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Dopamine neurons in culture express VGLUT2 explaining their capacity to release glutamate at synapses in addition to dopamine

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Abstract

Dopamine neurons have been suggested to use glutamate as a cotransmitter. To identify the basis of such a phenotype, we have examined the expression of the three recently identified vesicular glutamate transporters (VGLUT1-3) in postnatal rat dopamine neurons in culture. We found that the majority of isolated dopamine neurons express VGLUT2, but not VGLUT1 or 3. In comparison, serotonin neurons express only VGLUT3. Single-cell RT-PCR experiments confirmed the presence of VGLUT2 mRNA in dopamine neurons. Arguing for phenotypic heterogeneity among axon terminals, we find that only a proportion of terminals established by dopamine neurons are VGLUT2-positive. Taken together, our results provide a basis for the ability of dopamine neurons to release glutamate as a cotransmitter. A detailed analysis of the conditions under which DA neurons gain or loose a glutamatergic phenotype may provide novel insight into pathophysiological processes that underlie diseases such as schizophrenia, Parkinson's disease and drug dependence.

Keywords: culture, dopamine, glutamate, mesencephalon, vesicular glutamate transporters.

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Although neurons have long been known to have the capacity to release neuropeptides in addition to small-molecule neurotransmitters such as acetylcholine, glutamate and GABA, the co-release of two different small neurotransmitters by neurons has been less extensively investigated. However, data has been provided in support of the co-release of acetylcholine and ATP at the neuromuscular junction (Morel and Meunier 1981), of norepinephrine and acetylcholine by sympathetic neurons (Furshpan *et al.* 1976; Schweitzer 1987; Fu 1995) and of GABA and glycine in the brainstem and the spinal cord (Russier *et al*. 2002; Wu *et al*. 2002).

Central monoamine-containing neurons have also recently been proposed to release glutamate at synaptic contacts in addition to their standard neurotransmitter. For example, isolated serotonin (5-HT)-containing neurons in culture establish glutamate-releasing synaptic contacts (Johnson 1994a; Johnson 1994b). A similar observation has been made for mesencephalic DA neurons in culture which also establish glutamatergic synapses (Sulzer *et al*. 1998; Bourque and Trudeau 2000; Joyce and Rayport 2000; Sulzer and Rayport 2000; Rayport 2001; Congar *et al*. 2002).

The ability of monoamine neurons to release glutamate suggests that these cell's nerve terminals must have the capacity to package and release glutamate from synaptic vesicles in addition to DA or 5-HT. The recent cloning of three vesicular glutamate transporters, VGLUT1-3 (Ni *et al*. 1995; Bellocchio *et al*. 2000; Takamori *et al*. 2000; Bai *et al*. 2001; Fremeau *et al*. 2001; Hayashi *et al*. 2001; Herzog *et al*. 2001; Takamori *et al*. 2001; Gras *et al*. 2002; Schafer *et al*. 2002; Takamori *et al*. 2002; Varoqui *et al*. 2002) provides new molecular phenotypic markers for glutamate-releasing neurons. Expression of a VGLUT may be necessary and sufficient to allow synaptic release of glutamate. For example, overexpression of VGLUT1 in GABAergic neurons endows these cells with the capacity to release

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Abbreviations used: DA, dopamine; TH, tyrosine hydroxylase; VGLUT1-3, vesicular glutamate transporter1–3.

glutamate (Takamori *et al*. 2000). The localization of these transporters in the brain has lead to the surprising observation that 5-HT-containing neurons express one of these, namely VGLUT3 (Fremeau *et al*. 2002; Gras *et al*. 2002; Schafer *et al*. 2002). However, mRNA for none of these transporters appears to be detectable *in vivo* by *in situ* hybridization in DA-containing neurons of the ventral tegmental area (VTA) or substantia nigra (SN) (Fremeau *et al*. 2001; Herzog *et al*. 2001; Gras *et al*. 2002; Schafer *et al*. 2002). However, it should be noted that the presence of VGLUT3 mRNA has been suggested by one group (Fremeau *et al*. 2002), a finding not replicated by others (Gras *et al*. 2002; Schafer *et al*. 2002). Taken together, these findings suggest two possible explanations for the ability of DA neurons to release glutamate in culture. A first hypothesis is that these neurons express a fourth, currently unidentified vesicular glutamate transporter. An alternate hypothesis is that DA neurons possess a normally repressed ability to express one of the three cloned VGLUTs, and that this potential is revealed under culture conditions, and perhaps under physiopathological conditions.

To test these hypotheses, we have used immunocytochemistry and single-cell RT-PCR to localize and detect VGLUT1-3 in mesencephalic neurons in culture. We find that VGLUT2 is expressed in the majority of DA neurons in culture, providing an explanation for their ability to release glutamate.

Experimental procedures

Cell culture

Postnatal primary cultures were established from neonatal (P0 to P2) Sprague–Dawley rats, as previously described (Congar *et al*. 2002). Mesencephalic dopamine neuron cultures were prepared from small blocs of tissue containing the ventral tegmental area and substantia nigra. Serotonin neuron cultures were prepared by dissecting a small bloc of tissue containing ventral raphé nuclei. Most experiments were performed on 'microisland' cultures in which single or small groups of neurons grow together with a small number of pre-established mesencephalic astrocytes on small 100–200 μm diameter collagen/poly-L-lysine droplets (Congar *et al*. 2002). For our experiments, we selected single neurons that were confirmed to be dopaminergic by immunocytochemistry for tyrosine hydroxylase. Serotonin neurons were identified by immunolabeling against tryptophan hydroxylase. Culture medium for neurons was prepared from Neurobasal A together with B27 (Invitrogen, Burlington, Ontario, Canada) and contained 5% fetal calf serum (Hyclone Laboratories, Logan, UT, USA).

Immunocytochemistry

Single DA and 5-HT neurons were, respectively, identified by immunocytochemistry using tyrosine hydroxylase (TH) (1 : 1000) and tryptophan hydroxylase (TRH) (1 : 250) mouse monoclonal antibodies obtained from Sigma Chemicals Co. (St-Louis, MO, USA). In triple-labeling experiments, a goat polyclonal antibody (1 : 100) was used to label TH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody was less effective than the mouse monoclonal and only permitted the labeling of cell bodies and major dendrites. VGLUT1 (1 : 5000) and VGLUT2 (1 : 1000) antibodies were polyclonal rabbit antibodies and were obtained from Synaptic Systems Gmbh (Göttingen, Germany). The VGLUT3 (1 : 5000) antibody was a kind gift of Dr S. El Mastikawy (INSERM U513, Créteil, France). The SV2 (1 : 400) and glutamic acid decarboxylase (1 : 250) antibodies, developed by Dr K.M. Buckley and Dr D.I. Gottlieb, respectively, were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, The University of Iowa, Iowa City, IA, USA. Images were captured on an Olympus IX-50 microscope (Carsen Group, Markham, Ontario, Canada) using a point-scanning confocal microscope from Prairie Technologies LLC (Madison, WI, USA). Excitation was obtained using Argon ion (488 nm) and helium–neon (633 nm) lasers. Image stacks were collected with a 1-μm slice thickness and reconstructed using Metamorph software from Universal Imaging Corporation (Downington, PA, USA). Images were deconvolved using a nearest neighbor algorithm.

Electrophysiology

Patch-clamp recordings were obtained at room temperature from single isolated neurons in whole-cell mode using a PC-505 patch-clamp amplifier (Warner Instruments Corporation, Hamden, CT, USA), as previously described (Congar *et al*. 2002). Data was digitized using a Digidata 1200 board and Clampex 7 software from Axon Instruments (Foster City, CA, USA). Coverslips were mounted in a recording chamber and perfused with saline containing (in mM): NaCl (140), KCl (5), $MgCl_2$ (2), $CaCl_2$ (2), HEPES (10), sucrose (6), glucose (10), pH 7.35, 300 mOsm. To evoke synaptic currents, isolated neurons, 9–14 days old, were depolarized to +10 mV for 1 ms, thus generating an unclamped action potential that propagated along the axon to activate synapses established on the neuron's own dendrites (autapses). Recorded neurons were confirmed to be dopaminergic by postrecording immunocytochemical staining for tyrosine hydroxylase.

Single-cell collection and RT-mPCR

Borosilicate glass tubing was heat-sterilized and patch pipettes were pulled to a resistance of 4–6 MΩ. Recordings were obtained from cultured mesencephalic neurons and the cells' cytoplasm was aspirated as previously described (Cauli *et al*. 1997). The collected cytoplasm was immediately expelled into a prechilled PCR microtube containing 15 U Ribonuclease Inhibitor (Takara Biomedicals, Otsu, Japan) and 8.3 mM dithiothreitol, mixed, frozen on dry ice, and stored at − 80°C until use. Reverse transcription was carried out overnight at 42°C in the presence of 50 pmol of random hexamers (Applied Biosystems, Foster City, CA, USA), 1X First Strand Buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.5 mM dNTP, 100 U SuperScript II RNase H^- Reverse Transcriptase (all from Invitrogen), 20 U Ribonuclease Inhibitor and 10 mM dithiothreitol. After the denaturation of the reverse transcriptase at 70°C, the RNA complementary to the cDNA was removed by incubating 20 min with 2 U Ribonuclease H (Takara Biomedicals). For multiplex PCR, a two-step protocol modified from the one we have previously published was used (Puma *et al*. 2001). Briefly, the whole reverse transcription reaction was added to the first-round PCR which contained 10 μL 10X PCR buffer, 2 mM $MgCl_2$, 2 U

Platinum Taq DNA polymerase (all from Invitrogen), and 10 pmol of each of the five first-round primer pairs in a final volume of 100 μL. The first step of 20 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 35 s) was followed by an identical second round of 45 cycles except that specific cDNAs were amplified from 4% of the first round products using a single pair of primers in separate PCR microtubes in reactions containing 200 μM dNTP. The following oligonucleotide primers were used: for TH, 5′-CTGTCCGCCCGTGATTTTCTGG-3′ sense and 5′-CGCTGGATGGTGTGAGGGCTGT-3′ antisense (first round), and 5′-GGGGCCTCAGATGAAGAAATTGAAAAA-3′ sense and 5′-AGAGAATGGGCGCTGGATACGA-3′ antisense (second round); for VGLUT1, 5′-CCGGCAGGAGGAGTTTCGGAAG-3′ sense and 5′-AGGGATCAACATATTTAGGGTGGAGGTAGC-3′ antisense (first round), and 5′-TACTGGAGAAGCGGCAGGAAGG-3′ sense and 5′-CCAGAAAAAGGAGCCATGTATGAGG-3′ antisense (second round); for VGLUT2, 5′-TGTTCTGGCTTCTGGTGTCTTACGAGAG-3′ sense and 5′-TTCCCGACAGCGTGCCAACA-3′ antisense (first round), and 5′-AGGTACATAGAAGAGAGCATCGGGGAGA-3′ sense and 5′-CACTGTAGTTGTTGAAAGAATTTGCTTGCTC-3′ antisense (second round); for VGLUT3, 5′-AGGAGTGAAGAATGCCGTGGGAGAT-3′ sense and 5′-ACCCTCCACCAGACCCTGCAAA-3′ antisense (first round), and 5′-GATGGGACCAACGAGGAGGGAGAT-3′ sense and 5′-TGAAATGAAGCCACCGGGAATTTGT-3′ antisense (second round); and for GAD67, 5′-TTTGGATATCATTGGTTTAGCTGGCGAAT-3′ sense and 5′-TTTTTGCCTCTAAATCAGCTGGAATTATCT-3′ antisense (first and second rounds). All primer pairs (custom made by Medicorp, Montréal, QC, Canada or Invitrogen) were designed to flank at least one intron in rat or human genomic sequences according to the NCBI GenBank sequence database. Positive controls for all RT-PCR primers were obtained using mRNA extracted from adult rat striatum. The presence of TH mRNA (Melia *et al*. 1994) and of VGLUT1-2-3 mRNA (Gras *et al*. 2002; Danik *et al*. 2003) in this tissue has been demonstrated previously.

Drugs

Except otherwise stated, all drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Establishment of glutamatergic synaptic contacts by DA neurons

As previously reported (Sulzer *et al*. 1998; Bourque and Trudeau 2000; Congar *et al*. 2002), isolated DA neurons in primary culture establish synaptic terminals that have the capacity to release glutamate. Synaptic currents were measured by whole-cell patch-clamp recordings from isolated DA neurons (Fig. 1a). DA neurons were identified physiologically by the ability of the D2 receptor agonist quinpirole (1 μM) to presynaptically inhibit glutamate-mediated excitatory postsynaptic currents (EPSCs) (38.7 ± 7.6% of control; $p < 0.05$) (Fig. 1b) (Congar *et al*. 2002). In all such neurons ($n = 4$), evoked EPSCs were blocked by CNQX (5 μM), a AMPA-type glutamate receptor antagonist (Fig. 1b). Post-recording immunostaining for TH confirmed the dopaminergic phenotype of all quinpirole-sensitive neurons.

Immunolocalization of VGLUT2 in cultured DA neurons

Immunocytochemical labeling experiments were performed to determine whether the ability of cultured DA neurons to release glutamate is due to expression of one or more of the three recently identified VGLUTs. Double-labeling experiments were first performed on mature neurons (15–17 days in culture) to detect TH together with either VGLUT1, VGLUT2 or VGLUT3. The majority of isolated DA neurons (42 out of 55; 76%) displayed VGLUT2-like immunoreactivity (Fig. 1c). The labeling appeared as large numbers of small varicose-like puncta that decorated the neuron's thick dendritic-like processes (Fig. 1c). The vast majority of VGLUT2-labelled varicosities were also TH-positive (Fig. 1c,d). However, in many neurons, it was quite clear that a significant number of TH-positive/VGLUT2-negative varicosities could also be detected along presumed axonal processes emanating from the same neuron (Fig. 1d). These results suggest a partial segregation of TH-positive/VGLUT2-positive and TH-positive/VGLUT2-negative axonal segments and varicosities. It is to be noted that in many neurons, VGLUT2-positive terminals tended to be closer to the cell body than VGLUT2-negative/TH-positive varicosities (Fig. 1c).

A number of VGLUT2-positive TH-negative neurons were also detected in mesencephalic cultures (not shown). Although VGLUT1 could be detected in a number of TH-negative neurons (Fig. 2a), no detectable expression was found in DA neurons (Fig. 2b).

Immunolocalization of VGLUT3 in 5-HT but not DA neurons in culture

Considering the recently demonstrated expression of VGLUT3 in 5-HT neurons (Fremeau *et al*. 2002; Gras *et al*. 2002), the possibility of a similar expression in DA neurons needed to be determined. We found that no DA neurons were labeled by the anti-VGLUT3 antibody (Fig. 2c). However, as expected, cultured 5-HT neurons expressed abundant levels of VGLUT3 (Fig. 2d), not only in fine varicosities, but also at the somatodendritic level. No detectable immunolabeling either for VGLUT2 or for VGLUT1 (not shown) could be detected in isolated 5-HT neurons.

Mesencephalic GABA neurons are immuno-negative for vesicular glutamate transporters

To further examine the cell-specificity of VGLUT2 expression in cultured VTA/SN neurons, we next determined if VTA/SN GABAergic neurons also expressed VGLUT2. GABA-containing neurons were identified by immunostaining for glutamic acid decarboxylase (GAD-67). The labeling was mostly found in presumed axonal varicosities with very

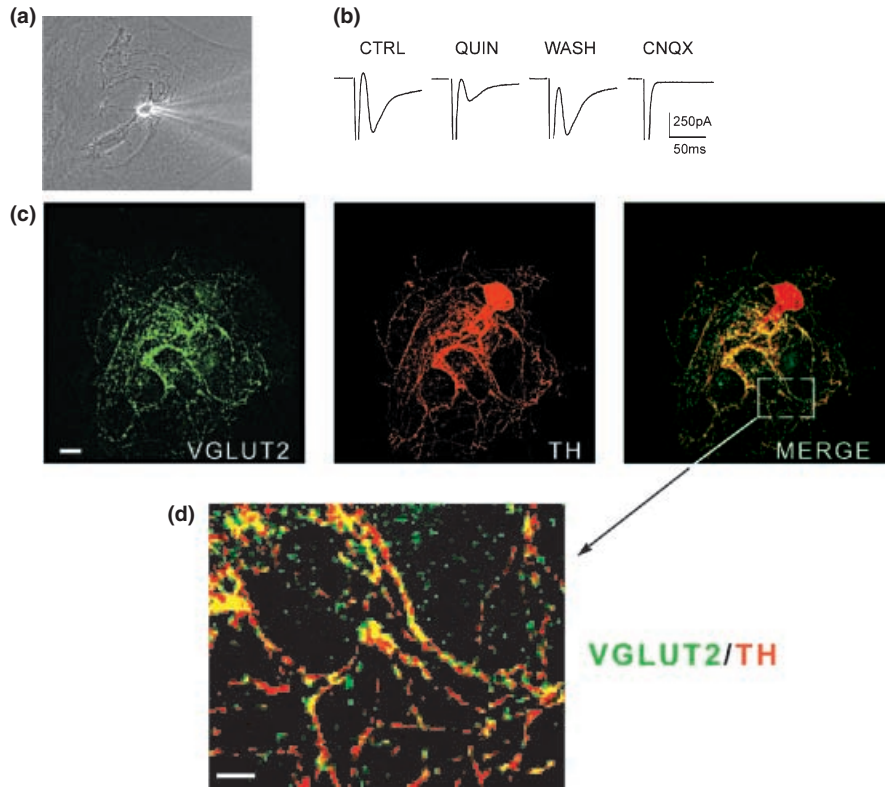

Fig. 1 Expression of VGLUT2 by isolated DA neurons in culture. (a) Phase contrast image of an isolated mesencephalic neuron. Note the presence of a patch pipette on the right, next to the phase-bright cell body of the neuron. (b) A single DA neuron in microculture was recorded by whole-cell patch-clamp. From a holding potential of −70 mV, a brief depolarization to +20 mV induced an unclamped action potential (clipped) followed by a rapid EPSC. The amplitude of this EPSC was inhibited by quinpirole, a D2 receptor agonist and completely blocked by CNQX (5 μM), a AMPA/kainate glutamate receptor antagonist. (c) Confocal image showing VGLUT2 immunolabeling (green) in a TH-positive (red) DA neuron in culture. The merged image (right) shows that most of the VGLUT2 staining is found on TH-positive processes (yellow). Note the punctate, non-somatic expression of VGLUT2. Scale bar: 15 μm. (d) Enlargement from the merged image shown in (c). Note that although most VGLUT2-positive structures were TH-positive (yellow), a number of TH-positive varicosities were VGLUT2-negative (red puncta). Scale bar: 4 μm.

little somatic staining (Fig. 3a). Double-labeling experiments were performed to identify GAD-67 together with VGLUT1 or VGLUT2. GABAergic neurons were found to express neither of these vesicular glutamate transporters (Fig. 3a,b). VGLUT3 was also absent (not shown). These findings are in accordance with the inability of these neurons to release glutamate, as shown by the complete absence of residual synaptic current after blockade of $GABA_A$ receptors (not shown) (Michel and Trudeau 2000).

Cellular coexpression of VGLUT2 and TH mRNAs

To confirm the expression of VGLUT2 in cultured DA neurons, we patch-clamped neurons, collected their cytoplasm and performed a multiplex RT-PCR amplification of the cell's mRNA to detect the phenotypic markers TH, VGLUT1, VGLUT2, VGLUT3, GAD65 and GAD67. Of 11 neurons analyzed, six were confirmed to express TH mRNA and to be negative for GAD65 or GAD67 and were thus considered dopaminergic. Detectable levels of VGLUT2 mRNA were found in three of those neurons (Fig. 4a). These cells were found to be negative for VGLUT1 and VGLUT3 transcripts (Fig. 4a), thus confirming the selective expression of VGLUT2 in DA neurons in culture. Five other neurons were found to contain VGLUT2 mRNA but no detectable levels of the other phenotypic markers (Fig. 4b), suggesting the presence of a subpopulation of purely glutamatergic neurons. Positive controls for all RT-PCR primers were obtained using mRNA extracted from adult rat striatum (Fig. 4c).

Time course of appearance of VGLUT2 expression in cultured DA neurons

The ability of DA neurons to express VGLUT2 was examined at time points from 1 to 21 days in culture. An expression at early time points would suggest that these neurons express this protein from the start, or can rapidly turn on this phenotype, while an expression at delayed time points only would be compatible with the hypothesis that VGLUT2 expression requires long-term withdrawal from

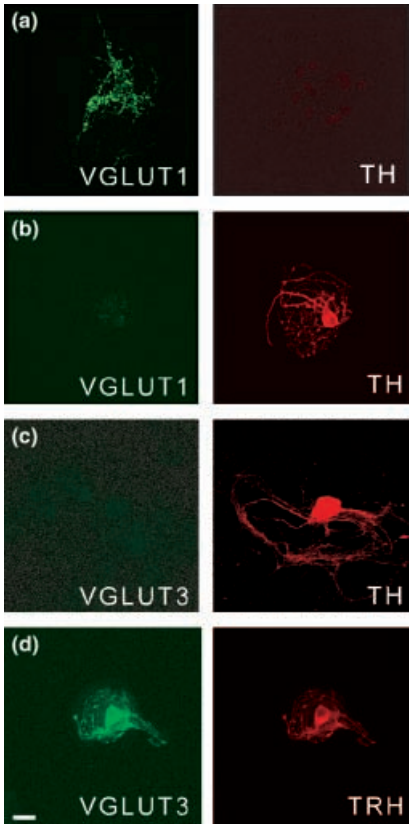

Fig. 2 Dopamine neurons in culture do not express VGLUT1 or VGLUT3. (a) Confocal image showing VGLUT1 immunoreactivity (green) in an isolated mesencephalic neuron. This neuron was TH-negative and thus not dopaminergic. (b) Absence of VGLUT1 immunoreactivity in an isolated DA neuron, confirmed to be dopaminergic by immunolabeling for TH (red). (c) Isolated DA neurons (TH) (red) are immunonegative for VGLUT3. (d) Serotonin neurons, identified by the expression of TRH (red), were double-labelled for VGLUT3 (green). Scale bar: 15 μm.

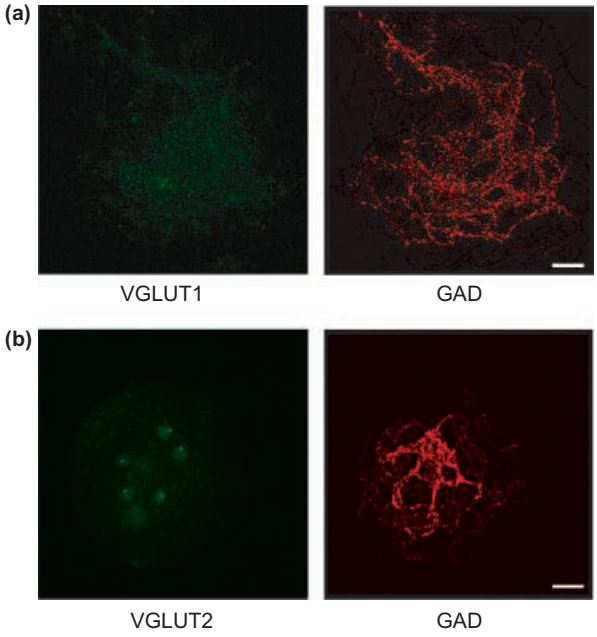

Fig. 3 Mesencephalic GABA neurons do not express vesicular glutamate transporters. (a) Confocal images showing expression of glutamic acid decarboxylase (GAD) in an isolated neuron (red). This neuron was immunonegative for VGLUT1 (left image). Note the exclusively punctate nature of the GAD-67 labeling in presumed GABAergic nerve terminals. Scale bar: 15 μm. (b) Confocal images showing expression of GAD in an isolated neuron (red). This neuron was immunonegative for VGLUT2 (left image). Scale bar: 15 μm.

factors present under *in vivo* conditions. When neurons were examined after only 24 h in culture, the proportion of DA neurons immuno-positive for VGLUT2 was already 51% (44 of 86 neurons examined). In addition, the labeling was mostly somatic, even though the neurons had already extended a limited number of neurites (Fig. 4d). At time points ranging from 3 to 21 days in culture (Fig. 4d,e), the majority of DA neurons expressed punctate VGLUT2 immunoreactivity (from 69 to 82% of DA neurons). At 3 days, VGLUT2 labeling was somewhat less abundant then at later time points, but it was already mostly varicose-like (Fig. 4d). After 6 days in culture and at later time points, only punctate labeling could be observed (not shown).

Expression of VGLUT2 in a subset of nerve terminals in cultured DA neurons

An important question is whether VGLUT2 is expressed in all nerve terminals established by DA neurons. To determine this, a triple-labeling experiment was performed to localize TH, VGLUT2 and SV2 (Bajjalieh *et al*. 1992), a ubiquitous synaptic vesicle protein (Floor and Feist 1989). We found that the vast majority of VGLUT2-positive varicosities in isolated DA neurons also express SV2 (Fig. 5a,b), thus confirming that VGLUT2 labeling mostly originates from nerve terminals. However, it was also clear that DA neurons expressed a large number of nerve terminals (SV2-positive) that were VGLUT2-negative (Fig. 5b), thus providing evidence for a partial segregation.

Discussion

The principal conclusion of this work is that postnatal DA neurons have the potential to express one of the vesicular glutamate transporters, namely VGLUT2, thus providing an explanation for their demonstrated ability to release glutamate in addition to DA at synapses in culture. The expression of VGLUT2 in DA neurons is selective inasmuch as VGLUT1 and VGLUT3 were not detected.

Our finding that 5-HT neurons, cultured under the same conditions and with the same mesencephalic astrocytes as the DA neurons, express VGLUT3 rather than VGLUT2, confirms recent data obtained in culture and *in vivo* (Fremeau

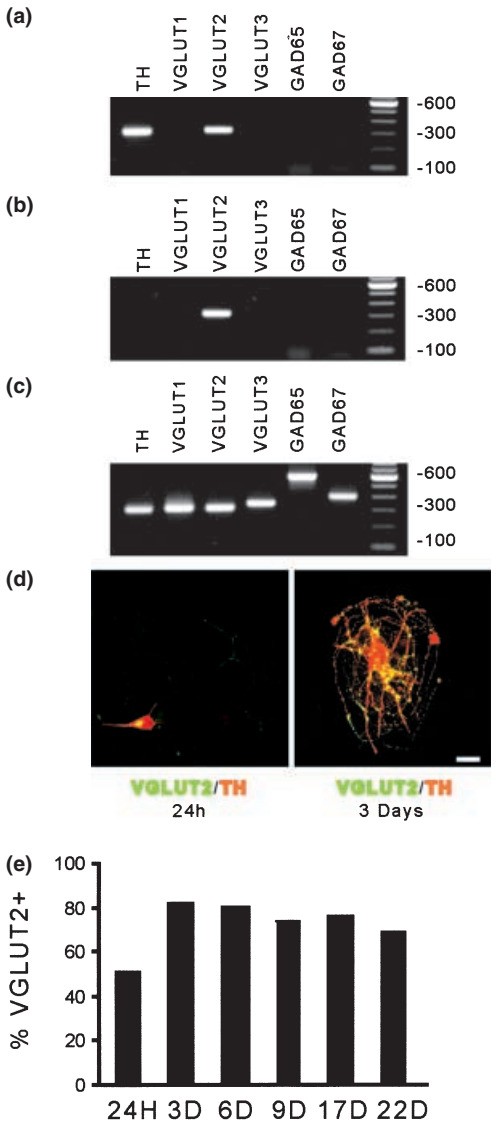

Fig. 4 Detection of VGLUT2 mRNA in DA neurons by single-cell RT-mPCR. (a) Expression profile of an individual cultured mesencephalic DA neuron. Fifteen μL of each second round PCR were analyzed on a 1.7% agarose gel with 1 μg of molecular size standards (100 bp DNA ladder; right lane). Expected size of PCR products in base pairs: TH (301), VGLUT1 (311), VGLUT2 (315), VGLUT3 (345), GAD65 (599) and GAD67 (401). Note that both TH and VGLUT2 mRNA could be detected in this neuron. (b) Putative glutamatergic mesencephalic neuron showing expression of VGLUT2 mRNA only. (c) Positive control obtained using mRNA extracted from adult rat striatum. (d) Confocal image showing expression of VGLUT2 (green) in an isolated TH-positive (red) dopaminergic neuron, 24 h after cell dissociation (left image). Note the somatic and apparently intracellular localization of the signal. The right image shows VGLUT2 expression (green) in a TH-positive neuron (red) after 3 days in culture. Note the predominantly punctate nature of the labeling. Scale bar: 15 μm. (e) Summary data representing the percentage of isolated DA neurons found to be immuno-positive for VGLUT2 after different times in culture.

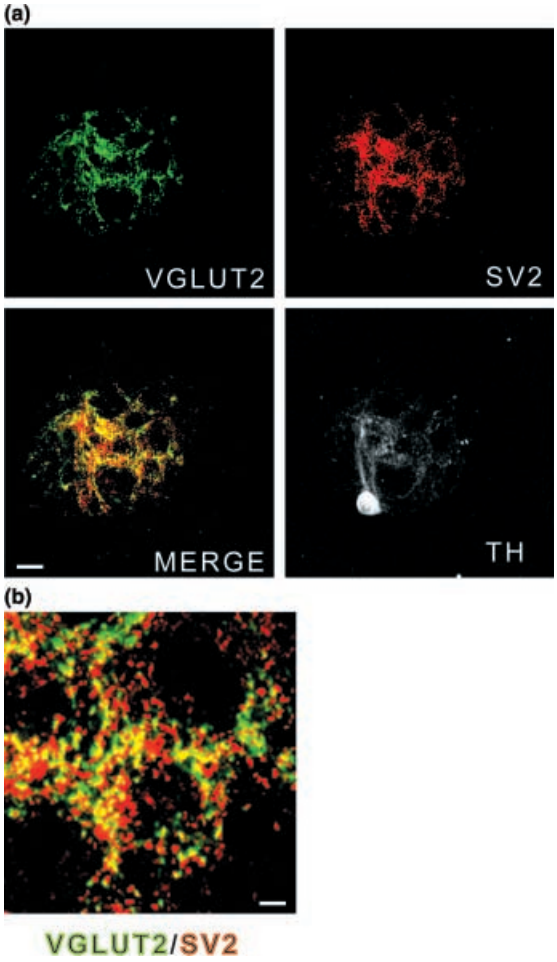

Fig. 5 Expression of VGLUT2 in a subset of nerve terminals established by DA neurons. (a) VGLUT2 immunoreactivity (green) in an isolated DA neuron (upper left image). SV2 immunoreactivity in nerve terminals established by the same neuron is shown in red (upper right image). The merged image shows the colocalization (yellow) of VGLUT2 (green) and SV2 (red) (lower left image). The lower right image provides a phenotypic identification of the same neuron by its immunoreactivity for TH. Scale bar: 15 μm. (b) Blow-up of a region of the merged image illustrating that most VGLUT2-positive structures are also SV2-positive (yellow). Note also that a number of SV2-positive terminals are VGLUT2-negative (red). Scale bar: 4 μm.

et al. 2002; Gras *et al*. 2002; Schafer *et al*. 2002). However, our finding that DA neurons in culture express VGLUT2 stands in relative opposition to the reports that mesencephalic DA neurons in adult rats do not express detectable levels of mRNA for this transporter, as shown by *in situ* hybridization (Fremeau *et al*. 2001; Herzog *et al*. 2001). However, northern blot analysis has reported the presence of VGLUT2 mRNA in substantia nigra (Aihara *et al*. 2000). These authors also reported that VGLUT2 mRNA was very abundant in fetal rat brain. Considering that our primary cultured neurons were prepared from neonatal (P0–P2) animals, one potential interpretation of this discrepancy is

that VGLUT2 is expressed early in development in DA neurons, with its mRNA levels decreasing gradually during the postnatal period. A detailed set of *in situ* hybridization experiments in developing brain will be required to test this hypothesis. An alternate hypothesis is that the ability of DA neurons to express VGLUT2 mRNA may require a perturbation of normal developmental conditions. As such, it will be interesting to evaluate the expression of VGLUT2 mRNA in pathophysiological animal models implicating DA neurons. Additional experiments both in culture and *in vivo* will also be necessary to identify some of the regulatory signals that control transcription of the VGLUT2 gene. Finally, it should be noted that although VGLUT2 mRNA levels are undetectable by standard *in situ* hybridization labeling in adult rodent brain, this by no means excludes that low level expression of this gene still occurs in adult animals at a level sufficient to maintain the capacity of at least a proportion of DA neurons to release glutamate in addition to DA. An ultrastructural analysis of the presence of VGLUT2 protein in the axon terminals of DA neurons in the striatum *in vivo* could help to test this hypothesis.

We found the majority of isolated DA neurons to be immunopositive for VGLUT2 protein. Modest immunostaining for VGLUT2 has also previously been reported in the SN compacta and VTA (Fremeau *et al*. 2001; Varoqui *et al*. 2002). However, this expression has not been demonstrated conclusively to be present in the cell bodies of DA neurons as opposed to synaptic terminals originating from other brain areas and terminating onto DA neurons or local GABA neurons. Considering the predominantly terminal nature of VGLUT2 expression, it may be extremely difficult to prove conclusively by immunostaining that any given neuron's cell body is positive for this transporter in intact tissue. In addition, the possibility that astrocytes are responsible for some of the immunoreactivity to VGLUT2 *in vivo* has also not been examined directly. A background level of VGLUT2 immunostaining in astrocytes was detected in our experiments (not shown), but this signal was much lower than the neuronal signal. RT-PCR and immunostaining experiments in purified astrocyte preparations might be useful to evaluate the specificity of this signal.

In the current set of experiments, we found that ~80% of isolated DA neurons display VGLUT2 immunoreactivity. However, in single-cell RT-PCR experiments, VGLUT2 mRNA could be detected in only half of the recorded DA neurons, although TH mRNA could be detected in all. This modest discrepancy most likely results from variable efficiency of cytoplasm aspiration, making it more difficult to detect less abundant mRNA species such as that for VGLUT2.

Our findings consolidate the concept of cotransmission in neurons releasing two small molecule neurotransmitters. The demonstration that only a subset of the neurons' synaptic terminals appear to express VGLUT2 argues for the hypothesis that DA neurons in culture can extend axonal segments that differentially contain or exclude VGLUT2. Our conclusion is compatible with previous data suggesting that glutamate immunoreactivity, although present in most processes of cultured DA neurons, is also found in a limited subset of TH-negative processes localized close to the cell body (Sulzer *et al*. 1998). This phenotypic heterogeneity between different sets of terminals expressed by a single neuron is an interesting biological problem. Although speculative, our observation that VGLUT2-positive terminals appear to be closer to the cell body than VGLUT2-negative varicosities, argues for the existence of some local instructive cues that may originate from the cell body and act in some paracrine fashion to promote the establishment of local glutamatergic terminals by the neurons. Such cues could perhaps drive the establishment of glutamate-releasing terminals by DA neurons *in vivo* during early development. Ultrastructural studies are required to more precisely evaluate the relationship between VGLUT2-positive and VGLUT2-negative terminals along specific axonal segments in cultured DA neurons and to determine the differential localization of VGLUT2-containing and vesicular monoamine transporter (VMAT2)-containing vesicles in single terminals.

In summary, our data provide a logical explanation for the ability of cultured DA neurons to release glutamate at synapses in addition to DA. Together with the recent demonstration that 5-HT neurons (Fremeau *et al*. 2002; Gras *et al*. 2002) and a subset of noradrenergic neurons (Stornetta *et al*. 2002a; Stornetta *et al*. 2002b) also express vesicular glutamate transporters, our findings support the hypothesis that glutamate cotransmission in monoaminergic neurons is a general phenomenon rather than an exception. A detailed analysis of the conditions under which DA neurons gain or loose the capacity to release glutamate may provide novel insight into pathophysiological processes that underlie diseases such as schizophrenia, Parkinson's disease and drug dependence.

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