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## Synapsins Differentially Control Dopamine and Serotonin Release

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### Abstract

Synapsins are a family of synaptic vesicle proteins that are important for neurotransmitter release. Here we have used triple knockout (TKO) mice lacking all three synapsin genes to determine the roles of synapsins in the release of two monoamine neurotransmitters, dopamine and serotonin. Serotonin release evoked by electrical stimulation was identical in substantia nigra pars reticulata slices prepared from TKO and wild-type mice. In contrast, release of dopamine in response to electrical stimulation was approximately doubled in striatum of TKO mice, both *in vivo* and in striatal slices, in comparison to wild-type controls. This was due to loss of synapsin III, because deletion of synapsin III alone was sufficient to increase dopamine release. Deletion of synapsins also increased the sensitivity of dopamine release to extracellular calcium ions. Although cocaine did not affect the release of serotonin from nigral tissue, this drug did enhance dopamine release. Cocaine-induced facilitation of dopamine release was a function of external calcium, an effect that was reduced in TKO mice. We conclude that synapsins play different roles in the control of release of dopamine and serotonin, with release of dopamine being negatively regulated by synapsins, specifically synapsin III, while serotonin release appears to be relatively independent of synapsins. These results provide further support for the concept that synapsin function in presynaptic terminals varies according to the neurotransmitter being released.

### Keywords

cyclic voltammetry; carbon-fiber microelectrode; cocaine; striatum; synapsin TKO; calcium

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Synapsins are phosphoproteins that bind to the cytosolic surface of synaptic vesicles and represent almost 10% of the total protein content of synaptic vesicles. There are three mammalian synapsin genes (synapsin I, II, and III), with alternative splicing creating 10 known synapsin isoforms. Synapsins are differentially expressed in nerve terminals, with synapsins I and II present in most areas of the adult brain while synapsin III expression is more restricted (Pieribone et al., 2002). Synapsins are thought to play a role in segregating vesicles into a reserve pool that is located away from the active zone, where exocytosis

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occurs (Greengard et al., 1993). This hypothesis is supported by observations showing that perturbing synapsins by microinjection of anti-synapsin antibodies (Pieribone et al., 1995) or synapsin peptides (Hilfiker et al., 2005) disrupts a distal, reserve pool of vesicles, sparing vesicles in the active zone. It is thought that mobilization of vesicles from the reserve pool is triggered by calcium-dependent phosphorylation of synapsins (Llinas et al., 1991; Greengard et al., 1993; Chi et al., 2003). More recent evidence suggests that synapsins are also important regulators of exocytosis, vesicle recycling, and vesicle integrity at the active zone (Fdez and Hilfiker, 2006; Evergren et al., 2007).

Synapsin knockout (KO) mice have provided further insights into the role of synapsins in the regulation of neurotransmitter release. While deletion of individual synapsin genes (Ferreira et al., 2000; Feng et al., 2002) or genes for both synapsins I and II (Rosahl et al., 1995; Lonart and Simsek-Duran, 2006) affect synaptic vesicle pool dynamics, interpretation of results from such genotypes are hindered by possible compensation caused by remaining synapsin isoforms and differential expression of synapsins across brain regions. These problems have been solved by generating triple knockout mice (TKO) that lack all three known synapsin genes. TKO mice exhibit reduced reserve pools in both glutamatergic and GABAergic nerve terminals, evident as changes in vesicle distribution and in neurotransmitter release during trains of action potentials (Gitler et al., 2004). However, when using single action potentials as stimuli to monitor release from the readily releasable pool of synaptic vesicles, release of GABA from this pool is reduced, while release of glutamate is not. This suggests that the function of synapsins at presynaptic terminals varies according to the type of neurotransmitter employed.

Here we have extended the analysis to the release of dopamine (DA) and serotonin (5-HT), comparing release of these two monoamine neurotransmitters in synapsin TKO mice both in brain slices and *in vivo*. DA release was enhanced in the absence of synapsins, while 5-HT release from brain slices was identical in the two genotypes. The altered DA release in TKO mice appeared to be due to deletion of synapsin III since release was similarly altered in mice with a synapsin III deletion. The dependence of DA release on extracellular calcium was found to differ between TKO and WT mice, with TKO mice being more sensitive to calcium. The ability of cocaine to increase release of DA was reduced in synapsin TKO mice, supporting the assertion that synapsins contribute to the presynaptic actions of cocaine (Venton et al., 2006). In contrast, cocaine did not affect release of 5-HT in either genotype. These findings demonstrate that synapsins play different regulatory roles in nerve terminals releasing DA and 5-HT.

## Materials and Methods

### Animals

Homozygous mutant synapsin triple knockout (TKO), synapsin III knockout (S3KO) and wild-type (WT) male and female mice were bred at Duke University as previously described (Gitler et al., 2004). Mice were age- and sex-matched and experiments were conducted with protocols approved by the Institutional Animal Care and Use Committees (IACUC) of both Duke University and the University of North Carolina at Chapel Hill.

### Electrochemistry

Cylindrical carbon-fiber microelectrodes (6  $\mu\text{m}$  diameter, Thornel T-650; Amoco, Greenville, SC) were fabricated as previously described (Bath et al., 2000). A carbon fiber was aspirated into glass capillary (1.2 mm  $\times$  0.68 mm, 4"; AM Systems, Carlsborg, WA.). The electrodes were pulled on a vertical capillary puller (Narishige) and the excess carbon fiber was trimmed to 50  $\mu\text{m}$ . Waveform application, current monitoring, and stimulus

application were all controlled by locally written software (Tarheel CV, Labview) through a home-built potentiostat (UEI, UNC electronics shop).

For DA detection, the carbon-fiber electrode was held at  $-0.40$  V versus an Ag/AgCl reference electrode between voltage scans. Detection in brain slices was achieved by application of a triangular waveform:  $-0.40$  V to  $1.00$  V to  $-0.40$  V with a scan rate of  $600$  V/s and repeated at  $60$  Hz. This waveform provided high temporal resolution (Bath et al., 2000). The waveform for *in vivo* use employed the same voltages, but was at  $300$  V/s and was repeated at  $10$  Hz; this method provided improved sensitivity (Baur et al., 1988). The current at the peak oxidation potential for DA ( $0.50$ – $0.70$  V vs. Ag/AgCl) was used to evaluate DA concentration changes.

For 5-HT detection the electrode potential between scans was  $0.20$  V vs Ag/AgCl. The cyclic voltammogram was obtained with an initial scan to  $1.00$  V, back to  $-0.10$  V, and then scanned to  $0.20$  V at  $1000$  V/s applied at  $10$  Hz. The positive rest potential minimizes electrode fouling from 5-HT reduction products (Jackson et al., 1995). In addition, the rapid scan rate enables side-reactions characteristic of electrochemical detection of 5-HT to be outrun. For both DA and 5-HT, the electrodes were calibrated in a flow injection system with a  $1$   $\mu$ M bolus of the analyte of interest. Calcium ions inversely reduce the sensitivity of electrodes in a linear manner. These changes in electrode sensitivity were accounted for in calibration (Kume-Kick and Rice, 1998).

### Slice Experiments

Mice were anesthetized with ether and decapitated. The brain was rapidly removed and placed on ice. Coronal brain slices ( $300$   $\mu$ m) containing the caudate putamen or substantia nigra, pars reticulata (SNr), were prepared in ice cold artificial cerebral spinal fluid (aCSF) using a Lancer Vibratome (World Precision Instruments, Sarasota, FL). The aCSF contained (in mM):  $20$  HEPES,  $2.4$   $\text{CaCl}_2$ ,  $1.2$   $\text{MgCl}_2$ ,  $1.2$   $\text{NaH}_2\text{PO}_4$ ,  $2.45$  KCl,  $126$  NaCl,  $11$  glucose, and  $25$   $\text{NaHCO}_3$ . All reagents were obtained from Sigma Aldrich. Cocaine, picrotoxin, CNQX, and mecamylamine were dissolved in doubly-deionized water at a concentration of  $2$  mM. Final dilutions were made in aCSF and applied to the slice for  $35$  min. prior to measurements. The pH was adjusted to  $7.4$  and the aCSF was saturated with  $95\%$   $\text{O}_2/5\%$   $\text{CO}_2$ . Slices were superfused in aCSF at  $37$   $^\circ\text{C}$  for  $35$ – $40$  min. prior to recording. Recordings were performed on a microscope (Nikon Fn-1, Gibraltar Stage) fitted with a slice perfusion chamber (Warner Instruments, Hamden CT).

Neurotransmitter release was evoked by local electrical stimulation delivered with a tungsten bipolar stimulating electrode placed on the surface of the slice (Frederick Haer Co., Bowdoinham, ME). The stimulus consisted of a computer generated, biphasic ( $2$  ms per phase), constant current ( $350$   $\mu\text{A}$ ) pulse. A single stimulation pulse was used to evoke striatal DA release in the caudate putamen while a  $20$  pulse,  $100$  Hz stimulation train was used to evoke 5-HT release in the SNr. The current pulse was optically isolated from the preparation (NL 800; Neurolog, Medical Systems, Great Neck, NY). Stimuli were given at regular intervals. Neurotransmitter release was detected with a carbon-fiber microelectrode placed  $75$ – $100$   $\mu$ m into the slice at a distance of  $100$ – $200$   $\mu$ m from the stimulating electrode. Electrode placements were made with micromanipulators and the aid of the microscope.

To determine the amount of DA release from each genotype, the maximal release at  $4$  locations in a single slice was recorded over a  $20$  minute period, and the values were averaged together. The responses from each genotype were pooled. To examine the effects of cocaine and  $\text{Ca}^{2+}$ , measurements were made at a single site that exhibited maximal DA release. The amount of 5-HT released was determined in a similar fashion to DA with the exception that three locations were used and data were pooled.

## **In Vivo Experiments**

Mice were anesthetized with urethane (1.5 g/kg i.p.) and placed in a stereotaxic frame. The coordinates relative to bregma for placement of the working electrode in the caudate-putamen were (in mm): AP +1.1, ML +1.2 and DV -2.2 (Paxinos et al., 1985). The stimulating electrode was placed in the medial forebrain bundle at AP -2.4, ML +1.1, DV -4.5. The dorsal-ventral placement of both the working and stimulating electrodes was adjusted in small increments to find maximal DA release. An Ag/AgCl reference electrode was inserted into the contralateral side of the brain.

## **High Pressure Liquid Chromatography**

Tissue samples were punched from slices (500  $\mu$ m thickness) with a 2.5-mm diameter cork borer. The tissue was blotted dry, weighed, and homogenized with a wand sonicator (Fisher Scientific) in 200  $\mu$ L 0.1N perchloric acid spiked with 1  $\mu$ M hydroquinone. Following centrifugation at 6,000 rpm for 10 min, the supernatant was removed and filtered with a 0.2  $\mu$ m syringe filter unit (Millex-LG). Injections (10  $\mu$ L) were made onto a reverse-phase column (C-18, 5 $\mu$ m, 4.6  $\times$  250 mm, Waters symmetry 300). The mobile phase (prepared in HPLC grade water) contained 0.1 M citric acid, 0.1 mM EDTA, and 1 mM hexyl sodium sulfate, pH 3.5. Catecholamines were detected with a thin-layer radial electrochemical flowcell (BASi, West Lafayette, IN USA), with the working electrode at 700 mV vs. the Ag/AgCl reference electrode. Catecholamine standards were prepared from 10 mM stock solutions in 0.1N perchloric acid.

## **Kinetic Modeling**

Nonlinear regression was used to extract kinetic constants associated with release and uptake of DA from the extracellular space (Wu et al., 2001). Concentration traces were fit using a Michaelis-Menten based model (Wightman et al., 1988). The nonlinear regression procedure used in conjunction with deconvolution of the measured responses from the electrode time constant (15–50 ms for a 60 Hz sampling rate) improves accuracy for release and uptake measurements (Garris and Wightman, 1995; Venton et al., 2002). The output of the regression is the amount of DA released for each stimulus pulse ( $[DA]_p$ ) during a train of stimuli and the  $V_{max}$  for uptake. The  $K_m$  value was fixed at 200 nM for DA (Near et al., 1988) and 170 nM for 5-HT (John et al., 2006). When competitive uptake inhibitors such as cocaine were used,  $V_{max}$  was fixed at predrug values to determine the apparent  $K_m$ .

## **Data Analysis**

Data are shown as mean  $\pm$  SEM. The n values are the number of animals used for a specific experiment. In addition to this replicate number, multiple locations and stimuli were used to reduce random error in the electrochemical measurements. Tests of significance included Student's t-test, one-way ANOVA with Student Newman-Keuls post-hoc, two-way repeated measures ANOVA with Bonferroni comparisons post-hoc, and three-way repeated measures with Bonferroni comparisons post-hoc where applicable.

## **Results**

### **Serotonin Release in Substantia Nigra Brain Slices**

The influence of synapsins on electrically evoked release of 5-HT (Bunin et al., 1998) was evaluated by comparing 5-HT release in slices prepared from the brains of TKO and WT mice. In these experiments, we used local electrical stimulation with a stimulus train consisting of 20 biphasic pulses (2 ms each phase) of 350  $\mu$ A at 100 Hz. 5-HT release was monitored by cyclic voltammetry and the voltammogram at the peak (Figure 1, inset) was used to positively identify the released substance. 5-HT release evoked under such

conditions was consistent over several hours if the stimulus trains were separated by intervals of 5 min or longer (Bunin et al., 1998). To minimize the contribution of spatial heterogeneity of 5-HT release sites, measurements were made at 3 different positions of the carbon-fiber electrode within each SNr slice and then averaged together.

In slices from both genotypes, 5-HT release began promptly at the onset of the stimulus and ended as soon as the stimulus stopped, with the concentration of 5-HT returning to prestimulus values over the next 1–2 sec (Figure 1). Measurements of the peak concentration of released 5-HT ( $[5\text{-HT}]_{\text{max}}$ ) from 10 animals of each genotype showed that the mean  $[5\text{-HT}]_{\text{max}}$  was  $130 \pm 8$  nM in WT mice and  $140 \pm 7$  nM in TKO mice; these values are not significantly different from each other ( $p > 0.05$ ). The maximal rate of 5-HT uptake ( $V_{\text{max}}$ ) was evaluated in WT and TKO slices by using nonlinear regression to model the decay of the traces (see Methods section). The  $V_{\text{max}}$  of 5-HT uptake for WT and TKO animals was  $450 \pm 70$  nM/sec and  $370 \pm 80$  nM/sec, respectively. These values are not significantly different from each other ( $p > 0.05$ ) and are similar to those reported previously for the SNr (John et al., 2006).

Tissue 5-HT content in the SNr was measured by high-performance liquid chromatography. Tissue punches taken from TKO and WT mice (4 animals/genotype) revealed that tissue levels of 5-HT were not different between genotypes (Table 1).

### Synapsins Affect Dopamine Release in Striatal Brain Slices

Local electrical stimulation was used to evoke DA release from terminals in the caudate-putamen of coronal brain slices from synapsin TKO and WT animals. In this case, a single biphasic current pulse (2 ms each phase) of 350  $\mu\text{A}$  was used as a stimulus, with cyclic voltammetry used to positively identify the signal as DA (Figure 2A inset). The peak oxidation potential of DA occurs at  $\sim 0.6$  V versus Ag/AgCl, similar to that shown for the peak oxidation of 5-HT. However, the cyclic voltammograms for the two substances differ on the reverse scan, with the peak for reduction of the dopamine-o-quinone occurring at  $\sim -0.3$  V with the waveform employed, whereas the reduction peak following 5-HT oxidation occurs at more positive potentials (Jackson et al., 1995).

DA release was evoked every 5 min at four different sites per slice, using the single biphasic pulse described above, and the values of maximal DA release ( $[\text{DA}]_{\text{max}}$ ) determined at each site were pooled for each genotype. In every experiment, DA release was observed at the onset of stimulation and then declined back to prestimulus values within 1 sec after cessation of the stimulation (Figure 2A). The  $[\text{DA}]_{\text{max}}$  was significantly greater ( $p < 0.01$ ) in brain slices from TKO mice than in slices prepared from WT mice, being  $1.31 \pm 0.09$   $\mu\text{M}$  ( $n = 6$ ) for WT and  $3.31 \pm 0.40$   $\mu\text{M}$  ( $n = 8$ ) for TKO slices (Figure 2B). The  $V_{\text{max}}$  for DA uptake was evaluated with the same procedure described above for 5-HT uptake. The analysis revealed that the  $V_{\text{max}}$  values for DA uptake were not significantly different between genotypes, at  $6.00 \pm 0.40$   $\mu\text{M}/\text{sec}$  for WT and  $6.20 \pm 0.50$   $\mu\text{M}/\text{sec}$  for TKO samples. These values are slightly higher than those reported previously in WT mice (Johnson et al., 2006).

Because synapsins I and II are lacking in DA terminals (Bogen et al., 2006), it is likely that synapsin III is the isoform that controls DA release. To test this prediction, DA release was compared in caudate-putamen slices of synapsin III KO (S3KO) and WT animals. Release of DA in S3KO brain slices was significantly higher ( $p < 0.05$ ) than in WT slices (Figure 2C), with  $[\text{DA}]_{\text{max}}$  being  $3.06 \pm 0.55$   $\mu\text{M}$  ( $n = 5$ ) in S3KO and  $1.22 \pm 0.13$   $\mu\text{M}$  in WT slices ( $n=5$ ) (Figure 2D). DA uptake values were not different between genotypes,  $5.97 \pm 0.58$   $\mu\text{M}/\text{sec}$  for S3KO ( $n=5$ ) and  $6.16 \pm 0.38$   $\mu\text{M}/\text{sec}$  for WT slices ( $n=5$ ). A comparison of the mean  $[\text{DA}]_{\text{max}}$  in TKO and S3KO groups showed that these values are not statistically different.



The similar amounts of DA release in TKO and S3KO slices indicate that a synapsin III gene product is responsible for regulating DA release.

To determine whether the previously reported changes in release of glutamate or GABA (Gitler et al., 2004) underlie the changes in DA release observed in TKO animals, we repeated our measurements in the presence of CNQX, to block AMPA receptors, or picrotoxin to block GABA<sub>A</sub> receptors. CNQX increased DA release while picrotoxin depressed it; however, the difference in [DA]<sub>max</sub> between genotypes remained unchanged (Supplementary Table S1). Thus, the difference between TKO and WT mice does not arise from differential regulation of DA release by glutamate or GABA. Because spontaneous release of acetylcholine also regulates DA release (Zhou et al., 2001), we further measured DA release in the presence of mecamylamine, an antagonist of nicotinic acetylcholine receptors. Consistent with previous reports, we found that mecamylamine reduced DA release. However, the degree of reduction in DA release was not different between genotypes (Supplementary Table S1). Thus, the increase in DA release in synapsin TKO and S3KO mice also is not a result of altered cholinergic signaling at DA terminals.

Dopaminergic neurons are known to release only a small fraction of their total stores to individual stimulus pulses (Gubernator et al., 2009). To determine whether the increased release of DA observed in TKO mice was due to the striatum containing greater amounts of DA, we used high-performance liquid chromatography to analyze the DA content of striatal tissue. No significant differences in the amounts of tissue DA in the striatum were found between the two genotypes (Table 1), indicating that the increased amount of DA release found in TKO mice was not due to larger DA stores, but instead is due to electrical stimulation releasing a larger fraction of these stores.

Given that synaptic depression is altered in synapsin TKO mice (Gitler et al., 2004), we asked whether loss of synapsins affects DA release during repetitive stimulation. With either 0.3 sec or 3 min intervals between stimuli, the amount of DA released per stimulus was depressed during repetitive stimulation and no differences in this activity-dependent decrement in DA release were observed between slices from WT and TKO mice (Figure 3). At stimulus intervals of 5 min, the amount of DA released per stimulus was constant in WT slices but there was a small reduction in DA release that was not significant (main effect of genotype,  $F=5.12$ ,  $p = 0.054$ ) in TKO slices after 6 or more stimuli. This might reflect the smaller size of the reserve pool of DA in TKO neurons (Venton et al., 2006).

### Synapsins Also Affect Striatal Dopamine Release In Vivo

We next assessed whether synapsins influence the amount of DA released *in vivo* by measuring DA release in the brains of anesthetized TKO and WT mice. In these experiments, DA release in the caudate-putamen was evoked by electrical stimulation of the medial forebrain bundle, rather than by the local electrical stimulation that was used in our brain slice experiments. As reported previously, the smaller amounts of DA released per stimulus *in vivo* made it necessary to deliver repeated stimuli to elicit detectable amounts of DA release (Venton et al., 2006). Consistent with the results of our slice experiments, 5 min inter-stimulus intervals were required to observe consistent amounts of DA release in response to each stimulus. Figure 4A shows averaged measurements of DA release measured in TKO and WT mice in response to a brief train of 20 Hz stimuli (0.5 sec duration, 300  $\mu$ A;  $n = 8$  for each genotype). At this frequency, trains of activity in WT animals led to a concentration change that approached steady-state during the stimulus, a response that has previously been documented in rats *in vivo* (Wightman et al., 1988).

The larger amounts of DA released from TKO terminals led to the different shapes of the DA concentration profiles seen in Figure 4A, where the DA concentration measured during

stimulation more closely approached a steady state during the stimulation in WT than in TKO animals. Transmission at dopaminergic synapses is a balance between DA release and uptake: because of this balance, DA concentration in WT animals reaches a steady plateau during the stimulus train. In contrast, the DA concentration continually increases when larger amounts of DA are released, because DA transport becomes saturated and thereby permits continued accumulation of extracellular DA (Wightman et al., 1988). Such behavior for DA release in the TKO mice presumably is due to the larger amounts of DA release in these mice.

Given that repetitive stimulation was required to elicit measurable DA release, we next evaluated how loss of synapsins affected the frequency dependence of DA release. At all stimulus frequencies tested, DA release was greater in TKO animals than in WT mice (Figure 4B; main effect of genotype,  $F = 6.86$ ,  $p < 0.05$ ). At a stimulus intensity of  $350 \mu\text{A}$ , the data could be described by a model that assumed that the amount of DA released per stimulus pulse ( $[\text{DA}]_p$ ) *in vivo* was constant.  $[\text{DA}]_p$  was found to be  $90 \pm 14 \text{ nM}$  for WT and  $150 \pm 18 \text{ nM}$  for TKO mice (Figure 4C). This difference between the two genotypes is significant ( $p < 0.05$ ). Although DA release from TKO animals approached a plateau at the same stimulus intensity ( $350 \mu\text{A}$ ) as in WT animals, the amount of DA released during 60 Hz stimulation was higher at all stimulus intensities tested (Figure 4D; main effect of genotype,  $F = 8.69$ ,  $p < 0.05$ ). Saturation of the relationship between DA release and stimulus intensity is thought to arise from saturating stimuli maximally depolarizing the membrane potential and maximally releasing DA from a restricted pool available for immediate release in response to such depolarization (Wiedemann et al., 1990). Given that the ability of the stimuli to depolarize should be similar between the two genotypes, the greater amount of DA released at saturation suggests a larger pool of readily releasable DA in TKO mice.

### Calcium Dependence of Dopamine Release

Given the essential role of calcium ions ( $\text{Ca}^{2+}$ ) in triggering neurotransmitter release (Katz, 1969; Augustine et al., 1987; Neher, 1998), we next determined whether loss of synapsins affected the calcium dependence of DA release. For this purpose, caudate-putamen slices first were equilibrated in conventional aCSF containing  $2.4 \text{ mM Ca}^{2+}$  and DA release produced by electrical stimuli (single biphasic pulse, 2 ms each phase,  $350 \mu\text{A}$ ) was measured at a single location. This location was maintained throughout the experiment while the  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]$ ) was varied. Treatment with aCSF lacking  $\text{Ca}^{2+}$  was then used to remove  $\text{Ca}^{2+}$  from the slice and the extracellular  $[\text{Ca}^{2+}]$  was subsequently increased incrementally from  $0.5 \text{ mM}$  to  $5 \text{ mM}$ . At each  $[\text{Ca}^{2+}]$ , slices were allowed to equilibrate for 35 min and then were stimulated to evoke DA release. To compare the absolute amount of DA released at each  $[\text{Ca}^{2+}]$  between genotypes, DA release at each  $[\text{Ca}^{2+}]$  was divided by the DA release at  $2.4 \text{ mM Ca}^{2+}$  in WT slices and the resulting ratio was termed ‘relative dopamine release’.

We found that the  $\text{Ca}^{2+}$  dependence of DA release differed between slices from TKO and WT animals. At all  $\text{Ca}^{2+}$  concentrations examined, DA release was significantly higher in slices from TKO mice than in WT slices ( $p < 0.001$ ,  $n = 5$  for each genotype; Figure 5A). To quantify the relationship between external  $[\text{Ca}^{2+}]$  and DA release, the data shown in Figure 5A were re-plotted in logarithmic coordinates (Dodge and Rahamimoff, 1967; Augustine and Charlton, 1986). In this case, the sigmoidal relationship between  $[\text{Ca}^{2+}]$  and DA release is linearized, with the slope of the line representing the amount of “cooperativity” in  $\text{Ca}^{2+}$  triggering DA release and the  $[\text{Ca}^{2+}]$  required for half-maximal DA release reflecting the apparent  $\text{Ca}^{2+}$  affinity,  $K_{\text{Ca}^{2+}}$  (Dodge and Rahamimoff, 1967). To determine these two parameters, we fit the data with the Hill equation. Although the shapes of these logarithmic plots were very similar for DA release from both genotypes, we found that the TKO data

were shifted toward lower  $[Ca^{2+}]$  (Figure 5B). This leftward shift was due to a significant difference in the apparent  $K_{Ca^{2+}}$  between the two genotypes:  $2.80 \pm 0.27$  mM for WT and  $1.34 \pm 0.04$  mM for TKO animals ( $p < 0.001$ ). The Hill coefficient was similar for both genotypes at  $2.11 \pm 0.20$  for WT animals and  $2.73 \pm 0.22$  for TKO animals ( $p > 0.05$ ). These values for the Hill coefficient are similar to those previously reported for depolarization-evoked DA release from synaptosomes (Nachshen and Sanchez-Armass, 1987). Thus, we conclude that the absence of synapsins raises the apparent  $Ca^{2+}$  affinity of the DA release process without changing the degree of cooperativity.

### Effects of Cocaine on Dopamine and Serotonin Release

The ability of cocaine to enhance DA release *in vivo* is reduced in synapsin TKO mice (Venton et al., 2006). We therefore compared the effects of cocaine on release of 5-HT and DA in these mice. The effect of cocaine on 5-HT release in SNr brain slices was evaluated by a 35 min application of cocaine (10  $\mu$ M), a concentration that causes competitive inhibition of 5-HT transport (John and Jones, 2007) and thereby increases the apparent value of  $K_m$ . Cocaine treatment caused dramatic changes in the shape of the 5-HT concentration profile produced by electrical stimulation (20 biphasic pulses, 2 ms each phase, 100 Hz, 350  $\mu$ A), prolonging the time course of these signals in slices from both WT and TKO mice (Figure 6, left panels). This prolongation is due to cocaine inhibiting 5-HT uptake. To estimate the apparent  $K_m$  for 5-HT uptake in the presence of cocaine (Jones et al., 1995), we again used nonlinear regression to evaluate the decaying phase of the 5-HT signal. The apparent  $K_m$  values were  $0.75 \pm 0.06$   $\mu$ M for WT and  $0.83 \pm 0.09$   $\mu$ M for TKO animals in the presence of cocaine ( $n = 7$  for WT,  $n = 6$  for TKO), values that were larger than the predrug value of 0.17  $\mu$ M (John et al., 2006). However, the  $K_m$  values measured in the presence of cocaine for each genotype were not significantly different ( $p > 0.05$ ) and were similar to previous measurements made for 5-HT uptake in the presence of 10  $\mu$ M cocaine (John and Jones, 2007). In addition, cocaine treatment did not significantly ( $p > 0.05$ ) alter the amount of 5-HT released by electrical stimulation in slices from either genotype: in the presence of 10  $\mu$ M cocaine, the amplitude of 5-HT release was  $110 \pm 6\%$  of predrug values in tissue from WT and  $112 \pm 8\%$  of predrug values in tissue from TKO animals.

We next considered the influence of synapsins on the ability of cocaine to increase DA release. The effect of cocaine on DA release evoked by electrical stimuli (single biphasic pulse, 2 ms each phase, 350  $\mu$ A) was compared in caudate-putamen slices prepared from the two mouse genotypes. These slices were treated for 35 min with 2  $\mu$ M cocaine, a dose known to inhibit DA uptake in mouse brain slices (Jones et al., 1995). Although uptake of DA was much faster than that of 5-HT (compare traces in left and right columns of Figure 6), cocaine still caused a significant slowing in the time course of the DA signal produced by electrical stimulation (Figure 6, right panels). This slowing indicates an inhibition of DA uptake. The apparent  $K_m$  values for DA uptake after application of cocaine were  $2.60 \pm 0.09$   $\mu$ M for WT and  $2.60 \pm 0.15$  TKO  $\mu$ M mice ( $n = 7$  for each genotype). While these values are not significantly different from each other, they are substantially larger than the value of 0.2  $\mu$ M measured in control conditions.

Cocaine treatment also caused an increase in the peak amplitude of the DA signals. This finding is similar to observations in rat (Jones et al., 1995) and mouse (Venton et al., 2006) brain tissue. The stimulatory effect of cocaine on DA was much greater in slices from WT mice than in slices from TKO mice (Figure 6, right panels). On average, cocaine increased electrically evoked DA release by  $89 \pm 4\%$  in slices from WT mice, but only  $49 \pm 3\%$  in TKO slices (Figure 6). This difference between the cocaine responses of the two genotypes is significant ( $p < 0.001$ ;  $n = 4$  for each genotype).



We also examined the influence of external  $\text{Ca}^{2+}$  on the ability of cocaine to enhance DA release. Over the entire range of  $[\text{Ca}^{2+}]$  considered, cocaine caused a greater enhancement of DA release from WT than from TKO slices ( $p < 0.001$ ). Figure 7A shows the relative DA release from both genotypes as a function of the external  $[\text{Ca}^{2+}]$  after all release values were normalized to the value measured for WT samples at 2.4 mM  $\text{Ca}^{2+}$ . The difference in  $\text{Ca}^{2+}$  sensitivity between WT and TKO animals shown in Figure 5 was diminished by 2  $\mu\text{M}$  cocaine. Hill equation fits to these data indicate that the apparent  $\text{Ca}^{2+}$  affinity,  $K_{\text{Ca}^{2+}}$ , was identical in the two genotypes, being  $1.07 \pm 0.15$  mM for WT and  $1.09 \pm 0.13$  mM for TKO. Likewise, the degree of cooperativity was identical, with Hill coefficients of  $2.19 \pm 0.43$  for WT and  $2.11 \pm 0.34$  for TKO. Even in the presence of cocaine, the maximum values for relative DA release were still higher in TKO mice. To further quantify the effects of cocaine on the  $\text{Ca}^{2+}$  dependence of DA release, we calculated the ratio of DA release measured in the presence and absence of cocaine relative to the value at 2.4 mM  $\text{Ca}^{2+}$  for each genotype (Figure 7B). This ratio was significantly greater than 1 when  $[\text{Ca}^{2+}]$  was below 2.4 mM for WT slices ( $p < 0.001$ ,  $n = 6$ ), while TKO slices only showed significant effects of cocaine at  $[\text{Ca}^{2+}]$  of 1 mM or less ( $p < 0.001$ ,  $n = 6$ ). Therefore, we conclude that cocaine preferentially enhances DA release from WT animals and that this enhancement depends upon  $\text{Ca}^{2+}$  and synapsins.

## Discussion

Our comparison of DA and 5-HT release from WT and synapsin-deficient TKO mice revealed differences in the effect of synapsin deletion on the release of these two monoamine neurotransmitters. The amount of DA released by electrical stimuli was almost twice as large in TKO mice as in WT controls. In contrast, 5-HT release was unaltered in TKO mice. While cocaine blocked uptake of both 5-HT and DA, raising the apparent value of  $K_m$  in both genotypes, it was able to enhance the release only of DA. The enhancement of DA release by cocaine was greater in WT than in TKO animals. The absence of synapsins increased the apparent  $\text{Ca}^{2+}$  affinity of the DA release process, an effect that was mimicked by cocaine in WT tissue. Thus, synapsins play very different roles in nerve terminals employing these two biogenic amine neurotransmitters.

### Synapsins Regulate Dopamine Release

Greater DA release was observed in intact TKO mice, in striatal brain slices prepared from these mice, and in S3KO slices. This establishes a role for synapsins in general, and synapsin III in particular, in dopaminergic presynaptic terminals. Although DA release was enhanced, uptake rates and tissue DA content were similar in TKO and WT mice. Thus, loss of synapsins caused a change in the ability of stimuli to release DA, rather than producing a change in the overall amount of DA or altering the function of DAT. Despite the increased amount of DA release in TKO mice, the activity-dependence of DA release during repetitive activity was minimally affected. This suggests that synapsins play only a minor role in replenishing the readily releasable pool of DA.

Synapsins have a wide range of actions at different nerve terminals. In the adult brain, loss of synapsins leads to a decrease in the amount of GABA released by single stimuli, an effect likely due to a reduction in the readily releasable pool of GABAergic vesicles (Gitler et al., 2004; Baldelli et al., 2007). This action of synapsins is thought to be mediated by synapsin I (Baldelli et al., 2007). While release of glutamate in response to single stimuli is unaffected by loss of synapsins, the kinetics of synaptic depression are accelerated (Gitler et al., 2004). This is thought to be due to the ability of synapsin IIa to maintain glutamatergic synaptic vesicles in a reserve pool (Gitler et al., 2008). Loss of synapsins increases the release of catecholamines from adrenal chromaffin cells by increasing the number of exocytotic events, an effect rescued by expression of synapsin IIa (Villanueva et al., 2006). These

findings suggest that synapsins play different roles in nerve terminals employing different types of neurotransmitters.

The results of our analysis of monoamine neurotransmitter release in synapsin TKO mice provide two additional lines of support for the hypothesis that synapsins play different roles in nerve terminals employing different types of neurotransmitters. First, release of 5-HT in response to brief trains of stimuli is unaffected by the absence of synapsins. While it is possible that different synapsins can have opposing effects on 5-HT release that are neutralized by deleting all 3 synapsin genes, the most parsimonious explanation for our results is that synapsins do not regulate 5-HT release. This is similar to the case for acetylcholine release at neuromuscular junctions, where transmission reportedly is normal in TKO mice (Gaffield and Betz, 2007). The absence of an effect of synapsins on 5-HT release may be due to the fact that 5-HT is mainly stored in large dense-core vesicles (Nirenberg et al., 1995), while glutamate, GABA and DA are primarily stored in small synaptic vesicles (Nirenberg et al., 1997). In synapses that contain both SSVs and LDCVs, synapsin I reportedly associates only with SSVs (Navone et al., 1984). Thus, the lack of effect on 5-HT release in TKO brain slices indicates that synapsins do not regulate 5-HT release. Although synapsin I mRNA is expressed in the SNr (Allen Institute for Brain Discovery), it is possible that this protein is not expressed in serotonergic nerve terminals. Secondly, we found that release of DA in response to single stimuli or brief trains of stimuli is enhanced in TKO animals. This is most likely due to loss of synapsin III, because DA release was enhanced in slices from S3KO mice. These results are consistent with the observation that the striatum is one of the few locations where synapsin III is found in the adult mouse brain (Pieribone et al., 2002). Even though the mRNAs for both synapsins I and II are present in the striatum (Allen Institute for Brain Discovery), neither isoform colocalizes with the vesicular monoamine transporter 2 (VMAT2) so it is likely that synapsin III is the synapsin isoform present in dopaminergic terminals (Bogen et al., 2006). The fact that DA released by single, brief stimuli was enhanced suggests that synapsin III serves as a negative regulator of the readily releasable pool of dopaminergic vesicles. This is reminiscent of the observation that S3KO mice have an increased recycling pool of glutamatergic synaptic vesicles (Feng et al., 2002), suggesting a general role for synapsin III as a negative regulator of neurotransmitter release. The regulation of DA release by synapsin III is even more intriguing in light of work implicating synapsin III in schizophrenia, a disorder in which altered DA signaling is thought to be important (Vawter et al., 2002; Porton and Wetsel, 2007; Porton et al., 2009).

In summary, it seems clear that different synapsin isoforms play distinct roles in nerve terminals employing different neurotransmitters. In some cases, synapsin I predominates, while in other cases synapsins II or III are the primary regulators of neurotransmitter release. Further, some synapsin isoform effects are limited to vesicles in the reserve pool, while in other cases they more directly influence exocytosis from the readily releasable pool of vesicles (see also Hilfiker et al., 1998; Coleman and Byhovskaia, 2009). Taken together, these findings extend the classical model of synapsins maintaining the reserve pool of vesicles through phosphorylation-sensitive tethers to the cytoskeleton (Greengard et al., 1993).

### Synapsin Removal Alters Calcium and Cocaine Responses

Increasing extracellular  $[Ca^{2+}]$  enhances neurotransmitter release by increasing  $Ca^{2+}$  entry into presynaptic terminals and subsequent accumulation of  $Ca^{2+}$  within the terminal cytoplasm (Augustine and Charlton, 1986; Nachshen and Sanchez-Armass, 1987). We found that terminals from TKO mice were more sensitive to extracellular  $[Ca^{2+}]$  than terminals from WT mice. The higher saturation value of DA release reached in TKO animals (Figure 5) provides another indication that synapsins serve as a negative regulator of the readily releasable pool of dopaminergic vesicles.

Cocaine inhibits the DA transporter, thereby increasing extracellular DA concentrations (Jones et al., 1996). Cocaine also facilitates DA release from presynaptic terminals (Lee et al., 2001) by a synapsin-dependent effect (Venton et al., 2006). The present work confirms this cocaine-induced enhancement of DA release and demonstrates that it is significantly less in tissue from synapsin TKO animals (Figure 6). In WT tissue, cocaine enhances both DA release and  $\text{Ca}^{2+}$  sensitivity (compare Figure 5A and Figure 7A) to values similar to those obtained in TKO tissue without cocaine. It is unclear how cocaine affects the  $\text{Ca}^{2+}$  sensitivity of release; cocaine can increase intracellular  $\text{Ca}^{2+}$  levels in brain cells (Du et al., 2006), which would be expected to increase the sensitivity of DA release to extracellular  $\text{Ca}^{2+}$  levels. This effect is not produced by methylphenidate, indicating that cocaine could increase DA release via a DAT-independent pathway (Du et al., 2006). Cocaine also has been proposed to alter calcium influx through calcium channels (Han et al., 2002), which would also produce an increase in the sensitivity of DA release to extracellular  $\text{Ca}^{2+}$ . The fact that cocaine can increase DA release in TKO slices at low  $[\text{Ca}^{2+}]$  (Figure 7B) indicates the presence of a second, minor pathway for enhancing DA release that is independent of synapsins. This effect may be minor because DA release is already close to saturation in TKO tissue.

Cocaine is also a potent inhibitor of the 5-HT transporter (Uhl et al., 2002; Uhl and Lin, 2003). Our measurements in the SNr confirm this action of cocaine by showing that the rate of uptake of 5-HT was lowered in the presence of the drug. However, unlike its effects on DA release, cocaine does not enhance 5-HT release (Figure 6). Furthermore, there is no difference in the cocaine sensitivity of 5-HT uptake between WT mice and synapsin TKO mice. These distinctions between the actions of cocaine on dopaminergic and serotonergic neurons may reflect the different roles that synapsins play in terminals employing DA and 5-HT.

In conclusion, the loss of synapsins alters DA release, but not 5-HT release, from presynaptic terminals. The similar enhancement of DA release that occurs in synapsin III KO mice emphasizes the predominant role of this synapsin isoform in regulating DA release. The ability of calcium to trigger exocytosis of DA was also enhanced by the absence of synapsins. Cocaine enhanced the  $\text{Ca}^{2+}$  sensitivity of DA release in WT slices to a level found in TKO tissue, further demonstrating the importance of synapsins for  $\text{Ca}^{2+}$  regulation of DA release. Taken together, our results indicate that synapsins have dramatically different functions at presynaptic terminals that employ different neurotransmitters. These findings should stimulate future efforts to understand the molecular mechanisms underlying the diverse actions of synapsins.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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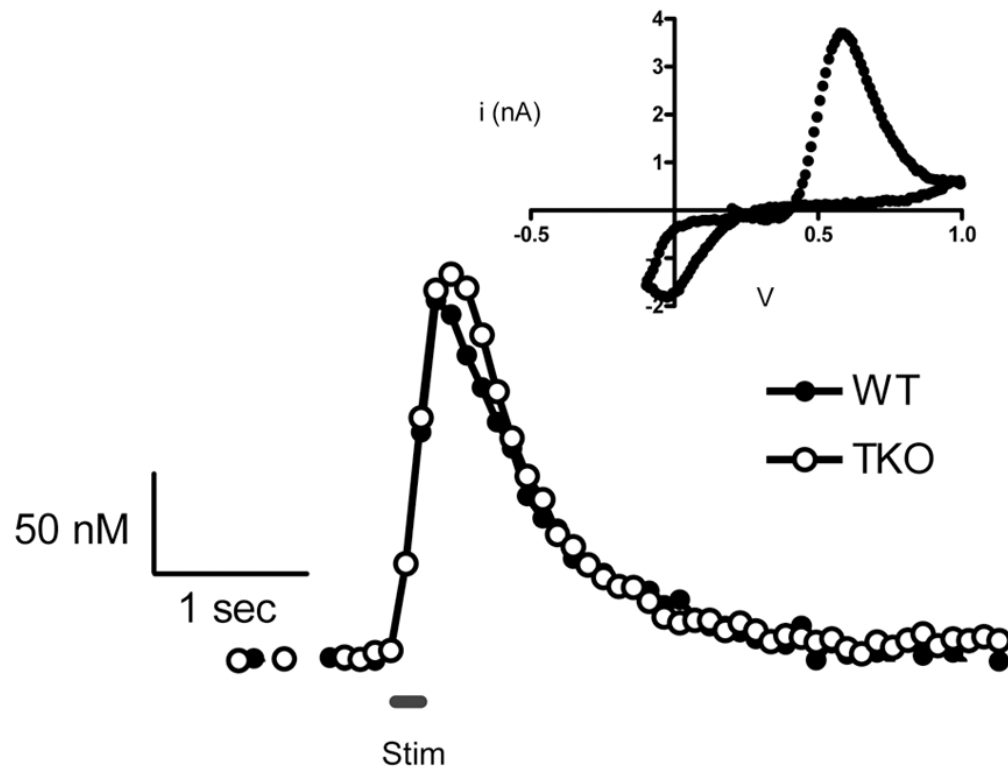
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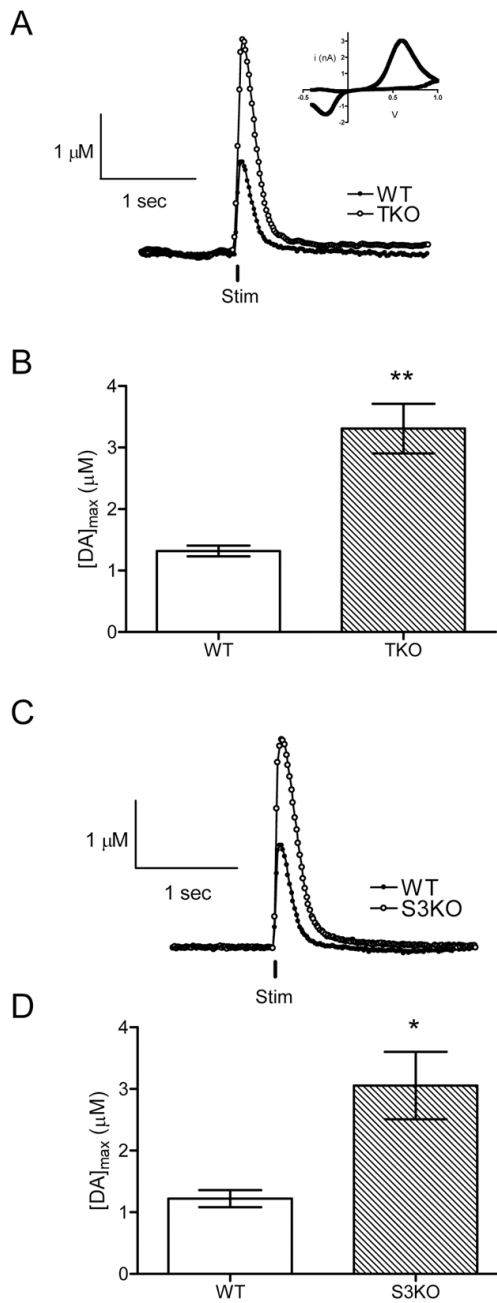
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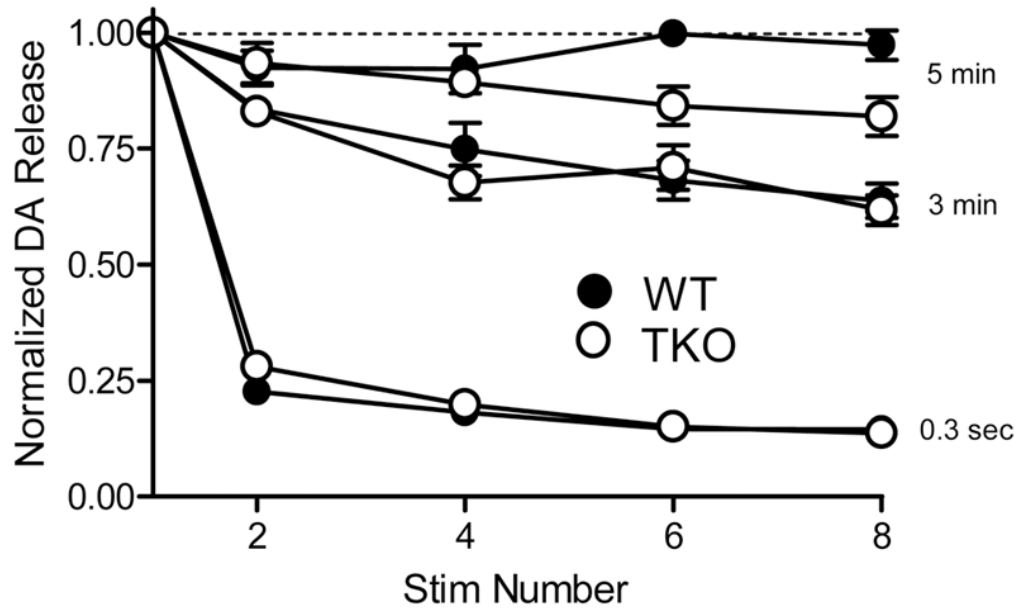


**Figure 1.** Representative traces of 5-HT release in coronal brain slices containing the SNr from WT and TKO mice evoked by a 100 Hz stimulation train (20 pulses, black line). Inset: the cyclic voltammogram is used for identification of 5-HT.

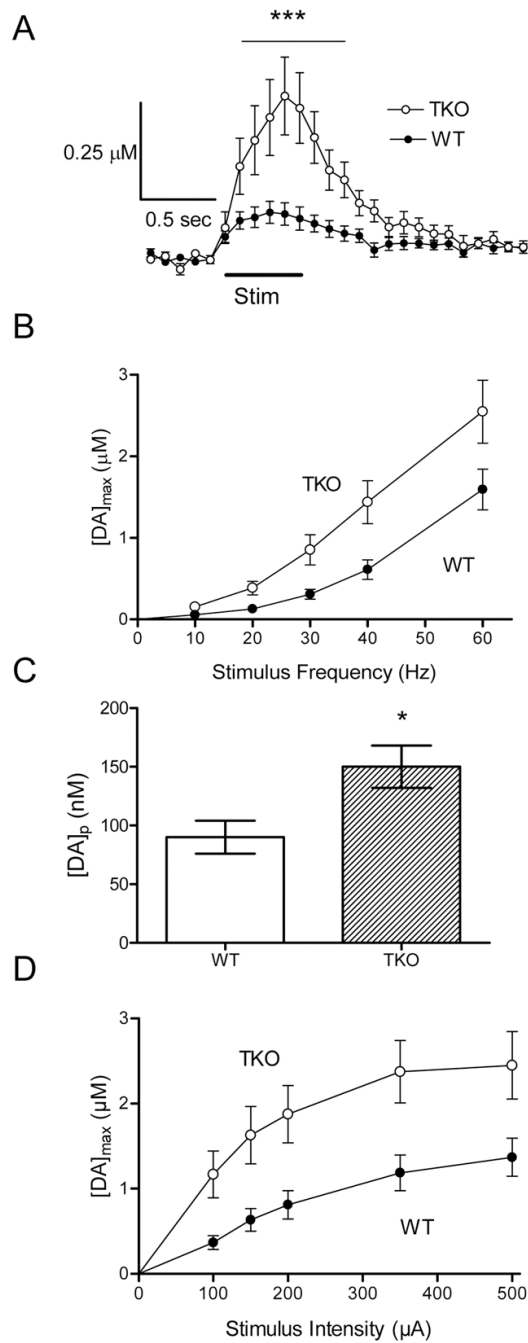


**Figure 2.**

DA release evoked by a single stimulation pulse (vertical black line) in striatal brain slices from WT or TKO animals. A) Representative responses of stimulated DA release; the time of stimulation is shown by the vertical bar. Inset: the cyclic voltammogram identifies the released neurotransmitter as DA. B) Mean  $[DA]_{max}$  from multiple locations of each genotype show an approximate two-fold difference in striatal DA release (\*\* indicates  $p < 0.01$ ,  $n = 8$  TKO,  $n = 6$  WT). C) Representative traces of DA release evoked by a single stimulus pulse in WT or synapsin III knockout (S3KO) animals. Time of stimulus is shown as a vertical black line. D) Measurements of DA release at multiple locations of each genotype indicate that S3KO slices have approximately double the amount of DA release measured in WT slices (\* indicates  $p < 0.05$ ,  $n = 5$  S3KO,  $n = 5$  WT).



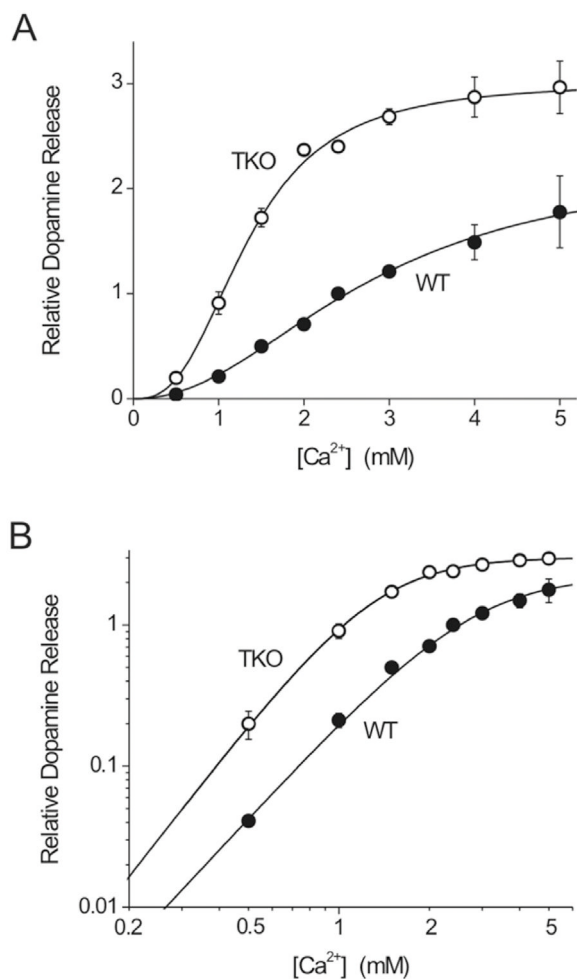
**Figure 3.** The effects of repetitive stimuli on DA release. Single stimuli were applied at intervals of 0.3 sec, 3 min, and 5 min, and the amount of DA released in response to each stimulus was normalized to the amount of DA released in response to the first stimulus.



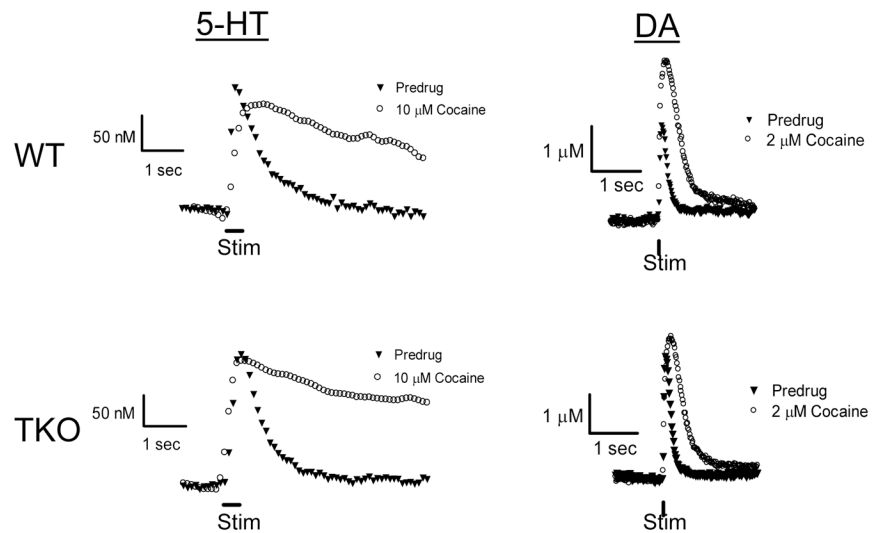
**Figure 4.**

Electrically stimulated DA release in the striatum measured *in vivo*. A) Average release profiles measured in WT and TKO mice ( $n = 8$  for each) in response to 0.5 sec stimulus train ( $300 \mu\text{A}$ , 20 Hz; \*\*\* indicates  $p < 0.001$ ). B)  $[\text{DA}]_{\text{max}}$  values exhibit similar frequency dependencies but release is uniformly greater in TKO animals. C)  $[\text{DA}]_{\text{p}}$  measured from the frequency dependent release shown in panel B (\* indicates  $p < 0.05$ ). D) Although similar responses to stimulus intensity (60 Hz stimulations) are found in both genotypes, DA release is greater in TKO animals.

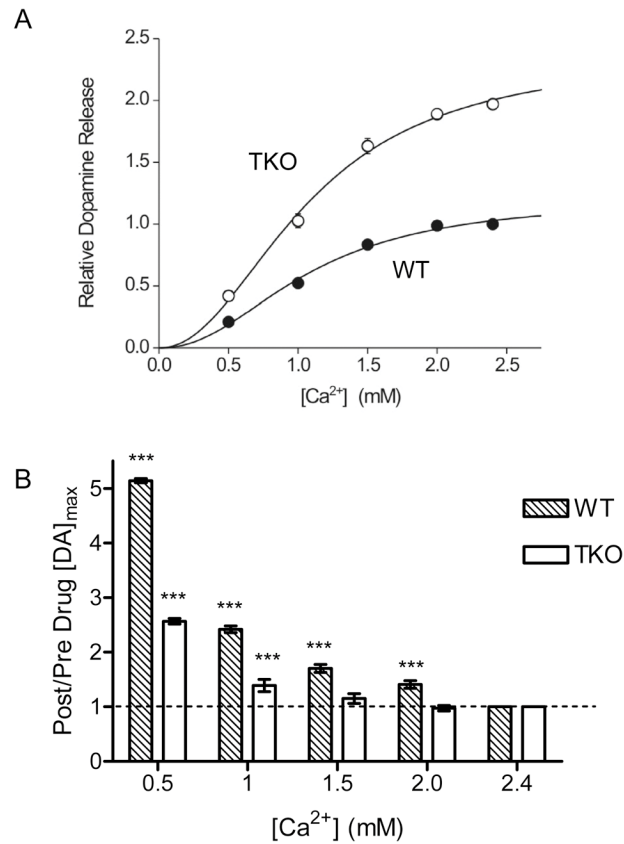




**Figure 5.** Calcium dependence of DA release in striatal slices. A) Relative  $[DA]_{\max}$  for TKO and WT animals at 0.5–5 mM extracellular  $[Ca^{2+}]$ . TKO animals release significantly more DA at all  $[Ca^{2+}]$ . B) Log-plot used to determine stoichiometric relationship between DA release and  $[Ca^{2+}]$  using a fit to the Hill equation.



**Figure 6.** Effects of cocaine on stimulated 5-HT and DA release and uptake in brain slices. Left traces: Representative responses of 5-HT release in SNr from WT (upper) and TKO (lower) mice in the presence and absence of 10  $\mu$ M cocaine for 35 min. Right Traces: Representative responses of DA release in striatal tissue from WT (upper) and TKO (lower) mice measured prior to and 35 min after the introduction of 2  $\mu$ M cocaine.



**Figure 7.**

The effect of 2  $\mu\text{M}$  cocaine on the  $\text{Ca}^{2+}$  dependence of DA release in striatal slices from WT and TKO mice. A) Relative  $[\text{DA}]_{\text{max}}$  in TKO and WT animals as a function of extracellular  $[\text{Ca}^{2+}]$  in the presence of 2  $\mu\text{M}$  cocaine. Data are normalized relative to the amount of DA release measured in WT slices at 2.4 mM  $\text{Ca}^{2+}$ . TKO slices release more DA at all  $[\text{Ca}^{2+}]$ , although little difference in the  $\text{Ca}^{2+}$  sensitivity of release is seen between the genotypes when cocaine is present. B) Ratio of release in the presence of cocaine to that in its absence as a function of external  $[\text{Ca}^{2+}]$  for WT and TKO animals ( $n = 6$ , each genotype); measured release was normalized to release at 2.4 mM  $\text{Ca}^{2+}$  for each genotype. In slices from WT mice, cocaine significantly increased electrically-stimulated DA release at all  $[\text{Ca}^{2+}]$  below 2.4 mM; however, TKO animals are only facilitated at low levels of  $[\text{Ca}^{2+}]$ . \*\*\* $p < 0.001$  indicates significant difference from 2.4 mM  $\text{Ca}^{2+}$ .

**Table 1**

Neurotransmitter content from tissue samples containing either the dorsolateral striatum (DA) or the substantia nigra pars reticulata (5-HT). Data is from four animals of each genotype and is expressed as mean  $\pm$  SEM. No statistical differences were found between genotypes for either neurotransmitter ( $p > 0.05$ , Student's t-test).

Animal	Tissue Striatal Dopamine Content ( $\mu\text{g/g}$ )	Tissue 5-HT Content SNr ( $\mu\text{g/g}$ )
<i>WT</i>	4.97 $\pm$ 0.96	1.03 $\pm$ 0.20
<i>TKO</i>	4.68 $\pm$ 0.60	0.74 $\pm$ 0.10