Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum

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Dopamine is vital for coordinated motion and for association learning linked to behavioral reinforcement. Here we show that the precise overlap of striatal dopaminergic and cholinergic fibers underlies potent control of dopamine release by ongoing nicotinic receptor activity. In mouse striatal slices, nicotinic antagonists or depletion of endogenous acetylcholine decreased evoked dopamine release by 90%. Nicotine at the concentration experienced by smokers also regulated dopamine release. In mutant mice lacking the β2 nicotinic subunit, evoked dopamine release was dramatically suppressed, and those mice did not show cholinergic regulation of dopamine release. The results offer new perspectives when considering nicotine addiction and the high prevalence of smoking in schizophrenics.

Dopaminergic mechanisms of the striatum are intimately involved in motor coordination, complex issues of behavioral reinforcement, and disorders such as schizophrenia and Parkinson's disease[1–7]. The striatum receives the densest dopaminergic innervation in the mammalian brain, which arises from neurons located in the substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain[8,9]. In addition, the striatum is densely innervated by local cholinergic interneurons[10,11] that are tonically active and release acetylcholine (ACh)[12,13]. Histochemical studies have indicated that nicotinic acetylcholine receptors (nAChRs) are expressed on dopaminergic nerve terminals in the striatum[14-17]. Exogenous nicotinic agonists affect dopamine release in the striatum[18,19], but the action of endogenous ACh release is not well understood.

To investigate cholinergic influence over dopamine release, carbon-fiber microelectrodes were placed into mice striatal brain slices, and fast cyclic voltammetry was used to monitor the concentration of action-potential-dependent dopamine release in real time. We found that cholinergic interneurons acting via nAChRs containing the β2 subunit potently regulate dopamine release. Furthermore, in the concentration range experienced by smokers, nicotine acts within the striatum and influences evoked dopamine release. Because the dopaminergic and nicotinic mechanisms of the striatum are so intimately linked, the results have broad implications for understanding nicotine addiction and the predilection for smoking by schizophrenic patients.

Results

Dopamine and ACh fibers overlap in the striatum

There is dense expression of tyrosine hydroxylase (TH, indicating dopamine synthesis), choline acetyltransferase (ChAT, indicating ACh synthesis), and acetylcholinesterase (AChE, indicating ACh degradation) in the striatum[20]. The anatomical distribution of TH, ChAT and AChE was determined in coronal sections (**Fig. 1**). The highest density of each enzyme was in the striatum, extending dorsally into the olfactory tubercle. There is precise overlap in the distribution of the enzymes, and at the highest magnification individual cholinergic interneurons are seen embedded within the intertwined dopamine and ACh fibers (**Fig. 1b**).

The precise overlap and close proximity of dopamine fibers and cholinergic enzymes led us to the following hypothesis. The high ChAT activity suggests ACh must be released often, and the high AChE indicates that ACh must be broken down extremely rapidly. The two enzymes together suggest fast nicotinic mechanisms. The nAChRs are very susceptible to desensitization[21], which can be avoided if the repeatedly released ACh is removed rapidly by the abundant AChE.

Depletion of ACh reduces dopamine release

Fast-scan cyclic voltammetry with carbon-fiber microelectrodes was used to monitor dopamine release on the subsecond time scale. A bipolar simulating electrode was placed in the striatum about 150 μm from the carbon-fiber microelectrode. Normally, dopamine release was electrically evoked every 2.5 min at 60% of the maximal response. Under those conditions, the dopamine signal was stable for over two hours. The evoked dopamine concentration at the tip of the carbon-fiber microelectrode was estimated to be 1.62 ± 0.12 μM (mean ± s.e.m., $n = 29$) in the dorsal striatum, 1.46 ± 0.13 μM ($n = 18$) in the nucleus accumbens (NAc) core, and 1.44 ± 0.13 μM ($n = 17$) in the NAc shell. In addition to electrically evoked dopamine release, we also measured spontaneous dopamine release in the slice. Both the spontaneous and evoked dopamine release was prevented by 0.5 μM tetrodotoxin ($n = 4$) and by removing Ca^{2+} ($n = 3$), indicating the release was Ca^{2+} dependent and action potential dependent.

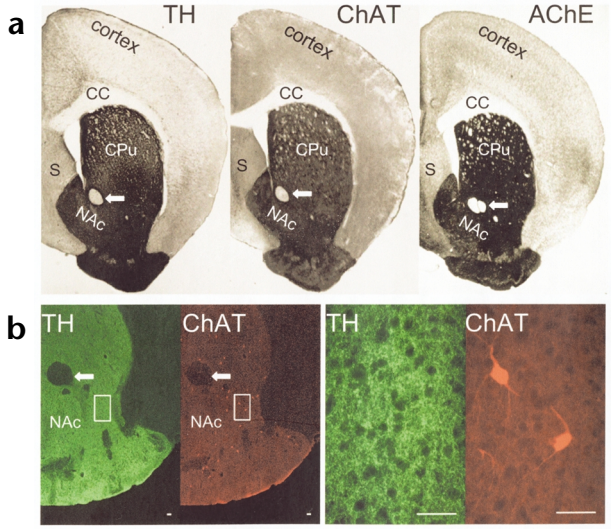

Fig. 1. Dense and overlapping distribution of ACh and dopamine in the striatum. (**a**) Bright-field photomicrographs show TH, ChAT and AChE in the NAc and dorsal striatum as demonstrated by TH and ChAT antibody staining and AChE histochemical staining. Arrows, anterior commissure. CC, corpus callosum, CPu, caudate putamen, NAc, nucleus accumbens, S, septum. (**b**) Pictures (left) taken from one slice show immunofluorescence double labeling for TH (green) and ChAT (red) at low magnification. The area in the white boxes is expanded to high magnification (right), revealing dense fiber tracts and two cholinergic interneurons. Scale bars, 50 μm.

To test for the importance of cholinergic activity, we prevented endogenous ACh release by depleting ACh stores with (±)-vesamicol, a well characterized inhibitor of vesicular ACh transport that consequently exhausts ACh release[22]. The (±)-vesamicol (2 μM) presumably diminished ACh release and inhibited evoked dopamine release by 81 ± 4.5% (n = 6; **Fig. 2a** and **b**). The maximum inhibition was 90.5 ± 4.3% in 5 μM or 10 μM (±)-vesamicol (n = 12). The median inhibitory concentration (IC_{50}) was estimated to be 1 μM (**Fig. 2c**). These results show that intact cholinergic activity is necessary for normal dopamine release induced by action potentials.

Nicotinic activity facilitates dopamine release

When muscarinic acetylcholine receptors were inhibited by 1 or 2 μM atropine, the evoked dopamine release slightly increased by 4.8 ± 3.2% (n = 6; **Fig. 3a**). In contrast, the non-specific nicotinic antagonist mecamylamine (1 μM) decreased evoked dopamine release by 83 ± 4.8% (n = 5). Maximal inhibition of dopamine release was by 92 ± 3.7% (n = 16) in 5 to 20 μM mecamylamine (**Fig. 3b**). Furthermore, mecamylamine's inhibition of dopamine release was not altered by atropine (n = 3).

In the absence of electrical stimulation, spontaneous, action-potential-dependent dopamine release could be monitored (**Fig. 3c**). When nAChRs were inhibited by mecamylamine (1 or 5 μM), the spontaneous dopamine release was inhibited below our resolution (n = 6).

Microdialysis studies and measures of radioactive dopamine release from minced striatal brain slices indicated that nicotine increases basal or ambient dopamine levels, and that increase was resistant to dihydro-β-erythroidine (DHβE) and to blockade of action potentials by tetrodotoxin[23]. Fast cyclic voltammetry measures dopamine release driven by action potentials on a fast time scale, which is different from the measures of basal dopamine obtained from samples taken over much longer times[24]. Because of the importance for addiction[25], we bath-applied nicotine and measured the effect on evoked dopamine. Under our experimental conditions, 50 nM nicotine decreased evoked dopamine release by 73 ± 4.8% (n = 4; **Fig. 4a** and **b**). The maximum inhibition was 90.5 ± 3.8% in 100 nM (n = 6) and 92.2 ± 3.5% in 500 nM (n = 5). The IC_{50} was estimated to be 30 nM (**Fig. 4c**). In the absence of electrical stimulation, the same concentrations of nicotine inhibited spontaneous, action-potential-dependent dopamine release (n = 4; **Fig. 4d**).

AChE inhibition reduces dopamine release

Based upon the distribution and the density of AChE in the striatum (**Fig. 1**), we reasoned that AChE might be important for the ongoing nAChR activity that enhances dopamine release. To test this idea, we used a potent AChE inhibitor, ambenonium[26]. Bath application of 0.1 μM (n = 6), 0.5 μM (n = 3) or 1 μM (n = 3) ambenonium gradually decreased dopamine release by 90.5 ± 2.6% (**Fig. 5**). The effect of ambenonium was reversed upon prolonged wash. This result demonstrates that AChE activity is essential for the ongoing nicotinic facilitation of dopamine release. By increasing extracellular ACh[27], AChE inhibition may increase nAChR desensitization, as suggested by the results with bath-applied nicotine. This interpretation was supported by results obtained with a puffer pipette containing 1 mM ACh that was positioned near to the carbon-fiber voltammetry electrode. Under control conditions (without inhibition of AChE), puffs of ACh applied just before and during the electrical stimulation decreased dopamine release by 16 ± 2.4% (n = 6). This result

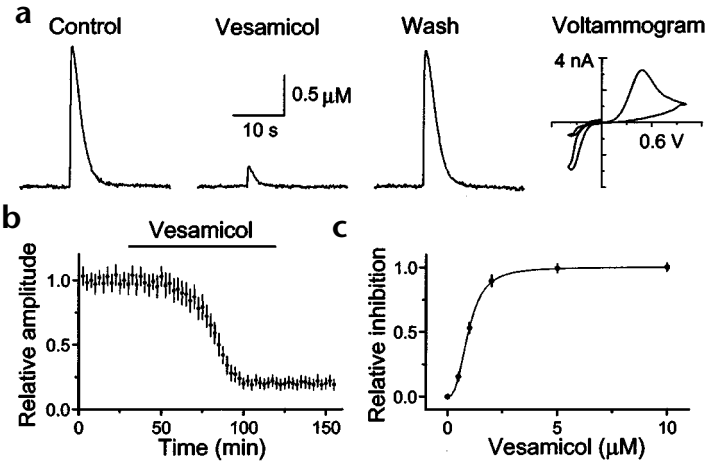

Fig. 2. Inhibition of ACh vesicular transport by vesamicol reduces dopamine release in the striatum. (**a**) The three dopamine responses were electrically evoked under control conditions, during 2 μM (±)-vesamicol application, and after recovery following 2 h of wash. The dopamine responses were constructed from voltammograms that were obtained at the rate of 10 Hz. The voltammogram (right) was obtained at the peak of evoked dopamine response under control conditions. The same results were obtained with or without atropine (1–2 μM, data not shown). (**b**) Summary of the effect of 2 μM (±)-vesamicol (n = 6). (**c**) The concentration dependence for inhibition of dopamine release produced by (±)-vesamicol was fitted by an Hill equation with IC_{50} of 1 μM.

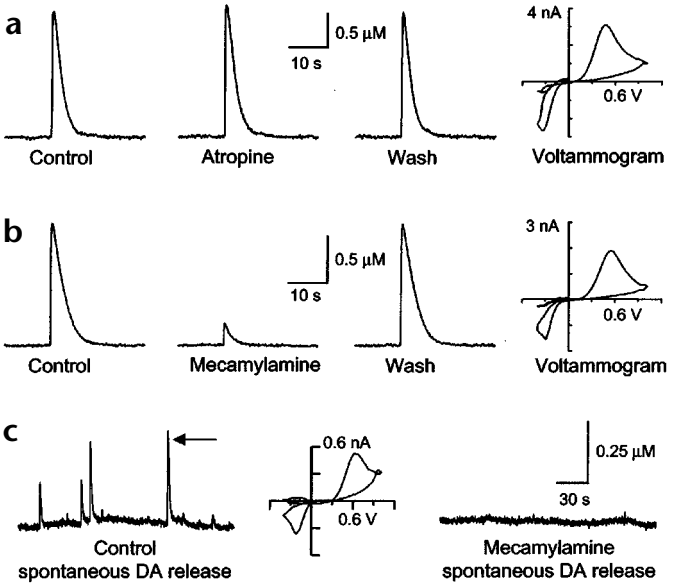

Fig. 3. Nicotinic but not muscarinic ACh receptors regulate dopamine release in the striatum. (**a**) The dopamine responses were evoked under control conditions, during 1 μM atropine application, and after recovery following a prolonged wash. The voltammogram (right) was obtained at the dopamine peak of the control trace. (**b**) The dopamine responses were evoked under control conditions, during 1 μM mecamylamine application, and after recovery following a prolonged wash. The voltammogram (right) is from the dopamine peak of the control trace. (**c**) Without electrical stimulation, spontaneous dopamine release is shown under control conditions and during application of 5 μM mecamylamine. The voltammogram was obtained at the peak of the event indicated by the arrow.

again is consistent with desensitization of nAChRs caused by the excess ACh.

Mecamylamine is a non-specific nAChR inhibitor (**Fig. 3**), but DHβE is a specific inhibitor of β2* nAChRs[28,29]. DHβE decreased dopamine release by 47 ± 5.3% (20 nM, $n = 4$) and 90 ± 5.2% (100 nM, $n = 4$; **Fig. 6a** and **b**), and maximum inhibition was 91.6 ± 4.3% (1,000 nM, $n = 4$). The IC_{50} was estimated to be 20 nM (**Fig. 6c**).

The results were verified using mutant mice lacking the β2 subunit[28,30]. It was much more difficult to electrically evoke dopamine release from β2-null mice (**Fig. 7a**). The evoked dopamine concentration was only 0.31 ± 0.07 μM ($n = 14$), which is an 80% decrease relative to wild-type littermates. Furthermore, 100 nM and 1,000 nM DHβE ($n = 4$), 10 μM mecamylamine ($n = 3$, data not shown), and 1,000 nM nicotine ($n = 6$) no longer had any effect (**Fig. 7b**). Although electrically evoked dopamine release was depressed by elimination of the nAChR β2 subunit, dopamine release induced by a depolarizing solution of 30 mM KCl produced the same dopamine signal in slices from β2-null mice (37.6 ± 3.8 μM, $n = 4$) and wild-type mice (38.2 ± 3.4 μM, $n = 6$). This result indicates that the dopamine content in β2-null mice was normal.

The effect of nAChRs is specific to the β2 subunit because inhibition of α7* nAChRs had no detectable effect. In the presence of the α7*-specific inhibitor, 20 nM methyllycaconitine[29], evoked dopamine release was 98.6 ± 2.3% of control level ($n = 3$).

Discussion

Striatal dopamine and ACh fibers form an intertwined meshwork that is the densest in the brain, and these fibers are associated with the densest expression of AChE[8,9,11]. Tonic activity of the cholinergic interneurons releases ACh[12,13], and the AChE rapidly terminates the ACh signal. This situation optimizes ongoing nAChR activity by avoiding desensitization. Our results show that dopamine release caused by action potentials is potently regulated by β2* nAChR activity.

Nicotine, at the concentrations achieved by smokers[31], also decreases action-potential-dependent dopamine release in the NAc, suggesting that nicotine is acting like an antagonist by causing desensitization[32]. This hypothesis is reasonable because the β2* nAChRs that regulate dopamine release have a high affinity for nicotine and are readily desensitized by those concentrations[33]. The finding was unexpected, however, because older studies using *in vivo* microdialysis or loading of slices with radiolabeled dopamine have shown that nicotine increases the basal level of dopamine[18,34]. That increase, however, is at a very low concentration of dopamine and is often action potential independent. Microdialysis is a slow process, often taking 10 minutes per sample. The sample is collected over a relatively large volume with probes of about 250–500 μm diameter. The radiolabeled and microdialysis samples report dopamine released from multiple sources and provide an average baseline dopamine concentration that escapes reuptake or breakdown, giving estimates near 4 nM (ref. 36).

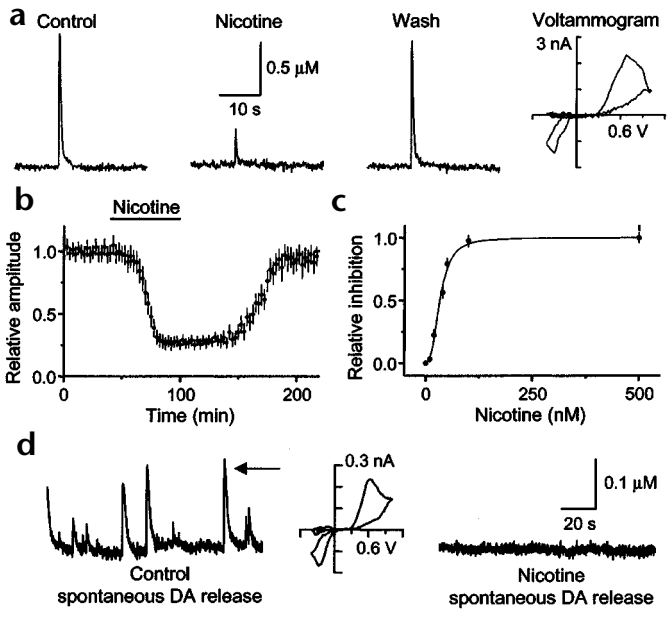

Fig. 4. Bath-applied nicotine reduces action-potential-dependent dopamine release. (**a**) The dopamine responses were electrically evoked under control conditions, during 50 nM nicotine application, and after recovery following a prolonged wash. The voltammogram (right) was obtained at the dopamine peak of the control trace. (**b**) Summary of the effect of 50 nM nicotine ($n = 4$). (**c**) The concentration dependence for inhibition of dopamine release produced by nicotine was fitted by an Hill equation with IC_{50} of 30 nM. (**d**) Without electrical stimulation, spontaneous dopamine release is shown under control conditions and during application of 50 nM nicotine. The voltammogram was obtained at the peak of the event indicated by the arrow.

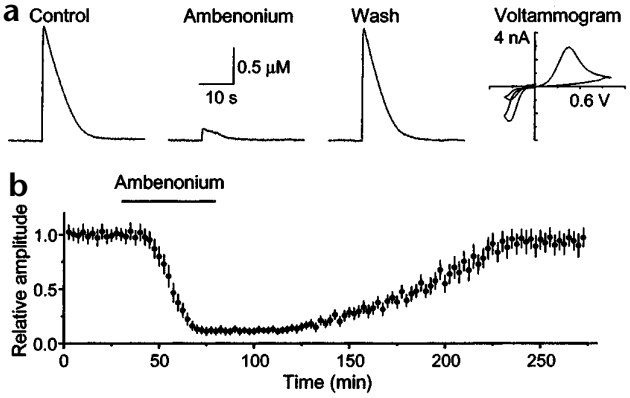

Fig. 5. An AChE inhibitor, ambenonium, reduces dopamine release in the striatum. (**a**) The dopamine responses were evoked under control conditions, during 100 nM ambenonium application, and after recovery following a prolonged wash. The voltammogram (right) was obtained at the dopamine peak of the control trace. (**b**) Summary of the effect of 100 nM ambenonium in 6 experiments, 3 of which lasted long enough to recover upon washing.

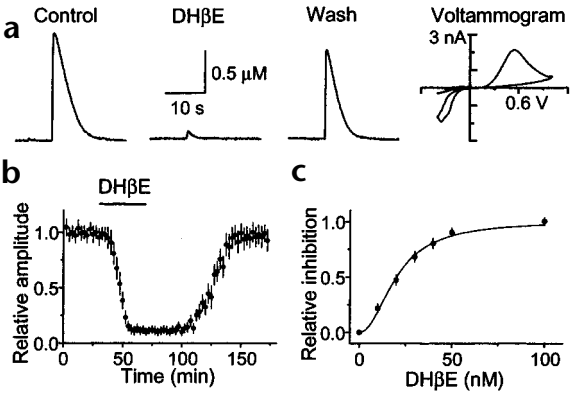

Fig. 6. A specific inhibitor of β2* nAChRs, DHβE, potently reduces evoked dopamine release. (**a**) The dopamine responses were evoked under control conditions, during 0.1 μM DHβE application, and after recovery following a prolonged wash. The voltammogram (right) was obtained at the dopamine peak of the control trace. (**b**) Summary of the effect of 0.1 μM DHβE (n = 5). (**c**) The concentration dependence for inhibition of dopamine release produced by DHβE was fitted by an Hill equation with IC_{50} of 20 nM.

Fast voltammetry in our present study detects dopamine that is released by action potentials, and the measurement estimates dopamine concentration before uptake and diffusion have much effect. The voltammetry measurement is from a smaller volume, with an electrode of 10 μm diameter, and it detects dopamine signals at speeds and concentrations that are indicative of direct neuronal activity. These two very different measurements of extracellular dopamine dynamics suggest that nicotine has multiple actions when analyzed in the dopamine target area of the NAc. The unexpected result that nicotine strongly inhibits action-potential-dependent dopamine release in the target has broad implications, particularly when considering nicotine addiction.

A simplification of a commonly held view of nicotine addiction is the following: nicotine elevates dopamine in the NAc, and that elevation reinforces continued use[32]. However, our understanding of dopamine participation in reinforcement processes is far from complete. It is clear that dopamine in the NAc is not a direct indication of reward. More sophisticated theories suggest that the dopamine signal conveys novelty and/or prediction error during the ongoing process of learning adaptive behaviors as an animal continually updates a construct of environmental saliency[1,7,32,34,37]. The complexity of the dopamine signal is exemplified by a recent study[38]. Rats learned to press a lever causing intracranial self-stimulation of the midbrain dopamine areas. After learning the task, the rats continued self-simulations as if it were pleasurable, but the self-simulations no longer increased the dopamine concentration at the target (NAc). Dopamine was released only during the initial phase while the rats were learning. Thus, even when the dopamine neurons are stimulated, other regulatory processes can ultimately control dopamine release. Our results identify a nicotinic cholinergic mechanism that regulates dopamine release at the target.

Ongoing β2* nAChRs activity in the NAc seems important for dopamine release driven by afferent action potentials, but nicotine desensitizes those β2* nAChRs[35]. Although this result is contrary to the simplest view of nicotine addiction, it offers a new clue to understand the high prevalence of smoking by schizophrenic patients. Schizophrenics have impaired voluntary or sustained attention, and the positive symptoms of schizophrenia, such as delusions and disorganized behavior, are associated with an excess of dopamine in the striatum[39]. Schizophrenic patients are usually treated with neuroleptics, which inhibit specific dopamine receptors and ultimately decrease dopamine signaling. Nicotine can transiently improve attention and some aspects of the positive symptoms in schizophrenic patients[40,41]. Such nicotine-induced improvements are thought to account, at least partially, for the extraordinarily high rate of smoking observed in schizophrenics[42,43]. However, it was difficult to explain why nicotine could help schizophrenics if it were increasing dopamine levels. Our finding

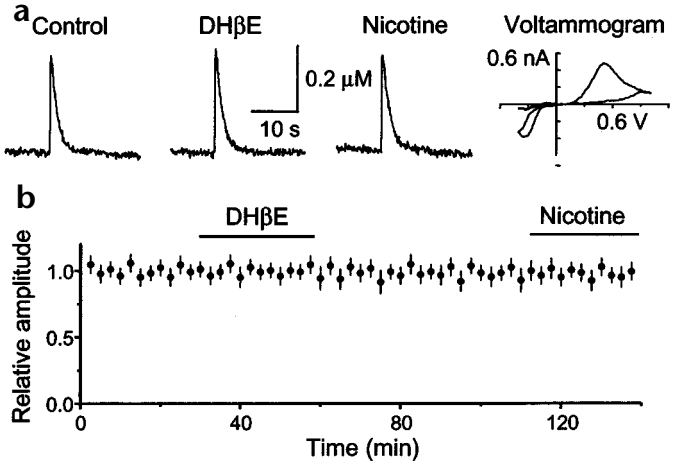

Fig. 7. β2-null mice have decreased dopamine release, and the release is not regulated by DHβE or nicotine. (**a**) The dopamine responses were evoked under control conditions, during 0.1 μM DHβE application, and during 1 μM nicotine application. The detected dopamine concentration was depressed in the β2-null mice to about 0.3 μM compared to about 1.5 μM in WT mice. The voltammogram (right) was obtained at the dopamine peak of the control trace. (**b**) Summary data showing the lack of effect by 0.1 or 1 μM DHβE and by 1 μM nicotine (n = 4).

that nicotine decreases action-potential-evoked dopamine release may underlie nicotine's transient positive influence for schizophrenic patients.

Methods

Wild-type C57BL/6J (Jackson Laboratory, Bar Harbor, Maine) and β2-null[28] mice were used at 3 to 6 months of age. Horizontal or coronal striatal slices (400 μm) were cut on a vibratome, held at room temperature, and studied at 30°C in 125 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 26 mM Na_2HPO_3, 1.25 mM $NaHCO_3$ and 10 mM glucose saturated with 95% O_2 and 5% CO_2. Horizontal slices appeared more robust than coronal sections, such that spontaneous dopamine events were recorded in about 10% of horizontal slices but none in the coronal slices. Animal care was in accord with Baylor College of Medicine's animal care committee.

Homemade carbon-fiber (P55S, Amoco Polymers, Greenville, South Carolina) microelectrodes were used for fast-scan cyclic voltammetry[44]. Stable, low-noise and sensitive electrodes were selected, especially for recording spontaneous dopamine release. An Axopatch 200B amplifier and pClamp 8 (Axon Instruments, Foster City, California) were used to acquire and analyze data. Scans of the microelectrode potential (12 ms duration, 10 Hz) were from 0 mV to –400 mV to 1,000 mV to –400 mV to 0 mV against an Ag/AgCl reference electrode at a rate of 300 mV/ms. The signal that formed the voltammogram was sampled at 50 kHz, and the net current due to the electrochemical reaction was obtained by digital subtraction before and after a stimulation. The voltammograms are of the proper form, having oxidation current peaks between 500 to 700 mV and the reduction current peaks between –230 to –330 mV. Dopamine is more than 90% of striatal monoamines[45], and the voltammetry signal was calibrated against fresh solutions of 1–10 μM dopamine.

Intrastriatal stimuli were delivered using bipolar tungsten electrodes with resistances of about 0.5 MΩ. The two tips of the stimulating electrode were about 100–200 μm away from each other. The tip of the recording electrode was about 150 μm away from the two tips of the stimulating electrode. For wild-type striatal slices, stimuli were 1 ms in duration and 2–6 V to achieve about 60% maximum amplitude, but the same results and conclusions were obtained with higher and lower stimulus intensities. For β2-null striatal slices, stimuli were 1 ms in duration and 6–12 V, and stimulation and recording sites were adjusted to obtain ≈ 80% maximum amplitude.

ChAT and TH immunohistochemistry and AChE histochemistry were adapted from published methods[46–48]. For immunohistochemistry, brains were fixed in 0.1 M phosphate buffer containing 4% (w/v) paraformaldehyde and 14% (v/v) picric acid. Coronal sections (30 μm) were cut on a cryostat, incubated with either goat anti-ChAT or rabbit anti-TH antibodies, followed by peroxidae-conjugated antibodies, and were finally treated with chromogen diaminobenzidine. For ChAT and TH double immunofluorescence, after treatment with the primary antibody mixture, the secondary antibody mixture containing Cy2-conjugated donkey anti-rabbit and rhodamine-conjugated donkey anti-goat IgG antisera was used. For AChE staining, sections were incubated for 15 h in 100 ml solution containing 50 mM sodium acetate, 4 mM copper sulfate, 16 mM glycine, 116 mg S-acetylthiocholine iodide and 3 mg ethopropazine. The sections were then rinsed and developed with 1% sodium sulphide. All images were captured with a digital camera and processed in Adobe PhotoShop.

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