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Basal somatodendritic dopamine release requires snare proteins

Gabriel D. Fortin,* Catherine C. Desrosiers,* Nobuharu Yamaguchi† and Louis-Éric Trudeau*

**Department of Pharmacology, Faculty of Medicine and †Faculty of Pharmacy, Université de Montréal, Montreal, Quebec, Canada*

Abstract

Dopaminergic neurons have the capacity to release dopamine not only from their axon terminals, but also from their somatodendritic compartment. The actual mechanism of somatodendritic dopamine release has remained controversial. Here we established for the first time a rat primary neuron culture model to investigate this phenomenon and use it to study the mechanism under conditions of non-stimulated spontaneous firing (1–2 Hz). We found that we can selectively measure somatodendritic dopamine release by lowering extracellular calcium to 0.5 mM, thus confirming the previously established differential calcium sensitivity of somatodendritic and terminal release. Dopamine release measured under these conditions was dependent on firing activity and independent of reverse transport through the plasma membrane. We found that treatment with botulinum neurotoxins A and B strongly reduced somatodendritic dopamine release, thus demonstrating the requirement for SNARE proteins SNAP-25 and synaptobrevin. Our work is the first to provide such direct and unambiguous evidence for the involvement of an exocytotic mechanism in basal spontaneous somatodendritic dopamine release.

Keywords: botulinum toxins, cell culture, dopamine, exocytosis, somatodendritic, rat, SNARE proteins.

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Somatodendritic dopamine release has been established as a biological fact in the late 1970s (Bjorklund and Lindvall 1975; Geffen *et al*. 1976; Cheramy *et al*. 1981). However, the mechanism by which dopamine is released from the soma and dendrites of neurons has remained controversial. Compatible with an exocytotic-like mechanism, numerous authors have confirmed that somatodendritic dopamine release is activity-dependent and sensitive to tetrodotoxin (Robertson *et al*. 1991; Kalivas and Duffy 1991; Santiago *et al*. 1992; Heeringa and Abercrombie 1995; Beckstead *et al*. 2004). Moreover, it is calcium-dependent (Beart and McDonald 1980; Elverfors *et al*. 1997; Chen and Rice 2001) and can be inhibited by blocking the vesicular monoamine transporter with reserpine (Elverfors and Nissbrandt 1991; Heeringa and Abercrombie 1995). Although these properties are characteristic of vesicular exocytotic release, observations of actual synaptic-like vesicles within dendrites have proven difficult (Hajdu *et al*. 1973; Wilson *et al*. 1977; Hattori *et al*. 1979; Wassef *et al*. 1981; Groves and Linder 1983). The endoplasmic reticulum (ER) and its associated tubulovesicles have been proposed as possible storage sites for somatodendritic dopamine. These structures indeed appear to contain dopamine (Groves and Linder 1983; Hattori *et al*. 1979), and similar structures have been shown to express proteins of the SNAP-receptor (SNARE) complex, otherwise known to be essential for exocytosis (Prekeris *et al*. 1999).

Falkenburger *et al*. (2001) have recently shown in a midbrain slice preparation that the stimulation of glutamatergic afferent fibres from the subthalamic nucleus, either with carbachol or with high frequency electrical stimulation, induced somatodendritic dopamine release through a mechanism requiring the plasma membrane dopamine transporter (DAT) (Falkenburger *et al*. 2001). On the contrary however, previous observers have reported that direct electrical stimulation of the substantia nigra in the slice or perfusion with high potassium induced extracellular dopamine accumulation that *increased* with DAT blockade (Elverfors *et al*. 1997; Chen and Rice 2001; Beckstead *et al*. 2004), implicating a mechanism that is completely independent from reverse transport. This apparent discrepancy is probably due to the fact that different stimulation conditions induce somatodendritic dopamine release through different mechanisms, including facilitation of reverse transport by glutamate

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Address correspondence and reprint requests to Dr Louis-Eric Trudeau, Department of Pharmacology, Faculty of Medicine, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Québec, Canada, H3C 3J7. E-mail: louis-eric.trudeau@umontreal.ca

Abbreviations used: BoNT, botulinum neurotoxin; DAT, dopamine transporter; EPSC and IPSC, excitatory and inhibitory postsynaptic currents; KRB, Krebs ringer buffer; SNARE, SNAP-receptor; TTX, tetrodotoxin; VAMP, synaptobrevin.

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(Lonart and Zigmond 1991; Rosales *et al*. 1994; Falkenburger *et al*. 2001; Leviel 2001; Bergquist *et al*. 2002).

An unresolved question is what mechanism mediates somatodendritic dopamine release when dopamine neurons fire at their normal basal firing rate. Unfortunately, established techniques like cyclic voltametry do not permit measurement of basal non-stimulated somatodendritic dopamine release and results from *in vivo* microdialysis are sometimes difficult to interpret due to indirect circuitry effects. To investigate the mechanism of somatodendritic release under basal firing activity, we took advantage of midbrain neuron cultures and detection of extracellular dopamine with HPLC. We show that calcium-dependent somatodendritic dopamine release can occur with spontaneous low frequency firing, and that the mechanism of release requires SNARE proteins.

Materials and methods

Neuron cultures

All experiments were performed on rat dopaminergic neuron cultures, prepared according to recently described protocols (Congar et al. 2002; Cardozo 1993; Sulzer et al. 1998; St-Gelais et al. 2004). Overall, the procedure involves plating neurons onto a pre-established monolayer of purified astrocytes covering a coated glass coverslip. Coverslips for standard neuron cultures were coated uniformly with collagen/poly-L-lysine, while coverslips for single neuron μ-cultures were sprayed with collagen (0.75 mg/mL) μ-droplets (50–150 μm in diameter).

Neonatal (P0 to P2) Sprague–Dawley rats were cryoanaesthetized and their brains were rapidly removed and transferred into ice-cold dissociation solution. For neuronal cultures, a 1 mm-thick slice was cut at the level of the midbrain flexure from which a block of tissue, approximately 2 mm^3 in size, was surgically isolated. The cells were dissociated and plated at a density of 350 000 living cells per millilitre. This density permitted stable and consistent measurements of extracellular dopamine and was optimal for neuronal viability. For single neuron μ-cultures, dissociated cells were plated at a density of 100 000 living cells per millilitres. Under such conditions, single neurons establish synaptic connections onto themselves (called autapses) and postsynaptic currents can be evoked and measured on the same cell (Bekkers and Stevens 1991; Congar et al. 2002). Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere for 12–24 days after neuronal plating, and contained on average 25% dopaminergic neurons. All experiments were performed at room temperature (22–23°C) in buffered saline under normoxic conditions. Experimental procedures were approved by the animal ethics committee of the Université de Montréal.

Dopamine sampling procedure and assay

Extracellular dopamine concentrations were measured using HPLC. Our dopamine sampling procedure was adapted from Rougé-Pont and colleagues (Rouge-Pont *et al.* 1999). At the start of the experiments, coverslips plated with the neuronal cultures were put in a well containing 400 μL of Krebs Ringer buffer (KRB), composed of (in mM): NaCl 140; KCl 5; $MgCl_2$ 2; $CaCl_2$ 2; Sucrose 6; Glucose 10; HEPES 10; pH adjusted to 7.35 with NaOH 5 N. We then drew 100 μL-samples every 3 min for the duration of the experiments (usually 50 min). The collected samples were aliquoted into numbered tubes and immediately replaced with an equivalent volume of fresh KRB. The previously prepared tubes contained 4 μL of preservative solution (pH 6.5) of ethylene glycol-bis-amino-ethyl ether-*N,N,N′*,N′-tetraacetic acid (95 mg/mL), glutathione (60 mg/mL), and 20 μL of distilled water. The data shown in this paper were corrected for this dilution and therefore represent actual dopamine content in the media (pg/mL). Samples were analyzed using an HPLC system (Gilson, Villiers-Le-Bel, France) coupled to a coulometric detector (Coulochem II; ESA, Bedford, USA). The detection limit for 100 μL-samples was 5 pg.

Drugs were either present in the bath from the start of the experiment, or were dissolved into the 100 μL replacement volumes and administered after the control period (usually 6 samples). The composition of the KRB needed to be modified in some experiments in order to maintain osmolarity or the concentration of divalent cations. NaCl concentration was lowered by 40 mM for the KRB containing 40 mM potassium. $MgCl_2$ was raised to 3.5 mM for experiments in 0.5 mM Ca^{2+}. Finally, the composition of the saline for experiments in 0 mM calcium was as follow (in mM): NaCl 140; KCl 5; $MgCl_2$ 4; EGTA 1; sucrose 4; glucose 10; HEPES 10; pH adjusted to 7.35 with NaOH 5 N.

Electrophysiology

We measured autaptic post-synaptic currents according to recently described procedures (Congar *et al*. 2002). Patch pipettes were prepared with borosilicate glass and had a resistance of 5.5 ± 0.5 MΩ. The intrapipette solution for autaptic current measurements contained (in mM): $KMeSO_4$ 120; KCl 20; EGTA 0.1; MgATP 2; GTP 0.5; HEPES 10; Phosphocreatine 10; NaCl 5; pH adjusted at 7.35 with KOH 5 N; 300 mOsm.

To evoke postsynaptic currents, we stimulated voltage-clamped isolated neurons every 15 s with a 1 ms-voltage command pulse to +20 mV from a holding potential of −40 to −60 mV. The presence of an autaptic inhibitory postsynaptic current (IPSC) following the un-clamped sodium spike indicated a GABAergic phenotype. Dopamine neurons in culture also release glutamate as a cotransmitter, hence detection of excitatory postsynaptic currents (EPSC) and inhibition of this response by 10 μM quinpirole, a D2 agonist, indicated a dopaminergic phenotype (Congar *et al*. 2002; Dal Bo *et al*. 2004).

Confocal imaging of terminals loaded with FM2-10

We loaded axonal terminals with FM2-10, a fluorescent indicator of vesicle cycling, and studied its release with confocal microscopy. Standard neuronal cultures on 25 mm coverslips were mounted in a closed bath imaging chamber with platinum stimulating electrodes from Warner Instruments (Hamden, USA). Cells were then exposed for 2 min to 200 μM FM2-10 (Molecular Probes, Eugene, OR, USA) diluted in KRB containing 40 mM potassium so as to depolarize neurons and induce exocytosis. They were subsequently rinsed for 5 min in normal KRB, and then 5 more min in either normal KRB, a solution containing 0.5 mM calcium, or in 0 mM calcium, depending on the calcium concentration tested in the experiment. This 10 min rinse eliminated the signal originating from the FM2-10 that was not

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internalized. Images were acquired every 10 s using a point-scanning confocal microscope from Prairie Technologies LLC (Middleton, WI, USA). Excitation was achieved using the 488 nm line of an argon ion laser and we measured FM2-10 emitted fluorescence between 505 and 545 nm. Images were analyzed using Metamorph software v4.5 from Universal Imaging Corp (USA). Release of the dye was elicited with a 60 s-electrical field stimulation delivered at 5 Hz (1 ms/pulse) using a S88 stimulator from Grass Instruments (Quincy, MA, USA). The field stimulation electrodes were placed on opposite sides of the imaging chamber to produce uniform stimulation of most of the coverslip surface. Preliminary experiments showed that the effect of the electrical stimulation was blocked by TTX 0.5 μM, thus indicating that it induced action potentials rather than directly depolarizing axon terminals (Pyle *et al*. 2000). Cells were finally exposed to high potassium saline to induce further exocytosis and empty all terminals of releasable FM2-10. Data are represented relative to the intensity of the first 6 images (1 min), prior to any treatment.

Immunocytochemistry

Neurons were fixed with methanol and immunostained using the following primary antibodies: TH (1 : 1000; Pelfrez Biological, AK, USA), rabbit polyclonal anti-DAT (1 : 500; Chemicon, Temecula, CA, USA) or rabbit polyclonal anti-SNAP-25 (1 : 1000; Synaptic Systems, Gottingen, Germany). Primary antibodies were detected using Alexa-488 or Alexa-647 (1 : 200) fluorescently labelled secondary antibodies (Molecular Probes Inc., Eugene, OR, USA).

Drugs and statistical analyses

Botulinum neurotoxin A (BoNT A) was obtained from Sigma (St Louis, MO, USA) and botulinum neurotoxin B (BoNT B) was obtained from Metabiologics Inc. (Madison, WI, USA). Neuron cultures were incubated overnight at 37°C with 30 nM of the toxin. Tetrodotoxin (TTX) was obtained from Alomone Laboratories (Jerusalem, Israel). All other drugs were obtained from Sigma (Oakville, ON, Canada). Most drugs were dissolved from a frozen stock solution into KRB. GBR12909 was prepared fresh each day in KRB and was used at 1 μM. Statistical analyses consisted either of analysis of variance (ANOVA) or of Student's *t*-test, as appropriate. Data are always shown as mean ± SEM.

Results

Dopamine release persists in low calcium concentrations and necessitates cell firing

Experiments were performed using rat postnatal dopamine neurons in primary culture (Fig. 1a). Dopamine neurons in

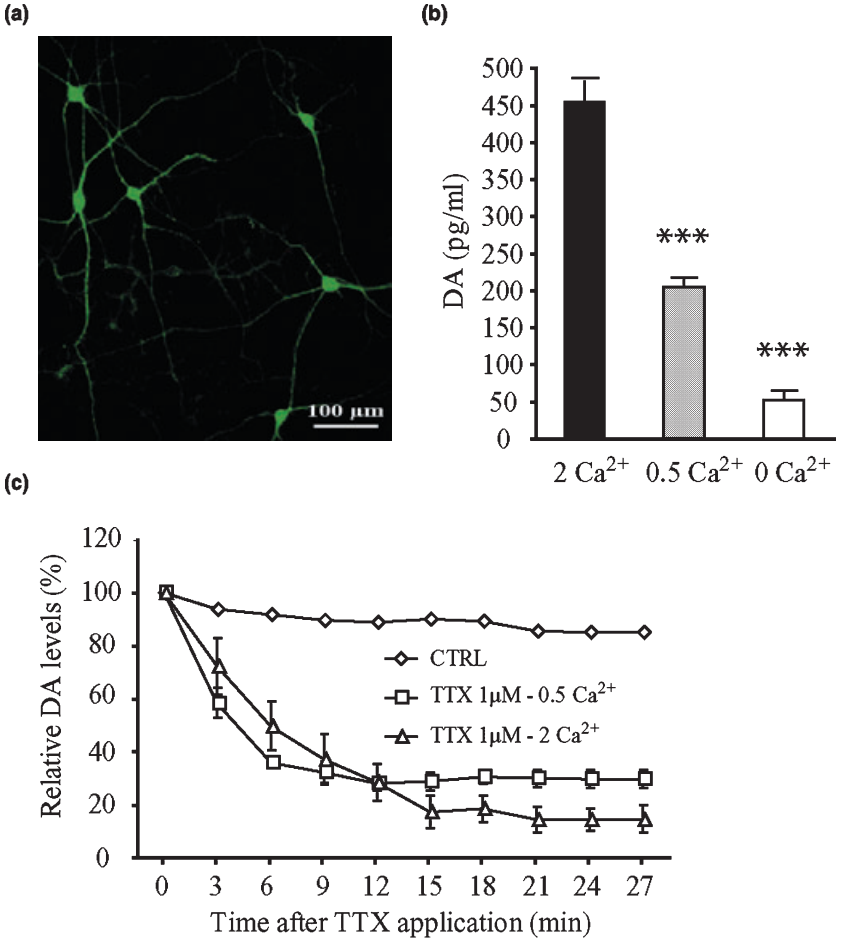

Fig. 1 Dopamine release persists at low calcium concentrations. (a) Immunocytochemistry for tyrosine hydroxylase identifies dopamine neurons (green) in primary culture. (b) The bar graph shows the average dopamine concentrations at three different extracellular calcium concentrations (2 mM: $n = 10$; 0.5 mM: $n = 14$; and 0 mM: $n = 9$). Average dopamine levels were significantly different between all three calcium concentrations (***$p < 0.001$). (c) Graph showing the action of 1 μM tetrodotoxin (TTX) on relative dopamine levels in both 2 mM ($n = 8$) and 0.5 mM calcium ($n = 10$). Data are expressed as a percentage of the mean dopamine levels before TTX application. The control trace was obtained in 2 mM calcium concentration and shows the stability of dopamine levels throughout the sampling protocol ($n = 52$). Some error bars are masked by symbols.

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our cultures are spontaneously active and discharge at a frequency between 1 and 2 Hz (St-Gelais *et al*. 2004). Considering a previous report demonstrating that in midbrain slices over 60% of somatodendritic dopamine release persists in 0.5 mM extracellular calcium while terminal dopamine release is completely abolished (Chen and Rice 2001), we started by evaluating extracellular dopamine levels at different extracellular calcium concentrations (2 mM, 0.5 mM and 0 mM). Lowering extracellular calcium concentrations from 2 mM to 0.5 mM reduced basal dopamine levels from 455 ± 30 pg/mL ($n = 10$) to 204 ± 20 pg/mL ($n = 14$). Dopamine levels dropped close to our detection limit when calcium was completely absent from the bath (52 ± 24 pg/mL; $n = 9$; Fig. 1b). Average dopamine levels were significantly different between all three calcium concentrations ($F = 61.50$; $p < 0.001$ for all comparisons). We next examined whether the measured dopamine levels necessitated cell firing. Application of the sodium channel blocker TTX (1 μM) significantly and drastically reduced dopamine levels in both 2 mM and 0.5 mM calcium ($F = 52.0$, $p < 0.001$; Fig. 1c)

Terminal release occurring during spontaneous firing is abolished in low calcium

Our results (Fig. 1) show that activity-dependent dopamine release occurs in 0.5 mM calcium. To confirm that this release comes from the somatodendritic compartment and not from terminals, we next attempted to verify whether there still was any residual terminal exocytosis in low calcium. For this we used FM2-10, an activity-dependent fluorescent indicator of synaptic vesicle cycling (Betz and Bewick 1992). First, we loaded terminals with FM2-10 using potassium depolarization (Fig. 2a). The dotted appearance of the fluorescent signal (Fig. 2a) can be attributed to the varicose nature of

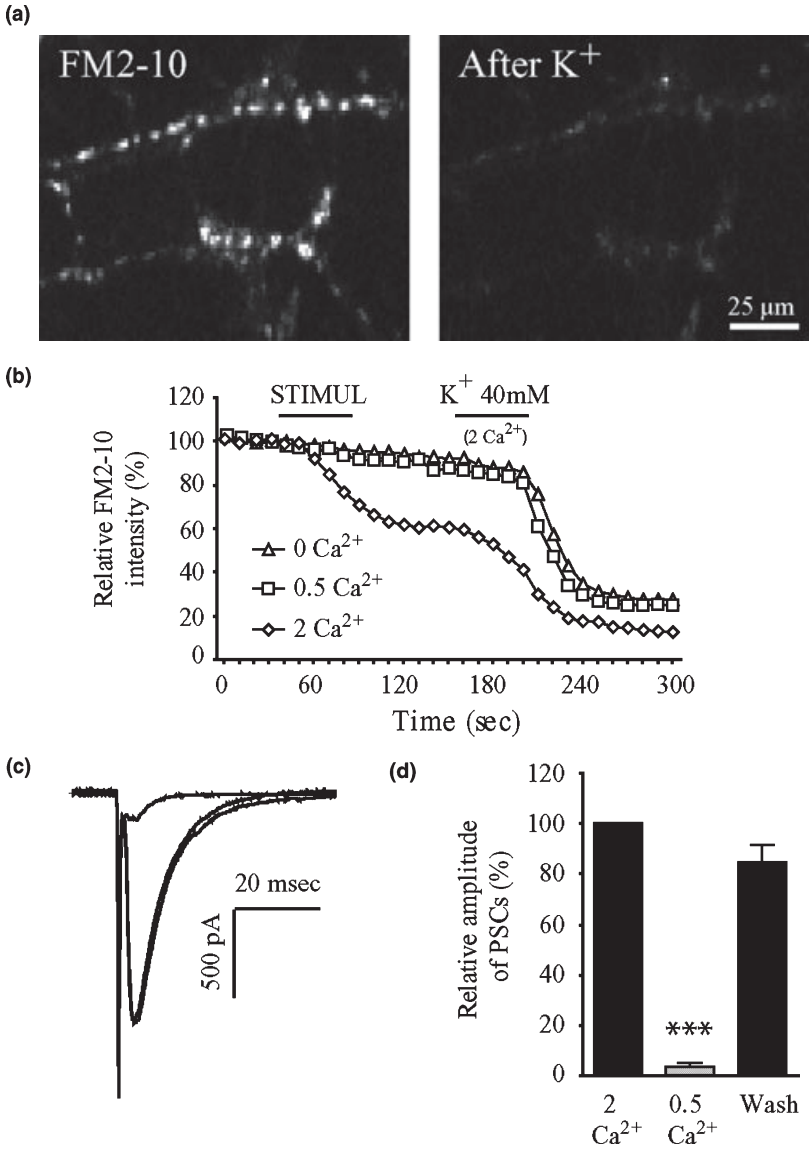

Fig. 2 Terminal release is abolished in low calcium. (a) Confocal fluorescence images showing the presence of the synaptic vesicle indicator FM2-10 in axon terminals (punctate structures in left image). This signal disappeared after a 1 min depolarization with 40 mM K^+ in 2 mM calcium (right image), indicating exocytosis of the dye and neurotransmitters. (b) Graph showing FM2-10 relative intensity over time. Electrical field stimulation at 5 Hz (1 ms/pulse) was used to trigger action potentials and evoke dye release at different calcium concentrations (2 mM: $n = 110$ terminals; 0.5 mM: $n = 132$; and 0 mM: $n = 110$). This was followed by stimulation with 40 mM K^+ in 2 mM calcium to empty axonal terminals. Error bars are masked by symbols. Note that electrically evoked FM2-10 release was prevented in low calcium. (c) Representative recording of autaptic excitatory postsynaptic currents in an isolated neuron growing in μ-culture. Postsynaptic currents were induced by a brief (1 ms) depolarizing voltage step to +20 mV from a holding potential of −60 mV. The postsynaptic current was nearly abolished in 0.5 mM calcium leaving only a small stimulation artefact. (d) Histogram comparing the average autaptic current amplitude in 2 mM calcium, 0.5 mM calcium and back to 2 mM calcium ($n = 9$). Data are expressed relative to the average amplitude in 2 mM calcium. (***$p < 0.001$).

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axonal terminals, where the dye accumulates within clusters of vesicles. We next applied low frequency electrical field stimulation (60 s at 5Hz) under varying calcium concentrations (2 mM, 0.5 mM and 0 mM) to generate action potentials and induce exocytosis from axon terminals. Figure 2(b) shows that electrical stimulation induced fluorescence loss in 2 mM calcium, and that it was completely ineffective in 0.5 mM and 0 mM calcium. Low frequency electrical stimulation was thus effective at stimulating terminal exocytosis in 2 mM calcium, but this was prevented at low calcium concentrations. Although dopaminergic axon terminals were only a subset of all terminals visualized in this experiment (not shown), the effects were uniformly observed across all loaded terminals and thus it is likely that both dopaminergic and non-dopaminergic terminals were equally affected.

We attempted to confirm these observations with an independent index of terminal activity. We used single neuron cultures to record autaptic postsynaptic currents in 2 mM and 0.5 mM calcium concentrations. As seen in Fig. 2(c,d), action potential-evoked postsynaptic currents were practically abolished in 0.5 mM calcium (3.7 ± 1% of control, $n = 9$). For statistical analyses, we averaged the amplitudes of postsynaptic currents in 2 mM calcium before and after washout of 0.5 mM calcium to account for rundown, and compared it to the average amplitude in 0.5 mM calcium with a Student's *t*-test ($t = 23.66$, $DF = 16$, $p < 0.001$).

Overall, the results from Fig. 2 show that action potential-evoked terminal activity was essentially absent in 0.5 mM calcium. Therefore, dopamine levels measured under these conditions represent somatodendritic dopamine release. These results confirm previous results from Chen and Rice in mesencephalic brain slices (Chen and Rice 2001).

The DAT is present and functional, but not involved in spontaneous somatodendritic dopamine release

Somatodendritic dopamine release could occur in part through reverse transport. We thus examined whether the DAT was indeed present and functional under our experimental conditions. Figure 3a shows images from immunocytochemistry against DAT and TH. Numerous neurons were strongly immunopositive for the DAT protein, and they were in all cases also positive for TH. We found that nearly all dopamine neurons in our culture express the DAT. In 2 mM calcium, application of 1 μM GBR12909, a selective DAT blocker that does not induce reverse transport (Eshleman *et al*. 1994), significantly increased basal dopamine concentrations from 455 ± 30 pg/mL ($n = 52$) to 855 ± 66 pg/mL ($n = 7$) (Student's *t*-test, $t = 4.680$, $DF = 57$, $p < 0.001$) (Fig. 3b). Dopamine reuptake thus contributes to establishing stable extracellular dopamine levels in our culture model. The application of 10 μM amphetamine, known to induce dopamine release by reverse transport (Sulzer and Rayport 1990; Khoshbouei *et al*. 2003), significantly increased dopamine levels to 233 ± 38% ($n = 11$) of basal values after 18 min (one-way ANOVA, $F = 8.12$, $p < 0.001$; Fig. 3c). These data show that the DAT is present and functional in our cultures.

Blocking the DAT with GBR12909 should *decrease* basal dopamine levels in 0.5 mM calcium if somatodendritic release relies on reverse transport. Alternately, the DAT blocker should *increase* dopamine levels if somatodendritic release relies on an independent mechanism, such as exocytosis. We applied 1 μM of GBR12909 in 0.5 mM calcium saline and measured dopamine levels. Basal dopamine levels in 0.5 mM calcium averaged 509 ± 52 pg/mL ($n = 8$) in the presence of 1 μM GBR12909 compared to 204 ± 20 pg/mL ($n = 14$) without GBR12909 (Fig. 3d). GBR12909 thus *increased* basal dopamine levels in 0.5 mM calcium (Student's *t*-test, $t = -4.28$, $DF = 20$, $p < 0.001$), as would be expected if somatodendritic dopamine release did not involve reverse transport.

Somatodendritic dopamine release requires intact SNARE proteins

Clostridial neurotoxins were used to evaluate the contribution of an exocytotic-like mechanism to somatodendritic dopamine release occurring in response to spontaneous firing. BoNT A and B cleave, respectively, SNAP-25 and synaptobrevin (VAMP), two proteins critically involved in the vesicular exocytosis of neurotransmitters. Cells were treated overnight with either of these toxins and then dopamine release was assessed. These experiments were performed in the presence of 10 μM CNQX and 5 μM SR95531 to block AMPA and $GABA_A$ receptors, respectively. This ensured that observed effects did not result indirectly from the blockade of afferent synaptic activity by the toxins. We found that basal somatodendritic dopamine release in 0.5 mM calcium was markedly reduced after treatment with BoNT, from 147 ± 1 pg/mL ($n = 22$) without the toxins to 66 ± 8 pg/mL ($n = 14$) and 52 ± 3 pg/mL ($n = 9$) with BoNT A and B, respectively ($F = 27.6$, $p < 0.001$) (Fig. 4a). Similarly, basal dopamine release in 2 mM calcium was reduced from 653 ± 47 pg/mL ($n = 19$) to 109 ± 14 pg/mL ($n = 11$) and 50 ± 2 pg/mL ($n = 9$) after treatment with BoNT A and B, respectively ($F = 73.7$, $p < 0.001$) (Fig. 4b). These experiments therefore show that SNAP-25 and VAMP are required for somatodendritic dopamine release. Compatible with a role of SNAP-25 in this process, immunocytochemical localization of this protein shows that in addition to being present in small axonal-like processes and varicosities, it is also abundant in the cell body and major dendrites of dopamine neurons (Fig. 5).

Discussion

Dopamine levels measured in cultures under normal calcium (2 mM) concentrations represent a combination of terminal as well as somatodendritic dopamine release. However, as

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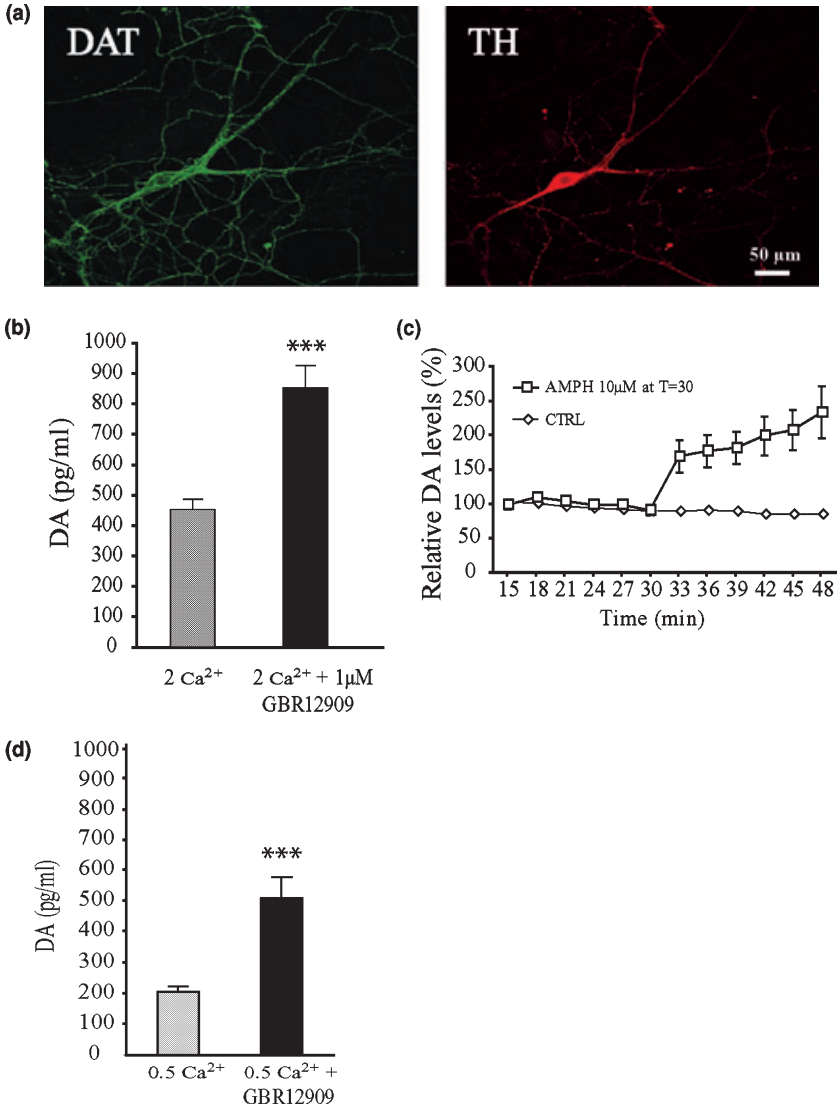

Fig. 3 The DAT is present and functional, but not involved in spontaneous somatodendritic dopamine release. (a) Immunocytochemical localization of the DAT (green) and tyrosine hydroxylase (TH; red). Note that neurons positive for DAT were also positive for TH. (b) Average dopamine levels measured in 2 mM calcium concentrations with ($n = 7$) or without ($n = 52$) 1 μM GBR12909, a DAT blocker. (c) Time course of extracellular dopamine concentrations in response to 10 μM amphetamine at t = 30 min ($n = 11$). For purposes of comparison, data from Fig. 1(c) are reproduced (gray) to show the stability of basal dopamine concentrations in 2 mM calcium ($n = 52$). Some error bars are masked by symbols. (d) Bar graphs comparing the basal dopamine concentrations in 0.5 mM calcium with and without 1 μM GBR12909. The increased dopamine levels indicate that reverse transport is not necessary for somatodendritic dopamine release. (***$p < 0.001$).

previously reported by Chen and Rice (Chen and Rice 2001), we found that lowering extracellular calcium to 0.5 mM practically eliminated terminal dopamine release, while somatodendritic dopamine release persisted. Our main finding is that basal somatodendritic dopamine release was significantly blocked by treatment with BoNT A and B, thus indicating the requirement for SNARE proteins SNAP-25 and VAMP. Moreover, we found no evidence for the involvement of reverse transport.

Differential calcium-dependency of terminal and somatodendritic dopamine release

As for most terminal exocytosis in the nervous system, dopaminergic terminal release requires normal levels of extracellular calcium (1–2 mM). Using two independent assays, we indeed found that terminal release was essentially absent in 0.5 mM calcium. First, release of FM2-10 from terminals in response to electrical field stimulation was abolished in 0.5 mM calcium. Second, autaptic postsynaptic currents measured in isolated neurons were abolished in 0.5 mM calcium. This clearly shows that lowering extracellular calcium to 0.5 mM practically eliminates terminal release, presumably because calcium influx near release sites is not sufficient to trigger exocytosis of synaptic vesicles. Under the same low calcium conditions however, the somatodendritic release mechanism is apparently still functional since extracellular dopamine levels remain significantly elevated.

Our results agree with previous findings showing that brief removal of extracellular calcium or exposure to lower calcium concentrations does not rapidly eliminate somatodendritic dopamine release (Hoffman and Gerhardt 1999; Chen and Rice 2001). Nonetheless, complete and prolonged absence of extracellular calcium abolishes somatodendritic dopamine release (Rice et al. 1997; Bergquist et al. 1998; Hoffman and Gerhardt 1999; Chen and Rice 2001). At least

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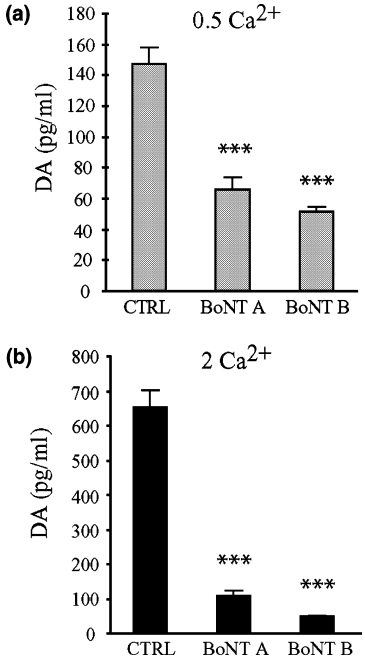

Fig. 4 Somatodendritic dopamine release requires intact SNAP-25 and synaptobrevin. Bar graphs comparing basal dopamine release in 0.5 mM and 2 mM calcium after overnight treatment with botulinum neurotoxins (BoNT) A and B. (a) Basal somatodendritic dopamine release measured in 0.5 mM calcium ($n = 22$) was significantly reduced after treatment with BoNT A ($n = 14$) and B ($n = 9$). (b) Similarly, basal dopamine release in 2 mM calcium ($n = 19$) was significantly reduced after treatment with BoNT A ($n = 11$) and B ($n = 9$). These experiments were performed in the presence of 10 μM CNQX and 5 μM SR95531 (***$p < 0.001$).

two hypotheses could account for this lower sensitivity to extracellular calcium concentrations. First, intracellular calcium stores, like the endoplasmic reticulum, could participate in somatodendritic dopamine release, perhaps within the context of a calcium-induced calcium release relay mechanism. Indeed, it has been demonstrated that mobilization of intracellular calcium stores could induce or modulate exocytosis of peptides and growth factors (Blochl and Thoenen 1995; Penner and Neher 1988). Second, the exocytotic-like process in the somatodendritic compartment could involve molecular machinery that does not require large intracellular calcium elevations to induce release. For instance, different isoforms of synaptotagmin, widely recognized as the main calcium sensor responsible for inducing exocytosis, could perhaps mediate terminal and somatodendritic dopamine release (Li et al. 1995; Schiavo et al. 1998).

Of course, these two hypotheses are not mutually exclusive. What seems clear however, is that induction of somatodendritic dopamine release requires less intracellular calcium than terminal release. This property is reminiscent of endocrine secretion, which requires intracellular calcium concentrations of around 5 μM, while terminal exocytosis of synaptic vesicles in neurons requires local calcium concentrations above 100 μM (Zucker 1996).

Although the use of low extracellular calcium levels represents a partial limitation of our model, it is currently the only strategy to isolate terminal from somatodendritic dopamine release. Lowering extracellular calcium could also potentially affect other cell parameters such as firing rate due to the calcium-dependence of many ionic channels. In the present work we have not directly evaluated the effect of lowering extracellular calcium on the spontaneous firing rate of dopamine neurons. Although we cannot exclude that spontaneous firing was affected, our data with TTX clearly shows that somatodendritic release measured under such conditions requires action potentials, thus confirming that dopamine neurons are still spontaneously active under our experimental conditions.

Mechanism of somatodendritic dopamine release

A number of stimuli such as K^+ depolarization, electrical stimulation or glutamate application have been used to stimulate somatodendritic dopamine release and facilitate its investigation. Studies testing the involvement of reverse transport under such diverse experimental conditions have lead to apparently contradictory results (Cragg *et al.* 1997;

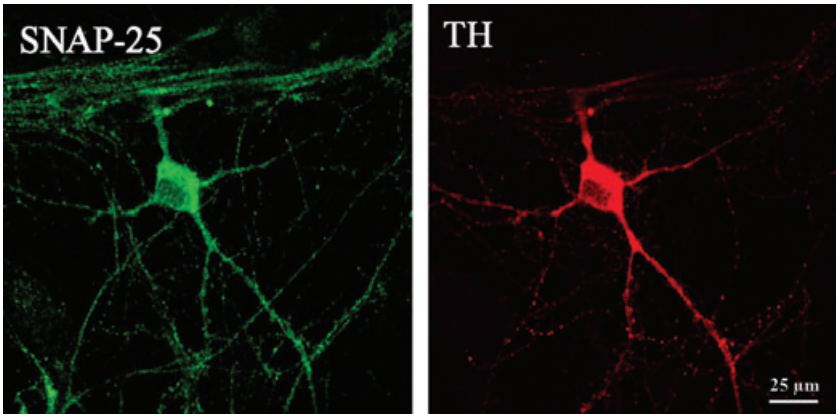

Fig. 5 SNAP-25 is found in the somatodendritic compartment of dopamine neurons. Immunocytochemical localization of SNAP-25 (green) and tyrosine hydroxylase (TH; red). Note that SNAP-25 was found in small calibre axonal-like processes, in varicosities and in the cell body and dendrites of TH-positive neurons.

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Elverfors *et al*. 1997; Falkenburger *et al*. 2001). Stimulation of dopamine release with high potassium in substantia nigra brain slices has been shown to be insensitive to DAT blockade (Elverfors *et al*. 1997). Similarly, somatodendritic dopamine release evoked by direct electrical stimulation of the substantia nigra slice is not prevented by DAT blockers (Cragg *et al*. 1997; Chen and Rice 2001). However, dopamine release from substantia nigra evoked either with the sodium channel opener veratridine, amphetamine, or with high concentrations of glutamate can be inhibited by DAT blockers (Elverfors *et al*. 1997; Falkenburger *et al*. 2001), thus suggesting that some conditions may favour somatodendritic dopamine release via reverse transport. These latter stimuli seem to mimic patho-physiological conditions rather than healthy basal activity, as even glutamate stimulation seems to require somewhat high concentrations (1 mM) to induce measurable somatodendritic release via reverse transport (Rosales *et al*. 1994; Falkenburger *et al*. 2001; Bergquist *et al*. 2002).

Our model allowed us to measure somatodendritic dopamine release occurring in response to low frequency spontaneous firing, thus circumventing the potential bias induced by the chosen stimulatory condition. We found that dopamine release measured under these conditions was not prevented by DAT blockade, but that measured dopamine levels were actually increased, as found in previous studies (Elverfors *et al*. 1997). Our findings are compatible with early *in vivo* microdialysis experiments on somatodendritic dopamine release. In such studies, DAT blockers were sometimes used to facilitate measurements since they increased dopamine levels (Kalivas and Duffy 1991; Robertson *et al*. 1991).

Moreover, we found that treatment of dopamine neuron cultures with BoNT A and B blocked somatodendritic dopamine release, thus providing for the first time an unambiguous direct evidence for the requirement of SNARE proteins, namely SNAP-25 and VAMP. Bergquist and colleagues, with *in vivo* microdialysis, had previously measured somatodendritic dopamine release after treatment with BoNTs, and also found that BoNT A blocked somatodendritic dopamine release (Bergquist *et al*. 2002). However, potential indirect circuitry effects complicate the interpretation of microdialysis experiments. In this case, the authors could not rule out the possibility that the toxins reduced somatodendritic dopamine release indirectly by acting on afferent inputs to dopamine neurons. Our experiments, performed in the presence of CNQX (10 μM) and SR95531 (5 μM) were free of such potential biases. Finally, we confirm with immunocytochemistry that SNAP-25 protein is present in the cell body and dendrites of cultured dopamine neurons.

We thus conclude that somatodendritic dopamine release in cultured dopaminergic neurons requires intact SNAP-25 and VAMP. An exocytotic mechanism of release is compatible with the physiological characteristics that were previously established for dopamine release in the substantia nigra or the ventral tegmental area (see Adell & Artigas (Adell and Artigas 2004) for a review), namely TTX-sensitivity (Kalivas and Duffy 1991; Robertson *et al*. 1991; Santiago *et al*. 1992; Heeringa and Abercrombie 1995), calcium-dependence (Beart and McDonald 1980; Elverfors *et al*. 1997; Chen and Rice 2001), and sensitivity to reserpine (Elverfors and Nissbrandt 1991; Heeringa and Abercrombie 1995). Our results are also compatible with the report of Jaffe and colleagues (Jaffe *et al*. 1998) who were able to detect amperometric events from somata of midbrain dopamine neurons. If somatodendritic dopamine release requires exocytosis, why was activity-dependent FM2-10 labelling not detected from dendrites in our experiments? A first important consideration is that while dendrites contain various pleiomorphic vesicular compartments, they are not known to contain vesicle clusters of a kind similar to what is found in axon terminals. Detection of diffuse FM2-10 signal due to labelling of single vesicles in dendrites would not be detectable under standard imaging conditions. A second point is that the standard FM2-10 loading protocol that we used is well known to stain only axonal terminals. Loading dendrites or somatic regions with styryl dies appears to be difficult and may require much longer dye exposure times that what is required to load axon terminals (Maletic-Savatic *et al*. 1998). We have thus not provided direct visualization of a dendritic vesicular compartment in the current work. However, our data, coupled with the converging results mentioned above, leave little doubt that the somatodendritic DA release measured under our experimental conditions arises from somatodendritic exocytosis.

In the end, let us note that some of our experimental conditions could have been unfavourable to reverse transport. For instance, our experiments were performed at room temperature, which is known to reduce DAT activity. Although we have shown that the DAT was fully functional in our cultures, perhaps raising ambient temperature could uncover a slight contribution for dopamine reverse transport. Identifying conditions where reverse transport contributes to somatodendritic release is an important task as many therapeutic drugs as well as drugs of abuse target the DAT. Another exciting avenue for future research will be to investigate the molecular machinery underlying the exocytotic mechanism of release in dendrites and its calcium sensitivity. The cell culture model that we have established, will be ideally suited to pursue such investigations because it will facilitate the use of various molecular, imaging, electrophysiological and electrochemical techniques. This model will also minimize the confounding influence of indirect circuitry effects.

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