Altered Dopamine Release and Uptake Kinetics in Mice Lacking D2 Receptors

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Dysregulation of dopamine transmission is thought to contribute to schizophrenic psychosis and drug dependence. Dopamine release is regulated by D2 dopamine autoreceptors, and D2 receptor ligands are used to treat psychosis and addiction. To elucidate the long-term effects of D2 autoreceptor activity on dopamine signaling, dopamine overflow evoked by single or paired-pulse stimulation was compared in striatal slices from D2-null mutant and wild-type mice. Quinpirole, a D2/D3 receptor agonist, had no effect on evoked dopamine release in D2 mutant mice, indicating that D2 receptors are the only release-regulating receptors at the axon terminal. Dopamine release inhibition by GABA receptors was unchanged in D2 null mutant mice, suggesting that other G-protein-coupled pathways remained normal in the absence of D2 autoreceptors. Paired-pulse stimulation revealed that autoinhibition of dopamine release was maximal 500 msec after stimulation and lasted <5 sec. In D2-null mutants, dopamine overflow in response to single stimuli was severely decreased. Experiments with the uptake inhibitor nomifensine indicated that this was caused by enhanced dopamine uptake rather than reduced release. Analysis of dopamine overflow kinetics using a simulation model suggested that the enhanced uptake was caused by an increase in the maximal velocity of uptake, Vmax. These results from D2-null mutant mice support the suggestion that D2 autoreceptors and dopamine transporters interact to regulate the amplitude and timing of dopamine signals.

Keywords: dopamine; D2 receptor; autoreceptor; dopamine transporter; release; paired-pulse depression; striatum

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animals had either a C57BL/6 × 129sv mixed genetic or a congenic C57BL/6 background. There were no differences with respect to DA release and reuptake between the two different genetic backgrounds (data not shown). All mice were between 8 and 16 weeks of age.

Mice were anesthetized with ketamine/xylazine and decapitated. Striatal brain slices were cut on a vibratome at 300 μm thickness. Recordings were obtained from the second to fourth frontal slice of caudate putamen (bregma = +1.54 mm to + 0.62 mm) (Franklin and Paxinos, 1997). Slices were allowed to recover for 1 hr in a holding chamber in oxygenated artificial CSF (ACSF) at room temperature; they were then placed in a recording chamber and superfused (1 ml/min) with ACSF (in m): 125 NaCl, 2.5 KCl, 26 NaHCO₃, 2.4 CaCl₂, 1.3 MgSO₄, 0.3 KH₂PO₄, and 10 glucose at 30°C. Nomifensine, (−)-sulpiride, quinpirole, and R(+)-baclofen were obtained from Sigma (St. Louis, MO).

Electrochemical recordings. Disk carbon fiber electrodes of 5 μm in diameter with a freshly cut surface (Kawagoe et al., 1993) were placed in the dorsal striatum −50 μm into the slice. For cyclic voltammetry (CV), a triangular voltage wave (−400 to +1000 mV at 300 V/sec vs Ag/AgCl) was applied to the electrode every 100 msec using a waveform generator (Model 39; Wavetek, Ltd., Norwich, Norfolk, UK). Current was recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), with a low-pass Bessel Filter setting at 10 kHz, digitized at 25 kHz (Instrunet board; GW Instruments, Somerville, MA), and acquired with the Superscope II program (GW Instruments). For amperometry, a constant voltage of +400 mV was applied via the Axopatch 200B. Amperometric traces were filtered with a digital hamming filter (125 Hz cutoff frequency). Striatal slices were electrically stimulated with a bipolar stimulating electrode placed −100 μm from the recording electrode using an Iso-Flex stimulus isolator triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). Background-subtracted cyclic voltammograms served to calibrate the electrodes and to identify the released substance.

Simulation model. To estimate DA release and uptake parameters from CV recordings of evoked DA overflow, we used a one-dimensional random walk/finite difference model of diffusion (Berg, 1983; Sulzer and Pothos, 2000), which incorporated a function for DA uptake according to Michaelis–Menten kinetics. A detailed description of the simulation has been described by Schmitz et al. (2001). Examples of spreadsheets that can be used to run this simulation model can be downloaded from our laboratory web site (http://www.columbia.edu/~ds43/download.html).

To find the best fit to an actual recording trace, we used the Mini Analysis Program (Synaptosoft, Decatur, GA), which contains a subroutine with our simulation model and uses a simplex algorithm to perform nonlinear regression.

HPLC analysis of catecholamine content in striatal slices. Corticostriatal slices from D₃−/− and WT mice were prepared as described above. The striatum was dissected and homogenized in 300 μl of 2% perchloric acid either immediately or after an incubation period in ACSF. Samples were sonicated, frozen, thawed, and again sonicated to ensure disruption of membranes and finally centrifuged at 15,000 × g at 4°C. The pellet was used to determine protein concentrations with a protein assay kit (Bio-Rad, Hercules, CA). The supernatant was kept frozen at −80°C. The HPLC system consisted of an ESA (Chelmsford, MA) Rad, Hercules, CA) with a column (250 μl of 2% perchloric acid) containing 10% methanol and (in mM): 50 sodium acetate, 0.05 EDTA, and 0.7 heptane-sulfonic acid. The HPLC system consisted of an ESA (Chelmsford, MA) Coulomb 5100A with a 5011 analytical cell and a BAS Biophase ODS column (250 × 4.6 mm; 5 μm).

Statistics. The two-tailed Student’s t test was used for pairs of data, whereas ANOVA with post hoc comparison (Newman–Keul test) was used to analyze groups of data (GB Stat software, Silver Spring, MD). Significance levels of p < 0.05 (*) or p < 0.01(**) are indicated in the figures. The best fit of simulations to DA overflow recordings was found by nonlinear regression, and the goodness of fit is reported by R² values (Schmitz et al., 2001).

RESULTS

Inhibition of DA release by G-protein-coupled receptors

DA overflow was recorded with carbon fiber disk electrodes in the dorsal striatum of corticostriatal slices using fast CV. DA overflow was evoked by single-pulse stimulation (1 msec, 400 μA) with a bipolar stimulation electrode placed −100 μm from the record-
G-protein-coupled heteroreceptors is altered as a result of compensatory adaptation. At somata of midbrain DAergic neurons, GABA_B receptors, which are coupled to the inhibitory G-proteins G_i and G_o (Kerr and Ong, 1995), affect the same inwardly rectifying potassium currents and voltage-gated calcium currents as the D_2 autoreceptor (Lacey et al., 1988; Cardozo and Bean, 1995). Therefore, we tested the effect of the GABA_B agonist R(+)-baclofen (100 µM) on evoked DA release in slices from WT and D_2−/− mice (n = 6) and WT mice (n = 8). As in a, the normalized peak response is plotted versus time. The gray area indicates the 10 min superfusion with R(+)-baclofen. There was no statistically significant difference between WT and D_2−/− mice (p > 0.05). Dotted lines indicate normalized amplitude = 1.

In summary, these results indicate that the axon-terminal D_2 receptor is the only release-regulating autoreceptor. Because the response to GABA_B agonists was unaffected, G-protein-mediated release inhibition by heteroreceptors appears to remain functional in D_2−/− mice.

**Evoked DA release and reuptake in D_2−/− mice**

We subsequently examined how the absence of D_2 autoreceptors affects axon-terminal DA release and uptake parameters. We found that in D_2−/− mice, DA overflow in response to a single electrical stimulus (1 msec, 400 µA) reached only 54% of the amplitude of evoked DA overflow recorded in slices from WT mice (Fig. 3a,b).

Because evoked DA overflow is determined by both DA release and reuptake, the reduced overflow in D_2−/− mice may be a result of either decreased release or increased uptake. To differentiate between the two, we recently developed a simulation...
model to estimate release and uptake parameters, which consists of a random walk/finite difference simulation of diffusion of DA in brain tissue corrected for DA uptake according to Michaelis–Menten kinetics (Schmitz et al., 2001). The simulation is fit to recordings of DA overflow using a simplex algorithm to perform nonlinear regression by varying four parameters: the initial DA concentration elicited by the stimulation, the maximal DAT uptake velocity ($V_{max}$), the apparent affinity ($K_m$), and the diffusion distance to the electrode (“dead volume”).

The solid lines in Figure 3a are the best fit simulations for the respective recording traces. The most striking difference in DA overflow parameters between D2−/− and WT mice was an increased $V_{max}$ of 11.1 μM/sec in D2−/− mice, compared with 7.3 μM/sec in WT mice ($+51\%$; $p < 0.05$). The initial DA concentration was slightly decreased in D2−/− mice (3 μM compared with 3.8 μM in WT mice, −20%, not significant). $K_m$ (0.9 μM in WT and 1 μM in D2−/−) and the estimated diffusion distance (7.3 ± 0.4 μm in WT and 8.2 ± 0.6 μm in D2−/−) were not significantly different. These results suggested that the reduced amplitude of DA overflow in D2−/− mice was primarily caused by enhanced uptake attributable to an increased $V_{max}$.

**Effects of uptake blockade**

To further test the possibility that the difference in DA overflow between D2−/− and WT mice was primarily caused by enhanced uptake in the mutants, we recorded DA overflow in slices from D2−/− and WT mice in the presence of the competitive uptake inhibitor nomifensine (Meiergerd and Schenk, 1994). In the dorsal striatum, nomifensine (5–10 μM) causes an increase in $K_m$, estimated to lie between 11 and 20 μM (Jones et al., 1995; Schmitz et al., 2001; Wu et al., 2001), resulting in DA overflow that is primarily determined by DA release and diffusion. Thus, if the difference between genotypes was attributable to reduced DA release in the D2−/− mutant, DA overflow in the presence of nomifensine would remain lower in D2−/− mice than in WT mice. In contrast, if the decrease in stimulated DA overflow in D2−/− mice was caused by increased DA uptake, nomifensine would be expected to eliminate the difference in DA overflow between D2−/− mice and WT mice.

DA overflow was stimulated once per minute, and slices were superfused for 20 min with 5 μM nomifensine, a concentration well above its estimated $K_m$ of 0.09–0.49 μM (Meiergerd and Schenk, 1994; Jones et al., 1995). Figure 4a shows an example of evoked DA overflow recorded before and after 20 min of nomifensine superfusion in a D2−/− and WT striatal slice. The plot of normalized amplitudes of DA overflow in Figure 4b shows that nomifensine caused a twofold increase in DA overflow in slices from WT mice and a fivefold increase in slices from D2−/− mice. This relatively greater enhancement of DA overflow in D2−/− mice resulted in identical absolute amplitudes of evoked DA overflow in the two genotypes (Fig. 4c; 2.41 μM in WT and 2.44 μM in D2−/−). These results thus confirm that DA release was nearly unaltered in D2−/− mice, whereas DA uptake was enhanced. The enhanced uptake could be attributable either to an increase in $V_{max}$ or to a decrease in $K_m$. As shown above (Fig. 3c), the simulation of evoked DA overflow suggested an enhanced $V_{max}$ in D2−/− mice.

**Striatal content of DA and metabolites**

To further test the possibility that altered DA release might contribute to the observed reduction of DA overflow in D2−/− mice, we measured overall DA tissue content in striatal slices immediately after sectioning and after 30 and 120 min of incubation in the holding chamber in ACSF at room temperature. The tissue content of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was determined in homogenates of striatal slices by HPLC analysis. Figure 5a shows that DA levels were not different in D2−/− and WT mice. There was a slight drop in DA levels after incubation for 30 min and a recovery to the original level after 2 hr of incubation for both genotypes. Although absolute DA levels were similar in both genotypes, the ratio of DA to DOPAC and HVA was slightly but not significantly elevated in D2−/− mice (Fig. 5b). We conclude that the observed reduced DA overflow in D2−/− mice was not caused by reduced tissue levels of DA.
Figure 5. HPLC analysis of tissue levels of DA and its metabolites DOPAC and HVA in striatal slices from WT and D2−/− mice. a, DA content of striatal slices (3 per animal) from WT mice (△, n = 6 for time 0 and n = 3 for 30 min and 2 hr) and D2−/− mice (○, n = 4 for time 0 and n = 2 for 30 min and 2 hr) was determined immediately after sectioning and after a 30 min and 2 hr recovery in the holding chamber at room temperature. There was no difference between WT and D2−/− mice (p > 0.05). b, Tissue content of the metabolites DOPAC and HVA determined in slices from WT mice (n = 6) and D2−/− mice (n = 4) immediately after sectioning, expressed as a percentage of DA content. There was no difference between WT and D2−/− mice (p > 0.05).

Time course of DA release autoinhibition

Although the inhibition of DA release by D2 receptors has been studied extensively using receptor agonists, there are few data on the autoinhibition by endogenous DA release itself. Furthermore, estimates of the duration of DA release autoinhibition have been especially divergent (see the introductory remarks). In rat striatal slices, D2 receptor antagonists were shown to affect paired-pulse depression (PPD) as long as 30 sec (Kennedy et al., 1992). We repeated this experiment with slices from WT mice and compared PPD before and after superfusion with the D2 receptor antagonist sulpiride (10 μM), but found no significant differences (n = 5, data not shown). Similarly, we compared PPD in slices from WT and D2−/− mice. Paired pulses were applied at intervals of 5, 10, 20, 30, and 60 sec (examples for 60, 10, and 5 sec in Fig. 6a). In Figure 6a (inset), the ratio of the maximal response amplitudes for the second and first stimulus (PPD) is plotted versus interpulse intervals. Consistent with the lack of effect of sulpiride, there was no significant difference in the response between the genotypes. The data were fit by two double exponentials with the time constants τslow = 20 sec (67% in D2−/− and 62% in WT mice) and τfast = 3 sec (33% in WT and 38% in D2−/−, respectively).

Because there was no difference in PPD between the genotypes for intervals in the second range, we subsequently tested the response to stimulation trains in the subsecond range. Figure 6b shows examples of CV recordings of DA overflow in response to 20 Hz train stimulations with increasing pulse number (1–10). The averaged normalized amplitude of DA overflow plotted versus pulse number in the stimulation train is shown below (Fig. 6c). In WT mice, there was only a slight increase in DA overflow with each additional pulse, whereas in D2−/− mice, DA overflow increased substantially with each pulse. A slight increase was
population trains of WT (Schmitz et al., 2001). Striatal slices from both genotypes were stimulated with trains of pulses at 2 Hz in a slice from a WT and a D2−/− mouse (arrows indicate peak of evoked DA overflow; vertical lines are stimulation artifacts). b. Average response (mean ± SEM) to pulse numbers 2–5 expressed as a fraction of the maximal response to the first pulse for stimulation trains with intervals of 1, 0.5, and 0.2 sec for WT mice (n = 5 for 1 and 0.5 sec intervals; n = 3 for 0.2 sec interval) and D2−/− mice (n = 8 for 1 and 0.5 sec intervals; n = 5 for 0.2 sec interval). Responses to all three stimulation trains were significantly different from WT (p < 0.01).

DISCUSSION

We studied long-term adaptation of DA axon terminals to the lack of D2 autoreceptors by comparing striatal DA release in D2−/− and WT mice. There was no compensatory expression of other release-regulating autoreceptors of the D2 receptor family. Thus, the D2 receptor appears to be the only release-regulating autoreceptor on nigrostriatal terminals. Heteroreceptor-mediated DA release inhibition was unaltered in D2−/− mice, because GABA_B receptor activation suppressed DA release to the same extent as in WT mice. Comparison of paired-pulse stimulation of DA overflow in slices from D2−/− and WT mice revealed that D2-mediated autoinhibition of DA release was maximal at ~500 msec after stimulation and lasted for <5 sec. PPD observed for longer interstimulus intervals (5–30 sec) was not mediated by autoreceptor activation. DA overflow evoked by a single stimulus was reduced in amplitude and duration in D2−/− mice compared with WT mice. Our results suggest that this reduced DA overflow was caused by an increase in the maximal velocity of uptake. This finding points to an interaction between the two principal regulators of extracellular DA levels, the D2 autoreceptor and DAT.

Autoreceptor and heteroreceptor-mediated DA release inhibition

Although mesencephalic dopamine neurons are known to express D2 receptors (Diaz et al., 2000), our experiments using the D2/D3 receptor agonist quinpirole confirmed conclusions of previous studies (Rubinstein et al., 1997; Koeltzow et al., 1998; L’hirondel et al., 1998) that the D2 receptor is the only release-inhibiting DA receptor on nigrostriatal terminals. We tested whether the lack of D2 receptors would result in a loss of inhibitory G-proteins or an increased response to G-protein-coupled heteroreceptors. GABA_B and D2 receptors affect the same potassium and calcium currents in DA somata (Lacey et al., 1988; Cardozo and Bean, 1995) and may use the same subset of inhibitory G-proteins (Kerr and Ong, 1995; Huff et al., 1998). However, the response to GABA_B receptor agonists was unchanged in D2−/− mice, indicating an intact set of inhibitory G-proteins and no compensatory changes in the response to heteroreceptor activation.

Autoinhibition of DA release

The inhibition of DA release by autoreceptors at axon terminals (Cubeddu and Hoffmann, 1982; Mayer et al., 1988; Limberger et al., 1991; Kennedy et al., 1992) and somata of DA neurons (Cragg and Greenfield, 1997) is well established. However, as emphasized in a recent publication by Benoit-Marand et al. (2001), the
reported time course of the autoreceptor effect has been quite variable, with estimates of the duration ranging from milliseconds to several seconds. A discrepancy appears to exist, especially between in vivo and in vitro studies. In vitro, in rat striatal slices using a paired-pulse stimulation paradigm, autoreceptor effects blocked by sulpiride were found to last as long as 30 sec (Kennedy et al., 1992), whereas in vivo, comparison of WT and D2−/− mice indicated a duration of maximally 600 msec (Benoit-Marand et al., 2001). This discrepancy may be attributable to the different behavior of stimulated DA release in vivo and in vitro. DA release in response to a single stimulus in vitro elicits ~10-fold more DA release than in vivo (Michael and Wightman, 1999). Furthermore, evoked DA release exhibits a marked PPD for interpulse intervals between 5 and 30 sec in vitro (Kennedy et al., 1992) that is not found in vivo (Chergui et al., 1994). These differences in response are not well understood (Michael and Wightman, 1999). Possible explanations include continuous spontaneous activity in vivo versus no activity in vitro, different release probabilities because of the different stimulation conditions, and/or release of an unknown inhibitory factor in vitro. Nevertheless, our estimates of the time course of autoreceptor effects in vitro, derived from experiments using the D2 receptor antagonist sulpiride in WT mice and from comparing D2−/− and WT mice, are in agreement with the results obtained in vivo (Benoit-Marand et al., 2001). In vivo, autoinhibition was maximal between 150 and 300 msec after stimulation and lasted for ~600 msec. In vitro, we found that the maximal effect occurred at 500 msec and lasted <5 sec. This slightly prolonged time course could be attributable to the larger amplitude and duration of DA overflow in vivo.

The time course of maximal autoinhibition appears to be suitable to enhance the DA signal in response to “meaningful” burst firing (Schultz, 1986), as opposed to baseline firing of substantia nigra neurons. In the rat, burst firing consists of two to six spikes at 15 Hz. Thus, all spikes occur before maximal release inhibition is reached. In contrast, tonic activity consists of single spikes with interpulse intervals (250 msec) that allow maximal autoinhibition (Grace and Bunney, 1984a,b). Beyond the physiological role of release-regulating D2 autoreceptors, this activity is likely to play an important role in situations when DA transmission is disturbed, as for instance in the short- and long-term response to psychostimulant drugs (Pierce et al., 1995; Schmitz et al., 2001).

Compensatory interaction between DAT and D2 autoreceptors

We found a striking reduction of DA overflow in response to a single-pulse stimulus in D2−/− mice. The simulation model indicated that this reduction was caused by an increased maximal velocity of uptake rather than by decreased DA release. Experiments with nomifensine confirmed this finding, because DA overflow was nearly identical in WT and D2−/− mice in the presence of the uptake inhibitor.

In contrast to our findings, in mice with a deletion mutation of the D2 receptor, the in vivo DAT activity was found to be decreased, as determined by the clearance rate of DA injections into the striatum (Dickinson et al., 1999). High potassium-evoked DA release and basal DA levels assessed by microdialysis were unaltered in these mice. The basis of these findings remains to be elucidated, because decreased DA uptake alone would result in higher basal DA levels. A possibility is that there are changes in DA release in this mutant that cannot be observed by microdialysis with its limited time resolution.

In another mouse line carrying a null mutation for the D2 receptor, Benoit-Marand et al. (2001) reported no change in DA overflow in response to three pulses at 100 Hz in vivo, suggesting at first glance that DA release and uptake are unaltered in these animals. However, in contrast to in vitro conditions, the baseline activity of DA neurons in vivo may result in a tonic activation of D2 autoreceptors. Accordingly, Benoit-Marand et al. (2001) reported that haloperidol increased the half life of DA overflow in vivo, suggesting that the basal level of autoreceptor activation in vivo stimulates DA uptake. Similar findings have been reported previously in vivo and in striatal homogenates in vivo (Meiergerd et al., 1993; Cass and Gerhardt, 1994; Batchelor and Schenk, 1998).

Therefore, it appears that in WT mice, DA uptake is enhanced by basal D2 activity, whereas, according to our results, DA uptake is enhanced because of the long-term absence of D2 activity in D2−/− mice. The short-term regulation of DAT by D2 receptors may involve DAT phosphorylation and/or trafficking (Batchelor and Schenk, 1998; Zahniser and Doolen, 2001), whereas long-term regulation may involve changes in DAT protein expression. The results of our simulation suggest that the increased DA uptake in D2−/− mice is because of increased Vmax (i.e., increased plasmalemmal DAT expression). This is in agreement with a recent immunocytochemistry study that found an increased staining for DAT in the striatum of D2−/− mice (Parish et al., 2001). Several studies have reported changes in DAT expression in response to D2 activity. An oocyte expression study suggested D2 receptor-induced upregulation of DAT expression by a voltage-dependent mechanism (Mayfield and Zahniser, 2001). An in vivo study found that D2 receptor agonists decreased DAT expression in the caudate putamen and increased DAT expression in the nucleus accumbens (Kimmel et al., 2001). From these studies, it appears that D2 activity may change DAT expression in either direction via mechanisms not yet elucidated.

However, DAT expression can also affect D2 autoreceptor function, because D2 autoreceptor activity is virtually absent in DAT mutant mice. Moreover, the tissue content of DA is severely reduced in these mutants, whereas DA metabolism is elevated (Giros et al., 1996; Jones et al., 1999). In D2−/− mice, however, we found no change in tissue levels of DA and only a slight increase in metabolite levels, also reported previously for these mice (Jung et al., 1999). We conclude that the absence of D2 autoreceptors results in only minor changes in DA synthesis and metabolism, whereas autoinhibition of DA release and reuptake are strongly affected.

In summary, several studies have provided evidence for an interaction between D2 autoreceptors and DAT activity and expression. Our results support such an interaction by demonstrating a compensatory regulation of DA uptake in D2−/− mice. This mutual regulation appears to ensure that DA signals are transmitted with the appropriate amplitude and timing. Short- and long-term effects of psychostimulant and antipsychotic drugs are therefore likely to include changes in both D2 autoreceptors and DAT.

Note added in proof. During the course of the publication of this manuscript, an in vitro study on wild-type mice was published that confirms the time course of D2 autoreceptor effects on DA release reported here (Phillips et al., 2002).

REFERENCES


