Corelease of Dopamine and Serotonin from Striatal Dopamine Terminals

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Summary

The striatum receives rich dopaminergic and more moderate serotonergic innervation. After vesicular release, dopamine and serotonin (5-hydroxytryptamine, 5-HT) signaling is controlled by transporter-mediated reuptake. Dopamine is taken up by dopamine transporters (DATs), which are expressed at the highest density in the striatum. Although DATs also display a low affinity for 5-HT, that neurotransmitter is normally efficiently taken up by the 5-HT transporters. We found that when extracellular 5-HT is elevated by exogenous application or by using antidepressants (e.g., fluoxetine) to inhibit the 5-HT transporters, the extremely dense striatal DATs uptake 5-HT into dopamine terminals. Immunohistochemical results and measurements using fast cyclic voltammetry showed that elevated 5-HT is taken up by DATs into striatal dopamine terminals that subsequently release 5-HT and dopamine together. These results suggest that antidepressants that block serotonin transporters or other factors that elevate extracellular 5-HT alter the temporal and spatial relationship between dopamine and 5-HT signaling in the striatum.

Introduction

The striatum is a large subcortical structure in the mammalian brain that is involved in motor coordination, cognitive functions, and complex processes associated with adaptive behaviors (Lang and Lozano, 1998; Berke and Hyman, 2000; McClure et al., 2003; Schultz et al., 2003; Zhou et al., 2003). A distinguishing feature of the striatum is its extremely dense dopamine (DA) innervation (Björklund and Lindvall, 1984). The dense DA axon terminals provide the strongest expression of DA transporters (DATs) in the brain (Ciliax et al., 1995; Coulter et al., 1996; Sesack et al., 1998). The striatum also receives more modest 5-HT innervation, and those fibers express the 5-HT transporters (Steinbusch, 1981; Pickel and Chan, 1999). Ultrastructurally, 5-HT terminals are in close proximity to DA terminals (Van Bockstaele and Pickel, 1993), providing an anatomical basis

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for the two systems to interact with each other. Furthermore, the extracellular 5-HT concentration is likely to be high near release sites because 5-HT is highly concentrated within releasable vesicles (Bruns et al., 2000) and there is no extracellular enzyme to degrade 5-HT. DATs have been shown to bind 5-HT with a low affinity, which is estimated to be about 1/15 of the affinity for DA (Giros et al., 1991; Pristupa et al., 1994; Eshleman et al., 1999). Evidence also suggests that depression and the mechanistic action of antidepressants are influenced by DA and by DA and 5-HT interactions in the striatum (Laasonen-Balk et al., 1999; D'Aquila et al. 2000; Meyer et al. 2001; Zangen et al. 2001; Willner 2002). Encouraged by that information, we examined the influence of DATs on 5-HT signaling in the striatum and found that under some conditions DATs uptake 5-HT into DA terminals. Subsequently, 5-HT and DA are released together, thereby altering the relationship between DA and 5-HT signaling and inducing some DA and 5-HT cosignaling.

Results

Elevated 5-HT Enters Dopamine Terminals

To determine whether 5-HT can accumulate in DA terminals, we used double immunostaining with antibodies against tyrosine hydroxylase (TH) and 5-HT to visualize their colocalization in striatal axon terminals (Figure 1). TH is the key enzyme for DA and norepinephrine synthesis. Norepinephrine (NE) axon terminals comprise a small minority of the TH-positive terminals in the striatum. Therefore, the TH labeling in the striatum can be assigned almost exclusively to DA terminals (Feuerstein et al., 1986; Sesack, 2002).

Under drug-free control conditions, the modest number of 5-HT terminals (Figure 1A1, red) and the dense DA terminals (Figure 1A2, green) were often next to each other, but did not generally overlap (Figure 1A3). This result is consistent with previous studies (Descarries and Mechawar, 2000). We increased the external 5-HT concentration by directly adding exogenous 5-HT to the bathing solution. After incubating a striatal slice in exogenous 5-HT (1–2 μM) for 2 hr, a different result emerged. There was a dramatic increase in the number of 5-HT-positive terminals (Figure 1B1, red), but the number of TH-positive DA terminals remained relatively constant (Figure 1B2, green). Superimposition of the images and counting of individual terminals indicated that the majority ($\sim$80%) of the 5-HT-positive terminals and the TH-positive terminals overlapped with each other (Figure 1B3, yellow). The result suggests that 5-HT has entered into DA terminals and that elevating external 5-HT caused DA terminals to acquire a dual neurochemical identity, containing both 5-HT and DA. Because DA terminals do not express 5-HT transporters (Pickel and Chan, 1999), DATs appeared to be responsible for uptaking 5-HT into the DA terminals.

To examine the role of the DATs, we specifically inhibited the DATs with GBR12909 (0.1 or 1 μM) (Pristupa et
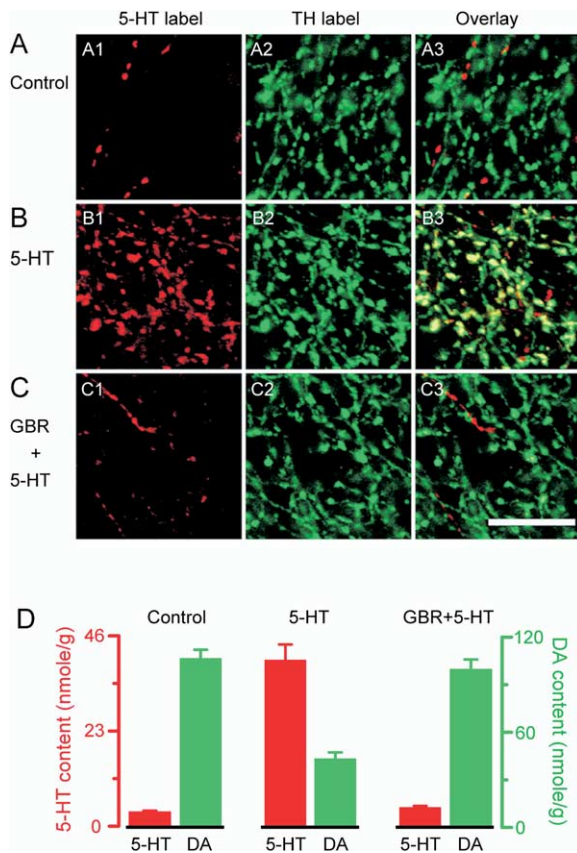
Figure 1. Anatomical Visualization of 5-HT Accumulation in Striatal DA Terminals

(A) Under control conditions, 5-HT axon terminals as detected by anti-5-HT antibody are relatively sparse (red, [A1]), and DA terminals as detected by anti-tyrosine hydroxylase (TH) antibody are dense (green, [A2]). The DA and 5-HT terminals generally do not overlap (absence of yellow, [A3]).

(B) Incubation with 2 μM 5-HT for 2 hr increases 5-HT-positive terminals (red, [B1]), and DA terminals are relatively unchanged (green, [B2]). Most of the 5-HT-positive terminals coincide with DA terminals (yellow, [B3]), suggesting that DA terminals in this case also contain 5-HT.

(C) Pretreatment with the DAT inhibitor GBR12909 (1 μM) blocks the effect of 2 μM 5-HT. 5-HT terminals remain sparse (red, [C1]), DA terminals are dense and unchanged (green, [C2]), and the 5-HT-positive terminals rarely coincide with TH-positive terminals (yellow, [C3]). Scale bar, 10 μm.

(D) The HPLC-measured striatal 5-HT content (red bars, left scale, in nmole/g wet tissue) is low under control conditions, increases after bathing the slice in 2 μM 5-HT, and is near the control value when DATs are inhibited by 1 μM GBR12909 before the 5-HT treatment (GRB + 5-HT). In contrast, the DA content (green bars, right scale, in nmole/g wet tissue) is high under control conditions, decreases after bathing the slice in 5-HT, and is near the control value when DATs are inhibited by GBR12909 before the 5-HT treatment (GRB+5-HT). Standard error bars are shown.

al., 1994) before and while applying 5-HT (2 μM) to the striatal slice preparation. With the DATs inhibited, the number of 5-HT-positive terminals was not increased (Figure 1C1, red). The DA terminals remained dense (Figure 1C2, green), and there was very little overlap between the 5-HT and DA terminals (Figure 1C3). These results suggest that there is a DAT-mediated uptake of 5-HT into striatal DA terminals when extracellular 5-HT is elevated.

To observe the influence of the DATs alone, fluoxetine and nisoxetine (each at 1 μM) were present in all of these experiments to block entry of exogenous 5-HT into intrinsic 5-HT and NE axons, respectively. However, the absence of 5-HT and NE transporter inhibitors did not cause an obvious difference in 5-HT fluorescence in DA terminals because the high concentration of exogenous 5-HT likely overwhelmed the 5-HT transporters in these experiments. Incubation with 0.1 and 0.5 μM 5-HT or shorter incubation times of 0.5–1 hr also increased the number of 5-HT-positive terminals, but to a lesser extent than 1–2 μM 5-HT incubated for 2 hr (data not shown).

Quantification of the Accumulation of Exogenous 5-HT into DA Terminals

To quantify the anatomically detected neurochemical changes, we employed high-performance liquid chromatography (HPLC) because of its precision and specificity. We determined the content of 5-HT and DA from striatal slices under the same experimental conditions used to obtain the images of Figures 1A–1C. Under control conditions (Figure 1D, Control), the 5-HT content in the dorsolateral striatum was 3.5 ± 0.2 nmoles/ g wet tissue, and the DA content was 105.4 ± 5.5 nmoles/g wet tissue (n = 20 samples from five mice).

After incubating striatal slices in 2 μM 5-HT for 2 hr and washing away the extracellular excess, the striatal 5-HT content increased 11-fold and the DA content decreased by 60% ± 4% (n = 16 samples from four mice) (Figure 1D, 5-HT). These results are consistent with the images of Figures 1A and 1B, and together they indicate that 5-HT was being taken into the DA terminals. Furthermore, striatal DA content was reduced by the 5-HT incubation, whereas the DA turnover rate, as measured by the ratio of 3,4-dihydroxyphenylacetic acid (DOPAC) to DA, increased from 0.08 ± 0.01 (n = 6) under control conditions to 0.14 ± 0.01 (n = 6) after 2 hr incubation in 2 μM 5-HT. These results suggest that 5-HT was displacing DA from DA vesicles in the striatal DA terminals such that relatively more DA was outside of DA vesicles and exposed to breakdown by monoamine oxidases.

To test the role of the DATs, they were inhibited by GBR12909 (1 μM) before adding 2 μM 5-HT (Figure 1D, GBR+5-HT). In that case, the 5-HT content was only 30% ± 4% higher than the control (n = 16 samples from four mice), as compared to the 1100% increase when DATs were not inhibited by GBR12909. This small increase in 5-HT content in the presence of GBR12909 was likely due to nonspecific uptake or binding (Krueger, 1990). Because 5-HT was not taken up into DA terminals under these conditions, the DA content was statistically unchanged from the control. Taken together, the results of Figure 1 suggest that elevated 5-HT is taken up into DA terminals and that 5-HT displaces some of the DA content within the terminals.

Identifiable DA and 5-HT Signals Measured Using Voltammetry

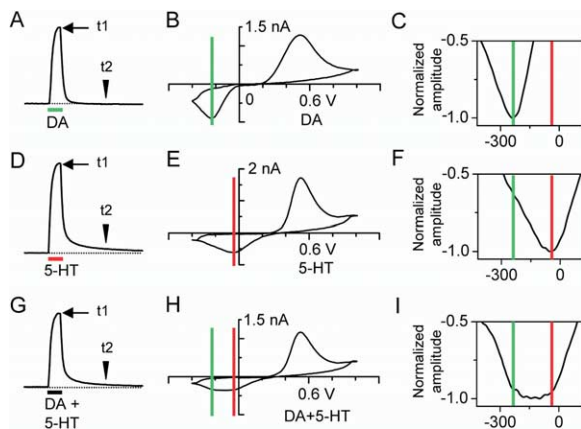The anatomical and HPLC data of Figure 1 suggested that 5-HT can colocalize with DA in DA terminals. We

Figure 2. Fast-Scan Cyclic Voltammetry Identifies DA and 5-HT Because They Have Different Potentials for Their Reduction Minima

In simple electrolyte solutions, this control experiment shows that applications of DA or 5-HT onto a carbon-fiber microelectrode produce identifiable signals. (A) The time course is shown for the carbon-fiber microelectrode response to an application of pure DA (2 μM, 1 s). (B) The complete voltammogram is shown at time t1, which is the peak of the response to the DA application. The oxidation-reduction profile is plotted as current versus the voltage. The reduction minimum is near –230 mV (green line). (C) The normalized and expanded DA reduction arm shows the DA reduction minimum more clearly (green line, near –230 mV) and shows the separation from the 5-HT reduction minimum (red line). (D) The time course is shown for the response to an application of pure 5-HT (1 μM, 1 s) onto the same carbon-fiber microelectrode. (E) The complete voltammogram is shown at time t1, which is the peak of the response. The reduction peak is near –30 mV (red line). (F) The normalized and expanded 5-HT reduction arm shows the 5-HT reduction minimum more clearly (red line, near –30 mV) and shows the separation from the DA reduction minimum (green line). (G) The time course is shown for the response to a mixture of DA and 5-HT (1 and 0.5 μM, 1 s) onto the same carbon-fiber microelectrode. (H) The complete voltammogram is shown at time t1, which is the peak of the response. The reduction minimum for the mixture is spread out through the range from –30 mV (red line for 5-HT) to –230 mV (green line for DA). (I) The normalized and expanded mixed DA/5-HT reduction arm shows the broad reduction peak ranging from –30 mV (red line) to –230 mV (green line).

next examined whether those neurotransmitters were subsequently released together. To monitor the real-time, physiological release of DA and 5-HT in striatal slices, we used fast-scan cyclic voltammetry to measure the identifiable oxidation-reduction profiles (or voltammograms) of DA and 5-HT (Kawagoe et al., 1993; Iravani and Kruk, 1997; Bunin and Wightman, 1998; Zhou et al., 2001). Measured with a carbon-fiber microelectrode, the distinct voltammograms of DA and 5-HT are particularly different near their reduction minima, which are near –250 mV (–220 to –280 mV) for DA and near –30 mV (0 to –50 mV) for 5-HT.

To test the sensitivity of this approach, we conducted control experiments in a simple bath solution without any neural tissue. In the electrolyte solution, pure DA was applied to the carbon-fiber electrode (solid bar, Figure 2A). The full voltammogram at the peak of the DA response (t1) is shown in Figure 2B, and the expanded reduction arm is shown in Figure 2C. The voltammogram shows the DA reduction minimum near –230 mV, as indicated by the vertical green line. There

is no significant signal for 5-HT at the reduction minimum of –30 mV (red line). Four seconds after the peak of the response (t2 in Figure 2A) there was no DA signal because DA had cleared from the carbon-fiber surface.

When pure 5-HT was applied in the same manner to the same carbon-fiber electrode (solid bar, Figure 2D), a longer-lasting signal resulted because 5-HT cleared from the carbon-fiber surface more slowly. The full voltammogram at the peak of the 5-HT response (t1) is shown in Figure 2E, and the expanded reduction arm is shown in Figure 2F. The voltammograms show the 5-HT reduction minimum near –30 mV, as indicated by the vertical red line. Four seconds after the peak of the 5-HT response (t2 in Figure 2D) there was still a small observable signal above the baseline because 5-HT clears from the carbon-fiber surface more slowly. Finally, when a mixture of DA and 5-HT was applied to the carbon-fiber microelectrode (solid bar, Figure 2G), the voltammogram displayed a broad reduction minimum that extended between the minimum for pure DA (green line) and the minimum for pure 5-HT (red line) (Figures 2H and 2I). At time t2 (Figure 2G), only the 5-HT signal was detected. An important distinguishing feature is that the clearance of 5-HT from the carbon-fiber microelectrode is much slower than the clearance of DA, such that the 5-HT signal can be recorded at the microelectrode after the DA signal has declined into the baseline. These properties were used later to identify exocytotic release of DA and 5-HT in striatal slices.

Real-Time Monitoring of 5-HT and DA Corelease in the Striatum

A bipolar simulating electrode was placed into dorsolateral striatal brain slices about 150 μm from the carbon-fiber microelectrode, and neurotransmitter release was electrically evoked every 2.5 min at 60% of the maximal response. Under control conditions, both spontaneous and electrically evoked release decayed rapidly, taking 1.1 ± 0.1 s (n = 12 recordings) to decline from 90% to 10% (Figure 3A). At the peak of the response (t1), a large DA signal was detected at the carbon fiber, as identified by its characteristic voltammogram and quantified in Figure 3B. Four seconds after the maximum (t2), the signal had declined to <10% of the peak value (Figure 3B). Along the baseline, small spontaneous events can be observed (Figure 3A), and analysis of the voltammograms showed these to be pure DA release (data not shown). A 5-HT component is not detected under normal conditions because the 5-HT content in control slices is very low and the physiologically released 5-HT is below the level of detection.

To examine the consequences of elevated external 5-HT, we bathed the striatal slices in 2 μM 5-HT and digitally subtracted the background 5-HT signal from our recordings. The presence of 5-HT greatly enhanced and prolonged both spontaneous and electrically evoked voltammetric signals (Figure 3C). The time for the evoked signal to decay (from 90% to 10%) increased 16 ± 1-fold (n = 12 recordings in 12 slices) over the control condition. At the peak of the response (t1), the signal was a mixture of DA and 5-HT showing an extended reduction peak (from –30 to –250) in the voltammogram (Figure 3C). The DA concentration at the peak decreased by 46% ± 7% compared to the control,
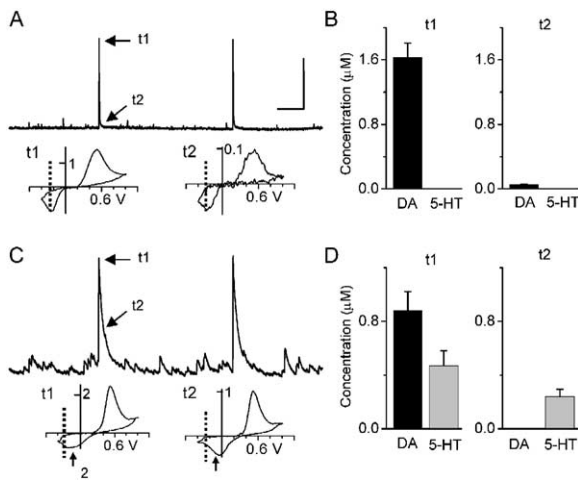
Figure 3. Fast-Scan Cyclic Voltammetry Reveals 5-HT and DA Corelease in the Striatum

(A) A control trace showing small spontaneous voltammetric events and two large electrically stimulated responses. The scale bars are 1 μM DA and 30 s. Time t1 indicates the peak of the signal. At time t2, 4 s after the peak, the signal is small but still detectable. The two voltammograms (below) from t1 and t2 are typical DA voltammograms with the oxidation peak near 500 mV and the reduction peak near −230 mV (dotted vertical lines). The y axis of the voltammograms varies and is labeled for comparison in nA. (B) Under control conditions at the maximum of the evoked release (t1 in panel [A]), the voltammogram analysis indicated a pure DA signal with no detectable 5-HT signal. The DA signal decayed rapidly, such that there was a barely detectable DA signal 4 s after the peak (t2 in panel [A]). (C) In the presence of 2 μM 5-HT after subtracting off the 5-HT background, both the spontaneous and evoked events are larger and longer lasting than in the control case. The voltammogram from t1 is a mixed DA/5-HT voltammogram with a broad reduction peak from −230 mV (dotted vertical line) to −30 mV (arrow), whereas the voltammogram from t2 is a predominantly 5-HT voltammogram with a reduction peak at −30 mV (arrow). (D) Voltammogram analysis indicates that, at t1, the signal is a mixture of DA and 5-HT. The peak DA concentration (t1 in panel [C]) was reduced by ∼50%, whereas the 5-HT concentration was increased from an undetectable level to ∼0.5 μM. Note that the ordinate scale has changed from that in panel [B]. At time t2, the 5-HT concentration decayed to ∼0.3 μM and the DA signal was no longer detectable. Standard error bars are shown.

but the 5-HT concentration increased from essentially 0 in the control to 0.5 ± 0.1 μM (n = 8 slices). Four seconds after the maximum (t2), only a 5-HT signal was detected (Figure 3D). This finding is consistent with the more rapid reuptake of DA by the DATs and slower clearance of 5-HT from the carbon fiber when compared to DA (see Figure 2).

Spontaneous Vesicular 5-HT and DA Corelease in the Striatum

Spontaneous vesicular neurotransmitter release is an important part of neuronal activity. Thus, we examined whether DA and 5-HT were released together spontaneously in the absence of electrical stimulation. Under control conditions, spontaneous DA release was observed in the baseline of the recordings (Figures 3A and 4A). These spontaneous events were fast, with rise times of 0.1 ± 0.01 s (10% to 90%) and decay times of
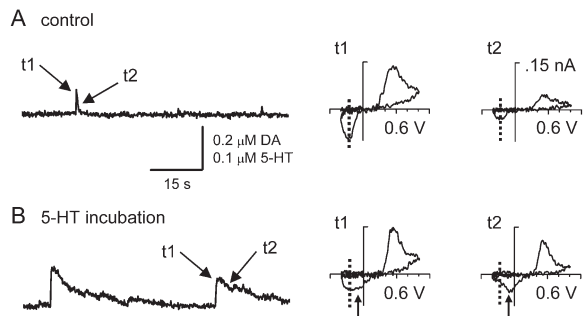

Figure 4. Incubation with 5-HT Induces Spontaneous, Vesicular 5-HT and DA Corelease from DA Terminals in the Striatum

(A) A segment of a recording of fast spontaneous events is shown under control conditions. The short duration of the spontaneous event is due to the efficient reuptake of DA by DATs. To the right, the voltammograms are shown at the maximum (t1) and at the decay foot (t2) of a spontaneous event. Both show the typical DA oxidation peak near 500 mV and reduction peak near −230 mV (dotted vertical lines). All the voltammograms are on the same scale.

(B) A segment of a recording of slower spontaneous events is shown after incubation with 2 μM 5-HT. The voltammogram at the maximum (t1) is a mixed DA and 5-HT voltammogram with a broad reduction peak ranging from near −230 mV (vertical dotted line) to near −30 mV (arrow). The voltammogram as the signal decays (t2) is predominately 5-HT with its voltammogram showing a reduction peak near −30 mV (arrow). The prolonged decay is likely due to the inefficient uptake of 5-HT by DATs. All spontaneous events are abolished by 0.5 μM TTX or 0 mM Ca^{2+}.

1.2 ± 0.1 s (90% to 10%). The voltammogram shapes of the spontaneous events were identical to those of evoked DA events, having oxidation and reduction peaks at the same potentials as DA standards (Figure 4A). After incubation with 2 μM 5-HT, the spontaneous events were prolonged (Figure 4B), similar to the prolongation of electrically stimulated events (Figures 3C).

To determine the chemical nature of these prolonged spontaneous events, we examined the voltammograms of 40 randomly chosen spontaneous events from eight different slices (five events from each slice). In all cases, the rising phase and the maximum of these spontaneous events were mixed signals of both DA and 5-HT (t1, Figure 4B). The falling phase of the events, however, was predominantly 5-HT with only a small DA component. Four seconds after the maximum response, only the 5-HT component was detected (t2, Figure 4B). These results indicate that DA and 5-HT were being released together spontaneously and that there is more rapid reuptake of DA by DATs and more rapid clearance of DA than 5-HT from the carbon-fiber microelectrode. If 5-HT and DA were being released from different terminals, then some spontaneous events would have contained only DA and some others would have contained only 5-HT.

All the spontaneous and electrically evoked events were reversibly blocked by 0.5 μM tetrodotoxin, a Na^+ channel blocker (n = 3), or by 0 mM extracellular Ca^{2+} (n = 2), indicating that these spontaneous signals arise from action potential-dependent vesicular or quantal release from DA terminals. Incubation with lower concentrations of 5-HT (0.05, 0.1, 0.5, and 1 μM) also induced 5-HT/DA corelease, but the 5-HT component
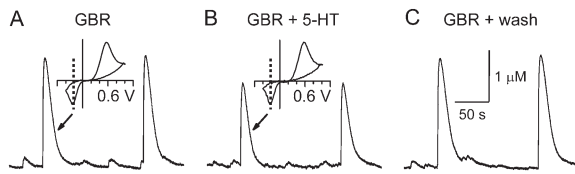
Figure 5. Dopamine Transporter Activity Is Required for the 5-HT-Induced Corelease of 5-HT and DA

(A) A trace is shown with small spontaneous and two large electrically evoked voltammetric events obtained in the presence of the DAT inhibitor GBR12909. DAT inhibition prolongs the decay of both evoked and spontaneous DA signals. The voltammograms used to construct the trace are all typical of DA with oxidation peaks near 500 mV and reduction peaks near –230 mV (vertical dotted line). An example voltammogram (inset) is shown from the time indicated by the arrow on the falling phase.
(B) With the DATs inhibited by 1 μM GBR12909, bath application of 2 μM 5-HT decreased the amplitude of the evoked and spontaneous DA signals. The voltammograms (inset) from the falling phase of the evoked event is indicative of pure DA without any 5-HT component because the inhibited DATs could not uptake 5-HT.
(C) After a prolonged wash out of the 5-HT, the electrically evoked DA signal returned to the initial amplitude. Sulpiride (1 μM) was present during the entire experiment to prevent D2 receptor-mediated autoinhibition of DA release.
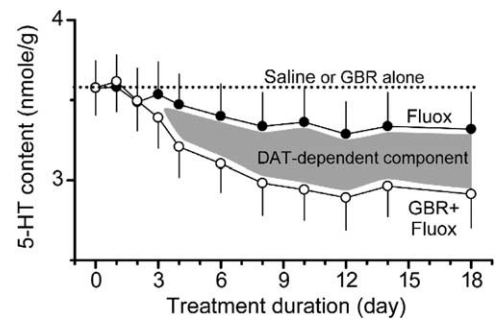

Figure 6. Chronic 5-HT Transporter Blockade by the SSRI Fluoxetine Induces DAT-Dependent Accumulation of Striatal 5-HT

Mice were given daily injections of 10 mg fluoxetine (Fluox) per kg body weight (filled circles) or 5 mg/kg GBR12909 with 10 mg/kg fluoxetine (open circles). Daily injections of saline or GBR12909 alone did not alter striatal 5-HT content (shown as a dashed baseline for clarity in nmole/g wet tissue). Inhibiting 5-HT transporters with fluoxetine (filled circles) slightly lowered 5-HT content. Inhibiting both 5-HT transporters and DATs with GBR12909 (open circles) reduced 5-HT content more, indicating that a portion of striatal 5-HT content during chronic fluoxetine treatment was being taken up by DATs (gray area).

was smaller than that seen with 2 μM 5-HT (data not shown).

5-HT Corelease with DA Requires Dopamine Transporter Activity

The evidence to this point indicates that elevated extracellular 5-HT induces 5-HT accumulation and, subsequently, corelease of DA and 5-HT from DA terminals. DA terminals express DATs, but they do not express 5-HT transporters (Pickel and Chan, 1999). Therefore, we examined whether the DATs were required for this uptake of extracellular 5-HT. We inhibited DATs before elevating 5-HT in the bathing solution. Striatal slices were first treated for 1 hr with the DAT inhibitor GBR12909 (0.1 or 1 μM), which caused the expected prolongation of both spontaneous and evoked DA release (Figure 5A). Under those conditions, application of 5-HT (2 μM) did not enhance or prolong either the spontaneous or the electrically evoked signals. On the contrary, with the DATs inhibited, 5-HT decreased DA release by 22% ± 2% (n = 5) (Figure 5B), which was likely mediated by events initiated by 5-HT receptor activity (Barnes and Sharp, 1999). In addition, analysis of the voltammograms showed clearly that the evoked and spontaneous release contained only a DA component, and no 5-HT component was detected throughout the time course of the signal (inset of Figure 5B). These results further confirm that DAT-mediated uptake of 5-HT into DA terminals is required for the vesicular release of 5-HT with DA (see Figures 3 and 4).

In Vivo Chronic Antidepressant Treatment Induces DAT-Dependent 5-HT Accumulation in the Striatum

Antidepressants such as fluoxetine are selective serotonin reuptake inhibitors (SSRIs) that prolong and elevate the extracellular 5-HT signals within hours after drug administration (Knobelman et al., 2000; Smith et

al., 2000; Wong et al., 1995). Therefore, we examined whether this antidepressant-induced elevation of extracellular 5-HT leads to uptake of 5-HT by DATs. This question is much more difficult because of the low level of endogenous 5-HT in the striatum (see Figure 1D). Pilot experiments indicated that immunostaining was not sensitive enough to resolve this question convincingly, but HPLC has much greater sensitivity.

As a control experiment, we daily injected mice with GBR12909 (5 mg/kg) alone. The treatment did not affect striatal 5-HT content, indicating that GBR12909 was not directly blocking 5-HT transporters and DATs are not normally important for 5-HT uptake. Then, we injected mice with saline, fluoxetine, or fluoxetine and GBR12909 and assayed striatal 5-HT content using HPLC. Treatment with fluoxetine alone (10 mg/kg body weight) modestly and gradually reduced striatal 5-HT content (Figure 6A, filled circles). Taken together, the 5-HT contents on day 6 to day 18 were significantly lower after fluoxetine treatment as compared to treatment with saline or GBR12909 alone (n = 15–30 samples from three to six mice, p = 0.02). This small decrease in 5-HT content likely arose from 5-HT lost from the tissue because fluoxetine prevented proper 5-HT reuptake into serotonergic terminals (Torres et al., 2003).

To examine the role of the DATs during SSRI treatment, we daily injected another set of mice with the combination of fluoxetine (10 mg/kg) and GBR12909 (5 mg/kg), a specific DAT inhibitor (Pristupa et al., 1994). With both the 5-HT transporters and the DATs inhibited (Figure 6, open circles), the 5-HT content of striatal tissue fell by 17% on average after 10 days (n = 20 samples from five mice, p = 0.01). Taken together, the 5-HT content from day 6 to day 18 was significantly lower after combined treatment as compared to treatment with fluoxetine alone (n = 15–25 samples from three to five mice, p = 0.05). This result suggests that there was

greater loss of 5-HT content when DATs were inhibited, preventing 5-HT from being taken into the DA terminals. Consistent with that interpretation, the area shaded in gray (Figure 6) indicates the content of 5-HT that was taken up by DATs when the 5-HT transporters were inhibited. In agreement with the previous results, these in vivo data indicate that during fluoxetine treatment 5-HT is taken up into DA terminals.

Endogenous 5-HT Release from DA Terminals after Fluoxetine Treatment

The HPLC experiments (Figure 6) suggest that 5-HT is taken into DA terminals in vivo after fluoxetine treatment. That hypothesis implies that we could detect the corelease of 5-HT and DA with voltammetry after the in vivo treatment with fluoxetine. The problem is that voltammetry does not have the high sensitivity obtained with HPLC. Despite the shortcomings, we cut slices from mice chronically injected with fluoxetine and examined the voltammograms. Under certain experimental conditions (see Experimental Procedures), we could detect the corelease of 5-HT and DA. To quantify the changes in the 5-HT signal induced by the different in vivo treatments and to normalize the sensitivity of the carbon-fiber electrodes, we calculated the ratio of the reduction current at –30 mV (5-HT reduction peak) over the total oxidation current peak (R/O ratio, Figure 7A). After 21 days of fluoxetine treatment with the last injection coming 2 hr before cutting dorsal striatal slices, a small 5-HT component could be seen at later times in the evoked signal (n = 7). When L-tryptophan (a 5-HT precursor) was injected with fluoxetine to further increase extracellular 5-HT, the 5-HT component of the evoked release was increased further (n = 8, Figure 7B). Addition of GBR12909 to the treatment regimen reduced the 5-HT signal (n = 7, Figure 7B). Overall, the results suggest that 5-HT collects slowly in the DA terminals and that ongoing fluoxetine treatment is necessary to maintain a small 5-HT component in the DA-containing vesicles of the dorsal striatum.

In slices cut to contain the nucleus accumbens (NAc) shell, we did not see significant corelease of 5-HT with DA after fluoxetine injections. Therefore, to boost the level of endogenous 5-HT, one group of mice was injected with L-tryptophan and clorgyline (a monoamine oxidase inhibitor) together with fluoxetine (see Experimental Procedures). After at least 8 days of this treatment, a small 5-HT component could be detected (Figure 7C). DA was still the overwhelmingly dominant component in the electrically stimulated signal. Addition of GBR12909 to the treatment of L-tryptophan, clorgyline, and fluoxetine significantly reduced the 5-HT component in a second group of mice (Figure 7D). For comparison, a voltammogram is shown at t2 from a drug-free mouse (bottom, left, Figure 7D).

In both the dorsal striatum and the NAc shell, the 5-HT component was small and highly contaminated by the DA signal. Therefore, we did not attempt to estimate the concentration of the 5-HT signal beyond simple detection and identification. The shape of the voltammograms were appropriate and similar to the ones obtained after in vitro incubation of striatal slices with 10–20 nM exogenous 5-HT for 2 hr. For DA standards

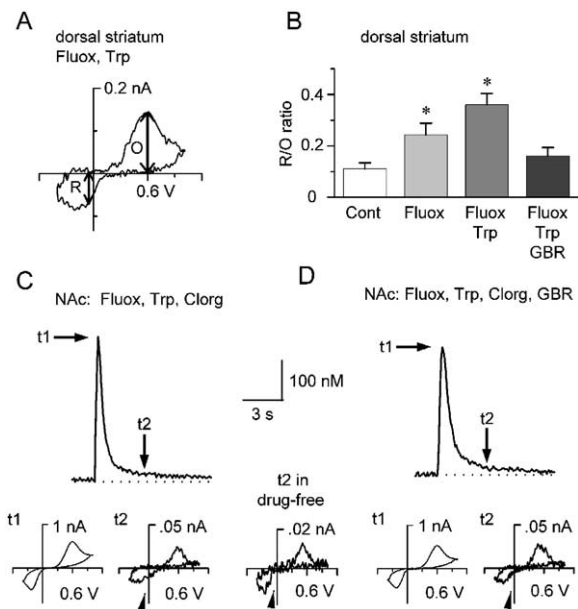
Figure 7. Vesicular Corelease of DA and 5-HT after Chronic In Vivo Treatments to Elevate Extracellular 5-HT

(A) A voltammogram at 3 s after the peak of a large signal obtained from a dorsal-striatum brain slice cut after injecting a mouse for 21 days with fluoxetine (Fluox) and L-tryptophan (Trp). The arrow (O label) near 550 mV represents the amplitude of the oxidation peak current, and the arrow (R label) near –30 mV represents the amplitude of the reduction current. The reduction current near –30 mV arises mainly from 5-HT.

(B) The ratio of the reduction current to the oxidation peak current (R/O ratio) in the dorsal striatum for mice injected with the following for ≥21 days: saline as a control (Cont), fluoxetine (Fluox), fluoxetine + L-tryptophan (Trp), or fluoxetine + L-tryptophan + GBR12909 (* indicates significantly different from the control with p < 0.05). Standard error bars are shown.

(C) An example of an electrically stimulated release measured in the NAc from a slice cut after treating a mouse for 14 days with fluoxetine, L-tryptophan, and clorgyline (Clorg). The signal is overwhelmingly caused by DA, as indicated by the DA voltammogram from the signal peak (t1). However, after the DA signal has substantially decayed, 4 s after the peak (t2), a small 5-HT component is clearly present, as revealed by the characteristic mixed DA/5-HT voltammogram with broad reduction peak. The arrowhead indicates –30 mV where the 5-HT reduction current was measured, but it is likely contaminated by DA.

(D) In another group of mice, the injections of fluoxetine, L-tryptophan, and clorgyline also contained GBR12909 to inhibit DATs. This treatment significantly reduced the 5-HT component compared to the non-GBR12909 treatment, as indicated by the smaller reduction current at –30 mV in the t2 voltammogram (arrow head). The evoked signal decays relatively slowly, probably due to residual GBR12909. In all of these cases, the small 5-HT component is contaminated by the larger DA component. The voltammogram on the left shows a pure DA voltammogram at t2 from a drug-free mouse where the reduction current at –30 mV (arrow) is virtually 0. Voltammograms are averages of 5 to 30 individual voltammograms to reduce noise. The voltammogram labeled as t2 in drug-free shows a common voltammogram seen in drug-free striatal slices.

and under drug-free conditions, the R/O ratio is always ≤0.15. The increased R/O ratios for the dorsal striatum experiments are given in Figure 7B. The R/O ratio in the NAc shell was 0.38 ± 0.04 (n = 12) during fluoxetine, tryptophan, and clorgyline treatment, and the ratio de-
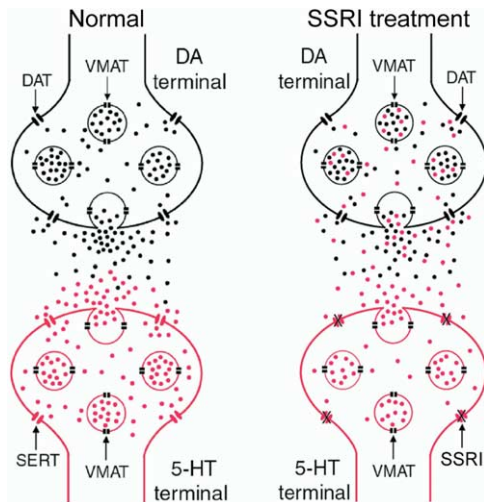
Figure 8. Schematic Representation of the Hypothesized DAT-Dependent 5-HT Accumulation in Striatal DA Terminals after Chronic SSRIs

(Left) Under normal conditions, when the 5-HT transporters (serotonin transporters, SERT) are functional, the released 5-HT is efficiently taken back into 5-HT terminals. Consequently, the extracellular 5-HT concentration remains low. DATs on DA terminals have little chance to uptake 5-HT. (Right) DATs may uptake a portion of endogenously released 5-HT into DA terminals when the extracellular 5-HT concentration is elevated after the SERTs are inhibited by SSRIs. Once inside the DA terminals, 5-HT is further sequestered into DA terminals through the vesicular monoamine transporter (VMAT), which has similar affinity for DA and 5-HT. Subsequently, 5-HT partially displaces DA from DA vesicles, and the two neurotransmitters are coreleased.

creased to 0.25 ± 0.03 (n = 11) when GBR12909 was also present (p < 0.01). GBR12909 was without significant effect when fluoxetine was left out of the treatment regimen. Overall, these results suggest the following: after blocking 5-HT transporters and elevating endogenous 5-HT, there is a small component of 5-HT corelease with DA only when DATs are active.

Discussion

In the striatum, the extremely dense DA fibers and DATs are located close to the sparser 5-HT terminals (Björklund and Lindvall, 1984; Coulter et al., 1996; Zhou et al., 2001). Because DATs have a low affinity for 5-HT (Giros et al., 1991; Pristupa et al., 1994; Shaskan and Snyder, 1970; Eshleman et al., 1999), they normally have little opportunity to bind 5-HT, which is efficiently recycled into serotonergic terminals by 5-HT transporters. When SSRIs block 5-HT transporters, extracellular 5-HT is elevated (Figure 8). It remains in the extracellular space longer and diffuses farther. Under those conditions, our results indicate that the striatal DATs are able to transport significant amounts of 5-HT into DA terminals. As suggested by our results with mice (Figure 6), this process likely proceeds slowly during SSRI treatment of patients, possibly contributing to the many days of SSRI treatment required before achieving anxiolytic benefits (Baldessarini, 2001; Nemeroff and Ow-

ens, 2002). Once inside the DA terminals, 5-HT is sequestered into DA vesicles through the vesicular monoamine transporter, which has similar affinities for DA and 5-HT (Yelin and Schuldiner, 2002). Our results are consistent with evidence suggesting that 5-HT may accumulate into DA cell bodies after genetically deleting an enzyme for 5-HT degradation or deleting the 5-HT transporters or after loading DA cells with 5-HT precursors (Cases et al., 1998; Jackson and Wightman, 1995; Zhou et al., 2002).

Despite the internal consistency of our results, other events could contribute to the effects we observed. During SSRI treatment, the richer innervation of the midbrain DA areas by serotonergic fibers could have altered the overall 5-HT and DA metabolism, and that could have contributed in a complex manner to the elevated 5-HT and decline in DA that we saw. Another potential problem is that systemic injection of fluoxetine may affect the activity of 5-HT neurons, which in turn may affect 5-HT synthesis and content. We indeed saw a small decline in striatal 5-HT content after fluoxetine treatment, which might have been caused by decreased 5-HT synthesis, but we have attributed that fall mainly to the decreased reuptake of 5-HT after 5-HT transporter inhibition.

When patients take fluoxetine (Prozac) and inhibit 5-HT reuptake into 5-HT terminals, our evidence suggests that there is a DAT-dependent uptake of 5-HT into DA terminals of the striatum. A DAT-dependent 5-HT signal was detected using HPLC (Figure 6), owing to its extremely high sensitivity. The vesicular release of this 5-HT could only be detected by voltammetry under specific experimental circumstances owing to the relatively low sensitivity of fast cyclic voltammetry, which can only detect signals that reach about 10–20 nM at the carbon fiber. Under particular conditions (Figure 7), the small 5-HT signal arising from vesicular release could be detected in the dorsal striatum after fluoxetine treatment. In the NAc shell, however, it was necessary to further boost the in vivo 5-HT concentration caused by fluoxetine by decreasing breakdown with a monoamine oxidase inhibitor (clorgyline) in combination with a 5-HT precursor (L-tryptophan). This difference between the dorsal striatum and NAc shell may have arisen from the greater density of DATs in the dorsal striatum, where 5-HT was more efficiently taken into DA terminals after fluoxetine treatment. These results suggest that the dense DA terminals and the highly expressed DATs may participate in 5-HT homeostasis and signaling in the striatum, a brain area important for reward and emotion as well as for motion control (Berke and Hyman, 2000; McClure et al., 2003; Schultz et al., 2003).

Functional Importance of DA and 5-HT Corelease
SSRIs, which block 5-HT reuptake, are among the most effective and prescribed antidepressant therapies, indicating the importance of serotonergic systems in the pathophysiology of depression (Baldessarini, 2001; Nemeroff and Owens, 2002; Delgado, 2000; Wong et al., 1995). Animal models and clinical findings also have implicated the mesoforebrain DA system in depression (D'Aquila et al. 2000; Zangen et al. 2001; Willner 2002).

For example, the striatal DA concentration and the density of striatal DATs were found to be abnormal in patients with major depressive disorder (Laasonen-Balk et al., 1999; Meyer et al. 2001). Our results suggest that DATs may influence 5-HT signaling, providing a basis for SSRI-induced interaction between these neurotransmitter systems. Our conclusion is also consistent with a study employing microdialysis that showed GBR12909 increased extracellular 5-HT in the striatum of 5-HT transporter knockout mice. The increased extracellular 5-HT may have arisen from GBR12909 inhibition of DAT-mediated 5-HT uptake (Shen et al., 2004). Our results further indicate that there is DA and 5-HT cotransmission in cases where extracellular 5-HT is sufficiently elevated.

Ectopic 5-HT plays vital roles in neuronal development (Gaspar et al. 2003). Blockade of 5-HT transporters with fluoxetine during early postnatal life induces depressive symptoms in adulthood (Ansorge et al. 2004). It is possible that during this chronic fluoxetine treatment a certain amount of 5-HT goes into DA terminals (particularly in the striatum) and contributes to the 5-HT signaling that is responsible for the behavioral abnormalities. In mature animals, we hypothesize that the ectopic storage of 5-HT into DA terminals and the consequent corelease of DA and 5-HT produce a number of consequences. The competition between DA and 5-HT for the DAT, for the vesicular monoamine transporter, and for the intravesicular space slightly reduces DA signaling, as indicated in Figure 1D. This 5-HT/DA competition at DA terminals also may occur during the potentially life-threatening serotonin syndrome. In this syndrome, elevated 5-HT activity is caused by dietary 5-HT precursor overloading or by the combined use of SSRIs and monoamine oxidase inhibitors (Sternbach, 1991). These abnormal situations would favor DAT uptake of 5-HT. DAT uptake of 5-HT and consequent corelease alters the timing, location, intensity, and interaction of 5-HT and DA signaling.

The striatum expresses a plethora of 5-HT receptors coupled to diverse signaling pathways (Barnes and Sharp, 1999; McMahon et al., 2001). The striatum also expresses multiple types of DA receptors that are crucial for the activity of the basal ganglia (Missale et al., 1998; Greengard, 2001). The relationship between DA and 5-HT signaling is likely vital for normal behavior and for the pathology that can be treated with SSRIs. The striatum, the ventral striatum in particular, is critically involved in the neuronal processes of reward and emotional functions (Berke and Hyman, 2000; McClure et al., 2003; Schultz et al., 2003). Thus, the enhanced participation of the striatal DA system in 5-HT signaling during chronic SSRI treatment may contribute to the therapeutic efficacy of SSRIs.

Our results do not connect the change in the relationship between DA and 5-HT signaling to a behavioral output. Recent evidence indicates that antidepressant treatment induces neurogenesis that is prerequisite for anxiolytic action (Santarelli et al., 2003). We can conclude, however, that the extremely dense DATs in the striatum are able to transport 5-HT into DA terminals when extracellular 5-HT is elevated. Normally, this process may be of little significance because of its low efficiency and the low extracellular 5-HT concentration. However, it may play a functional role when 5-HT is elevated for prolonged times during treatment with SSRI-type antidepressants.

Experimental Procedures

Mice, Striatal Brain Slices, and Voltammetry

Wild-type C57BL/6J mice were used at 3–6 months of age. Under deep anesthesia, mice were decapitated, and the brains were rapidly removed following the methods of Zhou et al. (2001). Horizontal 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$_2$, 1.25 mM Na$_2$HPO$_3$, 25 mM NaHCO$_3$, and 10 mM glucose saturated with 95% O$_2$ and 5% CO$_2$.

Homemade carbon-fiber microelectrodes were used for fast-scan cyclic voltammetry. The microelectrodes were calibrated after the experiment using fresh standards: 0.5–5 μM DA, 0.2–2 μM 5-HT, and mixtures of 0.5–2 μM DA and 0.01–1 μM 5-HT (Zhou et al., 2001). An Axopatch 200B amplifier and pClamp software (Axon Instruments) were used to acquire and analyze the data. Scans (10 or 20 Hz) of the microelectrode potential were from 0 mV to −400 to 1000 to −400 to 0 at a rate of 300 mV/ms and were sampled at 50 kHz. The net current due to the electrochemical reaction was obtained by digital subtraction of the ohmic background currents. Intrastriatal stimuli (1–4 V in amplitude and 1 ms in duration) were delivered using 0.5 MΩ bipolar tungsten electrodes. Recording and stimulation were made in the dorsolateral striatum and the core and shell of the nucleus accumbens. Because the dorsolateral striatum has denser DA terminals and DATs than the nucleus accumbens (Coulter et al., 1996; Descarries and Mechawar, 2000), its DA signal is generally larger in amplitude and faster in decay. Despite these differences in the signal, the overall conclusions were generally the same in these two regions of the striatum.

Immunostaining

Immunohistochemistry was performed according to published methods (Pickel and Chan, 1999; Zhou et al., 2001). Tyrosine hydroxylase (TH) and 5-HT staining were conducted with control slices, slices incubated in 2 μM 5-HT for 2 hr, or slices pretreated with 1 μM GBR12909 for 1 hr followed by 2 hr incubation with 2 μM 5-HT and 1 μM GBR12909. Fluoxetine and nisoxetine (each at 1 μM) were present under all conditions to block 5-HT and norepinephrine transporters, respectively. Afterward, slices were thoroughly washed. The slices were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 6 hr at 4°C and then rinsed thoroughly in PBS. Sections of 25 μm were cut on a cryostat and incubated overnight with goat anti-5-HT antibody and rabbit anti-TH antibody. They were then reacted with a mixture of Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Cy3-conjugated donkey anti-goat IgG antisera. Microscopic observations and digital microphotography were made on a Bio-Rad MRC1024 confocal microscope using LaserSharp 2000 software. Digital images were further processed using NIH ImageJ and Adobe Photoshop.

In Vivo Drug Administration to Mice

Drugs were dissolved in 0.2 μm filtered saline and administered to male mice (3–6 months of age) intraperitoneally once a day (at 10 AM) for up to 18 days for the data displayed in Figure 6 (Bymaster et al., 2002). The mice were divided into four groups, each receiving injections of saline or fluoxetine (10 mg/kg body weight) or GBR12909 (5 mg/kg body weight) or a mix of fluoxetine (10 mg/kg) and GBR12909 (5 mg/kg). To obtain striatal tissue samples, the mice were sacrificed 4 hr after the last drug injection. For each of the four groups, three to six mice were sacrificed on each chosen day of the time course, and four striatal samples were taken from each mouse for HPLC analysis.

For voltammetry detection of 5-HT in the dorsal striatum (Figures 7A and 7B), mice were divided into five groups, each receiving one injection a day: saline, fluoxetine (10 mg/kg body weight), fluoxetine (10 mg/kg) + L-tryptophan (50 mg/kg), GBR12909 (5 mg/kg), or fluoxetine (10 mg/kg) + L-tryptophan (50 mg/kg) + GBR12909 (5 mg/kg). At least three mice from each group were sacrificed, and striatal brain slices were cut on the 7th, 14th, or 21st day of treat-

ment. To significantly detect the 5-HT component, the injections had to continue for many days, with the last injection coming only 2 hr before the slices were cut. The 5-HT component was largest within the first hour after cutting the slice. We reasoned that the released synaptic vesicles initially contain DA and 5-HT, but as time passed vesicles that were released were refilled only with DA because the SSRI was no longer present and the 5-HT was no longer elevated in those slices.

A slightly different protocol was used to increase the extracellular 5-HT concentration for voltammetric detection in the NAc shell (Figures 7C and 7D). One group of mice was treated twice a day with L-tryptophan (100 mg/kg), clorgyline (1 mg/kg), and fluoxetine (7.5 mg/kg). A second group of mice was treated twice a day with GBR12909 (4 mg/kg) as well as L-tryptophan, clorgyline, and fluoxetine. A third group of mice was treated with L-tryptophan, clorgyline, and GBR12909 but no fluoxetine. A saline group was used as the control. Two mice from each group were sacrificed, and striatal brain slices were cut on the 4th, 8th, 12th, and 16th day of treatment. A small 5-HT component was only detected beyond 8 days of injections.

HPLC Analysis for DA and 5-HT

DA and 5-HT content in dorsolateral striatal brain samples was determined by challenging with 200 mM KCl followed by several rounds of physical disruption by trituration. The disrupted slices were then freeze-thawed, sonicated, and filtered at 0.2 μm. DA and 5-HT contents were analyzed using an HPLC-EC system (ESA, Chelmsford, MA) (Yuan et al., 2002; Pidoplichko et al., 2004). On the HPLC chromatograms, DA and 5-HT were separate peaks that were quantified by comparing their heights to those of fresh DA and 5-HT standard curves using linear regression analysis. DOPAC arising from the metabolic breakdown of DA was also detected by HPLC and compared to DOPAC standards.

Statistics

Descriptive statistics were performed using OriginLab (OriginLab Corporation, Northampton, MA) or StatMost (Dataxiom Software Inc., Los Angeles, CA). Comparisons of the distributions of DA and 5-HT contents from individual samples were made using Kolmogorov-Smirnov (K-S) test with StatMost, and p values ≤ 0.05 were considered statistically significant.

Supplemental Data

The authors' conflict of interest statement can be found online at http://www.neuron.org/cgi/content/full/46/1/65/DC1/.

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