Long-lasting Modulation of Glutamatergic Transmission in VTA Dopamine Neurons after a Single Dose of Benzodiazepine Agonists

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Initial effects of drugs of abuse seem to converge on the mesolimbic dopamine pathway originating from the ventral tegmental area (VTA). Even after a single dose, many drugs of abuse are able to modulate the glutamatergic transmission activating the VTA dopamine neurons, which may represent a critical early stage in the development of addiction. Ligands acting on the benzodiazepine site of the inhibitory γ-aminobutyric acid type A (GABA$_A$) receptors are known to be rewarding in animal models and have abuse liability in humans, but notably little evidence exists on the involvement of the mesolimbic dopamine system in their effects. Here we report that single *in vivo* doses of benzodiazepine-site agonists, similar to morphine and ethanol, induce a modulation in the glutamatergic transmission of VTA dopamine neurons. This is seen 24 h later as an increase in the ratio between α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor-mediated excitatory currents using whole-cell patch-clamp configuration in mouse VTA slices. The effect was due to increased frequency of spontaneous miniature AMPA receptor-mediated currents. It lasted at least 3 days after the injection of diazepam, and was prevented by coadministration of the benzodiazepine-site antagonist flumazenil or the NMDA receptor antagonist dizocilpine. A single injection of the GABA$_A$ receptor α1 subunit-preferring benzodiazepine-site ligand zolpidem also produced an increase in the AMPA/NMDA ratio in VTA dopamine neurons. These findings suggest a role for the mesolimbic dopamine system in the initial actions of and on neuronal adaptation to benzodiazepines.
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INTRODUCTION

Benzodiazepine-site ligands (BZs) of the γ-aminobutyric acid type A (GABA$_A$) receptors, such as diazepam (DZ) and zolpidem (ZOL), are commonly used to treat anxiety disorders and sleep disturbances, respectively. Although BZs are clinically effective, they have, unfortunately, reinforcing properties and abuse potential in both humans and animal models of addiction (Griffiths and Weerts, 1997), with a subset of users developing drug dependence, tolerance, and withdrawal syndrome (Petursson, 1994; Ashton, 2005).

Neural mechanisms underlying BZ tolerance and withdrawal symptoms associated with BZ dependence are not well known. Although the GABA$_A$ receptors are the direct targets of BZ ligands, studies focusing on alterations in GABAergic systems have not been able to present a clear consensus. Actually, it has been suggested that the

excitatory glutamatergic transmission is enhanced as a compensatory mechanism to chronic enhancement of the inhibitory GABAergic transmission by BZs (Stephens, 1995). Regulation of the glutamatergic systems by chronic BZ treatments is well established in some limbic and cortical areas (Izzo *et al*, 2001; Van Sickle *et al*, 2002; Allison and Pratt, 2006). Particularly, in rats withdrawing from chronic flurazepam treatment, neurotransmission mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)-type glutamate receptors in the hippocampal CA1 pyramidal neurons is enhanced due to increased membrane incorporation of GluR1 subunit-containing AMPA receptors (Song *et al*, 2007). This enhanced glutamatergic transmission in the hippocampus is also associated with anxiety-like behavior (Xiang and Tietz, 2007). Furthermore, it has been shown that the development of DZ tolerance and dependence can be prevented by concurrent treatment with the N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine (Steppuhn and Turski, 1993). All these findings propose a role for various glutamatergic receptor systems of the brain in BZ tolerance and withdrawal. However, the neural basis for BZ reinforcement has not been clarified by these studies.

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Regardless of their specific target molecules and general behavioral manifestations, the effects of drugs of abuse seem to converge at the mesolimbic dopamine (DA) pathway originating from the ventral tegmental area (VTA). This is seen, eg in drug-induced DA release in the nucleus accumbens, the target area of the pathway (Di Chiara and Imperato, 1988; Koob, 1992). The DA pathways are important in reinforcement and reward processing (Wise, 1996; Schultz, 1998).

The role of mesolimbic DA projections in the effects of BZs is still unclear. Systemically administered BZs decrease extracellular DA in the nucleus accumbens (Finlay et al, 1992). On the other hand, blocking DA receptors with the antipsychotic drug haloperidol or lesioning the nucleus accumbens prevents DZ-induced conditioned place preference (Spyraki and Fibiger, 1988). In addition, intravenously administered DZ increases the firing of VTA DA neurons (O'Brien and White, 1987), which can be explained by the finding that intravenously or microiontophoretically administered BZs inhibit GABAergic interneurons in the VTA leading to disinhibition of VTA DA neurons (O'Brien and White, 1987). The $\gamma2$ subunit of $GABA_A$ receptor is obligatory for BZ sensitivity (Pritchett et al, 1989). This subunit is expressed in the VTA both in DA and non-DA cells (Korpi et al, 2002; Okada et al, 2004), making it possible for all VTA neurons to be direct targets of BZs.

Glutamatergic synapses on VTA DA neurons can undergo several forms of plasticity, such as long-term potentiation (LTP) and depression (Bonci and Malenka, 1999). Long-lasting alterations in synaptic strength are thought to underlie memory formation and learning (Huang et al, 1996). It has been suggested that the process of addiction is founded on a very persistent, but maladaptive, neural plasticity that resembles the plasticity associated with natural reward learning and memory (Kauer, 2004; Jones and Bonci, 2005). This may take place particularly in the mesolimbic DA system (Fitzgerald et al, 1996; Thomas et al, 2001). Importantly, even single in vivo injections of several different drugs of abuse induce a modulation in the excitatory synapses at VTA DA neurons seen in vitro by electrophysiological methods (Ungless et al, 2001; Saal et al, 2003). The quick and persistent drug-induced plasticity may be a critical early stage in a chain of molecular/cellular events that could facilitate development of addiction. Because of the unclear and controversial nature of the mesolimbic DA system in BZ dependence, we have studied the effects of single in vivo doses of the classical BZ agonist DZ and the novel subtype-preferring BZ-site agonist ZOL on glutamatergic transmission in VTA DA neurons, and found that these drugs induce long-lasting alterations in the currents mediated by AMPA-type glutamate receptors.

MATERIALS AND METHODS

Animals and Drug Injections

C57BL/6NHsd female and male mice were purchased from Harlan BV (Horst, the Netherlands) and maintained for up to three generations at our facility. We used the offspring aged between 21 and 29 days for all experiments. Animals were weaned 1 or 2 days before the drug injections. They were group-housed in standard cages with woodchips covering the bottoms, in a ventilated Uniprotect cabinet (Ehret, Emmendingen, Germany) under 12 h light/dark conditions (lights on at 6 a.m.) with food (Harlan Teklad, Oxon, UK) and water available ad libitum. Mice were injected intraperitoneally between 8 and 9 a.m., 24–30 h before preparing VTA slices. The drug doses used were: DZ (5 or 20 mg/kg), morphine (10 mg/kg), ethanol (2 g/kg), ZOL (5 mg/kg), flumazenil (FLU; 10 and 15 mg/kg), and dizocilpine (MK-801; 0.1 mg/kg). FLU was injected at two time points: first, the dose of 15 mg/kg 10 min before DZ injection and second, the dose of 10 mg/kg 30 min after DZ, a schedule that prevented any visible sedating effects of DZ. Animals injected with vehicle (saline or ClinOleic 20% emulsion) were used as controls throughout the experiments. Drugs were injected at a volume of 100 μl per 10 g of body weight. All experiments were approved by the Helsinki University Animal Care Committee.

Spontaneous Locomotor Activity

To measure spontaneous locomotor activity after DZ treatment, mice were injected with DZ (5 mg/kg, i.p.) or vehicle 24 h before testing the activity. Mice were adapted to the experimental room, in their home cages, for 1 h before testing. Locomotor activity was measured for 30 min in Makrolon cages ($42 \times 26 \times 20$ cm; Tecniplast, Buguggiate, Italy), in which the distance moved (in cm) by the mouse was registered by Ethovision Color-Pro 3.0 video-tracking software (Noldus Information Technology, Wageningen, The Netherlands).

Slice Preparation

Animals were decapitated 24–30 h after the in vivo injections of the drugs (or 24 h to 5 days after DZ injections, when determining the time course of the DZ effect). The brains were rapidly removed and rinsed in ice-cold carbogen-bubbled cutting solution (containing in mM: 60 NaCl, 2 KCl, 8 $MgCl_2$, 0.3 $CaCl_2$, 30 $NaHCO_3$, 1.25 NaH_2PO_4, 140 sucrose, and 10 D-glucose). A block of tissue containing the midbrain was sliced horizontally into 225-μm-thick VTA-containing slices using a vibratome (vibratome 1000 Plus, Vibratome, St Louis, MO, USA). The slices were then let to recover at $+32^\circ$C in carbogen-bubbled artificial cerebrospinal fluid (ACSF, in mM: 126 NaCl, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 18 $NaHCO_3$, and 11 D-glucose) for at least 1 h before recordings.

Electrophysiological Recordings

The recordings were made in carbogen-bubbled ACSF that was perfused over the slice in a recording chamber (RCP-6T; Dagan, Minneapolis, MN, USA) at a rate of 2 ml/min. Cells were visualized using an upright microscope (Olympus BX51WI, Hamburg, Germany) with infrared illumination and a digital camera (Hamamatsu C8484, Hamamatsu City, Japan). Whole-cell voltage-clamp recordings were conducted with an Axopatch 200B patch-clamp amplifier and digitized with a Digidata 1322A analog-to-digital converter and pClamp 9.0 software (Axon Instruments,

Union City, CA, USA). The recordings were low-pass filtered at 2 kHz and digitized at 20 kHz. Electrodes had a resistance of 3–5 MΩ when filled with internal solution containing (in mM): 130 cesium methanesulfonate, 10 HEPES, 0.5 EGTA, 8 NaCl, 5 QX314, 4 MgATP, 0.3 MgGTP, and 10 BAPTA (pH adjusted to 7.2–7.25 with CsOH, osmolarity 278 ± 5 mOsm). The series and input resistances were monitored throughout the experiments. Series resistance of < 20 MΩ was accepted, and if it changed more than 20% during the recording, the cell was discarded from the analysis.

Identification of Dopaminergic Neurons in VTA

In horizontal midbrain slices, the VTA was recognized as the area medial to the substantia nigra compacta and medial to the terminal nucleus of the accessory optic tract. A neuron was identified dopaminergic if a clear hyperpolarization-activated cation current (I_h current) emerged after hyperpolarizing the neuron from −70 to −130 mV in 10 mV steps immediately after break-in. I_h current is a good marker for dopaminergic neurons in the mouse midbrain, being present in more than 90% of them (Cameron et al, 1997; Neuhoff et al, 2002; Wanat et al, 2008). Recent findings in rats, on the other hand, have questioned whether its presence could unequivocally identify DA cells (Margolis et al, 2006). However, in previous studies (Ungless et al, 2001; Saal et al, 2003; Faleiro et al, 2004; Wanat et al, 2008) as well as in the present study, this criterion was sufficient to obtain clear differences between the cells from control and experimental animals.

AMPA/NMDA Ratio

The whole-cell ratio for the AMPA and NMDA receptor-mediated currents (AMPA/NMDA ratio) was calculated to assess the synaptic strength of glutamatergic synapses in VTA DA neurons (Ungless et al, 2001; Saal et al, 2003). This measurement offers the advantage that it is independent of the number of synapses activated. In brief, excitatory postsynaptic currents (EPSCs) were evoked by electrical pulses delivered at 0.1 Hz frequency by a stimulator (S-900; Dagan) through a bipolar stimulus electrode placed on the slice 100–300 µm rostral to the recording site to stimulate the glutamatergic afferents to VTA. Stimulus intensity was set at the lowest level that evoked stable EPSCs (usually about 100 pA) with no failures. Picrotoxin (100 µM) was added to block the GABA_A receptor-mediated inhibitory currents. The recordings were made in voltage-clamp configuration at + 40 mV as described earlier (Ungless et al, 2001). After recording stable baseline EPSCs for 5–10 min, 50 µM D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) was added to perfusion to prevent the NMDA receptor-mediated currents. After 5 min perfusion with D-AP5, the remaining AMPA receptor-mediated current was recorded for 5–10 min. To calculate the AMPA/NMDA ratio the average AMPA current (the average response in the presence of D-AP5) was subtracted from the average baseline EPSC, revealing the average NMDA current. The AMPA/NMDA ratio was calculated from the peak amplitude of the currents.

Miniature Excitatory Postsynaptic Currents and Paired-Pulse Ratios

Spontaneous AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded in VTA DA neurons clamped at −70 mV, in the presence of 100 µM picrotoxin, 1 µM tetrodotoxin (TTX), and 50 µM D-AP5, at + 32°C. Spontaneous mEPSCs were analyzed with Mini Analysis program software (Synaptosoft Inc., Decatur, GA, USA). Briefly, mEPSCs were screened by using an amplitude threshold of 8 pA, and based upon the shape of the event (rise < 1.5 ms and decay < 3.5 ms), they were visually either accepted or rejected by an examiner blind to the experimental conditions. Electrical noise was typically around 3 pA in amplitude. For each neuron the mEPSCs were recorded for a 5 min period.

Paired-pulse ratios were recorded in VTA DA neurons at −70 mV in the presence of 100 µM picrotoxin and 50 µM D-AP5. The excitatory afferents were stimulated electrically with paired pulses to evoke two responses with short time intervals: 20, 50 and 100 ms at 0.1 Hz. The paired-pulse ratio was calculated between the peak amplitudes of the second and the first evoked excitatory current.

Statistical Analyses

Only two or three VTA slices per animal were obtained and only one recording per slice was made. The recordings from the same animal were averaged out, and this average was used in the analysis. All values are expressed as means ± SEM. Statistical significance was assessed with two-tailed t-test, or one-way ANOVA with Tukey's or Dunnett's post-test using Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Drugs

Diazepam (Stesolid Novum, Yliopiston Apteekki, Helsinki, Finland) was suspended in ClinOleic 20% emulsion (Baxter Oy, Helsinki, Finland). Morphine-HCl (Yliopiston Apteekki) and dizocilpine maleate (MK-801; Tocris Bioscience, Bristol, UK) were diluted with saline. Ethanol (Altia Oyj, Rajamäki, Finland) was mixed with saline (10% w/v). ZOL tartrate (Stilnoct, Yliopiston Apteekki) was dissolved with saline. FLU (Sigma-Aldrich Finland Oy, Helsinki, Finland) was dissolved with Tween 80 and diluted with 1% Tween 80 in saline. Stock solutions of picrotoxin, TTX, and D-AP5 (Tocris Bioscience) were diluted with ACSF, and added to perfusion medium when needed.

RESULTS

One In Vivo Dose of Diazepam Leads to Increased AMPA/NMDA Ratio of VTA DA Neurons

In agreement with Saal et al (2003), we found that single doses of morphine and ethanol led to a significant increase (morphine, $p < 0.01$; ethanol, $p < 0.001$) in the AMPA/NMDA ratio measured from VTA DA neurons 24 h after the drug injection (Figure 1). We also found that a single injection of DZ at a dose of 5 or 20 mg/kg, i.p., significantly
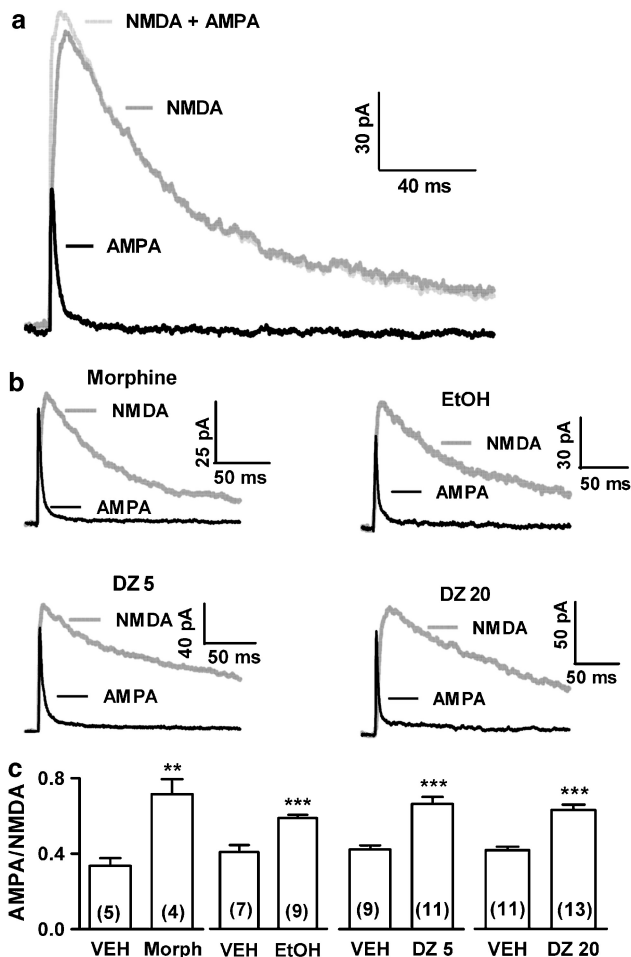
Figure 1 Modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/*N*-methyl-ᴅ-aspartate (AMPA/NMDA) current ratios of ventral tegmental area (VTA) dopamine (DA) neurons measured *ex vivo* 24 h after single doses of drugs of abuse. (a) Example traces showing AMPA and NMDA components after vehicle administration. (b) Representative AMPA and NMDA receptor-mediated current traces recorded after single doses of morphine, ethanol (EtOH) and two doses of diazepam (DZ). (c) Bars (± SEM, the number of animals tested in parentheses) showing the average AMPA/NMDA ratio after one *in vivo* injection of morphine hydrochloride (10 mg/kg), EtOH (2 g/kg), and DZ (5 or 20 mg/kg). All drug doses increased the AMPA/NMDA ratios compared to the corresponding vehicle administration. **$p < 0.01$ and ***$p < 0.001$ (*t*-test).

Figure 2 The average α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/*N*-methyl-ᴅ-aspartate (AMPA/NMDA) current ratios (± SEM, the number of animals tested in parentheses) in the ventral tegmental area (VTA) dopamine (DA) neurons 24 h after diazepam (DZ, 5 mg/kg) and zolpidem tartrate (ZOL, 5 mg/kg). (a) The drug treatment increased the AMPA/NMDA ratio ($F(2, 38) = 11.15$, $p < 0.0002$), but ZOL had a smaller effect. *$p < 0.05$, ***$p < 0.001$ (Tukey's test). (b) Cluster plot of AMPA/NMDA ratios for individual neurons after vehicle, DZ and ZOL treatments. From several animals, more than one neuron was recorded, each from a different midbrain slice. The data suggest that fewer neurons had altered glutamatergic transmission after ZOL as compared to DZ.

($p < 0.001$) increased the AMPA/NMDA ratio in VTA DA neurons (Figure 1). The effect was similar at both doses, and also very similar to that produced by morphine and ethanol. Behaviorally, the mice showed heavy sedation (loss of locomotor activity) within a few min after the DZ (5 mg/kg, i.p.) injection, and they started to recover around 60 min afterward, with no visible sedation anymore after 2–3 h (based on visual observations of the mouse activity induced by moving the home cage and the lid).

In another group of mice, we determined spontaneous locomotor activity in a novel environment 24 h after the administration of 5 mg/kg DZ or vehicle. The distance traveled by the mice in 30 min was 5178 ± 135 (mean ± SEM, $n = 5$) and 5464 ± 230 cm ($n = 7$) for the vehicle and DZ groups, respectively ($p > 0.05$).
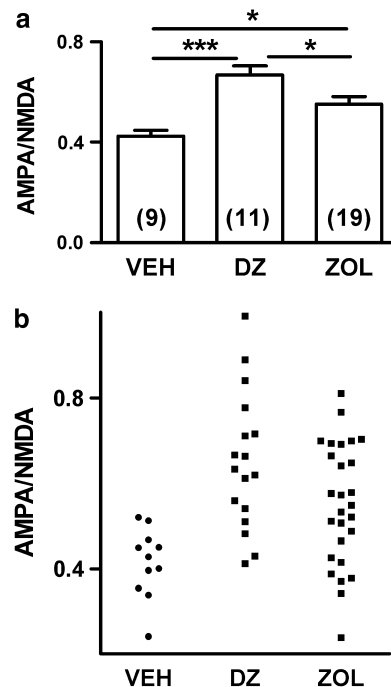
Zolpidem Increases the AMPA/NMDA Ratio

GABA$_A$ receptor BZ sites show pharmacologically significant heterogeneity (Lüddens *et al*, 1995). DZ shows relatively little selectivity in binding affinity between GABA$_A$ receptors containing α1, α2, α3, or α5 subunits, although its efficacy is slightly affected by the α variant (Ducic *et al*, 1993). The imidazopyridine ZOL, in contrast, is an α1βγ2 subtype-preferring BZ-site ligand, showing 10- to 20-fold selectivity in affinity for GABA$_A$ receptors with α1 subunits over those with α2 or α3 subunits and even a greater selectivity difference for α1 over α5 subunit-containing receptors (Faure-Halley *et al*, 1993; Sieghart, 1995). To assess the effects of ZOL, we injected the mice with ZOL at a dose of 5 mg/kg 24 h before recording the AMPA/NMDA ratio in the VTA DA neurons. At this dose, the effects of ZOL are specifically mediated by α1 subunit-containing GABA$_A$ receptors (Crestani *et al*, 2000). ZOL was able to significantly ($p < 0.05$) increase the AMPA/NMDA ratio, but this effect seemed to be less pronounced than that of 5 mg/kg DZ ($p < 0.05$; Figure 2).

Modulation is Long Lasting and Dependent on NMDA Receptors

We next studied the time course of the DZ-evoked increase in the AMPA/NMDA ratio of VTA DA neurons. The
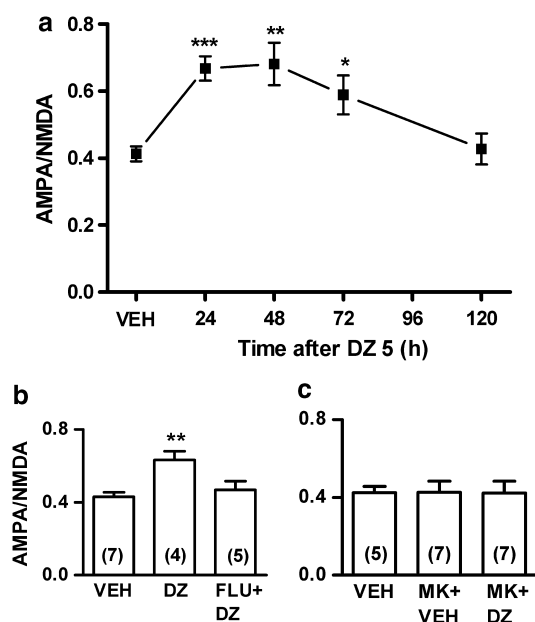
Figure 3 Time course and pharmacological manipulations of diazepam (DZ)-induced increase in the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid/N-methyl-D-aspartate (AMPA/NMDA) current ratio of ventral tegmental area (VTA) dopamine (DA) neurons. (a) Points (± SEM, $n = 7–11$) showing the average AMPA/NMDA ratios 24 h to 5 days after a single injection of DZ (5 mg/kg). The ratio is increased for 3 days after the DZ ($F(4, 40) = 8.174$, $p < 0.0001$) returning back to the baseline in 5 days. (b) The benzodiazepine receptor antagonist flumazenil (FLU) prevented the DZ-induced potentiation in the AMPA/NMDA ratio of VTA DA neurons, when injected 10 min before (15 mg/kg) and 30 min after (10 mg/kg) the DZ injection (5 mg/kg). (c) The NMDA receptor noncompetitive antagonist dizocilpine (MK-801, 0.1 mg/kg) prevented the DZ-induced potentiation in the AMPA/NMDA ratio when co-injected with DZ (5 mg/kg). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (Dunnett's test).

phenomenon was still evident 3 days after the injection, but after 5 days the AMPA/NMDA ratio had returned back to the baseline (Figure 3a).

To ascertain that the effect of DZ is indeed mediated through the BZ-binding site of GABA$_A$ receptors, we injected the mice with the selective BZ-site antagonist FLU (Hunkeler et al, 1981) 10 min before (15 mg/kg) and 30 min after (10 mg/kg) the DZ (5 mg/kg) injection. With this double-dosing schedule, FLU completely prevented the effect of DZ on the AMPA/NMDA ratio (Figure 3b). Interestingly, a single 15 mg/kg dose of FLU 10 min before the 5 mg/kg DZ injection alone was not effective in preventing the DZ's effect on VTA DA neurons (the AMPA/NMDA ratios were 0.42 ± 0.03 ($n = 5$), 0.62 ± 0.05 ($n = 4$) and 0.57 ± 0.09 ($n = 5$) for animals treated with vehicle, DZ and FLU + DZ, respectively; $p > 0.05$ for the difference between DZ and FLU + DZ).

Activation of NMDA receptors is a requirement for a single in vivo injection of cocaine to induce the potentiation of the AMPA/NMDA ratio in VTA DA neurons (Ungless et al, 2001). We found now that the NMDA receptor antagonist dizocilpine (0.1 mg/kg) itself had no effect on the ratio, but when it was co-injected with DZ (5 mg/kg), the DZ-induced increase in the ratio was fully prevented (Figure 3c).
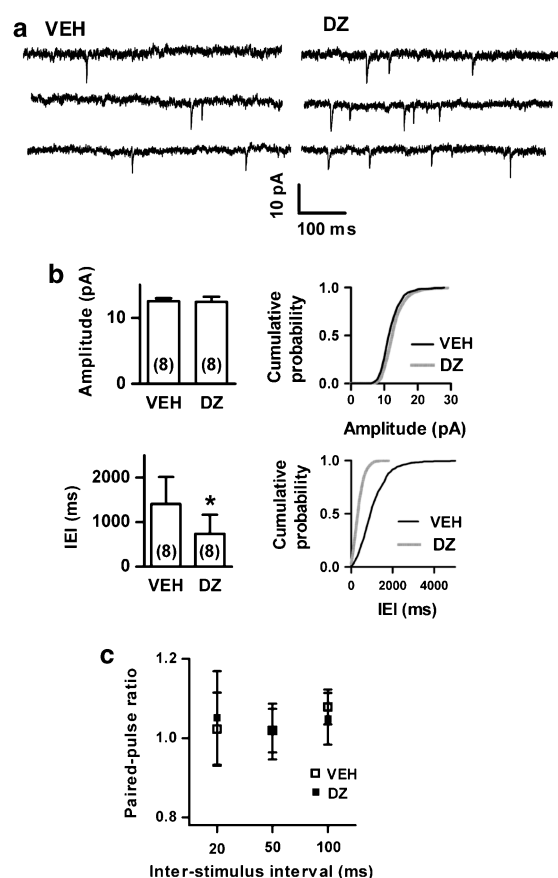

Figure 4 Spontaneous α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) and paired-pulse ratios in ventral tegmental area (VTA) dopamine (DA) neurons 24 h after a single injection of diazepam (DZ, 5 mg/kg). (a) Representative traces recorded from DA neurons after vehicle or DZ treatments. (b) Means and cumulative probability plots for the amplitudes and interevent intervals (IEI) of mEPSCs recorded from the VTA DA neurons of vehicle- or DZ-treated animals. DZ had no effect on the mEPSC amplitudes ($p > 0.05$), but it decreased the IEI. *$p < 0.05$ (t-test). (c) Paired-pulse ratios at various interstimulus intervals did not reveal any differences between groups ($p > 0.05$, t-test). Data are means ± SEM for eight animals in both groups for both experiments.

Increased Frequency of AMPA Receptor mEPSCs in VTA DA Neurons after DZ Treatment

To study the mechanism of potentiation of the AMPA/NMDA ratio after DZ treatment, we recorded AMPA receptor-mediated spontaneous mEPSCs in VTA DA neurons 24 h after DZ (5 mg/kg). The interevent interval (IEI) was shorter in the DZ-treated mice than in the vehicle-treated mice ($p < 0.05$), indicating that DZ treatment had increased the frequency of mEPSCs (Figure 4a). The treatment did not change the amplitudes of the miniature currents.

To test whether a change in the probability of neurotransmitter release from glutamatergic nerve terminals was behind the increased frequency of AMPA events, we recorded AMPA receptor-mediated currents evoked by closely time-wise paired electrical pulses and calculated a so-called paired-pulse ratio in VTA DA neurons. The paired-pulse ratio is used to compare the probability of

neurotransmitter release under control conditions and following drug treatments or experimental manipulations. An increase in release probability is typically associated with a decrease in paired-pulse ratio, and *vice versa* (Dobrunz and Stevens, 1997). However, DZ treatment of the mice did not change the paired-pulse ratios in VTA DA neurons (Figure 4b).

DISCUSSION

Different drugs of abuse can induce plasticity changes in the excitatory synapses of VTA DA neurons after a single dose of the drug. BZs are known to have abuse potential, but the role of mesolimbic DA system in BZ dependence and addiction has remained unclear. Here we show that similar to strongly addicting opioid morphine and alcohol, a single *in vivo* dose of the classical GABA$_A$ receptor-positive modulator BZ DZ induced a modulation in the glutamatergic transmission to VTA DA neurons, seen as an increase in the AMPA/NMDA current ratio.

The increase in the AMPA/NMDA ratio after DZ was a relatively long-lasting modulation of synaptic transmission, being detectable at least for 3 days after the injection. On the basis of the literature, we can estimate that DZ at the dose of 5 mg/kg will be quickly metabolized to its active metabolites *N*-desmethyldiazepam and oxazepam in mice, and that hardly any DZ should be present in the brain after 2 h (Coutinho *et al*, 1969; Greenblatt and Sethy, 1990). *N*-desmethyldiazepam, which has roughly the same affinity to BZ receptors and the same hypnotic potency as DZ (but less anticonvulsant potency than diazepam) (Frey and Löscher, 1982; Gobbi *et al*, 1987; Klockowski and Levy, 1987), disappears from the brain at around 12 h, whereas oxazepam has the longest elimination half-life of the active DZ metabolites and might be detectable at a very low level still at 24 h but unlikely to be detected at 48 h (Greenblatt and Sethy, 1990). However, oxazepam has clearly less hypnotic and anticonvulsant efficacy than DZ (Frey and Löscher, 1982; Klockowski and Levy, 1987). Thus, it is unlikely that the possible residual oxazepam is directly involved in the glutamate receptor modification 24–72 h after a single injection of DZ. This is also supported by the finding that DZ by potentiating the GABA$_A$ receptor-mediated inhibition *in vitro* prevents, and not facilitates, the LTP induction in VTA DA neurons of chronically cocaine-treated rats (Liu *et al*, 2005). Interestingly, repeated, but not a single, exposure to cocaine leads to a reduction in GABA$_A$ receptor-mediated inhibition in VTA DA neurons, which facilitates LTP induction (Liu *et al*, 2005; Pan *et al*, 2008). Although the GABA$_A$ receptor targets are present in the VTA in both DA and non-DA neurons (Korpi *et al*, 2002; Okada *et al*, 2004), at least in anesthetized rats BZs seem to act by inhibiting the non-DA neurons more strongly and thus leading to a disinhibition of the DA neurons of the area (O'Brien and White, 1987). Further experiments are warranted to ascertain the possible long-lasting alterations in the GABA$_A$ receptor function as an after effect of a single dose or repeated doses of BZs, both in the DA and non-DA neurons of the VTA.

The effect of DZ on AMPA/NMDA ratio was mediated through the BZ site of GABA$_A$ receptor, as the selective antagonist FLU prevented it. This suggests that the acute enhancement of GABA$_A$ receptor-mediated transmission by DZ eventually leads to a modulation of the glutamatergic control of VTA DA neurons. However, we needed two high doses of FLU to block the modulation, indicating that the modulation happens in response to the initial strong action of DZ (and/or its metabolites) in the first few hours, which coincides with the clear behavioral effects. The elimination half-life of FLU in the rodent brain is short (8–16 min) and it is no longer detectable at 90 min (Lister *et al*, 1984; Mandema *et al*, 1991; Atack *et al*, 1999). This explains why one dose of FLU failed to prevent the emergence of the visible sedative effect of DZ.

The GABA$_A$ receptor α1 subunit-preferring BZ-site ligand ZOL has less abuse liability than DZ (Darcourt *et al*, 1999; Hajak *et al*, 2003; Griffiths and Johnson, 2005). Here we found that ZOL at the dose (5 mg/kg) that acts specifically via α1 subunit-containing receptors (Crestani *et al*, 2000) increased the AMPA/NMDA ratio in VTA DA neurons. Even if its effect seemed to be weaker than that of 5 mg/kg DZ, we cannot directly compare the efficacy of the two drugs to induce a long-lasting neurobiological change when using only one dose. Although the affinities of ZOL and DZ for the α1 subunit-containing receptors might be similar or differ slightly (about twofold) in recombinant α1β2/3γ2 GABA$_A$ receptors (Wieland and Lüddens, 1994; Atack *et al*, 1999) and in α1 subunit-enriched regions of the rat brain (Arbilla *et al*, 1986; Benavides *et al*, 1988), the most important difference might be in the time course of action. ZOL is a short-acting hypnotic, with *in vivo* occupancy of the GABA$_A$ receptor BZ sites lasting only about 15–30 min after i.p. injection of 10 mg/kg in mice (Benavides *et al*, 1988). As discussed above with regard to FLU antagonism, the BZ effect may need to last longer than 15 min to induce the full and consistent modulation in the glutamate synapses of the VTA DA neurons. To reach that long an effect, higher ZOL doses would probably have been required. In any case, our data are in agreement with the results of self-administration of several subtype-selective BZ ligands by rhesus monkeys indicating that the activation of α1 subunit-containing GABA$_A$ receptors is sufficient for abuse potential of BZ-type drugs (Rowlett *et al*, 2005).

The increase in AMPA/NMDA ratio may happen as a result of an enhancement in AMPA receptor-mediated neurotransmission at existing excitatory synapses and/or emergence of newly formed synapses. We found here that DZ injection increased the frequency of spontaneous miniature AMPA currents in VTA DA neurons, although the amplitudes remained unaltered. This could be interpreted as a presynaptic alteration, suggesting that the transmitter release probability has changed. Depending on synapses and experimental techniques, BZs may directly or indirectly either enhance or reduce glutamate release (Schmid *et al*, 1999; Khan *et al*, 2000; Harte and O'Connor, 2004). However, we found no differences in paired-pulse ratios between the treatments, indicating that the release probability of glutamate in the excitatory synapses of VTA DA neurons after DZ remained unchanged.

The increased frequency of the miniature AMPA events without a change in the amplitude may result from a postsynaptic alteration. If DZ treatment induced formation of new glutamatergic synaptic contacts, increased AMPA receptor-mediated responses would ensue in the DA

neurons. This idea is intriguingly supported by the recent finding of increased dendritic spine density in those VTA DA neurons that exhibited increased AMPA/NMDA ratio in rats after a single injection of cocaine in vivo (Sarti et al, 2007). In the hippocampus, it has been shown that the exocytosis of recycling endosomes containing AMPA receptors could be the mechanism behind new spine formation (Park et al, 2006) and this would nicely support the idea of enhancement of AMPA transmission in these neurons. Interestingly, the increase in the AMPA/NMDA ratio after DZ was prevented if the NMDA-receptor antagonist dizocilpine was co-injected with DZ, even though this drug has a relatively short half-life in rodents (elimination $t_{\frac{1}{2}}$ for dizocilpine approximately 60–75 min in rats (Hucker et al, 1983)). Therefore, systemic DZ administration has to lead to activation of NMDA receptors, to enable the modulation of excitatory synapses of VTA DA neurons to occur during the 24 h. In agreement, also after cocaine injection the activation of NMDA receptors is needed for the AMPA potentiation to develop in the VTA DA neurons (Ungless et al, 2001). Thus, the phenomenon we observed shows similarity to the classical model of synaptic plasticity, the NMDA-dependent LTP. It is set in motion by NMDA receptor activation and intracellular calcium signaling, leading to exocytosis of recycling AMPA receptors and recycling endosomes, finally resulting in increased synaptic strength and spine formation and growth (Lüscher et al, 2000; Park et al, 2006). Learning and long-lasting synaptic modifications are dependent on gene expression and protein synthesis (see for reviews Sutton and Schuman, 2006; McClung and Nestler, 2008), whereas many functional synaptic changes such as the rapid entry and exit of the AMPA receptors to/from cell surface are not (Park et al, 2004). It remains to be studied whether the increased AMPA/NMDA ratio induced in the VTA DA neurons by in vivo administration of drugs of abuse is dependent on novel gene expression and/or protein synthesis, or rather due to other protein modifications such as receptor subunit phosphorylation/dephosphorylation or altered receptor recycling. BZs are not known to be potent inducers of gene expression. At a high acute dose of 30 mg/kg, DZ produces brain-region-dependent reduction in the expression of genes such as c-Fos, CaMKII, and BDNF (Huopaniemi et al, 2004), believed to be essential for structural/functional modifications of synaptic organization. In attempt to clarify the molecular mechanisms of BZ-induced changes in synaptic glutamate transmission, the present study could be extended, eg by VTA DA neuron-specific gene expression profiling.

An important question is what this plasticity phenomenon means? Only the classical drugs of abuse are able to induce the enhancement of the excitatory transmission in VTA DA neurons, whereas two psychoactive, but nonaddictive drugs, fluoxetine and carbamazepine, fail to alter these synapses (Saal et al, 2003). In this study we show that DZ and ZOL, that also have abuse potential, are able to induce a long-lasting modulation of the excitatory transmission in these synapses. DZ treatment produced increased frequency of AMPA receptor mEPSCs in the VTA DA neurons. This modulation did not alter spontaneous locomotor or exploratory activity in mice (the present study), in agreement with the finding that overexpression of GluR1 subunit of the AMPA receptors by viral gene-transfer in the VTA does not affect spontaneous locomotor activity in rats (Carlezon et al, 1997). Increased VTA AMPA receptors may, however, be facilitatory for morphine- and stimulant-induced locomotor sensitization (Carlezon and Nestler, 2002). It could also be hypothesized that the specific effect that drugs of abuse, including the BZs, have on the excitatory synapses of VTA DA neurons would play a part in the reinforcing properties of those drugs. The mesolimbic DA system operates in reward expectation and processing, reward-based learning, and reinforcement (Wise, 1996; Waelti et al, 2001). Drug-induced enhancement of synaptic strength at excitatory synapses on the DA cells could facilitate further drug-induced alterations in the mesolimbic DA system, which could lead to excessive wanting of the drugs and thus to addictive behaviors. So far, the activation of AMPA receptor function has been linked primarily to the withdrawal state from chronic BZ treatment (see 'Introduction'), representing a homeostatic neuro-adaptation in the form of upregulation of excitatory mechanisms in response to increased inhibition, and we cannot exclude that our findings might simply reflect the AMPA receptor upregulation in response to strong sedation by a single dose of BZs. On the other hand and more interestingly, our results may also stress a direct addiction potential for BZ-site agonists in addition to mere negative reinforcing capacity, such as alleviation of drug-withdrawal symptoms. The role of glutamatergic mechanisms and glutamate/DA interactions in rewarding and positive reinforcing effects of BZs should thus be further explored.

DISCLOSURE/CONFLICT OF INTEREST

The authors have no conflicts of interest.

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