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Dopamine Neurons Make Glutamatergic Synapses *In Vitro*

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Interactions between dopamine and glutamate play prominent roles in memory, addiction, and schizophrenia. Several lines of evidence have suggested that the ventral midbrain dopamine neurons that give rise to the major CNS dopaminergic projections may also be glutamatergic. To examine this possibility, we double immunostained ventral midbrain sections from rat and monkey for the dopamine-synthetic enzyme tyrosine hydroxylase and for glutamate; we found that most dopamine neurons immunostained for glutamate, both in rat and monkey. We then used postnatal cell culture to examine individual dopamine neurons. Again, most dopamine neurons immunostained for glutamate; they were also immunoreactive for phosphate-activated glutaminase, the major source of neurotransmitter glutamate. Inhibition of glutaminase reduced glutamate staining. In single-cell microculture, dopamine neurons gave rise to varicosities immunoreactive for both tyrosine hydroxylase and glutamate and others immunoreactive mainly for glutamate, which were found near the cell body. At the ultrastructural level, dopamine neurons formed occasional dopaminergic varicosities with symmetric synaptic specializations, but they more commonly formed nondopaminergic varicosities with asymmetric synaptic specializations. Stimulation of individual dopamine neurons evoked a fast glutamatergic autaptic EPSC that showed presynaptic inhibition caused by concomitant dopamine release. Thus, dopamine neurons may exert rapid synaptic actions via their glutamatergic synapses and slower modulatory actions via their dopaminergic synapses. Together with evidence for glutamate cotransmission in serotonergic raphe neurons and noradrenergic locus coeruleus neurons, the present results suggest that glutamatergic cotransmission may be the rule for central monoaminergic neurons.

Key words: glutamate; cotransmission; mesolimbic; nigrostriatal; cell culture; ventral tegmental area

Ventral midbrain (VM) dopamine (DA) neurons play a pivotal role in the organization of movement and behavior (Iversen, 1995; Williams and Goldman-Rakic, 1995; Montague et al., 1996). Degeneration of substantia nigra (SN) DA neurons gives rise to Parkinson's disease, whereas aberrant activity of ventral tegmental area (VTA) DA neurons appears to underlie psychosis in schizophrenia (Egan and Weinberger, 1997). Natural rewards are potent activators of VTA DA neurons, so that psychostimulants that cause supraphysiological release of DA may reinforce their own use, accounting in part for their addicting properties (Robinson and Berridge, 1993; Di Chiara, 1995; Mirenowicz and Schultz, 1996). Thus, the synaptic actions of DA neurons have been the focus of considerable interest; however, they have been difficult to resolve (Grenhoff and Johnson, 1997). DA appears to be released in more of a paracrine than a synaptic manner. Single DA neuron spikes evoke overflow of DA beyond the synapse (Garris et al., 1994), and DA receptors as well as the DA transporter are often found at a distance from release sites (Pickel et al., 1996), together raising the question as to the role of the synaptic specializations of DA neurons.

Several lines of evidence suggest that DA neurons release an excitatory amino acid such as glutamate (GLU). An early study showed that SN stimulation evoked fast EPSPs in striatal (STR) neurons (Kitai et al., 1976), although this was later ascribed to the collateral activation of cortical afferents (Wilson et al., 1982). In a recent study, stimulation of DA neuron axons in the median forebrain bundle evoked fast non-DAergic excitation as well as slower DAergic excitation (Gonon, 1997). Although the fast response could result from attributable to activation of fibers of passage, in SN–STR cortex slice cocultures in which such fibers should be lacking, stimulation of the SN also evoked fast excitatory responses in STR neurons (Plenz and Kitai, 1996).

Because most DA neurons immunostain for phosphate-activated glutaminase (PAG), the biosynthetic enzyme (EC 3.5.1.2) for neurotransmitter GLU, DA neurons may also be GLUergic (Kaneko et al., 1990). Single DA neurons examined at the ultrastructural level appear to have not only DAergic terminals, identified by staining for the DA synthetic enzyme tyrosine hydroxylase (TH), that have symmetric synaptic specializations (associated with inhibitory actions), but also non-DAergic terminals, identified by orthograde [^{3}H]leucine transport, that have asymmetric synaptic specializations (associated with excitatory actions) (Hattori et al., 1991). In the nucleus accumbens (nAcc),

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a major mesolimbic target, immunostaining for DA itself reveals terminals with symmetric as well as asymmetric specializations (Ikemoto et al., 1996). Finally, 6-hydroxy-DA lesions of DA neuron cell bodies reduce the number of terminals with asymmetric specializations in the STR by 20% (MacMillan et al., 1997), possibly reflecting the loss of non-DAergic terminals of DA neurons. These morphological observations are consistent with the possibility that some DA neuron synapses mediate fast excitation.

To address this issue, we have first shown that most DA neurons in the intact brain as well as postnatal VTA cell culture immunostain for GLU. GLU immunostaining appears to reflect neurotransmitter GLU, based on a comparative analysis of neurotransmitter immunostaining and on demonstrating that inhibition of PAG leads to a reduction in GLU immunostaining (as would be expected if the GLU visualized reflects neurotransmitter GLU). In dual-immunostained single-cell microcultures, we have seen that DA neurons give rise to two sets of varicosities, one set that is both DAergic and GLUergic and another set that appears to be mainly GLUergic. At the ultrastructural level, single DA neurons give rise to synapses with both DAergic symmetric and non-DAergic asymmetric synaptic specializations. Stimulating DA neurons in microculture elicited strong GLUergic autaptic excitation that was modulated by concomitant DA release via a presynaptic mechanism. Together with morphological studies in the intact brain (Hattori et al., 1991), these observations show that DA neurons release GLU and may do so selectively at a subset of their synapses.

MATERIALS AND METHODS

Preparation of brain sections. Following animal protocols approved by Columbia University, NYS Psychiatric Institute, and the University of Rochester, adult male rats and old world monkeys (*Macaqua nemestrina*) were deeply anesthetized with ketamine and perfused with 4°C heparinized saline followed by 0.3% glutaraldehyde and 4% paraformaldehyde; 0.1 mg/ml ketamine was added to the saline to maintain GLU blockade during fixation, which markedly reduced background GLU staining. Free-floating cryostat sections (50 μm) were double fluorescence immunostained as described below.

Cell culture. Mass cultures were prepared from the VTA, ventral midbrain, nAcc, cerebellum, and hippocampus of postnatal day 2 (P2)–P4 rat pups using our previously established methodology for VTA and nAcc neurons (Rayport et al., 1992; Shi and Rayport, 1994). Animal protocols were approved by the Institutional Animal Care and Use Committees of Columbia University and the NYS Psychiatric Institute. On the first of 2 culture days, two pups were anesthetized with ketamine and then chilled in ice chips; their cerebral cortices were enzymatically dissociated as a source of astrocytes. One hour before use, microwell dishes that had been prepared in advance (by making 12-mm-diameter circular holes in the bottoms of Petri dishes and attaching poly-D-ornithine-coated coverslips to form 100 μl microwells) were coated with laminin. Dissociated cortical cells were then plated; 1 hr later, they were washed vigorously with cold medium to dislodge most cells, leaving only tightly adherent astrocytes. Astrocytes reached near confluence after ~1 week; further division was then inhibited with fluorodeoxyuridine.

On the second of 2 culture days, 20 pups were prepared as described above. A 2-mm-thick midline sagittal slice was made, and the VTA was isolated in a 2 × 2 × 2 mm cube following established landmarks (Rayport et al., 1992, their Fig. 4). This cube was further divided, and the resulting 1 × 1 × 1 mm segments were incubated in papain at 32°C under continuous oxygenation with gentle agitation for 90 min. The papain was quenched with 10% calf serum, and the tissue segments were dissociated by gentle trituration in the presence of DNase. Neurons were resuspended in serum-free media (to which 1% serum was added to ensure glial longevity) and plated onto the preestablished cortical astrocytic monolayers in the microwells. Cultures were maintained in a total volume of 2.5 ml, which filled the whole dish, and were never fed. Except as noted, 0.5 mM kynurenate (KYN) (Sigma, St. Louis, MO) was included in the culture medium to block excitotoxicity.

Microcultures were prepared following established methods (Segal and Furshpan, 1990). Briefly, coverslips were coated with agarose to create a substrate unfavorable for cell attachment and then mounted to make microwell dishes. Collagen (Vitrogen 100, Collagen Corporation) was applied as an aerosol to form substrate islands (50–150 μm in diameter) that were favorable for cell attachment. On the first culture day, dissociated cortical cells were plated to form a glial substrate on the collagen-coated areas; unattached cells were washed away with cold medium after 2 hr. Astrocytes grew to confluence on the collagen dots after ~1 week; a typical microwell had ~50 glial islands. On the second culture day, dissociated VTA cells were plated at a density titrated to maximize the number of single neuron microcultures.

Glutaminase inhibition. Glutaminase inhibition studies were performed on mass cultures. The 6-diazo-5-oxo-norleucine (DON) enantiomers (Sigma) were applied for 20 hr at a concentration of 5 mM. Cultures were then fixed for TH–GLU immunocytochemistry. Occasionally very intense GLU^+ cells were seen, possibly resulting from upregulation of PAG after inhibition with L-DON (Kaneko et al., 1992); this reduced the overall diminution in GLU staining. Consistent with this, there was massive GLU-mediated cell death in L-DON-treated cultures if GLU receptors were not blocked pharmacologically. Therefore, this series of experiments was performed using the standard concentration of kynurenate and 10 μM CNQX (Tocris).

Immunocytochemistry. For immunostaining, cells were fixed with 0.3% glutaraldehyde and 4% paraformaldehyde and permeabilized with 1% Triton X-100. This relatively high concentration of Triton X-100 maximized penetration of antisera, so that in the case of TH staining we found stained cells throughout the depth of sections and in cultures saw that cell bodies (typically the thickest parts of the culture) were stained completely. Primary antisera were applied overnight in the culture microwells at 4°C with slow agitation. Secondary antibodies were applied at room temperature for 1 hr. We used fluorescein or rhodamine secondary antisera at 1:200 (Chemicon, Temecula, CA) or the ABC method with diaminobenzidine (DAB) as the chromagen (Vectastain Elite kit). For double or triple staining, we used the following antibody combinations: a 1:200 dilution of a polyclonal anti-TH antiserum (Chemicon) and a 1:2000 dilution of a monoclonal anti-GLU antiserum (Glu2, 1:2000; Incstar, Stillwater, MN) (McDonald et al., 1989) with fluorescence; 1:10,000 anti-TH polyclonal with DAB followed by 1:50 anti-synaptophysin monoclonal (Chemicon) and 1:2000 anti-GLU by fluorescence; 1:2 anti-TH monoclonal (Boehringer Mannheim, Indianapolis, IN) and 1:1000 polyclonal anti-GABA (Sigma); 1:200 anti-TH and 1:250 anti-PAG monoclonal IgM (a gift from Takeshi Kaneko, University of Kyoto) (Kaneko et al., 1990) with fluorescence. For cell counts, scaled images (see below) were displayed using NIH Image software 1.61 (Wayne Rasband, National Institutes of Health; http://rsb.info.nih.gov/nih-image) with a 32-color pseudocolor scale. Representative fields were examined to identify cells that were clearly positive and ones that were clearly negative. Using these levels of staining for reference, other fields were then scored.

Imaging. Both Nomarski differential interference contrast and epifluorescence images were acquired with a chilled CCD digital camera (Star1 Camera, Photometrics; IP-Lab Spectrum 3.1 software, Signal Analytics, running on a Power Macintosh). Throughout a given experiment, imaging parameters were held constant, the epifluorescence field iris was stopped down to just outside the region of interest to reduce background light scattering, and 2 or 5 sec exposures were made with the camera in the high-gain mode. Varicosity staining was resolved by digital deconvolution of stacks of images to obtain confocal slices using MicroTome 2.0 (VayTek) running under IP-Lab Spectrum. For display, the 12 bit IP-Lab images (4095 shades of gray) were converted to 8 bit images (256 shades of gray), scaling the extremes of the image intensity range to the full 8 bit dynamic range. Color images and merges were made by placing the individual 8 bit monochrome images in the red or green red–green–blue channels of 24 bit color images (NIH Image software or IP-Lab Spectrum). Plates were made using Adobe Photoshop 4.0 and Macromedia FreeHand 7.

Electron microscopy. For electron microscopy, cultures were stained for TH using the ABC reaction and DAB and then dehydrated and embedded following established protocols (Harris and Rosenberg, 1993). Dishes were inspected at the light microscopic level to find compact single-neuron microcultures, which were then serial sectioned. The relatively high Triton X-100 concentration assured antibody penetration, as was reflected at the ultrastructural level in TH staining throughout DA neuron cell bodies. Although this approach unavoidably damaged mem-

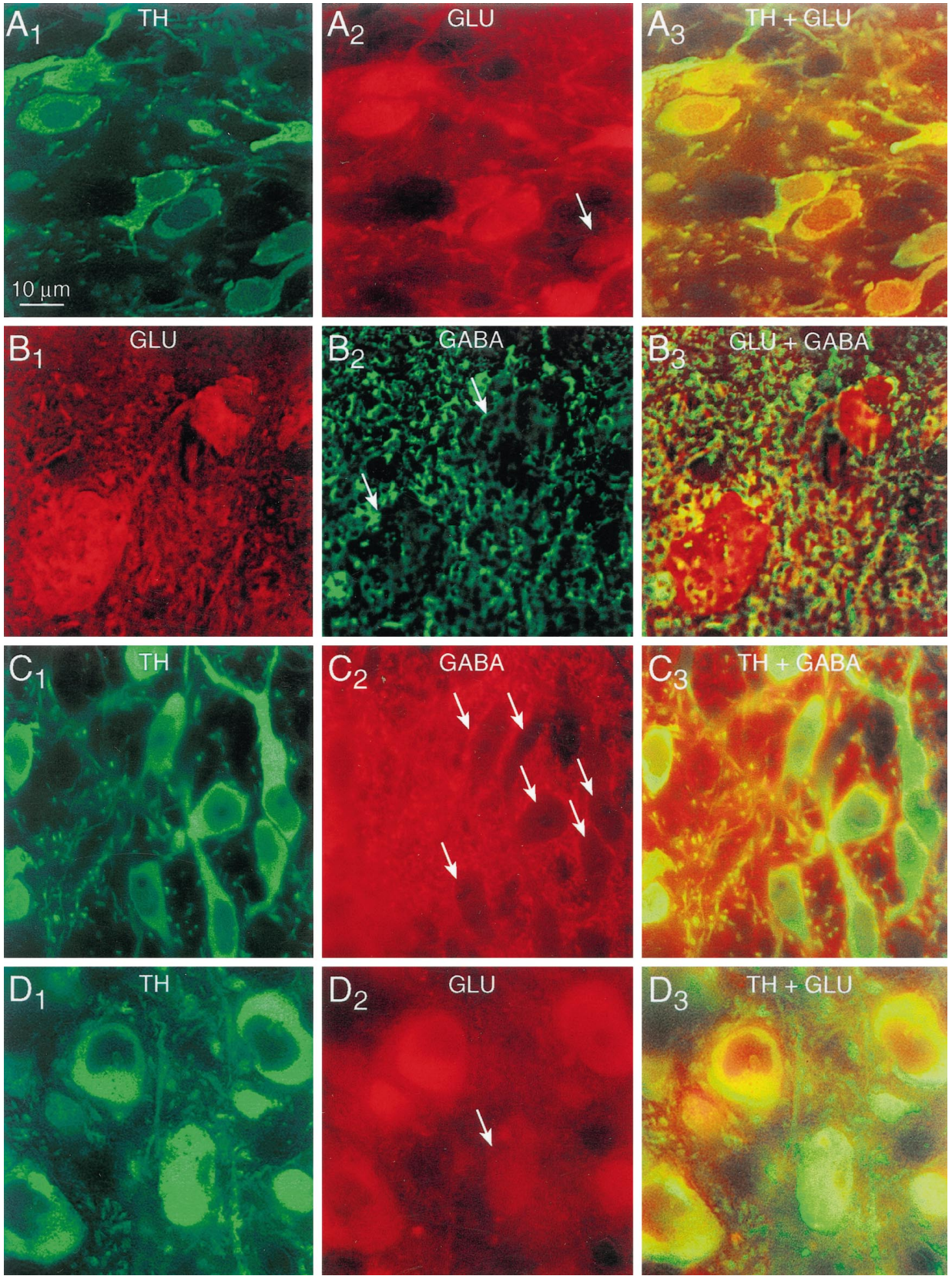

Figure 1. Immunostaining of DA neurons in VM sections for GLU and GABA. Coronal sections of rat and monkey VM were double-immunofluorescence-stained for the DA synthetic enzyme TH and GLU or GABA. *A*, In rat VM, the majority of DA neurons (A_1) were GLUergic (A_2); occasional DA neurons (A_2, *arrow*) were non-GLUergic. In a color merge (A_3), in which colocalization appears *yellow*, neuronal nuclei appear *red*, reflecting selective GLU staining because TH is cytoplasmic. The dense cortical GLUergic projection to the DA cell groups accounts for the strong GLUergic staining of the neuropil. *B*, GLUergic (B_1) neurons are not GABAergic (B_2), arguing that precursor GLU does (*Figure legend continues*).

branes, compared with conventional electron microscope preservation techniques (cf. Sulzer and Rayport, 1990; Rayport et al., 1992), presynaptic and postsynaptic specializations were well preserved.

Electrophysiology. For recordings, cultures were placed on the stage of an inverted microscope (Zeiss), and the medium was replaced with oxygenated extracellular solution containing (in mM): 135 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.35, at room temperature. The bath was perfused continuously in some experiments using a gravity flow system. Electrodes were pulled on a Flaming-Brown P-80 PC micropipette puller (Sutter). The intracellular solution contained (in mM): 140 gluconic acid, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 ATP-Na_2, 0.1 GTP-Na, and 10 HEPES, pH 7.25, with KOH. Electrode resistances were 4–7 MΩ. After formation of a gigaohm seal, whole-cell mode was achieved with brief suction. In some experiments, cells were recorded using the nystatin perforated patch technique (Korn et al., 1991). Voltage and current signals were recorded using an Axoclamp 2A or Axopatch 200 interfaced to a Pentium PC (TL1–25 interface; Axon Instruments, Foster City, CA) running pClamp 6.0 (Axon) or a Power-Macintosh (Instrutech ITC-16 interface) running Pulse Control 4.7 (Richard J. Bookman, University of Miami; http://chroma.med.miami.edu/cap) under IgorPro 3.0 (Wavemetrics). Off-line data analysis was performed using Microsoft Excel and IgorPro. Numerical data are expressed as mean ± SEM, and significance of differences were evaluated by *t* test. Drugs were applied by local perfusion using a Y-tube system (Greenfield and Macdonald, 1996). At the end of experiments, cells were fixed on the stage of the microscope, their *x,y* coordinates were noted, the field was imaged, and a circle was scribed on the underside of the coverslip (Zeiss objective maker) to facilitate relocation of recorded cells after immunocytochemistry.

RESULTS

GLU staining of DA neurons *in situ*

Because known GLUergic neurons display strong cytoplasmic GLU immunoreactivity (Storm-Mathisen and Ottersen, 1990), we double immunostained VM sections (coronal sections including both the SN and VTA) for TH and GLU (Glu2 monoclonal) to determine whether DA neurons were GLU^+. In rat, we found that 91 ± 4% of DA neurons were GLU^+ (n = 1551 neurons in 13 sections from four rats) (Fig. 1*A*). The incidence of colocalization in SN and VTA was not significantly different, so the data were combined. The presence of GLU^- DA neurons suggests that metabolic GLU, which should be present at the same level in all DA neurons, does not contribute significantly to the GLU staining of DA neurons *in vivo*. To rule out staining of GLU that acts as a GABA precursor, we double-stained sections for GLU and GABA; within the SN and VTA, $GABA^+$ cell bodies were always GLU^- (Fig. 1*B*). Moreover, double staining for TH and GABA showed that TH^+ neurons were always $GABA^-$ (Fig. 1*C*), as previously reported (Kosaka et al., 1987). In the monkey, 86 ± 6% of DA neurons were GLU^+ (n = 714 neurons in four sections from four monkeys) (Fig. 1*D*). We were unable to assess how the GLU immunostaining of DA neurons compared with that of known GLUergic neurons in the hippocampus and cortex, because afferent staining was so intense in those areas that cell body staining could not be resolved.

GLU staining of DA neurons *in vitro*

We then used postnatal cell cultures made from restricted dissections of the VTA, in which 50% of the neurons are DAergic (Rayport et al., 1992) and the others are almost entirely GABAergic (L. Lin and S. Rayport, unpublished observations), to ask whether the GLU immunoreactivity reflects neurotransmitter GLU. As in brain sections, we found by double immunostaining that *in vitro* 84 ± 5% of VTA DA neurons were GLU^+ (n = 1503 neurons in 12 cultures prepared on five separate culture days) (Fig. 2*A*). We obtained similar levels of colocalization in SN cultures. We corroborated these results using a polyclonal GLU antiserum (Arnel, New York, NY) (Hepler et al., 1988); moreover, a recent EM study using this antibody (Smith et al., 1996) revealed significant GLU staining of DA neuron dendrites in the intact VM.

We used cell cultures from brain regions with well characterized cell types to verify further the specificity of the Glu2 GLU antiserum. In cultures from cerebellum, in which small cells are GLUergic granule cells and large cells are GABAergic Purkinje cells, we found that granule cells were GLU^+ and $GABA^-$. Purkinje cells were $GABA^+$, but they were also GLU^+ (Fig. 2*B*). Similarly, in cultures from hippocampus (Fig. 2*C*) and nucleus accumbens (data not shown), most GLU^+ cells were $GABA^-$, consistent with their being bona fide GLUergic neurons, whereas $GABA^+$ cells were almost always GLU^+. This indicates that *in vitro*, Glu2 recognizes both GLUergic neurons and GABAergic neurons, in which GLU is likely to be present as a GABA precursor. This differs from the situation in the intact brain (Ottersen and Storm-Mathisen, 1984; Conti et al., 1987), presumably because neurons in culture are quiescent so that precursor GLU levels build up to immunocytochemically detectable levels.

Contrary to the situation in the intact VTA and SN, some DA neurons appear to be GABAergic. In a previous study, 2% of SN and 0.6% of VTA DA neurons in high-purity postnatal cultures were $GABA^+$ (Masuko et al., 1992). In our cultures (Fig. 2*D*), 11 ± 1.6% of DA neurons were $GABA^+$ (n = 299 cells in eight cultures). These $TH^+/GABA^+$ VM neurons may derive from a minority population of SN reticulata neurons that send collateral projections to both the tectum and the striatum and contain both DA and GABA (Campbell et al., 1991). In contrast, hypothalamic DA neurons are extensively GABAergic (Schimchowitsch et al., 1991). Subtracting the fraction of DA neurons that are GABAergic (in which GLU staining may reflect precursor GLU) from the fraction that are GLUergic (reported above) yielded a corrected incidence of 73% of DA neurons that are GLUergic.

To examine a marker more specific to neurons using GLU as a transmitter, we double stained DA neurons for PAG (Fig. 3). We found that 51.2% of DA neurons were PAG^+ (n = 78 neurons). In nAcc cultures, which do not contain intrinsic GLUergic neurons, there was no PAG staining (Fig. 3*B*), whereas in hippocampal cultures, in which the majority of neurons are GLUergic, many neurons stained for PAG (Fig. 3*C*). If in fact PAG activity gives rise to the neurotransmitter GLU visualized by immunostaining, then inhibition of PAG should reduce the incidence of GLU staining of DA neurons (Fig. 4). So, we pretreated cultures with the irreversible PAG inhibitor 6-diazo-5-oxo-L-norleucine (L-DON) and its inactive enantiomer D-DON. L-DON reduced the incidence of GLU colocalization in DA neuron cell bodies from 89 ± 7% to 59 ± 12%, whereas D-DON had no effect (91 ± 8%) (n = 50 cells in each of three cultures per condition in three

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not account for GLU staining; this is shown as a color merge in B_3. In this field, both GLU^+ neurons are $GABA^-$ (B_2, *arrows*). *C*, Furthermore, DA neurons (C_1) are never GABAergic (C_2); this is seen more clearly in the color merge (C_3). In this field, all nine DA neurons are $GABA^-$ (some are indicated by C_2, *arrows*). *D*, In monkey VM, the majority of DA neurons (D_1) were GLUergic (D_2). Occasional DA neurons (D_2, *arrow*) were non-GLUergic. This is shown as a color merge in *D3*.

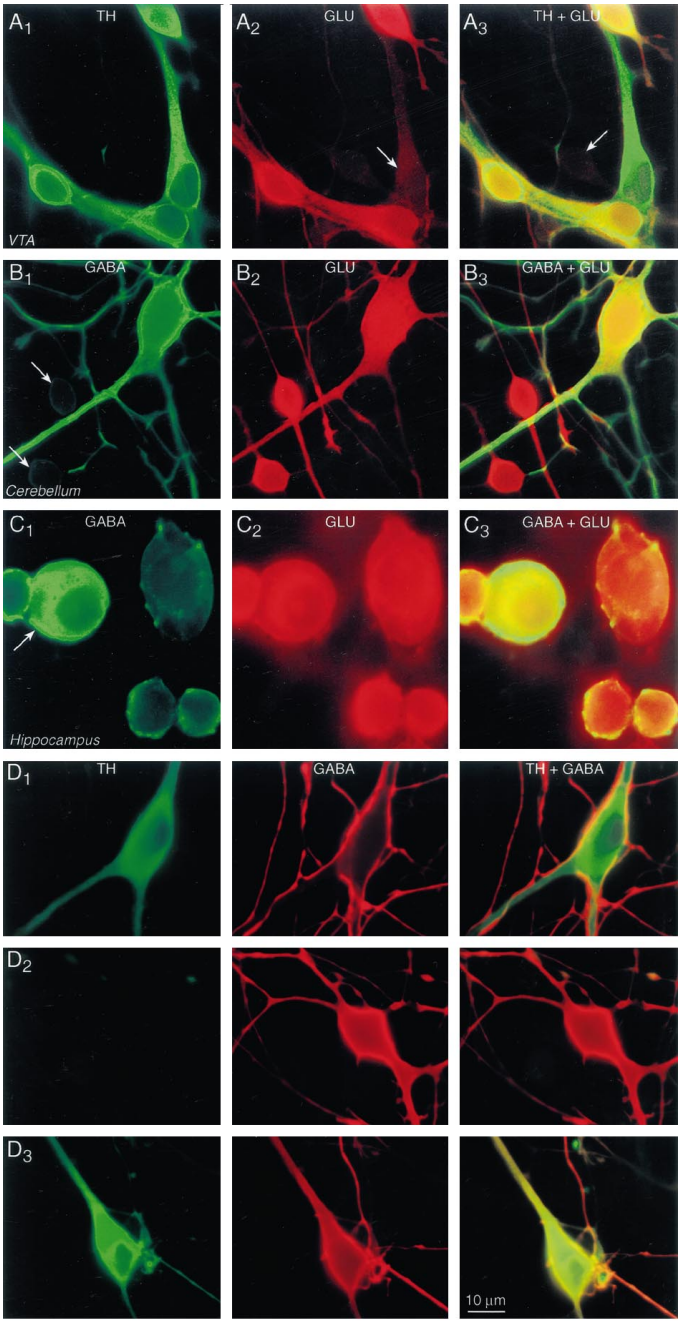

Figure 2. Immunostaining of DA neurons for GLU *in vitro*. To evaluate GLU staining of DA neurons, mass cultures of VTA, cerebellum, and hippocampus were immunostained for GLU and GABA. *A*, *In vitro* the majority of DA neurons in VM cultures (A_1) were GLUergic (A_2); occasional DA neurons (A_2, *arrow*) were non-GLUergic. In the color merge (A_3), in which colocalization appears *yellow*, neuronal nuclei appear *red*, reflecting selective GLU staining. A neuron that is neither TH^+ nor GLU^+ is seen (A_3, *arrow*). *B*, In a cerebellar culture in which granule cells, which are small and GLUergic, can be distinguished from Purkinje cells, which are large and GABAergic, only the putative large Purkinje cell stains for GABA (B_1), whereas the two granule cells do not stain (*arrows*). However, both the Purkinje cell as well as the granule cells appear GLUergic (B_2). This is seen more clearly in the color merge (B_3). In this experiment, all large neurons were $GABA^+$ and GLU^+ ($n = 16$), whereas all small neurons were only GLU^+ ($n = 40$). This indicates that *in vitro* GABA neurons contain appreciable GLU, which is likely to be present as a precursor to GABA. *C*, Hippocampal neurons are either GLUergic (majority) or GABAergic (minority). In this culture, occasional cells stained for GABA (C_1, *arrow*), whereas most stained for GLU (C_2). In this experiment, 100% of $GABA^+$ neurons were also GLU^+, whereas 27% of GLU^+ neurons were $GABA^+$ ($n = 38$). So again, GLU staining appears to identify cells that are GLUergic as well as GABAergic cells, whereas GLU is likely present as a precursor to GABA. *D*, Immunostaining of precursor GLU was not so much of a confound in VTA cultures because most DA neurons were not GABAergic (D_1) and most GABA neurons were not DAergic (D_2). However, occasional DA neurons were GABAergic (D_3) (see Results for incidence).

experiments). GLU staining of thin processes and varicosities (which we have shown previously to be axonal) was largely eliminated by L-DON (Fig. $4C_2$).

Identification of two sets of synaptic varicosities

To examine the relationship between the DAergic and GLUergic synapses of single DA neurons, we first TH immunostained single VTA neurons in microculture. We found that the intensity of TH staining varied considerably both in the processes and varicosities of single DA neurons, consistent with the possibility that the cells have non-DAergic release sites (Fig. 5*A*). Second, we immunostained the same microcultures for the intrinsic synaptic vesicle membrane protein synaptophysin (SYN); this revealed a number of TH^-/SYN^+ release sites. Third, we immunostained for GLU; this revealed that the TH^- release sites were GLU^+. Such $TH^-/SYN^+/GLU^+$ release sites were found in 75% of the single cell microcultures so examined ($n = 8$). They were invariably near the cell body, regardless of the size of the microculture. In single DA neuron microcultures that were double-immunofluorescence-stained for TH and GLU, the majority of varicosities stained for both transmitters, whereas a minority stained for GLU alone (Fig. 5*B*). TH^-/GLU^+ sites were seen near the cell body overlaying the proximal dendrites, whereas the TH^+/GLU^+ sites were more peripherally distributed in the microculture, and in most instances not in contact with dendrites.

We examined single TH^+ neurons in compact microcultures (Fig. 6, *inset*) at the ultrastructural level ($n = 4$). We used a high detergent concentration to maximize antibody penetration. Although this unavoidably damaged membrane preservation, presynaptic and postsynaptic specializations were, in fact, more easily discerned. Somatic TH staining was patchy. Regions of intense staining as well as regions of light staining each gave rise to lightly and intensely TH-stained processes that intermingled in the neuritic field (Fig. 6*A*). Within individual processes, TH staining sometimes abruptly started and stopped (Fig. 6*B*). Synaptic specializations were mainly found near the cell body. Rare TH^+ presynaptic terminals made symmetric synapses (Fig. 6*C*); most presynaptic terminals were TH^- and made asymmetric synapses (Fig. 6*D*). In each of the four single-cell islands examined, there were two or three symmetric specializations and six to eight asymmetric specializations. Invariably, symmetric specializations were made by TH^+ axonal varicosities, whereas asymmetric specializations were made by TH^- terminals; this association was highly significant ($\chi^2 = 21.5$; df = 1; $p < 0.0001$). Axo-axonic synapses were not seen.

DA neurons make GLUergic autapses

To test for synaptic release of GLU, we recorded from single VTA neurons in microcultures. In a series of 52 consecutive VTA neurons, 28 were DA neurons (TH^+) closely matching their incidence in our routine VTA cultures (Rayport et al., 1992). Individual action potentials sometimes evoked reverberatory activity similar to the epileptiform-like activity described in single

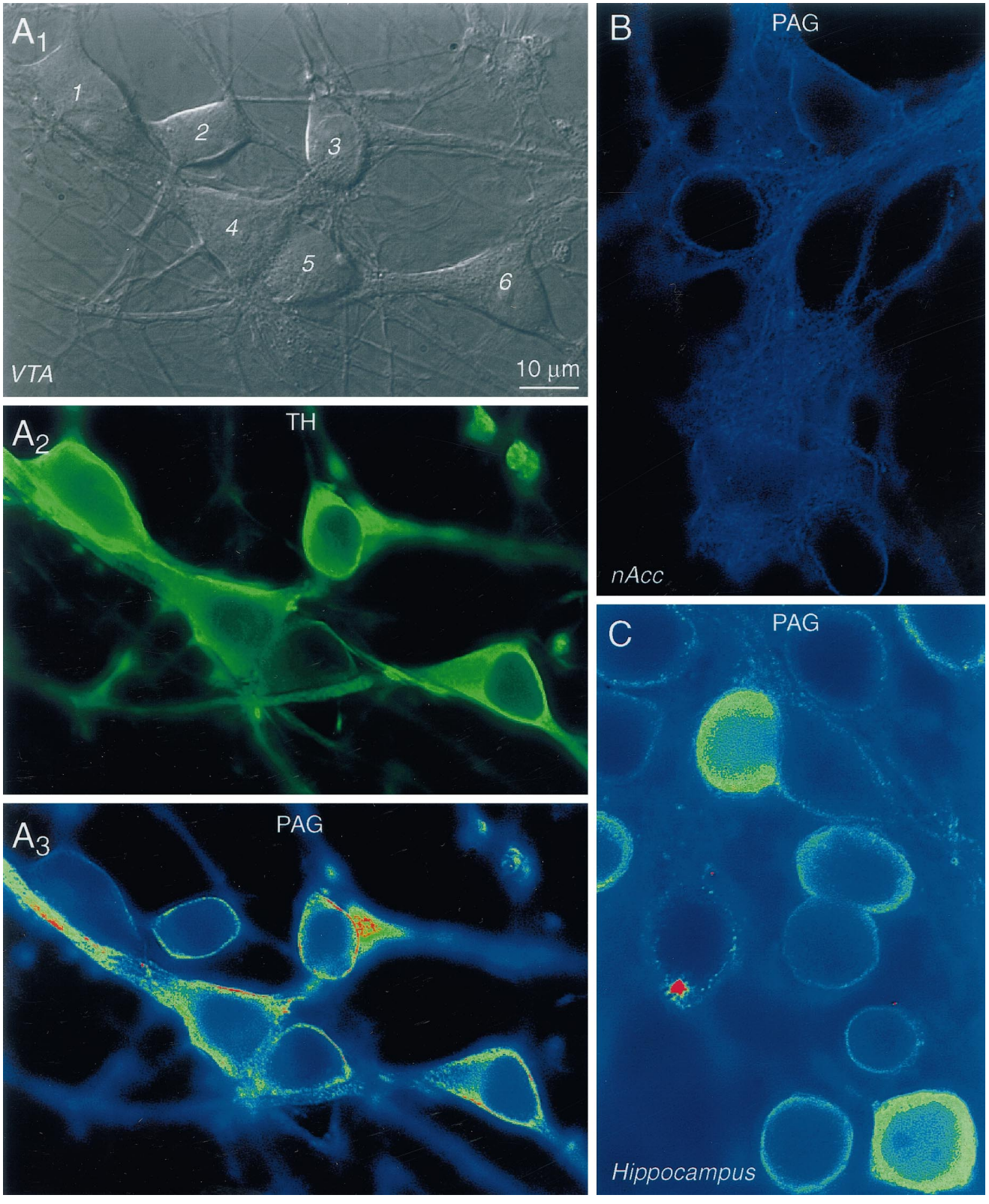

Figure 3. PAG immunostaining *in vitro*. *A*, In a VTA culture, six neurons are shown (A_1, *numbered*). All the neurons in the field except for neuron *2* are DA neurons (A_2); the level of TH staining varies *in vitro* as it does *in vivo* (Bayer and Pickel, 1990). In A_3 and in subsequent panels, staining intensity is shown on a pseudocolor scale in which warmer colors reflect more intense staining. Of the DA neurons, all except for neuron *1* show high levels of immunoreactivity for PAG. The non-DA neuron (neuron *2*) is PAG^+. *B*, In nAcc, which is composed principally of GABAergic neurons (with a minority population of cholinergic neurons) and has no GLUergic neurons, there was no PAG staining. In this field, all six neurons are PAG^-. *C*, In contrast, in hippocampus in which most neurons are GLUergic, most neurons stain for PAG. Here the 13 neurons in the field show varying degrees of PAG staining.

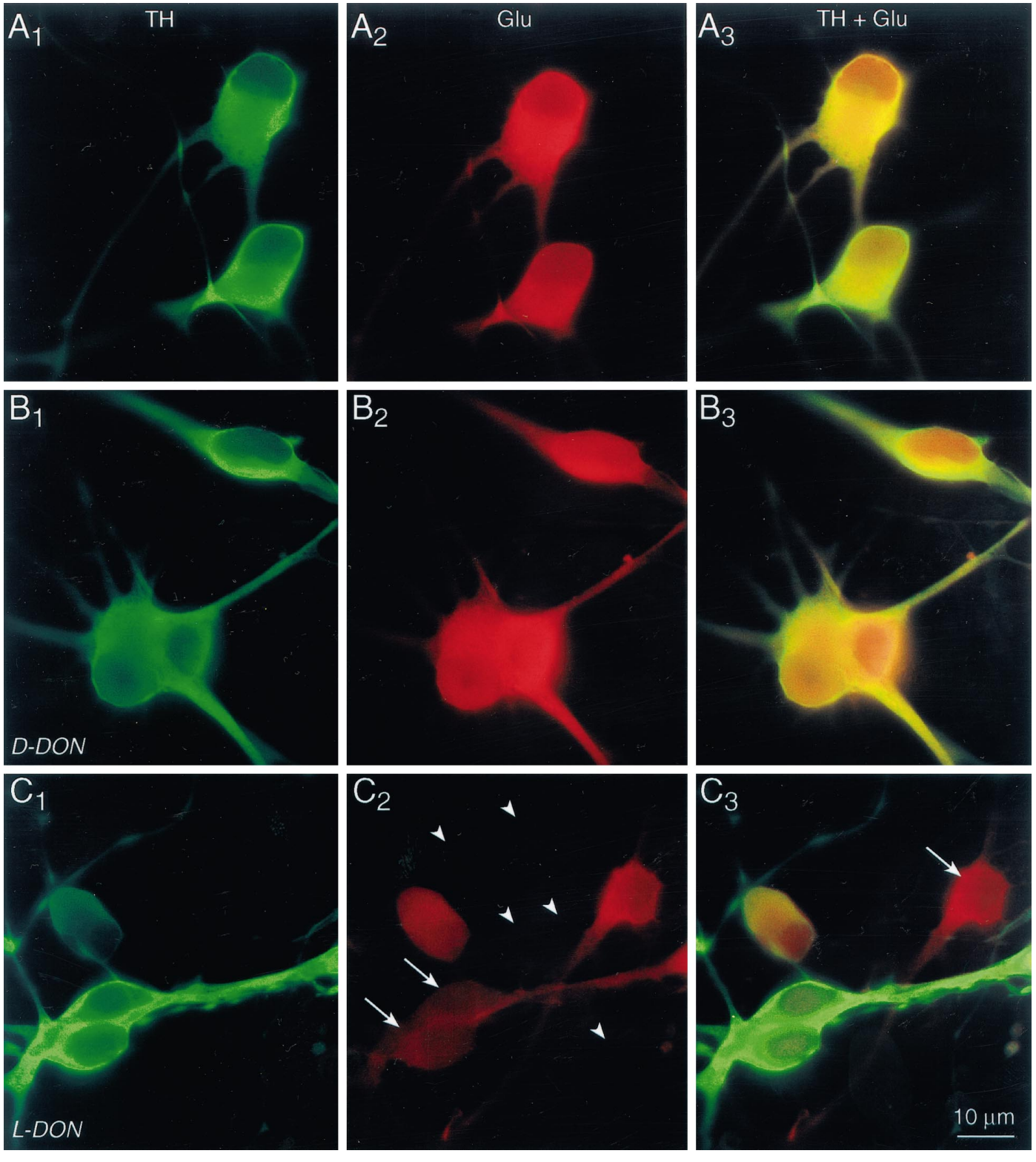

Figure 4. PAG inhibition reduces GLU immunostaining. In an untreated culture (*A*), a field is shown with two DA neurons (A_1), both of which are GLU^+ (A_2, A_3). GLU staining was not diminished when cultures were pretreated with D-DON (5 mM for 20 hr), the inactive enantiomer of the irreversible PAG inhibitor (*B*); here three of three TH^+ cells (B_1) are GLU^+ (B_2, B_3). In contrast, with L-DON (5 mM for 20 hr), the active enantiomer, there was a significant reduction in the GLU staining of DA neurons; here two of the four DA neurons in the field (C_1) were GLU^- (C_2, *arrows*). Although the reduction in cell body staining is not complete, there was an almost complete loss of GLU staining in DA neuron processes (C_2, *arrowheads*; C_3). This field also contains one TH^-/GLU^+ neuron (C_3, *arrow*), which most likely was GABAergic (see Results) and, as would be expected, showed strong GLU staining after PAG inhibition.

GLUergic hippocampal neurons in microcultures (data not shown) (Segal, 1991). In most cells, large autaptic EPSPs were seen. These were almost completely blocked by the GLU antagonist KYN and completely blocked by removal of extracellular Ca^{2+} (Fig. 7*A*).

We found that 61% of DA neurons in these microcultures made excitatory autapses ($n = 17$ of 28) but never inhibitory ones. Application of the D2 antagonist sulpiride (1 μM) revealed no DA-dependent synaptic components ($n = 14$). Furthermore, we saw no DAergic component in the synaptic response with perforated patch recordings ($n = 10$), arguing against a washout problem. We found that 8% of TH^- neurons made autaptic EPSPs ($n = 2$ of 24; these might have been DA neurons that were so disrupted after recording that they were spuriously deemed negative); another 8% of TH^- neurons made autaptic IPSPs ($n = 2$ of 24). Excitatory autapses could be blocked with either APV or CNQX (Fig. 7*B*), whereas inhibitory autapses were blocked with the $GABA_A$ antagonist bicuculline (10 μM; data not shown). Although some VTA DA neurons immunostained for GABA (see above), the absence of autaptic IPSPs in DA neurons argues that GABA is not a cotransmitter in these neurons.

The incidence of excitatory autapses was increased by growing cultures in 0.5 mM KYN, as was done in most of the experiments reported. In a separate series of cultures grown without KYN, we found excitatory autapses in 25% of DA neurons ($n = 2$ of 8), showing that excitatory autapses did not arise as an artifact of growing cells in KYN. Arguing against a presynaptic change, we found no significant difference in the incidence of GLU immunostaining of DA neurons between cultures grown in KYN (76 ± 15%) and control cultures (88 ± 3%), nor were there differences in the incidence of DA neurons with TH^-/SYN^+ synapses. Most likely KYN upregulates GLU receptors (Furshpan and Potter, 1989) and thus facilitated the detection of autapses. Growing cultures under D2 blockade with sulpiride did not, however, reveal any DAergic synaptic components.

Presynaptic modulation of GLU release

To see whether released DA might exert modulatory actions, we voltage clamped VTA DA neurons (identified by subsequent TH immunostaining) in single-cell microcultures (Fig. 8). Cells were stimulated every 10 sec with a brief depolarizing step to elicit a stable EPSC. Application of the D2 antagonist sulpiride augmented autaptic EPSCs ($n = 4$ of 4; 123 ± 3% of control), whereas the D2 agonist quinpirole inhibited EPSCs ($n = 4$ of 4; 40 ± 13%). Sulpiride could be blocking the action of ambient DA; however, two of these experiments were performed with continuous local perfusion so that ambient DA should have been washed away. Therefore, the DA appeared to be released by the cell itself.

We examined this in detail in DA neurons identified by the presence of an excitatory EPSC that increased in amplitude with sulpiride application (because D2 receptors are mainly found on DA neurons in VTA cultures; Rayport et al., 1992; Rayport and Sulzer, 1995; Kim et al., 1997; Rayport, 1998). As before, quinpirole inhibited (65 ± 5%) and sulpiride augmented (111 ± 5%) autaptic EPSCs ($n = 10$ cells). We then rested cells for a minimum of 2 min and examined the first two EPSCs in a stimulation series. Under control conditions (saline), the second EPSC (evoked 10 sec later) was significantly smaller than the first, whereas in the presence of sulpiride there was no significant difference between the two EPSCs (Fig. 9). Sulpiride did not affect the amplitude of the initial EPSC, ruling out a role for ambient DA and showing that DA action is mainly attributable to activity-dependent release. We repeated this experiment in reserpinized cells (90 min of 1 μM reserpine, which depletes >90% of DA content in VTA cultures; Sulzer et al., 1996) and found no decrement in the autaptic EPSC at the second stimulation ($n = 9$ cells).

The lack of a DAergic PSC component suggests that the DA action is presynaptic. To test this, we examined the effects of DA on the paired pulse ratio (PPR); an increase in the PPR during inhibition indicates a presynaptic mechanism (Davies et al., 1990; Manabe et al., 1993). Cells were rested for 2 min and then stimulated with a pair of depolarizing pulses separated by 35 msec. Quinpirole increased the PPR (Fig. 10), whereas in sulpiride the PPR did not change (data not shown). To show that activity-dependent DA release presynaptically inhibits GLU release, we compared the PPR at two paired stimulations separated by 10 sec (Fig. 11). In saline, the PPR increased with the second stimulation, whereas in sulpiride there was no change (the PPR was 1.02 ± .04 in saline vs 0.93 ± .05 in sulpiride; $p < 0.05$ using *t* test). Therefore, DA released during the first stimulation apparently increased the PPR at the second stimulation.

DISCUSSION

We have found that GLU appears to be a cotransmitter in DA neurons. DA neurons immunostain for GLU both in rat and monkey brain, arguing that this coincidence of staining is phylogenetically conserved. DA neurons *in vitro* stain similarly for GLU. Immunostaining single DA neurons in microcultures reveals both DAergic–GLUergic and purely GLUergic synapses. At the ultrastructural level, non-DAergic synapses of DA neurons show asymmetric synaptic specializations of the kind associated with excitation, whereas rarer DAergic synapses show symmetric synaptic specializations. Stimulation of single DA neurons in microcultures evokes Ca^{2+}-dependent EPSPs mediated by both NMDA and AMPA receptors, indicating that GLU is synaptically released. Although the neurons also release DA, it has no appreciable postsynaptic effect but rather presynaptically inhibits GLU release.

Does GLU content imply that a neuron is GLUergic?

We have seen that most DA neurons immunostain for GLU, confirming the original observations of Ottersen et al. (1984). GLU could, however, be either neurotransmitter, precursor, or metabolic GLU. The observation that GLU immunostaining of DA neurons is comparable in intensity to that of known GLUergic neurons in hippocampal cultures suggests that DA neurons are GLUergic, because the highest levels of GLU immunostaining appear to reflect neurotransmitter GLU (Storm-Mathisen and Ottersen, 1990). Although neurotransmitter content is not synonymous with release, with sufficient cytoplasmic GLU content, non-GLUergic neurons show exocytic GLU release (Dan et al., 1994). The presence of PAG, the principal synthetic enzyme for neurotransmitter GLU (Kaneko et al., 1995), in DA neurons in the intact brain (Kaneko et al., 1990) and in culture argues that the GLU visualized is in fact neurotransmitter GLU (Hamberger et al., 1979; Kaneko and Mizuno, 1994) and is not metabolic. Furthermore, it argues against the GLU being precursor to GABA, because PAG is not found in GABAergic neurons (Kaneko and Mizuno, 1994). Inhibition of PAG reduces GLU immunostaining of cell bodies and largely eliminates GLU immunostaining of axons and axonal varicosities, consistent with previous observations that

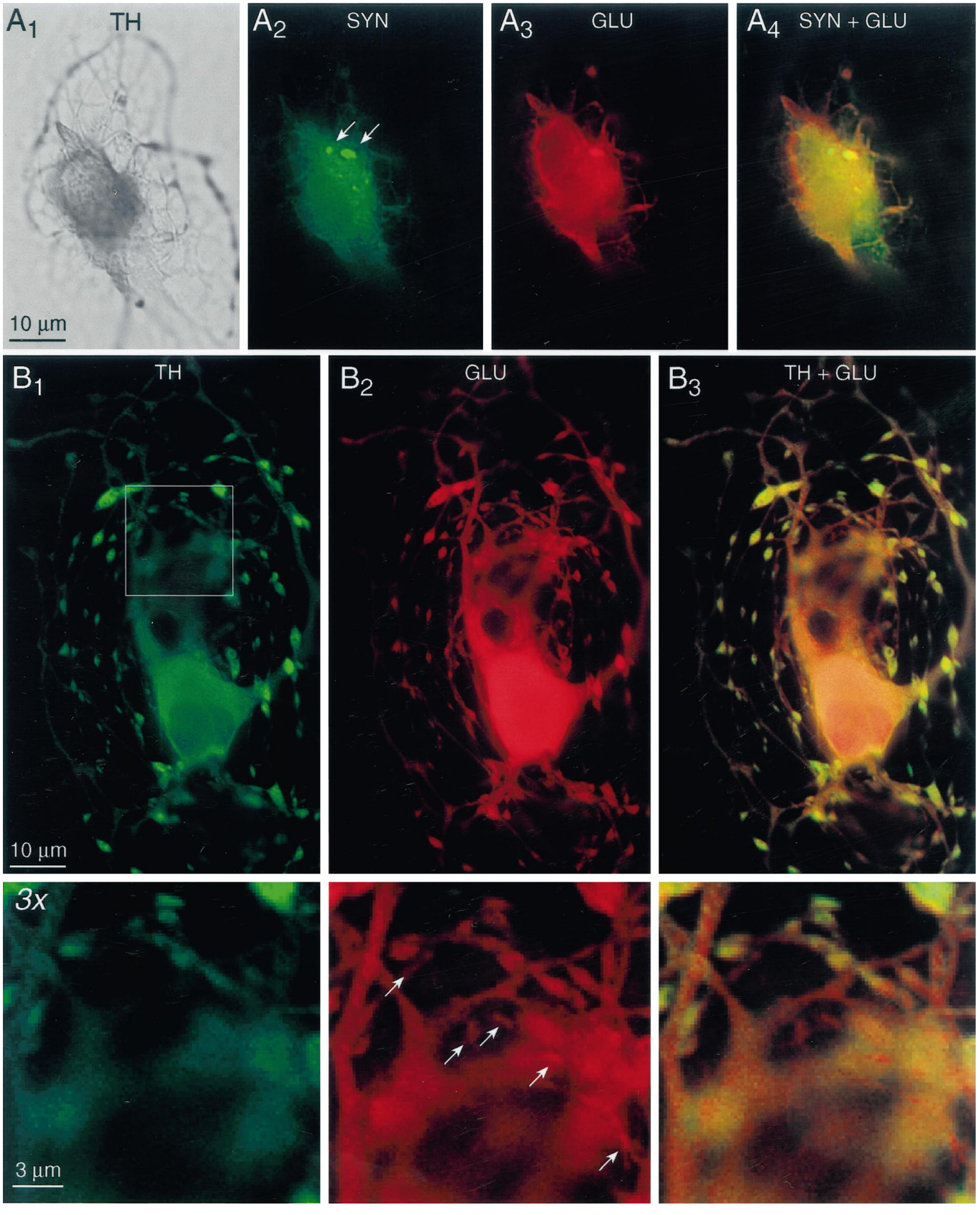

Figure 5. DA neurons have two overlapping sets of synaptic varicosities. *A*, Single DA neurons in microcultures were identified after TH staining with DAB (A_1). The culture was subsequently stained for SYN to identify presynaptic sites and then for GLU. As the DAB reaction product obscured any fluorescence immunostaining, subsequent fluorescence immunostaining was consequently restricted to TH^- areas; this revealed several SYN^+ presynaptic sites near the cell body (A_2; the two most prominent ones are identified by *arrows*). Immunostaining for GLU revealed that these sites were GLU^+ (A_3, A_4). *B*, To examine the relationship between putative DAergic and GLUergic synaptic sites, we double (*Figure legend continues*).

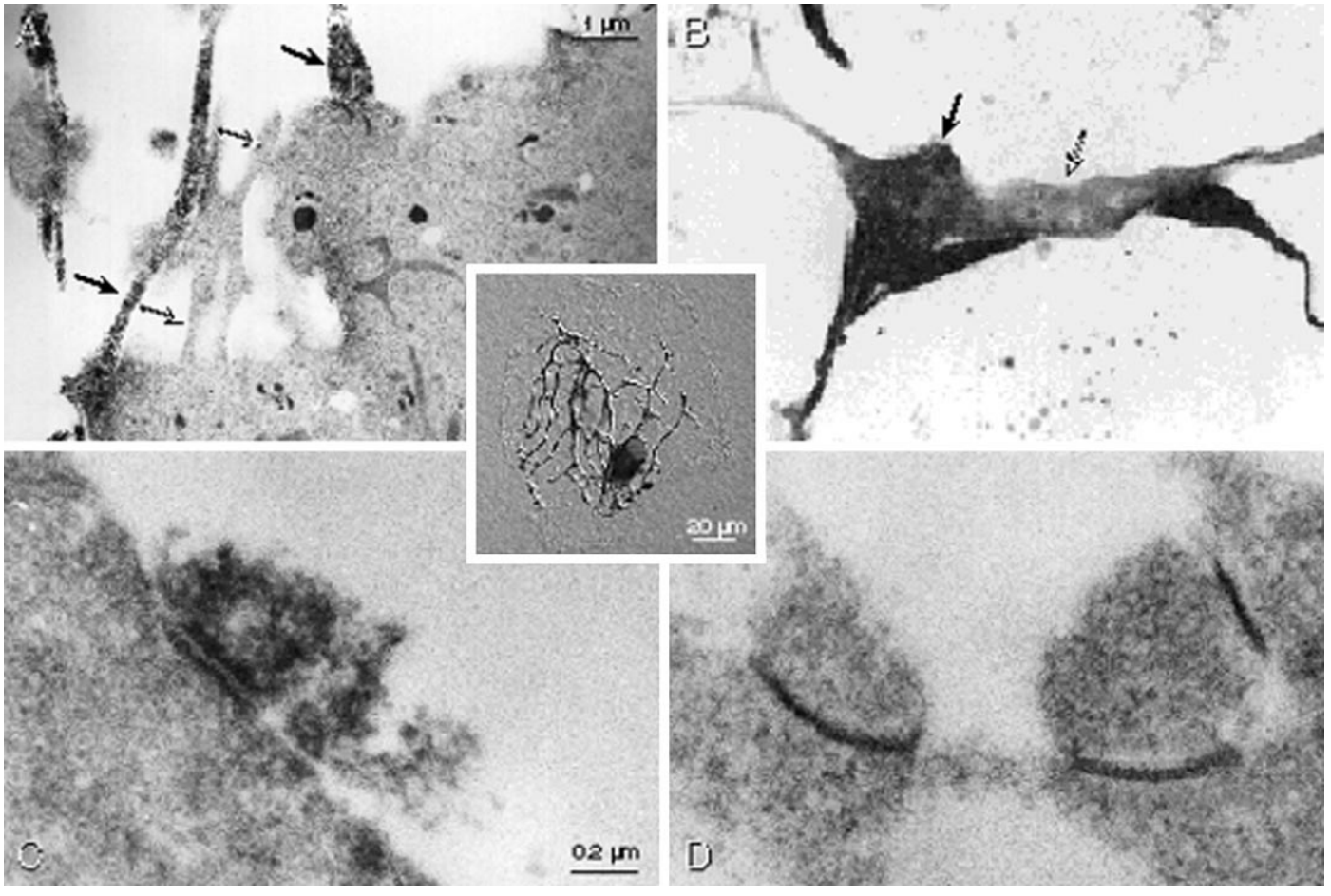

Figure 6. Ultrastructure of a DA neuron in single cell microculture. Sections are shown from a single DA neuron grown in microcultures and TH-stained using DAB (*inset*). To maximize antibody penetration, we used a relatively high detergent concentration. Although this solubilized membranes, resulting in an apparent degradation of the quality of ultrastructural preservation, it enhanced the visualization of synaptic specializations. *A*, In the cell body, TH staining was distributed in a patchy pattern throughout weakly stained cytoplasm. TH^+ processes emerged from intensely stained regions (*filled arrows*); nearby, TH^- processes emerged from unstained regions (*open arrows*). *B*, Within the neuropil, distinctly stained and unstained processes intermingled with each other; in some cases within a single process, a stained portion (*filled arrow*) was clearly distinguishable from an unstained portion (*open arrow*). *C*, Single TH-immunoreactive neurons formed morphological synapses on themselves (autapses). Those autapses were in close proximity to the cell body (as seen at the light level; Fig. 5). In this cell, a total of eight autapses with clear postsynaptic specializations were identified after serial sectioning; one autapse showed presynaptic TH staining and had symmetric synaptic membrane specializations. *D*, The other autapses had asymmetric synaptic specializations with no detectable immunostaining of the presynaptic elements. Two of the seven TH^- boutons (data not shown) made asymmetric synaptic contacts with TH^+ dendritic elements. TH^+ varicosities at a distance from the cell body had accumulations of synaptic vesicles but lacked presynaptic or postsynaptic densities (data not shown).

GLU in axonal processes is more susceptible to activity-dependent depletion (Osen et al., 1995) and therefore reflects neurotransmitter GLU.

DA neurons have two sets of terminals

The possibility that DA neurons make two morphologically distinct types of synapses has been extensively debated (for review, see Hattori, 1993; Groves et al., 1994). On one hand, terminals with asymmetric synaptic specializations of the kind classically associated with excitatory actions have been identified by degeneration after 6-hydroxy-DA SN lesions or by orthograde radiolabeling from the SN. On the other hand, immunostaining for TH or DA has identified terminals with symmetric specializations classically associated with inhibitory actions (Pickel et al., 1981). Both kinds of terminals have been identified with uptake of the false transmitters α-methylnorepinephrine (Kaiya and Namba, 1981) and 5-hydroxy-DA (Groves et al., 1994), which produce electron-dense deposits in monoaminergic synaptic vesicles. A recent examination of DA-immunostained processes in the medial nAcc of the monkey revealed synapses with asymmetric specializations in contact with dendrites and dendritic spines as well as en passant profiles with rarer synaptic specializations (Ikemoto et al., 1996). Whether terminals with asymmetric specializations belong to DA neurons has been questioned (Groves et al., 1994); however, given that the nigrostriatal projection is

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immunofluorescence stained single DA neurons in microcultures for TH and GLU. Many varicosities double stained for TH (B_1) and for GLU (B_2). Just above the cell body (the outlined region is shown 3× enlarged in each *bottom panel*), a cluster of smaller varicosities (*bottom panel* of B_2, *arrows*) stains primarily for GLU. Thus, DA neurons appear to have varicosities that are both DAergic and GLUergic and others that are just GLUergic.

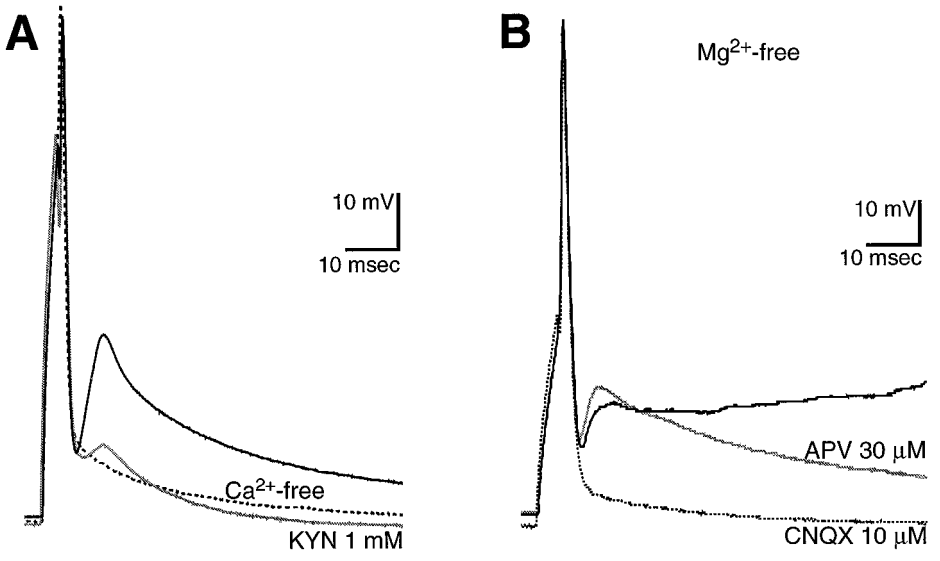

Figure 7. DA neurons make GLUergic EPSPs in microculture. *A*, Under whole-cell current clamp, a single VTA neuron in a microculture was stimulated with brief depolarizing current pulses (traces shown are averages of 6 stimulations). A large EPSP was evoked with fixed latency (*solid line*), which was completely blocked by local perfusion with Ca^{2+}-free saline (*dashed line*), and recovered fully in physiological saline (data not shown). KYN (at a high concentration that completely blocks NMDA receptors via action at the allosteric glycine site and competitively blocks AMPA receptors with lesser potency) significantly attenuated the EPSP (*gray line*). *B*, In another cell recorded in Mg^{2+}-free saline, a large fixed latency EPSP was evoked, which was followed by a prolonged depolarization; in some traces this went on to trigger recurrent spikes (i.e., epileptiform-like activity). This EPSP was largely blocked by CNQX; APV attenuated the later phase of the EPSP.

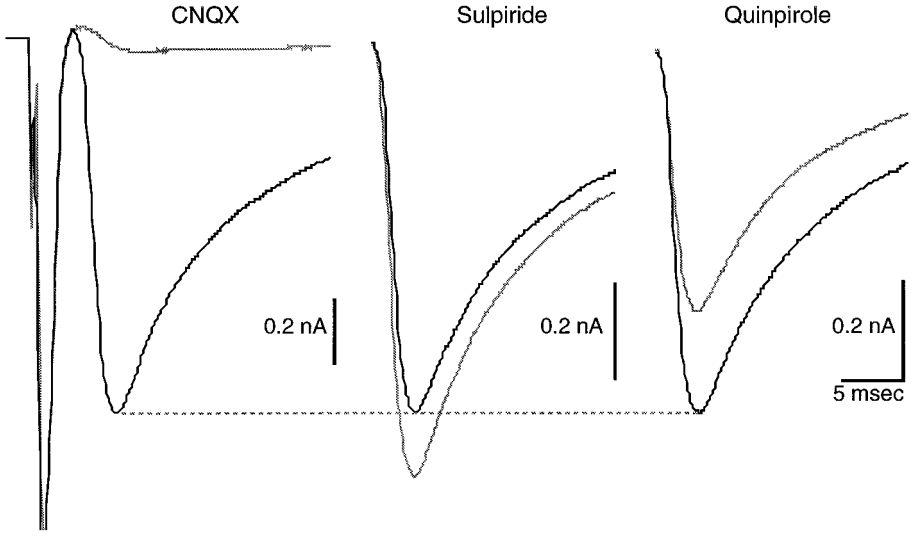

Figure 8. D2 modulation of GLUergic EPSC. In a neuron, subsequently shown to be TH^+, a large autaptic EPSC was recorded under voltage clamp. This was almost completely blocked by CNQX (EPSC was 4% of control; traces shown are averages of 10 stimulations; traces during drug application are shown in *gray*). The reversible D2 antagonist sulpiride enhanced the EPSC (117%; shown here and in subsequent traces without the initiating action current), whereas the D2 agonist quinpirole markedly attenuated the autaptic EPSC (76%). This suggests that concomitant DA release modulates the GLUergic EPSC.

~95% DAergic (van der Kooy et al., 1981; Silva et al., 1990), degeneration after chemical lesions of the SN or orthograde labeling from the SN most likely identifies DA neuron terminals (Hattori, 1993). Hattori et al. (1991) addressed this issue directly using orthograde labeling with [^{3}H]leucine and immunocytochemical staining for TH and showed that there are two sets of varicosities, one set that is double-labeled and has symmetric specializations and a second set that is solely radiolabeled and has asymmetric synaptic specializations.

Our morphological observations in single-cell microcultures, in which we can be assured that all the processes arise from a single neuron, indicate that DA neurons indeed have two types of chemical synapses with distinct synaptic morphologies (Fig. 12). The synapses are segregated to different postsynaptic domains, with GLUergic terminals localized to proximal dendrites and the TH–GLU varicosities more peripherally distributed and apparently not contacting major dendritic branches. Taken together with the synaptic physiology, our morphological observations indicate that DA neurons make DAergic varicosities that are involved in volume transmission and make GLUergic varicosities that mediate rapid excitatory transmission. Supporting this conclusion, Gonon (1997) showed that stimulation of DA neuron axons in the median forebrain bundle evokes either fast non-DAergic excitation or delayed D1-mediated excitation. If this dual action results from activation of both GLUergic and DAergic terminals of DA neurons, then the two sets of terminals would appear to have different postsynaptic targets. In contrast, serotonergic raphe neurons in single-cell microculture, which also release GLU as a cotransmitter, show slow serotonergic inhibition as well as fast GLUergic excitation (Johnson, 1994) and have a single set of synapses with two different vesicle types (Johnson and Yee, 1995). So although both DAergic and serotonergic neurons appear to use GLU as a cotransmitter, they do so in strikingly different ways.

Excitatory autapses of DA neurons

Autaptic EPSPs show both NMDA and AMPA components, consistent with our immunocytochemical observations that GLU itself is the neurotransmitter. Other excitatory amino acid candidates that have been seen in DA neurons such as *N*-acetyl-aspartyl-glutamate (Sekiguchi et al., 1992) or the spontaneously occurring DA breakdown product trihydroxyphenylalanine (Rosenberg et al., 1991) are more selective agonists (Trombley and Westbrook, 1990; Newcomer et al., 1995). The neuropeptides

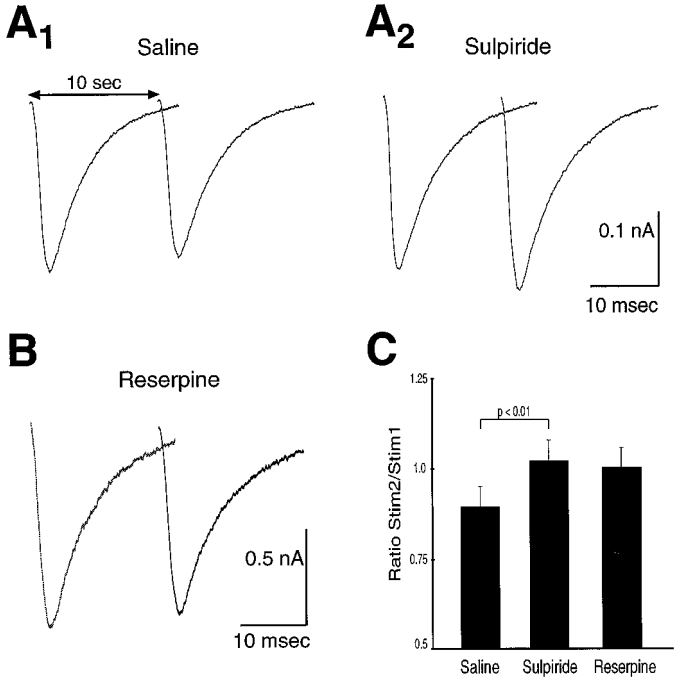

Figure 9. Activity-dependent DA release modulates the EPSC. *A*, When two stimulations were delivered separated by 10 sec, the second EPSC was significantly smaller (A_1) in contrast to the same stimulation in the presence of 1 μM sulpiride (A_2). Cells were rested for a minimum of 2 min between experimental trials. *B*, In another cell after exposure to reserpine (1 μM for 90 min) there was no significant reduction in EPSC size at stimulation 2. *C*, Overall in 10 such experiments, there was a significant DA-dependent inhibition at stimulation 2.

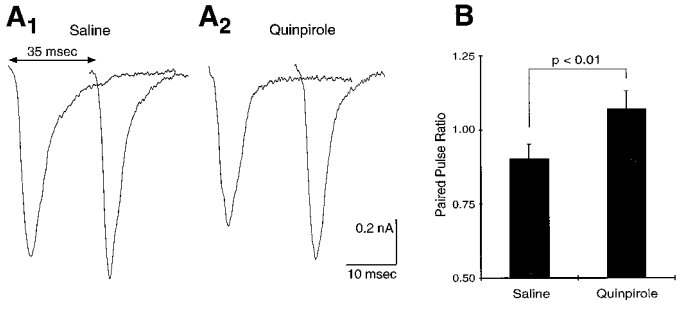

Figure 10. DAergic presynaptic inhibition of GLU release. *A*, To determine whether the locus of the D2 inhibition was presynaptic, we examined the effects of quinpirole on paired pulse responses. In saline, there was modest increase in the PPR (A_1, 112%). Quinpirole both diminished the size of the response (A_2), in this case to 71% of the response in saline, and increased the PPR (137%). *B*, In 10 experiments quinpirole significantly increased PPR favoring presynaptic action.

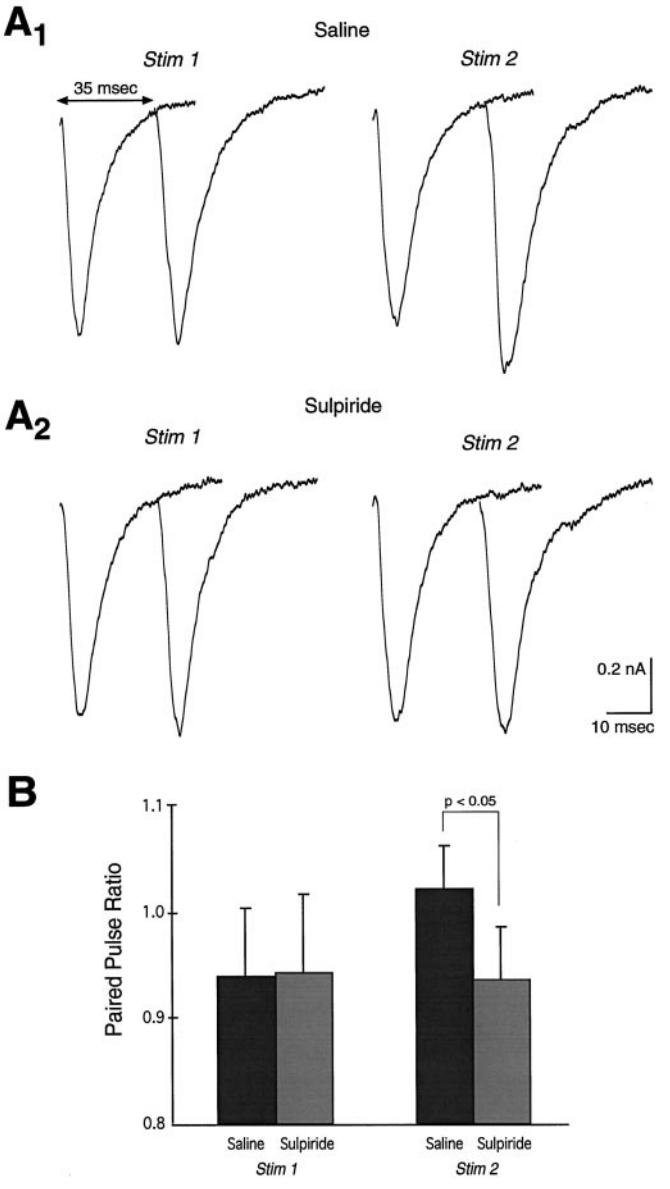

Figure 11. Activity-dependent presynaptic inhibition. *A*, A cell was stimulated with two sets of paired pulses separated by 10 sec. In saline (A_1), there was an increase in the PPR from stimulation 1 to stimulation 2, as well as a decrement in the first response at stimulation 2, whereas in sulpiride (A_2) there was neither an increase in the paired pulse ratio between stimulation 1 and stimulation 2 nor a decrease in the first response of the pair at stimulation 2. *B*, In 10 experiments, there was a significant difference in the PPR between saline and sulpiride at stimulation 2, consistent with activity-dependent D2-mediated presynaptic inhibition.

cholecystokinin and neurotensin have been found in rat DA neurons (Hökfelt et al., 1984) and might account for the excitatory actions; however, they do not have GLU receptor activity. Moreover, their expression may be superfluous in the rat (Bowers, 1994), because they are not found in DA neurons in primates (Savasta et al., 1990; Berger et al., 1991).

In contrast to the strong excitatory responses, we saw no direct DAergic responses, although VTA neurons in culture express D2-like DA receptors (Rayport and Sulzer, 1995) and are inhibited, just as in the slice (Lacey et al., 1988) by the D2 agonist quinpirole (Rayport et al., 1992; Kim et al., 1997). Furthermore, the cells show electrochemically detectable quantal DA release from axonal varicosities (Pothos et al., 1998). If the DA release were from the same varicosities mediating the excitatory response, which show close synaptic appositions, then one must postulate that the DA receptor density on the proximate postsynaptic membranes is not sufficient to mediate a measurable action. There may also have been subtle modulations of membrane currents that went undetected in our experiments. However, our ultrastructural observations indicate that synapses with asymmetric specializations that putatively mediate the excitatory response are invariably TH^-. Furthermore, TH^+ symmetric synapses are rare, arguing that most DA release emanates from nonsynaptic

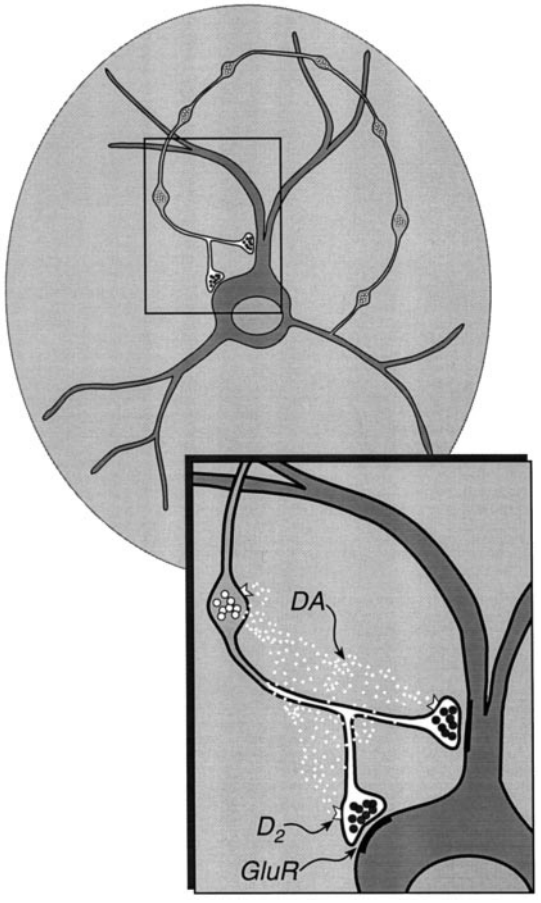

Figure 12. Relationship of DA neuron terminals in microculture. To illustrate the relationship between the two sets of DA neuron synapses, a schematic of a single DA neuron in microculture is shown. Regions of TH staining are shaded *gray*; DAergic synaptic vesicles in DAergic varicosities are shown in *white*, whereas GLUergic vesicles in GLUergic terminals are shown in *black*. DA neuron axons commonly arise from dendrites (Hausser et al., 1995). The area outlined by the *rectangle* is expanded as an *inset* that shows DA release (*small white dots*) from a DAergic varicosity. This overflows to TH^- GLUergic terminals, binds to D2 receptors, and mediates presynaptic inhibition. D2 autoreceptors are also present on DAergic varicosities (Rayport, 1998), which would inhibit DA release (Cragg and Greenfield, 1997). GLU receptors (*GluR*) are shown as forming the postsynaptic densities of asymmetric synaptic specializations.

sites, either the more peripheral varicosities in the microcultures or from somatodendritic regions.

Somatodendritic DA release (Cheramy et al., 1981) might contribute to the modulation of the GLUergic EPSC. However, VMAT staining is mainly seen in axonal varicosities, both in the intact brain (Nirenberg et al., 1996) and *in vitro* (Pothos et al., 1998), making somatodendritic release a less likely source. Furthermore, the observation that some DA cells with autaptic EPSCs are inhibited by quinpirole but do not show a response to sulpiride argues that the DA release is not as reliable as one would expect if the release were from immediately adjacent dendrites. Therefore, it appears more likely that the released DA derives from overflow from DAergic varicosities that are at some distance from the GLUergic synapses, much as it does in the intact brain (Garris et al., 1994). Depending on the spatial relationships and the functional status of the DAergic and GLUergic varicosities, the released DA might or might not modulate GLU release.

The inhibition of autaptic excitation by DA could be attributable to either postsynaptic modulation of GLU receptor sensitivity or to presynaptic modulation of GLU release. Postsynaptic modulation appears unlikely for three reasons. First, DA responses show rapid washout under whole-cell recording conditions (Rayport et al., 1992), whereas quinpirole modulation persisted for the duration of most experiments. Second, in paired pulse facilitation experiments quinpirole increased facilitation, consistent with a presynaptic locus of action (Davies et al., 1990; Manabe et al., 1993). Third, stimulation of DA neurons caused an increase in paired pulse facilitation, showing that evoked DA release presynaptically inhibits GLU release.

Implications

The idea that monoaminergic neurons as a class might release GLU was originally suggested by Kaneko et al. (1990), who showed that monoaminergic neurons in each of the three major CNS monoaminergic cell groups immunostain for PAG. Not only do serotonergic raphe neurons make GLUergic EPSPs in microcultures (Johnson, 1994), but noradrenergic neurons also immunostain for GLU and mediate excitatory actions (Liu et al., 1995). Thus, GLU colocalization appears to be the rule for the major CNS monoaminergic projections, so that the cells may exert rapid synaptic as well as slower modulatory actions.

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