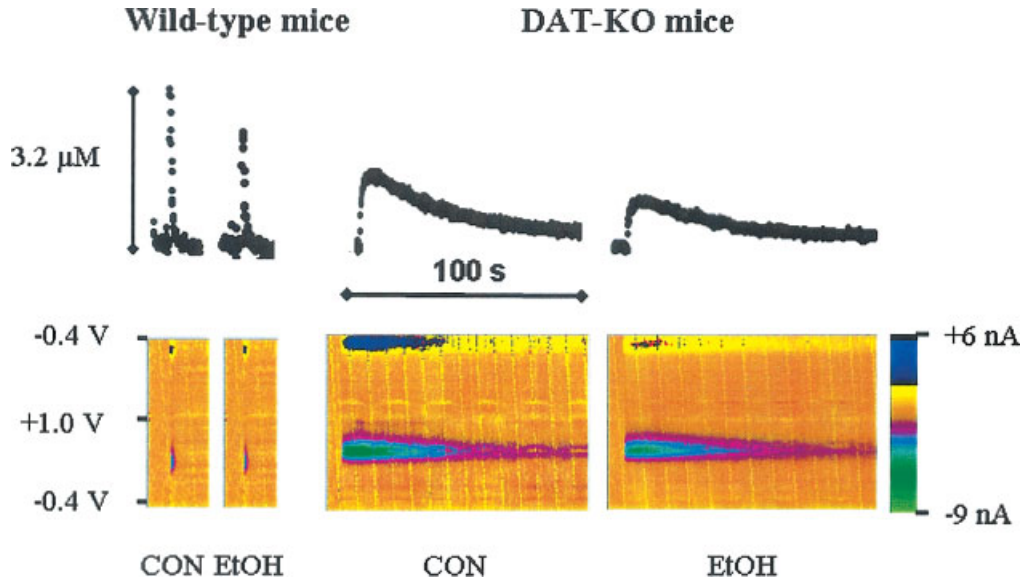


Fig. 2. Effect of ethanol on representative electrically evoked (1 pulse) DA signals in caudate-putamen slices from wild-type and DAT-KO mice. Top panel: Control (CON) and ethanol (EtOH) data points are voltammetry DA measurements taken every 100 ms. Note that the DA release was decreased by $\sim 25\%$ in both wild-type

and DAT-KO mice when 200 mM ethanol was applied to the slice. Ethanol did not alter DA uptake in caudate-putamen slices from either genotype. Bottom panel: representative color plots. The voltammetric current (encoded in color in the z-axis) is plotted against the applied potential (y-axis) and the acquisition time (x-axis).

nol, we conclude that DAT does not play an important role in the interaction of ethanol with the DA system.

In the present study, microdialysis and voltammetry were used to examine the effects of an acute dose of ethanol on DA release and clearance. Microdialysis is a sampling technique that measures baseline DA concentrations with high selectivity and sensitivity (Bungay et al., 2003; Kehr, 1993; Ungerstedt and Pycock, 1974). However, there are disadvantages associated with *in vivo* microdialysis techniques, including (1) the relatively large size of the probe ($0.24 \times 2 \text{ mm}^2$), (2) the damage associated with inserting the microdialysis probe into the brain region under study (Clapp-Lilly et al., 1999), and (3) the temporal resolution, which is on the order of minutes. Because of these weaknesses, microdialysis does not thoroughly evaluate changes in DA uptake. In contrast, voltammetry is unable to measure basal DA levels, but can provide real-time analysis of DA kinetics, such as release and uptake. Therefore, the use of both microdialysis and voltammetry yields complementary data on extracellular DA levels, release and uptake, which allows for a more complete understanding of the complex relationship between ethanol, extracellular DA levels, and the role of DAT.

Previous microdialysis studies in mice have shown 50–170% increases in extracellular DA levels in response to an acute dose of ethanol (Hungund et al., 2003; Olive et al., 2000; Tang et al., 2003). The microdialysis results obtained in wild-type mice presented here are in agreement with these values. In addition, administration of 2 g/kg ethanol evoked a similar increase in DA levels ($\sim 80\%$) in DAT-KO and wild-type mice,

suggesting that ethanol does not interact with the DAT to produce changes in DA levels. Zero net flux is a microdialysis method that provides an indirect estimate of changes in DA uptake by measuring E_d , known as the extraction fraction, which is the slope of the zero net flux regression line (Justice, 1993). A previous study by Yim and Gonzales (2000) using the method of zero net flux also demonstrated that ethanol did not alter DAT function.

To evaluate a physiologically relevant ethanol concentration on electrically stimulated DA release and uptake, voltammetry was performed in caudate-putamen slices. We report here that 20 mM ethanol produced no change in stimulated DA release in the caudate-putamen of wild-type or DAT-KO mice. This is in agreement with previous brain slice voltammetry studies performed with monkeys, rats, and mice, where low concentrations ($<100 \text{ mM}$) of ethanol did not cause a change in stimulated release or uptake (Budygin et al., 2000, 2001b, 2003). The divergence between ethanol-induced increases in DA release measured by *in vivo* microdialysis and no effect on release measured by voltammetry in brain slices is due to the preparation used. The slice preparation used for voltammetry requires the removal of the cell bodies, which are an essential ethanol target; therefore, there is no ethanol-induced increase of DA release in the terminal fields. These *in vitro* voltammetry results are also consistent with microdialysis findings, where the local perfusion of ethanol at low and moderate concentrations failed to modify basal DA levels in the striatum of freely moving rats (see also Yan, 2003, Yim et al., 1998). The local

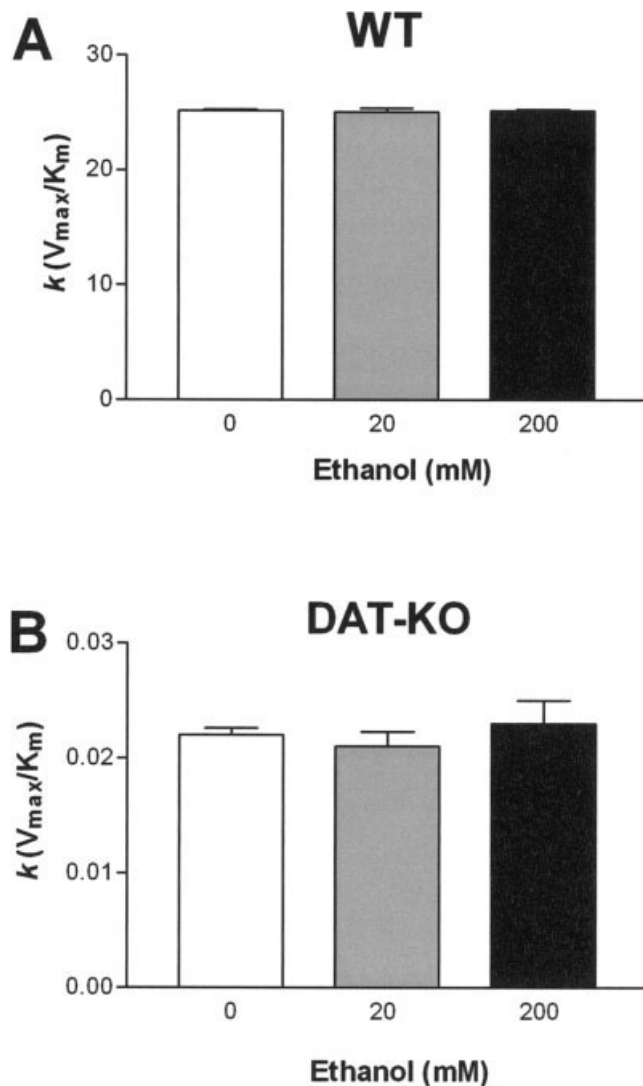


Fig. 3. Effect of ethanol on DA clearance in wild-type (WT) and DAT-KO mice. Locally evoked DA signals are measured by voltammetry during 0, 20, or 200 mM ethanol-bath application. k is the rate constant for DA clearance. **A:** Comparison of 0, 20, and 200 mM ethanol in wild-type mice ($k = 25.1 \pm 0.2$, 25.1 ± 0.3 , 25.1 ± 0.2 s⁻¹), $n = 4$ –5 animals. **B:** Comparison of 0, 20, and 200 mM ethanol in DAT-KO mice ($k = 0.02 \pm 0.002$, 0.02 ± 0.001 , 0.02 ± 0.002 s⁻¹), $n = 4$ –5 animals. DA clearance rates in caudate–putamen slices from wild-type mice are faster than in DAT-KO mice, note the different k -values (y -axes). Ethanol did not alter clearance rates in either genotype ($P < 0.05$).

effects of ethanol measured with either microdialysis or voltammetry suggest that DA terminals in the caudate–putamen are insensitive to low and moderate concentrations of ethanol (Budygin et al., 2001b, 2003; Yim et al., 1998). This is in agreement with the idea that rewarding doses of ethanol act by directly exciting the cell bodies of dopaminergic neurons, resulting in elevated DA release in the terminal fields (Brodie et al., 1999).

Previous voltammetry studies have shown that a supraphysiological concentration of ethanol reduces

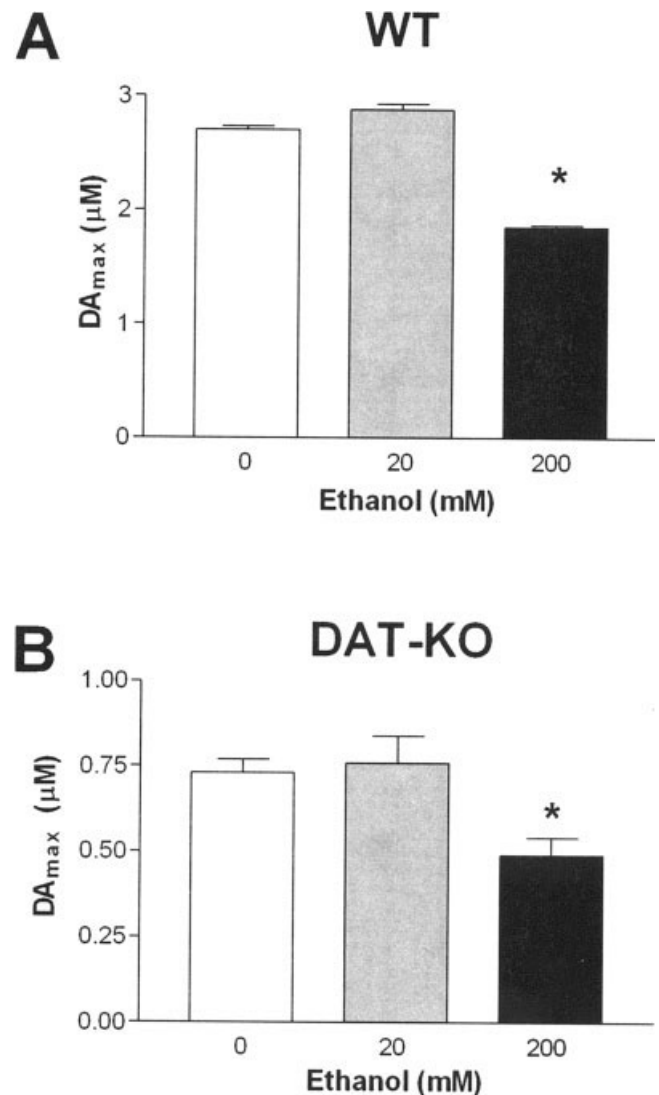


Fig. 4. Effect of ethanol on DA release in caudate–putamen slices from wild-type (WT) and DAT-KO mice. Locally evoked DA signals are measured by voltammetry during 0, 20, or 200 mM ethanol-bath application. **A:** Comparison of 0, 20, and 200 mM ethanol in wild-type mice (2.7 ± 0.03 , 2.9 ± 0.05 , and 1.9 ± 0.02 μM), $n = 4$ –5 animals. **B:** Comparison of 0, 20, and 200 mM ethanol in DAT-KO mice (0.7 ± 0.04 , 0.8 ± 0.08 , and 0.5 ± 0.05 μM), $n = 4$ –5 animals. Ethanol (200 mM) decreased DA_{max} equally in wild-type and DAT-KO mice.

electrically evoked DA release (Budygin et al., 2001b, 2003). In the present study, a high concentration (200 mM) of ethanol produced a robust attenuation of stimulated DA release in both wild-type and DAT-KO mice, demonstrating that DAT is not involved in this ethanol effect (Fig. 2). The percent decrease in stimulated DA release was the same between genotypes, although the absolute magnitude of baseline DA levels was higher in DAT-KO mice, as had been previously reported (Jones et al., 1998). Although the ethanol-induced decrease in electrically evoked DA signals could be a result of increased extracellular DA levels

activating DA autoreceptors, microdialysis studies have shown that high doses of ethanol decrease extracellular DA levels in the caudate–putamen (Blanchard et al., 1993). Therefore, the activation of presynaptic DA autoreceptors is most unlikely under the present conditions. It is more likely that ethanol decreases DA release by decreasing calcium influx, either directly or indirectly (Budygin et al., 2001b). For example, ethanol increases extracellular adenosine (Nagy et al., 1990), which inhibits DA release by suppressing calcium influx (Dunwiddie and Masino, 2001; Fredholm et al., 1998). In addition, ethanol can significantly modify the level of brain gangliosides (Klemm and Foster, 1986; Klemm et al., 1988), which are important controllers of intracellular calcium equilibrium (Ningaraj et al., 2001). Ethanol also affects the function of multiple receptors and ion channels (Crews et al., 1996). Thus, ethanol has many substrates that could potentially lead to DA release inhibition at presynaptic terminals at high concentrations.

These results further highlight the differences between cell systems expressing DAT in the rodent brain. Recent studies by Mayfield et al. (2001) have shown that, in cells overexpressing DAT, ethanol enhanced DA uptake by increasing the number of DAT on the cell surface. On the other hand, the mouse brain is apparently resistant to these effects of ethanol on the DAT.

In agreement with our *in vitro* results, an *in vivo* voltammetry study using mice revealed no changes in DA clearance with ethanol (Yavich and Tiihonen, 2000). However, a recent study using rats reported that a single dose of 2.5 g/kg ethanol or greater decreased DAT function in dorsal and ventral striatum, both *in vivo* and *in vitro* (Robinson et al., 2005). In addition, Lin and Chai showed that, when ethanol was locally applied to the striatum of rats *in vivo*, DA clearance was prolonged (Lin and Chai, 1995). The discrepancies between these two *in vivo* voltammetry ethanol studies could arise from (1) species variations, (2) ethanol concentrations in the brain, and (3) distinct stimulation parameters. In the reports made by Robinson et al. (2005), Lin and Chai (1995), and Yavich and Tiihonen (2000), it is important to note that the results were obtained in anesthetized animals, which may have changed the pharmacological effects of ethanol. There have been only two studies using microelectrodes to evaluate the effects of ethanol in awake animals (Budygin et al., 2001a; Sabeti et al., 2003). An increase in the clearance of exogenous DA was reported following ethanol administration, using chronoamperometry and exogenously applied DA in freely moving rats (Sabeti et al., 2003). However, no ethanol-induced changes in the clearance of electrically stimulated endogenous DA release were found in rat striatum, using fast scan cyclic voltammetry (Budygin et al., 2001a). Our previous voltammetric findings in brain slices

from rat and monkey show no changes in DA uptake (Budygin et al., 2001b, 2003) and are in agreement with previous *in vivo* cyclic voltammetry results (Budygin et al., 2001a). In addition, a zero net flux microdialysis study in freely moving rats supports the hypothesis that acute doses of ethanol do not alter transport (Yim and Gonzales, 2000). Combined, these studies suggest that acute ethanol effects on DA clearance may be different for synaptically released and experimenter-applied DA. Therefore, the acute ethanol-induced elevation in extracellular caudate–putamen DA is most likely the result of increased DA release without any uptake contribution. If the DAT contributed significantly to the dopaminergic effects of ethanol, then marked differences would have been observed between wild-type and DAT-KO mice. However, we found no changes between DAT-KO and wild-type mice with respect to ethanol-induced increase in extracellular DA levels or stimulated DA release and uptake. Although the behavioral effects of ethanol are somewhat modified in DAT-KO mice (Savelieva et al., 2002), the neurochemical effects appear to be identical. These data from DAT-KO mice are in agreement with the majority of *in vivo* and *in vitro* voltammetry and chronoamperometry studies (Budygin et al., 2001a,b; Samson et al., 1997; Yavich and Tiihonen, 2000), illustrating that there is no change in DA clearance after acute ethanol administration.

In conclusion, DAT-KO mice provide a way to address the very important question of whether ethanol interacts with DAT to produce its effects. Using *in vivo* microdialysis and *in vitro* voltammetry in wild-type and DAT-KO mice, an identical augmentation of the dopaminergic system after ethanol was demonstrated. Finally, this study using DAT-KO mice illustrates that while ethanol has many neurochemical targets *in vivo*, caudate–putamen DA uptake is not one of them.

ACKNOWLEDGMENTS

The authors express their gratitude to Marc Caron for the generous gift of DAT-KO mice.

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