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BRAIN RESEARCH REVIEWS

Review

Glutamate in dopamine neurons: Synaptic versus diffuse transmission

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ABSTRACT

There is solid electron microscopic data demonstrating the existence of dopamine (DA) axon terminals (varicosities) with or without synaptic membrane specializations (junctional complexes) in many parts of the CNS, and notably in neostriatum and nucleus accumbens. The dual morphological character of these DA innervations has led to the suggestion that the meso-telencephalic DA system operates by diffuse (or volume) as well as by classical synaptic transmission. In the last decade, electrophysiological and neurochemical evidence has also accumulated indicating that monoamine neurons in various parts of the CNS, and particularly the mesencephalic DA neurons, might release glutamate as a co-transmitter. Following the identification of the vesicular transporters for glutamate (VGluT), *in situ* hybridization and RT-PCR studies carried out on isolated neurons or standard tissue cultures, and more recently *in vivo*, have shown that VGluT2 mRNA may be expressed in a significant proportion of mesencephalic DA neurons, at least in the ventral tegmental area. A current study also suggests that the co-expression of tyrosine hydroxylase (TH) and VGluT2 by these neurons is regulated during embryonic development, and may be derepressed or reactivated postnatally following their partial destruction by neonatal administration of 6-hydroxydopamine (6-OHDA). In both 15 day-old and adult rats subjected or not to the neonatal 6-OHDA lesion, concurrent electron microscopic examination of the nucleus accumbens after dual immunocytochemical labeling for TH and VGluT2 reveals the co-existence of the two proteins in a significant proportion of these axon terminals. Moreover, all TH varicosities which co-localize VGluT2 are synaptic, as if there was a link between the potential of DA axon terminals to release glutamate and their establishment of synaptic junctions. Together with the RT-PCR and *in situ* hybridization data demonstrating the co-localization of TH and VGluT2 mRNA in mesencephalic neurons of the VTA, these observations raise a number of fundamental questions regarding the functioning of the meso-telencephalic DA system in healthy or diseased brain.

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Contents

1. Introduction

The concept of diffuse or volume transmission was proposed in the nineteen seventies, on the basis of electron microscope autoradiographic observations on the serotonin and the noradrenaline innervations in adult rat cerebral cortex (Descarries et al., 1975, 1977; Beaudet and Descarries, 1978). It was then demonstrated that many of these axon terminals or varicosities lacked the plasma membrane specializations that are the hallmark of morphologically defined synapses (Peters and Palay, 1996). These axonal boutons displayed the characteristic clusters of small vesicles implicated in the storage and release of transmitters, but often no junctional complex, that is, no small zone of plasma membrane thickening on either side of a slightly enlarged extracellular space, where receptors for transmitters were then assumed to be confined. It was inferred from these unexpected findings that the transmitter released from such non-junctional or non-synaptic terminals might diffuse in the extracellular space and reach remote targets, allowing for effects at a distance, on a variety of cellular elements in a relatively large tissue volume (reviewed in Descarries et al., 1991).

This initial proposal had considerable merit. It provided an explanation for: i) some general, prolonged and/or indirect actions attributed to noradrenaline and serotonin in the cerebral cortex, and which came to be designated as «modulation» to distinguish them from classical, point-to-point, short-acting, excitatory or inhibitory synaptic transmission (Reader et al., 1979); ii) the existence of so-called «pre-synaptic» effects of transmitters on their own release or that of other transmitters, which took place in the obvious absence of axo-axonic synapses (Vizi, 1984; Vizi and Labós, 1991); iii) the frequent mismatch between the regional distribution of many receptors for different transmitters and that of the corresponding innervations (Herkenham, 1987; Jansson et al., 2001); iv) the extrasynaptic localization of many transmitter receptors, as visualized by immuno-electron microscopy (e.g. Pickel and Sesack 1995; Caillé et al., 1996; Delle Donne et al., 1997; Aoki et al., 1998), and v) effects of monoamine and other transmitters on non-neuronal targets, such as astrocytes and microvessels (e.g., Kalaria et al., 1989; Hansson, 1991), endowed with receptors often remote from the corresponding release sites. The implications of diffuse transmission for the understanding of normal and abnormal brain function as well as pharmacotherapeutics have since been the subject of extensive reviews (Agnati et al., 1992, 1995; Zoli and Agnati, 1996; Zoli et al., 1998, 1999).

The electron microscopic evidence that many axon terminals, boutons or varicosities issued from different kinds of transmitter-defined neurons in mammalian CNS do not form junctional complexes is now incontrovertible (reviewed in Descarries and Mechawar, 2000). In recent years, this information has mostly been derived from immunocytochemical studies based on the use of specific antibodies against the neurotransmitters themselves or their biosynthetic enzymes, or else against the specific membrane transporters ensuring their reuptake. In some of these studies, the immunostained axon terminals have been examined in serial as well as single ultrathin sections, allowing scrutiny of their entire volume and direct determination of the frequency with which they made a synaptic junction (synaptic incidence). Most often, however, the frequency of junctions was extrapolated from observations in single ultrathin sections, by means of a stereological formula taking into account the average size of the sectional profiles, the average length of visible junctions and the thickness of the sections (Beaudet and Sotelo, 1981). The validity of this formula has since been verified experimentally in several studies where the same pool of axon varicosities was sampled in serial as well as single ultrathin sections (e.g., Umbriaco et al., 1994).

The partly asynaptic nature of the three monoamine innervations (dopamine, noradrenaline, serotonin) in adult rat cerebral cortex was thus confirmed, and similar findings were made in other CNS regions and in different species, including monkey and human (reviewed in Descarries and Mechawar, 2000; see also Smiley, 1996). Other examples of largely asynaptic innervations include the histamine innervation of the rat cerebral cortex (Takagi et al., 1986), as well as the cholinergic innervation of neocortex (Umbriaco et al., 1994; Mechawar et al., 2002), hippocampus (Umbriaco et al., 1995; Aznavour et al., 2005) and neostriatum (Contant et al., 1996; Aznavour et al., 2003). Of all transmitter-defined CNS systems thus far examined for the synaptic or asynaptic nature of their axon terminals or varicosities, the mesencephalic dopamine (DA) neurons innervating the striatum have however received the most attention.

2. Dual character, asynaptic and synaptic, of the dopamine innervation in striatum

Early electron microscopic descriptions after specific cytochemical and autoradiographic labeling have led to divergent conclusions about the synaptic features of neostriatal DA

terminals. Early experiments on tissue slices fixed with potassium permanganate after preloading or not with false transmitter (Hökfelt, 1968; see also Tennyson et al., 1974) gave no indication of the presence of junctional complexes on neostriatal boutons showing small granular vesicles and thus positively identified as containing dopamine or serotonin. A few years later, the first description by Pickel et al. (1976) of neostriatal terminals immunostained with specific antibodies against the biosynthetic enzyme tyrosine hydroxylase (TH) seemed to confirm this finding in aldehyde-fixed tissue. In 1980, however, a preliminary report from our laboratory indicated that many single ultrathin sections from DA terminals of adult rat neostriatum labeled by uptake of [^{3}H]DA *in vivo* exhibited small, symmetrical, synaptic membrane specializations on dendritic branches or spines, suggesting a relatively high synaptic frequency (Descarries et al., 1980). Another TH immunocytochemical study from Pickel's laboratory (1981), also carried out in single ultrathin sections, then suggested the existence of two categories of neostriatal DA terminals: a restricted one with larger varicosities that were deprived of synaptic membrane specializations, and a more abundant one with smaller varicosities exhibiting specializations that were indeed symmetrical and found occasionally on neuronal somata as well as on dendritic branches and spines (Pickel et al., 1981; see also Kaiya and Namba, 1981; Arluison et al., 1984; Triarhou et al., 1988). In 1984, polyclonal antibodies directed against DA–glutaraldehyde–protein conjugate became available (Geffard et al., 1984), followed by monoclonal antibodies against this same antigen (Chagnaud et al., 1987). These allowed for a better morphological preservation of tissue processed for immunocytochemistry, as they were compatible with the use of higher concentrations of glutaraldehyde for primary fixation of the brain. Using these antibodies, Voorn et al. (1986) described DA varicosities in the ventral striatum of an adult rat as making small symmetrical synaptic contacts with dendritic shafts and spines.

Meanwhile, in our laboratory, further autoradiographic experiments on neostriatal terminals labeled *in vivo* with [^{3}H]DA (Fig. 1A) revealed a synaptic incidence in the order of 30–40%, as extrapolated stereologically from single ultrathin sections (Descarries et al., 1996). However, this sampling was limited to the paraventricular portion of neostriatum because of the restricted penetration of the tracer which had to be administered intraventricularly. For this reason, as soon as the DA antibodies became available, it was decided to pursue this study with immunocytochemistry (Fig. 1B) in a mediodorsal region of neostriatum. Moreover, an effort was made to obtain direct evidence by examining a great number of these DA terminals in long, uninterrupted, series of ultrathin sections (e.g. Fig. 2).

The results obtained through these various approaches are summarized in Table 1. They allowed us to conclude that, in an adult rat, approximately 30–40% of DA terminals, in both a paraventricular and a more central zone of neostriatum were endowed with a synaptic membrane specialization (Descarries et al., 1996). Similar values were later reported by Antonopoulos et al. (2002), who showed that in a 21 day-old rat, 63% of DA-immunolabeled varicosities from the striatal patches, but only 35% from the matrix, were synaptic. These authors also examined the ventral striatum in a 21 day-old rat, and found that 47% and 71% of DA varicosities were synaptic in the core and the shell of nucleus accumbens (nAcb), respectively. As mentioned below, in our own material from the core of adult rat nAcb, we observed a junctional frequency of 11% in single thin sections, for a synaptic incidence of 34%, as extrapolated to the whole volume of these varicosities.

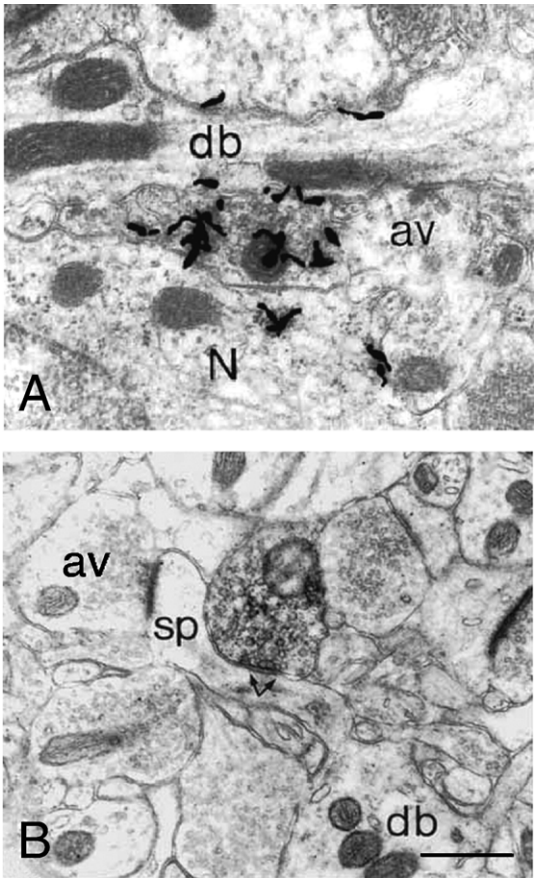

Fig. 1 – *Electron micrographs of DA axon terminals (varicosities) in adult rat neostriatum, identified by high resolution autoradiography after in vivo uptake of tritiated DA (A), or by immunocytochemistry with primary antibodies against DA–glutaraldehyde–protein conjugate (B).* In A, the elongated profile of the [^{3}H]DA-labeled varicosity displays the characteristic array of small vesicles associated with a mitochondrion, but no zone of plasma membrane differentiation suggestive of a synaptic contact. This varicosity is directly apposed to a neuronal cell body (N), a dendritic branch (db) and another axonal varicosity, unlabeled (av). In B, the DA-immunostained profile is one from a series of 10 thin sections across the same varicosity. In this and 2 adjacent sections, this DA varicosity is seen to make a small symmetrical synapse (between arrows) with the neck of a spine (sp) observed in continuity with its parent dendrite (db). Note that this spine also receives a relatively large asymmetrical synapse on its head from the unlabeled varicosity on its left (av). Scale bars: 0.5 μm. (Reproduced with permission from Wiley-Liss (J. Comp. Neurol., 1996, 375:167–186)).

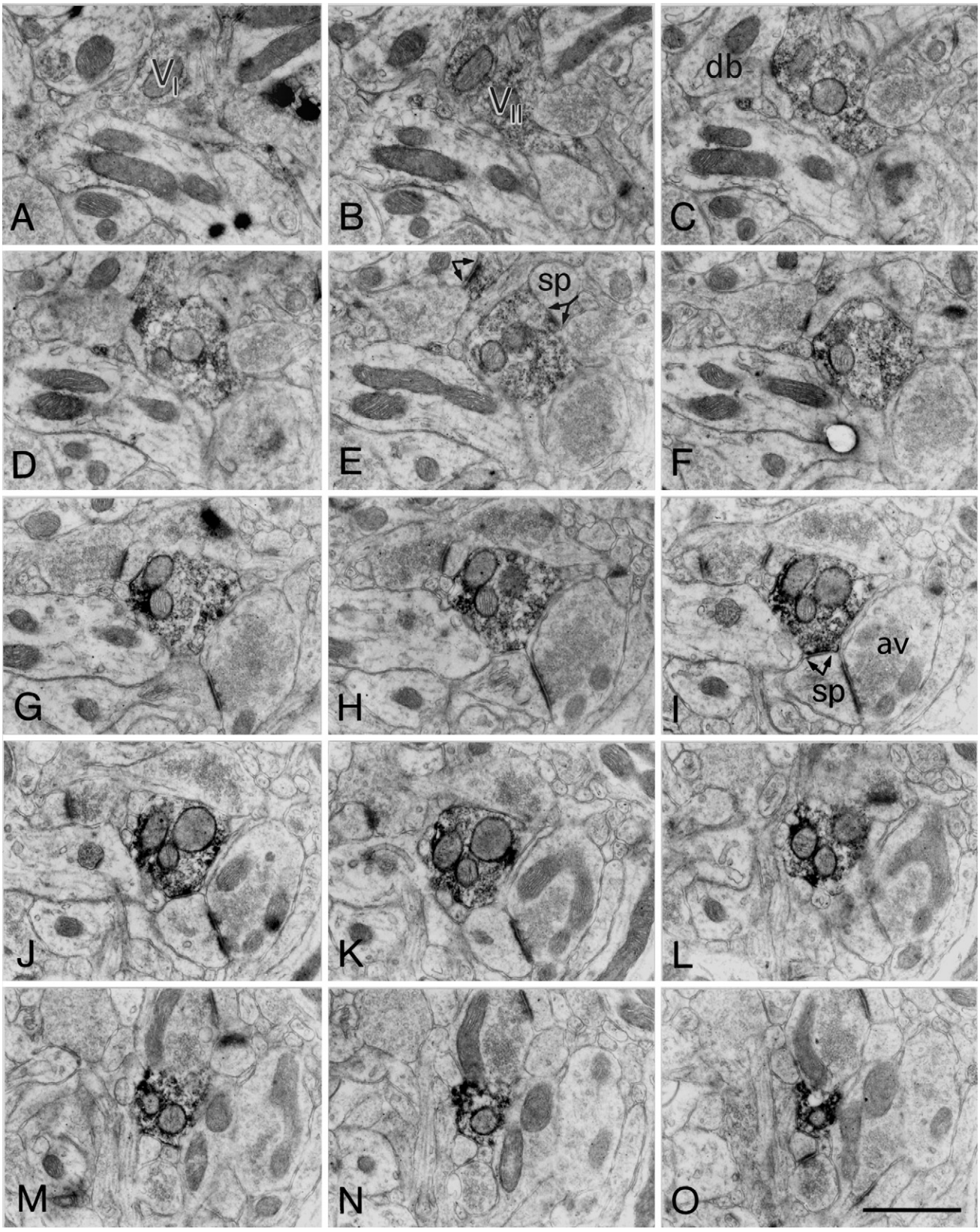

Fig. 2 – *Series of 15 consecutive thin sections across the entire volume of a DA immunoreactive varicosity (V_{II} in B) from the neostriatum*. Part of another DA varicosity (V_I) is also visible in A to F. Both the DA varicosities form small, symmetrical synaptic contacts (between arrows in E and I). In E, V_I is seen to make synapse with the dendritic branch (db) in C, whereas V_{II} displays a first synapse with a dendritic spine (sp). In I and J, V_{II} makes a second synapse with a dendritic spine (sp in I), which also receives a large asymmetrical synaptic contact from an adjacent unlabeled varicosity (av in I). Scale bar (in O): 1 μm. (Reproduced with permission from Wiley-Liss (J. Comp. Neurol., 1996, 375:167–186)).

3. Dopamine–glutamate co-transmission by mesencephalic dopamine neurons

The observation that DA neurons are endowed with asynaptic as well as synaptic axon varicosities raises the question as to what distinguishes these two classes of terminals in terms of function. Although a definitive answer to this question is not presently available, the hypothesis has been raised that the synaptic junctions established by DA neurons are the sites at which these neurons release glutamate as a co-transmitter (Trudeau, 2004).

3.1. *Converging evidence that CNS monoamine neurons use glutamate as a co-transmitter*

The first indirect evidence that CNS monoamine neurons might release glutamate as a co-transmitter was the demonstration that a proportion of monoamine neurons, including DA, noradrenaline and serotonin neurons, were immunopositive for glutamate, in both rat and monkey (Ottersen and Storm-Mathisen, 1984; Nicholas et al., 1992; Fung et al., 1994a,b; Liu et al., 1995; Sulzer et al., 1998). These same cell populations were also shown to be immunopositive for phosphate-activated glutaminase, the glutamate biosynthetic enzyme (Kaneko et al., 1990). Glutamate immunoreactivity was not sufficient evidence to prove that glutamate was being used as co-transmitter. However, electrophysiological data was then obtained demonstrating that rapid excitatory synaptic responses could be evoked in striatal neurons by extracellular stimulation in DA cell body areas or in the medial forebrain bundle (Hull et al., 1970, 1973; Kitai et al., 1975). Moreover, recent work has shown that local application of a D2 receptor agonist at the site of stimulation inhibits the generation of glutamatergic EPSPs in striatal neurons, suggesting that D2 responsive, putative DA neurons (and not fibres of passage), are indeed responsible for such EPSPs (Chuhma et al., 2004). Similar responses in the anesthetized rat have also been reported to disappear after chemical ablation of the DA neurons, strongly arguing that they were indeed generated by these neurons (Lavin et al., 2005). Rapid excitatory synaptic events sensitive to the AMPA/kainate glutamate receptor antagonist CNQX have also been shown to be evoked in spinal cord ventral horn motoneurons after extracellular stimulation of presumed noradrenergic neurons (Fung et al., 1994a,b; Liu et al., 1995). Excitatory responses were also generated in striatal neurons and ventral horn motoneurons following extracellular stimulation of presumed serotonin neurons in raphe nuclei (Park et al., 1982; Holtman et al., 1986; Fung and Barnes, 1989).

A direct test for glutamate release by monoamine neurons *in vivo* would require paired recordings between, for example, a DA neuron and a target cell in a projection area such as the striatum. These experiments are hardly feasible, considering the low synaptic connectivity between these cells and the large distance between the cell bodies of the pre- and post-synaptic neurons. Further evidence for the synaptic release of glutamate by monoamine neurons was therefore sought *in vitro*. Using a microculture system in which isolated postnatal serotonin neurons of the rat raphe nuclei develop on small (100–500 μm) microdroplets of substrate, Johnson (1994) studied the synaptic development of serotonin neurons. Under such conditions, isolated neurons establish synaptic contacts onto a limited subset of neighbouring neurons, or, if the neuron is alone, onto its own dendrites (i.e. "autapses"). Recordings from such isolated cells revealed that ~60% of serotonin neurons generate fast autaptic EPSPs that can be blocked by CNQX. This demonstrated clearly that these serotonin neurons indeed release glutamate as a co-transmitter (Johnson, 1994). Similar observations were then made by Sulzer et al. (1998) in isolated rat DA neurons. A single action potential in a DA neuron evoked an EPSC that was blocked by AP5, an antagonist of NMDA glutamate receptors and by CNQX (Sulzer et al., 1998; Bourque and Trudeau, 2000). Together with the results of glutamate and TH double-immunostaining experiments, these observations led Sulzer et al. (1998) to propose that a proportion of axon terminals formed by DA neurons could be specialized for glutamate release (Fig. 3). Comparable results were also obtained in co-cultures of ventral tegmental area (VTA) DA neurons together with GABAergic medium spiny neurons of the nAcb (Joyce and Rayport, 2000), indicating that glutamate release by DA neurons was not restricted to isolated neuron cultures.

Table 1 – Synaptic incidence of dopamine axon terminals in adult rat striatum

	Showing a synapse (single sections)	Observed (serial sections)	Extrapolated
NEOSTRIATUM			
Descarries et al. (1996)			
[^{3}H]DA autoradio.	9.6%		39%
DA immuno.	12.3%	35%	40%
Antonopoulos et al. (2002)			
DA immuno.			
Patch	22%		63%
Matrix	11%		35%
NUCLEUS ACCUMBENS			
Antonopoulos et al. (2002)			
DA immuno.			
Core	14%		47%
Shell	20%		71%
Bérubé-Carrière et al. (2007)			
TH immuno.			
Core	11%		34%

3.2. *Vesicular glutamate transporters in transmitter-defined neurons of CNS*

The physiological and immunocytochemical evidence of glutamate co-release by monoamine neurons implies that these neurons are able to package glutamate in synaptic vesicles. In the last few years, the identification of vesicular transporters for glutamate (VGluT) has provided new means to investigate the glutamatergic phenotype of neurons (Ni et al., 1994; Takamori et al., 2000). The first of these transporters to be identified, VGluT1 (BNPI) was shown to be present at high concentration in the synaptic vesicles of a subset of CNS glutamatergic neurons (Bellocchio et al., 1998, 2000; Takamori et al., 2000). Overexpression studies in cell lines proved that VGluT1 is a *bona fide* vesicular glutamate transporter that acts to load synaptic

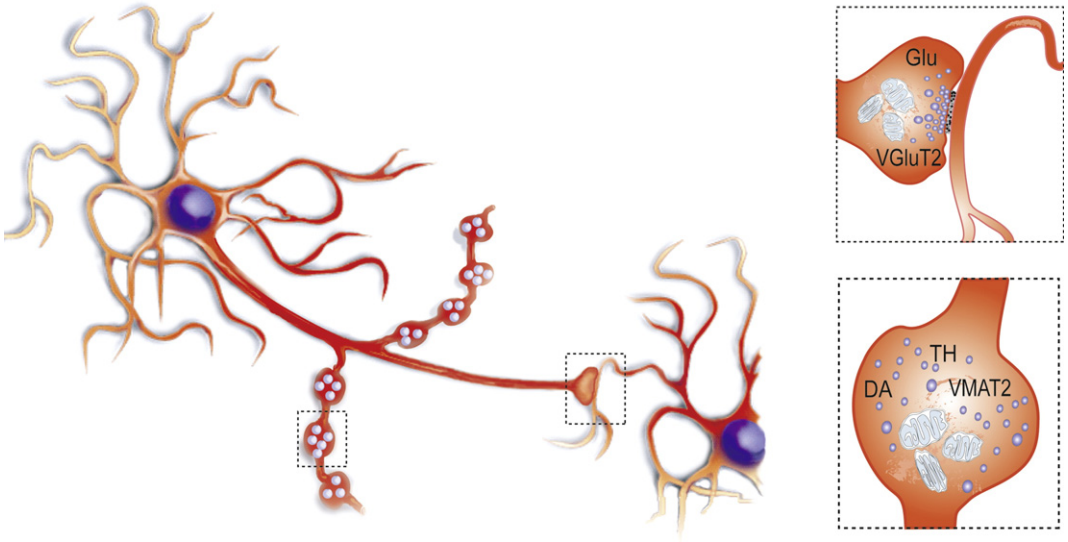

Fig. 3 – *Schematic representation of the synaptic and non-synaptic axon terminals established by DA neurons*. DA neurons are known to establish two morphologically distinguishable axon terminals: some that are non-synaptic and others that are synaptic. The non-synaptic terminals (varicose-like structures) display no obvious pre- and post-synaptic specialization (see lower right illustration showing a magnified view of a single non-synaptic terminal), contain TH and could be specialized for the release of DA. The synaptic terminals, displaying a more classical active zone, post-synaptic density and synaptic cleft, could be the site of VGluT2 expression and of glutamate (Glu) release (see upper right illustration showing a magnified view of a synaptic axon terminal). Adapted from Trudeau and Gutierrez (2007). (Reproduced with permission from the American Society of Pharmacology and Experimental Therapeutics (Mol. Interv., 2007, 7:138–146)).

vesicles with glutamate (Bellocchio et al., 2000; Takamori et al., 2000).

A close homologue of VGluT1, VGluT2, acts as another important vesicular glutamate transporter (Aihara et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Hayashi et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002). Interestingly, the expression patterns of VGluT1 and VGluT2 in the brain are mostly complementary, with VGluT1 mRNA being widely expressed by pyramidal neurons of the neocortex and hippocampus, and in the cerebellar cortex, whereas VGluT2 mRNA is more abundantly produced in diencephalic and other subcortical nuclei, in deep cerebellar nuclei and in the brainstem (Ni et al., 1995; Hisano et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001). Closer examination of VGluT2 mRNA in the brainstem showed that this transcript is present in most noradrenaline neurons of the A2 group in the nucleus tractus solitarii complex (Stornetta et al., 2002a), and most adrenaline neurons of the C1, C2 and C3 groups in the medulla (Stornetta et al., 2002b). It is noteworthy that over-expression of either VGluT1 or VGluT2 in cultured GABA neurons allowed these neurons to co-release glutamate, providing support for the idea that expression of a vesicular glutamate transporter is necessary and sufficient to permit vesicular glutamate release by neurons (Takamori et al., 2000, 2001).

A third vesicular glutamate transporter (VGluT3) was also cloned more recently, showing a more restricted expression limited to a number of neurons not classically thought of as glutamatergic. In particular, VGluT3 mRNA and protein were found in most serotonin neurons of the raphe, as well as in cholinergic neurons of the striatum and forebrain (Gras et al., 2002; Schafer et al., 2002; Takamori et al., 2002; Harkany et al., 2003). Together with the work showing that monoamine neurons establish functional glutamate-releasing terminals in culture and contain glutamate immunoreactivity *in vivo*, these data support the view that glutamate co-transmission might be widespread in monoamine neurons of the CNS.

3.3. *Expression of VGluT2 in DA neurons in vitro and in vivo*

VGluT2 mRNA was first reported to be present in rat substantia nigra compacta (SNc) in a Northern blot experiment (Aihara et al., 2000). However, initial *in situ* hybridization studies at low resolution failed to detect significant amounts of VGluT2 mRNA in mesencephalic DA neurons from adult animals (Gras et al., 2002). Considering the physiological evidence for glutamate release by DA neurons, we initiated efforts to better characterize the expression of VGluTs in DA neurons both *in vitro* and *in vivo*. We first evaluated the presence of VGluTs in postnatal rat mesencephalic neurons in primary culture. Using immunocytochemical labeling, we found that ~80% of isolated DA neurons were immunopositive for VGluT2 (Dal Bo et al., 2004), while VGluT1 and 3 were not detected. The VGluT2 labeling was punctate in nature and particularly concentrated close to major dendrites and the cell body (Fig. 4A). Interestingly, although most VGluT2 positive varicosities were also TH positive, many neurons displayed long thin axonal-like segments bearing multiple TH positive/VGluT2-negative varicosities (Fig. 4A, lower right). In addition,

in a triple labeling experiment, it was apparent that a number of nerve terminals identified by the presence of the synaptic vesicle protein SV2 were VGluT2 negative (Fig. 4B). These findings were compatible with the hypothesis that DA neurons can establish distinct sets of terminals, a subset of which having the ability to co-release glutamate (Sulzer et al., 1998).

The expression of VGluT2 mRNA in cultured mouse and rat DA neurons was then confirmed by single-cell RT-PCR (Fig. 4C). This expression of VGluT2 was specific for the DA neurons since GABA neurons in the same cultures did not express VGluT2. Another series of single-cell RT-PCR experiments was carried out on mesencephalic DA neurons acutely dissociated from mice at different ages. In these experiments, we took advantage of transgenic mice expressing GFP under the control of the TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002; Jomphe et al., 2005), thus allowing easy visual identification of DA neurons. Using this approach, we found that VGluT2 mRNA, but not VGluT1 nor VGluT3 mRNA, was present in 25% of GFP-expressing DA neurons acutely isolated from P0 pups (Mendez et al., 2005; Mendez and Trudeau, unpublished observations). Interestingly, this proportion appeared to diminish with age, decreasing to 13% at P45.

To determine whether the dual dopamine–glutamate phenotype was also expressed by mesencephalic DA neurons *in vivo*, double *in situ* hybridization experiments for TH and VGluT2 were performed on rat brain tissue obtained during embryonic development and at various postnatal ages, up to P15 (Dal Bo et al., 2005). At embryonic days 14 and 15, there was a striking regional overlap in the labeling of TH and VGluT2 mRNA in the ventral mesencephalon, which was no longer found at late embryonic stages (E18–E21) and postnatally. In normal pups at P6–P15, only 1–2% of neurons containing TH mRNA in the ventral tegmental area (VTA) and SNc, also displayed VGluT2 mRNA. In a similar study carried out in adult rats, DA neurons co-expressing VGluT2 mRNA were reported to be rare (approximately 0.1%) in the VTA of adult rats (Yamaguchi et al., 2007), but neurons expressing VGluT2 mRNA relatively abundant, suggesting the existence of purely glutamatergic neurons in the VTA (Kawano et al., 2006). In our own studies, the much higher proportion of mesencephalic DA neurons expressing VGluT2 mRNA, as observed by single-cell RT-PCR (~25%) rather than *in situ* hybridization (1–2%) supported the latter findings and presumably reflected the advantage of RT-PCR for detecting low abundance mRNAs.

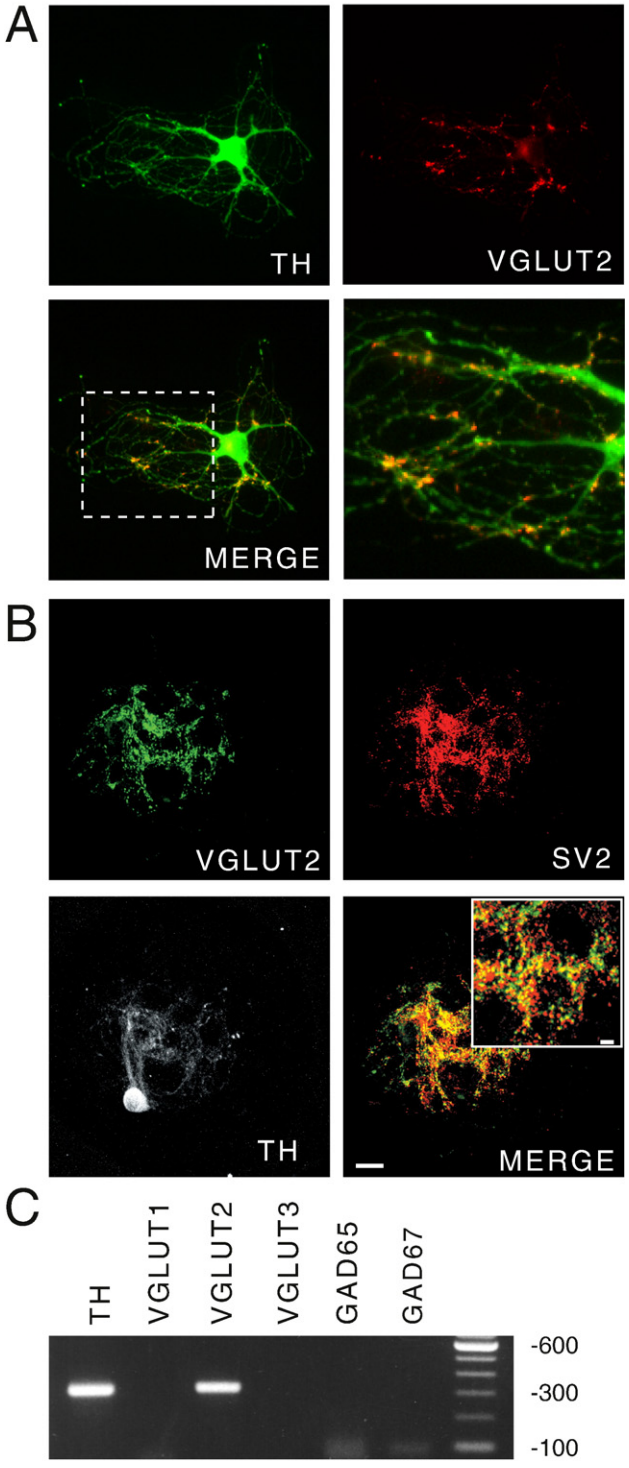

Fig. 4 – *Confocal imaging (A,B) and RT-PCR detection (C) of the presence of VGluT2 in isolated DA neurons in culture.* In A, the isolated neuron displays TH (green, upper left) as well as VGluT2 immunofluorescence (red, upper right). The merged image in the lower left panel demonstrates the co-localization of VGluT2 mainly in TH positive processes (yellow). In the enlarged view of the boxed area from this panel (lower right), it is apparent that there are varicose-like punctae immunopositive for both VGluT2 and TH (yellow) and others for TH only (green) along the same processes. In B, the upper panels illustrate the immunoreactivity to VGluT2 (green, upper left) and to the synaptic vesicle protein SV2 (red, upper right) in a subset of nerve terminals from the isolated TH positive neuron (lower left). The merged image (lower right) demonstrates the co-localization of VGluT2 and SV2 (yellow). The enlargement in inset shows that most VGluT2 positive structures are also SV2 positive (yellow), but that there are SV2-positive terminals which are VGluT2 negative (red). All scale bars: 15 μm. C represents the mRNA expression profile of an individual mesencephalic DA neuron in culture. Both TH and VGluT2 mRNA were found in this neuron. Expected size of PCR products in base pairs: TH (301), VGluT2 (315). (Reproduced with permission from Blackwell Publishing (J. Neurochem., 2004, 88: 1398–1405)).

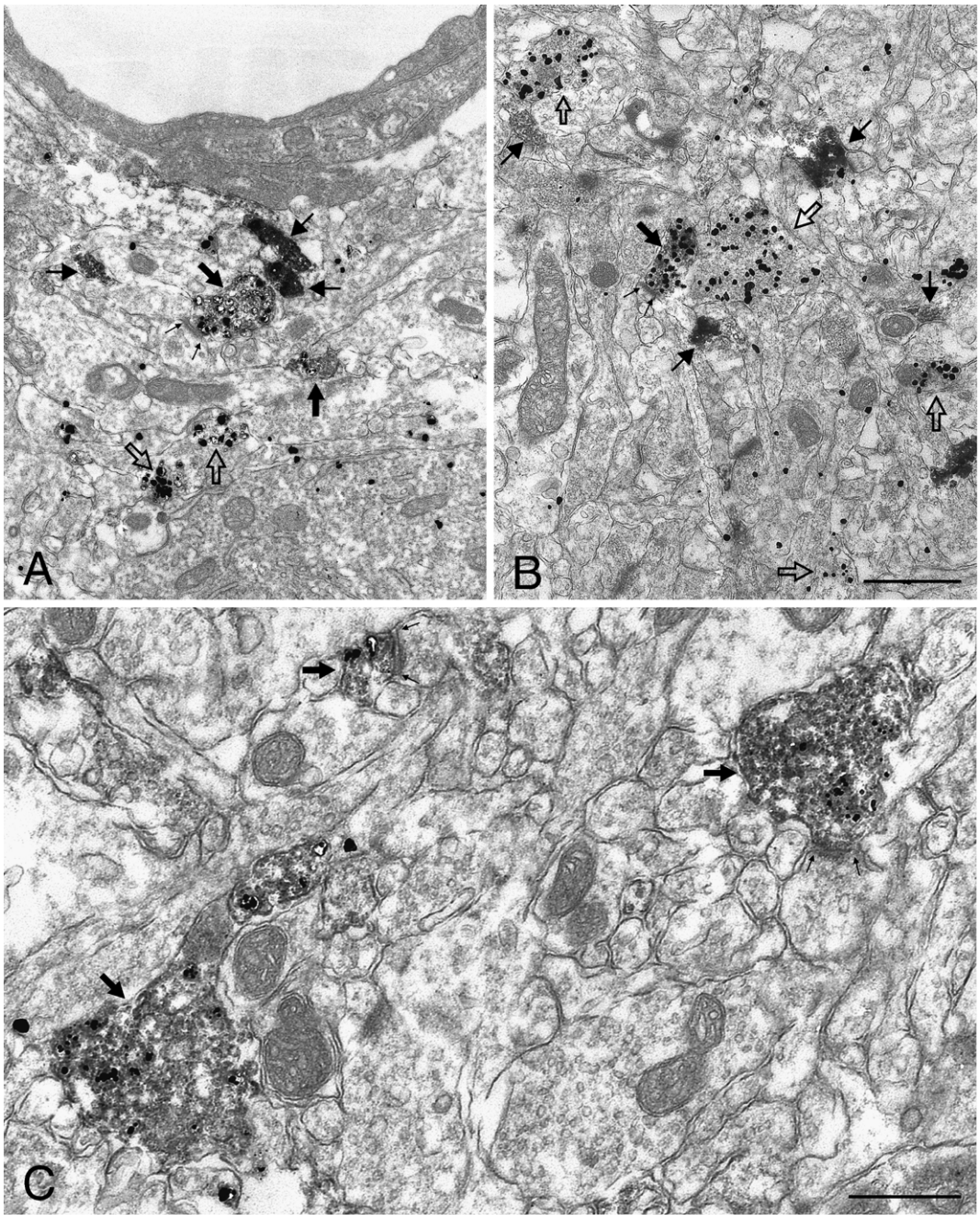

Fig. 5 – *Electron micrographs of TH and VGluT2 immunoreactive axon terminals (varicosities) in the nAcb of normal (A,B) or 6-OHDA-lesioned (C) 15 day-old rats*. TH and VGluT2 were respectively labeled with the immunoperoxidase technique (fine DAB precipitate) and the immunogold technique (silver-intensified, gold particles). In A, two doubly labeled varicosities (thick black arrows), as well as three varicosities labeled for TH only (thin black arrows) and two for VGluT2 only (empty arrows) are visible. The larger of these doubly labeled varicosities makes synapse (between small arrows) with a dendritic spine. In B, a doubly labeled terminal lies between two other varicosities, one unlabeled, on its left, and the other labeled for VGluT2 only, on its right. Note the synaptic contact (between small arrows) made by the doubly labeled varicosity on a small dendritic spine. Scale bar (for A and B): 1 μm. In C, three doubly labeled terminals can be seen, two of which display a synaptic contact (between small arrows) with a dendritic spine. Scale bar: 0.5 μm.

In the objective of evaluating whether VGluT2 expression by DA neurons was regulated, we also examined tissue from postnatal rats cerebroventricularly injected with 6-hydroxydopamine (6-OHDA) at P4 to partially lesion DA neurons. Intraventricular infusion of 6-OHDA in a neonatal rat destroys most of the DA neurons in SNc, but a significant proportion of the DA neurons in VTA are spared. In contrast to the low expression found in normal rodents, 25% of surviving DA neurons in the VTA of P6–P15 rats co-expressed VGluT2 after the administration of 6-OHDA at P4, suggesting an induction

Table 2 – Number of singly and doubly labeled axon terminals (varicosities) in the core of nucleus accumbens of normal or 6-OHDA-lesioned rats at P15 and P90

	TH (total)	TH/VGluT2	TH	VGluT2
P15 (n=7)				
Normal	161±12	45±6 (28%)	115±6	82±21
6-OHDA	99±7***	37±4 (37%)*	63±7***	46±9
P90 (n=3)				
Normal	68±4	8±5 (12%)	60±2	106±11
6-OHDA	36±11	14±8 (39%)	22±4	114±4

Data for an equivalent ultrathin tissue section area of 1665 μm^2 in each rat. *P<0.05; ***P<0.001 by unpaired Student's *t* test.

or derepression of the glutamatergic phenotype (Dal Bo et al., 2005). Together with the enhanced expression of VGluT2 mRNA in cultured DA neurons (80% versus 25% in acutely dissociated neurons), these results support the exciting hypothesis that expression of the glutamatergic phenotype by mesencephalic DA neurons is a regulated phenomenon that might be induced or reactivated by injury. Additional studies are now required to directly address this possibility.

4. Co-localization of TH and VGluT2 in axon terminals of the nucleus accumbens

To seek *in vivo* evidence for the co-localization of TH and VGluT2 in axon terminals of DA neurons, the nAcb of P15 rats was examined by electron microscopy after double, pre-embedding immunolabeling for the two proteins (Bérubé-Carrière et al., 2006). Knowing that the expression of VGluT2 was increased after the neonatal 6-OHDA lesion, this study was conducted in normal as well as 6-OHDA-lesioned rats. In these experiments, VGluT2 was always labeled first with immunogold, and then TH, with the immunoperoxidase technique. Doubly as well as singly labeled axon terminals were readily identified in the nAcb of both normal and 6-OHDA-lesioned rats (Fig. 5). When determined in an equivalent surface of thin section surface in each rat, the mean number of axon varicosity profiles labeled for TH alone or for TH together with VGluT2 was markedly reduced after the 6-OHDA lesion (Table 2). In normal rat, VGluT2 was co-localized in 28% of all TH-labeled terminals. After the lesion, this proportion of dually labeled terminals was slightly but significantly increased (37%), in keeping with the hypothesis of an activation or reactivation of the glutamatergic phenotype of DA neurons following injury. An apparent decrease in the number of terminals labeled for VGluT2 alone was not statistically significant. Similar findings have recently been made in adult (P90) rats (Bérubé-Carrière et al., 2007). In normal adults, the dually labeled terminals appeared to be less frequent than at P15 (12%), but again, after the 6-OHDA lesion, their percentage over all TH+ appears to be increased (Table 2).

In these studies, particular attention was also paid to the synaptic incidence of the singly and dually labeled terminals (Fig. 6). As extrapolated stereologically from the observations in single thin sections, in both normal and 6-OHDA-lesioned rats and at both ages, all terminals which co-localized TH and VGluT2 appeared to be synaptic, at variance with those labeled for TH alone or even for VGluT2 alone at P15, and for TH alone at P90. Moreover, at both ages, the proportion of axon terminals labeled for TH alone and displaying a synapse appears to be greater after the 6-OHDA lesion (Bérubé-Carrière et al., 2007). Whether synaptic TH terminals of the nAcb preferentially resist 6-OHDA lesioning or develop a synaptic phenotype as consequence of the lesion remains to be determined.

5. Concluding remarks

1) In an adult as well as postnatal rat, a significant number of axon terminals in the nucleus accumbens co-localize TH and VGluT2 and should therefore be capable of releasing glutamate in addition to DA as neurotransmitter. Together with the single-cell RT-PCR and dual *in situ* hybridization data demonstrating the co-localization of TH and VGluT2 mRNA in mesencephalic neurons of the VTA, these ultrastructural observations raise a number of fundamental questions regarding the functioning of these neurons. Under what circumstances is glutamate released from DA terminals in the nAcb, and with what consequences on the

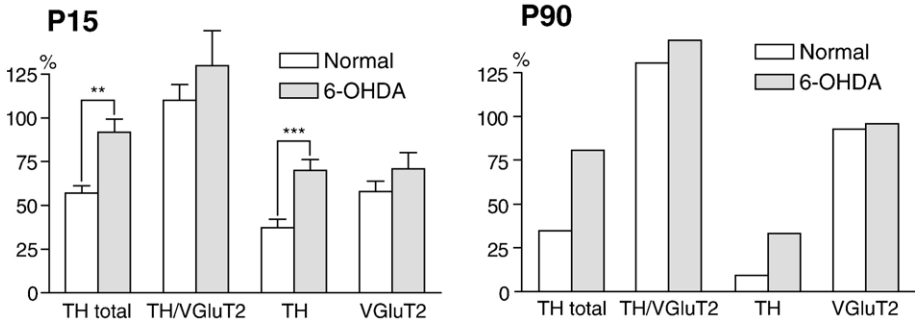

Fig. 6 – *Frequency distribution histograms of the synaptic incidence of singly (TH or VGluT2) or doubly (TH/VGluT2) labeled axon varicosities in the nAcb of P15 and adult rats, normal or subjected to neonatal administration of 6-OHDA.* Means±sem from 7 rats in each group at P15. At P90, only 3 rats were examined. As stereologically extrapolated to the whole volume of varicosities, values above 100% suggest that some of the corresponding varicosities make more than one synaptic contact, which is indeed occasionally observed in single ultrathin sections for electron microscopy. **P<0.01; ***P<0.001.

functioning of this brain region? In this regard, it should be noted that the release of glutamate by DA neurons projecting to the prefrontal cortex has already been proposed to account for a rapid component in the response of pyramidal neurons and cortical interneurons to DA neuron activation (Lapish et al., 2006, 2007).

2) It is also clear that all axon terminals endowed with the dual DA–glutamate phenotype are synaptic, at variance with the terminals containing TH only. This finding suggests a link between the potential of DA terminals to release glutamate and their establishment of synaptic junctions. Whether the presence of VGluT2 and the co-release of glutamate are one of the factors involved in the formation of synapses by axon varicosities is an open question. It should be interesting to determine if the proportion of DA varicosities co-localizing VGluT2 is higher in the shell as opposed to the core of nAcb, where the proportion of synaptic DA axon varicosities appears to be higher (Antonopoulos et al., 2002), or in other parts of the brain such as the cerebral cortex, where the proportion of synaptic DA varicosities differs from one region to another (Séguéla et al., 1988). It will also be interesting to evaluate whether the association of synaptic junctions to glutamate co-release applies to other monoamine neurons, and more generally to all those operating with both diffuse and synaptic transmission. Another key question is what distinguishes at the molecular level the synaptic and non-synaptic junctions established by DA neurons. For example, whether the same complement of SNARE proteins involved in exocytosis will be found in both types of terminals needs to be determined.
3) Finally, it may be asked how and what regulates the dual DA–glutamate phenotype during development and in disease. And, more specifically, whether and how this dual transmitter phenotype might be implicated in pathological conditions affecting the DA neurons innervating the nAcb.

Further studies will obviously be needed to answer these questions. Meanwhile, it should be feasible to determine whether similar characteristics and properties are shared by DA neurons innervating other brain regions, such as the neostriatum and the prefrontal cortex, and whether they prevail in higher species, including man!

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