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Glutamate co-transmission as an emerging concept in monoamine neuron function

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Converging research efforts over the last 4 decades have established beyond a doubt that many, if not most, neurons release more than 1 neurotransmitter. Although much attention has been paid to the co-release of small-molecule neurotransmitters with neuropeptides, a number of examples of co-release of 2 small-molecule neurotransmitters have now been described. It has been suggested recently that monoamine neurons use glutamate as a co-transmitter. First, both serotonin (5-HT) and dopamine (DA) neurons in culture establish functional glutamatergic synapses in addition to classic terminals that release 5-HT or DA. Second, immunocytochemical work has provided evidence for the presence of neurotransmitter pools of glutamate in DA, 5-HT and noradrenergic neurons. Third, the recent cloning of 3 vesicular glutamate transporters (VGLUT1–3) has led to the discovery that noradrenergic neurons contain VGLUT2 mRNA, whereas 5-HT neurons contain VGLUT3 mRNA. Finally, although VGLUT2 mRNA does not appear to be abundant in DA neurons in the adult brain, DA neurons cultured from neonatal animals express VGLUT2, suggesting that these neurons may have the capacity to express this protein under specific conditions. Taken together with recent work describing the capacity of neurons to change neurotransmitter phenotype during development or in an activity-dependent manner, the finding of glutamate co-transmission in monoamine neurons may lead to significant revisions of current physiologic models of monoamine neuron function. In addition, the possible role of glutamate co-release in physiopathologic models of diseases that implicate central monoamine pathways, such as schizophrenia, must now be seriously considered.

Des recherches convergentes réalisées au cours des quatre dernières décennies ont établi au-delà de tout doute qu'un grand nombre, sinon la plupart, des neurones libèrent plus d'un neurotransmetteur. Même si l'on a accordé beaucoup d'attention à la libération simultanée de neurotransmetteurs à petites molécules et de neuropeptides, on a maintenant décrit un certain nombre d'exemples de libération simultanée de deux neurotransmetteurs à petites molécules. On a suggéré récemment que les neurones à monoamine utilisent le glutamate comme cotransmetteur. Tout d'abord, les neurones à sérotonine (5-HT) et à dopamine (DA) en culture établissent des synapses glutamatergiques fonctionnelles en plus des terminaisons classiques qui libèrent la 5-HT ou la DA. Deuxièmement, des études immunocytochimiques ont démontré la présence de fortes concentrations de glutamate dans les neurones à DA, à 5-HT et noradrénergiques. Troisièmement, suite au clonage récent de trois transporteurs vésiculaires du glutamate (VGLUT1–3) il a été démontré que les neurones noradrénergiques contiennent de l'ARNm de VGLUT2, tandis que les neurones à 5-HT contiennent de l'ARNm de VGLUT3. Enfin, même si l'ARNm de VGLUT2 ne semble pas abondant dans les neurones à DA dans le cerveau d'adulte, les neurones à DA en culture, préparés à partir d'animaux néonataux, libèrent du glutamate et expriment VGLUT2, ce qui indique que ces neurones peuvent exprimer cette protéine dans des conditions précises. Conjuguée aux résultats de travaux récents qui décrivent la capacité des neurones à modifier leur phénotype pendant le développement ou en réponse à des changements de leur

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activité, la découverte de la capacité des neurones à monoamine à libérer le glutamate pourrait entraîner une révision importante des modèles physiologiques courants du fonctionnement des neurones à monoamine. En outre, il faut maintenant envisager sérieusement le rôle possible de la co-libération de glutamate dans les modèles physiopathologiques de maladies, comme la schizophrénie, qui mettent en cause les voies centrales des monoamines.

Introduction

Considering the complexity of information transmission in the nervous system, it is obviously tempting to ignore the fact that most, if not all, neurons in the central and peripheral nervous system synthesize and release more than a single type of neurotransmitter. However, it is now well established that co-transmission of small-molecule neurotransmitters such as acetylcholine (ACh), glutamate or γ-aminobutyric acid (GABA) together with neuropeptides, such as calcitonin gene-related peptide (CGRP), enkephalins, substance P, neurotensin or cholecystokinin, is a general phenomenon, both in vertebrates and invertebrates.[1] Ultrastructural examination of axon terminals has revealed that many contain large, dense core vesicles of various dimensions in addition to the small, clear vesicles that contain small-molecule neurotransmitters. These larger vesicles are usually more distant from synaptic zones than small vesicles and are thought to contain neuropeptides poised for release upon the appropriate signal, which is usually thought to be high-frequency action potential firing.[2,3] Although much is known currently about the regulation and roles of neuropeptides in the nervous system,[1,4] especially in invertebrates,[5,6] it is still fair to say that our understanding of the functions of neuropeptides when acting as co-transmitters is still fragmentary, and that much additional work is required to begin to understand the physiologic and physiopathologic roles of co-transmission. An obvious complication of research on neuropeptide release is the fact that, unlike typical small-molecule neurotransmitters such as glutamate, ACh and GABA, neuropeptide receptors are not ionotropic and, therefore, neuropeptide release at synapses cannot be readily studied with the same high-resolution techniques, such as patch–clamp synaptic current recordings, that have been responsible for much of the progress in our understanding of fast neurotransmission. In addition to the co-release of a classic transmitter and a neuropeptide, co-transmission through the use of 2 small-molecule neurotransmitters is also now gradually being accepted as a general phenomenon.

Co-transmission of 2 small-molecule neurotransmitters in the nervous system

Co-release of ATP and ACh

A first example of such co-transmission is the co-release of adenosine 5′-triphosphate (ATP) together with ACh and other neurotransmitters. Early work provided data suggesting that ATP can be released from sensory nerves by electrical stimulation.[7] This nucleotide was actually identified as a constituent of cholinergic synaptic vesicles in the electric organ of the Pacific electric ray *Torpedo californica* in the early 1970s.[8–11] At the same time, it was also found to be co-released with ACh from the neuromuscular junction,[12] a finding that has been replicated and extended.[13] There is now abundant evidence indicating that ATP can be released in an activity-dependent manner with ACh or norepinephrine (NE) from various components of the sympathetic nervous system.[14–16] Evidence for the co-release of ATP and ACh from the terminals of striatal cholinergic neurons, and of ATP and GABA from spinal neurons in culture, has also been provided.[17,18]

Co-release of glutamate and GABA at mossy fibre terminals in the hippocampus

Perhaps even more surprising than co-release of ACh and ATP is the gradually emerging concept that single neurons can release both a typically excitatory neurotransmitter (glutamate) with a typically inhibitory neurotransmitter (GABA). Investigations of such a phenomenon were initiated following work by Ottersen and Storm-Mathisen[19,20] that showed that, at the light-microscope level, mossy fibre-like terminals in the stratum lucidum of the rat hippocampal formation appeared to be immunoreactive for GABA. This observation was surprising and raised skepticism, because these fibres were otherwise known to be excitatory and glutamatergic. An ultrastructural investigation, however, subsequently confirmed these initial findings by showing that mossy fibre terminals in contact with CA3 pyramidal neurons were not only GABA immunopositive but also glutamate immunopositive, as shown in serial thin sections.[21] The presence of the GABA biosynthetic enzymes GAD-65 and GAD-67 was then confirmed in mossy fibres and granule neurons of rats, mice and the monkey *Macaca nemestrina*.[22,23] These provocative findings obviously raised the question as to why and when would excitatory neurons co-release glutamate and GABA. A partial answer is that the GABAergic phenotype of these neurons may be preferentially expressed under conditions of increased activity. Schwarzer and Sperk[22] showed that kainic acid-induced seizures caused an elevation of GAD-67 mRNA and protein in granule neurons and mossy fibres. Sloviter et al[23] also showed that perforant path stimulation for 24 hours caused a pronounced upregulation of GAD-65 and GAD-67 mRNA and protein in granule neurons. These authors proposed that GABA release by granule neurons may represent a compensatory mechanism that could serve to partially react to excessive activity within the context of epileptic seizures. The functional nature of this GABAergic phenotype by granule neurons was recently demonstrated by showing that stimulation of granule neurons indeed evokes a GABA_A receptor-mediated, inhibitory postsynaptic current (IPSC) in CA3 pyramidal neurons.[24,25] This IPSC can only be revealed in the presence of ionotropic glutamate receptor blockers, because the glutamate-mediated excitatory postsynaptic current (EPSC) evoked in the same postsynaptic neurons is more than an order of magnitude bigger than the IPSC. In addition, the IPSC appears to be seen under basal conditions only in juvenile animals.[25,26] In adult animals, no residual synaptic current can be detected after ionotropic glutamate receptor blockade.[26] However, in such adult animals, a kindling stimulation protocol that is as short as 3 hours can induce a gradual increase in the GABAergic component of mossy fibre synaptic currents, an effect that is dependent on protein synthesis and accompanied by increased levels of GAD-67 immunolabelling.[26] The neurotransmitter phenotype of granule neurons is thus highly plastic, raising the possibility of its implication in development, synaptic plasticity and pathologic processes.

Co-release of GABA and glycine in the spinal cord

The ability of spinal cord inhibitory interneurons to release both GABA and glycine provides another striking example of co-transmission. Co-localization of glutamic acid decarboxylase (GAD)-positive nerve terminals together with postsynaptic glycine receptor clusters in the ventral horn of rat spinal cord provided the first indication that interneurons in this structure, which were otherwise known to be glycinergic, could perhaps also release GABA as a neurotransmitter.[27] About 10 years later, Jonas et al[28] used paired recordings from neurons in spinal cord slices to demonstrate that, indeed, a minor component of evoked IPSCs in this preparation is mediated by biccuculine-sensitive GABAergic receptors. Moreover, a population of miniature IPSCs recorded in these neurons appeared to be mediated by coactivation of GABA and glycine postsynaptic receptors, suggesting that perhaps these 2 transmitters could be contained in the same vesicles.[28] The possibility that GABA and glycine can be stored in the same vesicles is supported by the fact that the cloned vesicular inhibitory amino acid transporter (VIAAT) can transfer both GABA and glycine into synaptic vesicles.[29–31] The fact that the first recordings showing co-release of GABA and glycine were obtained from rats that were 6–7 days old suggests that, perhaps, this form of co-release is a phenomenon limited to early brain and spinal cord development.[32] However, evidence for the co-release of GABA and glycine has also been obtained from spinal cord slices from rats that were 30–60 days old.[33] Lastly, the phenomenon may not be restricted to the spinal cord, because it has been shown recently that IPSCs recorded from brain-stem motoneurons and from Golgi cells in the rat cerebellum can also share a mixed GABA and glycine phenotype.[34,35]

Basal forebrain neurons may release both ACh and glutamate

Cholinergic basal forebrain neurons project to a number of cortical structures including the entorhinal cortex, to which they provide a dense innervation. It is thought that this projection contributes to the regulation of memory formation. Investigating the hypothesis that other neurotransmitters are synthesized and released by these neurons, Manns et al[36] evaluated the expression of phosphate-activated glutaminase (PAG), an enzyme critical for the synthesis of neurotransmitter glutamate, in the rat basal forebrain. They reported that most cholinergic neurons were PAG positive, thus raising the possibility that these cholinergic neurons may have the capacity, as yet undemonstrated, to release both ACh and glutamate. Additional support for such a hypothesis is provided by the recent demonstration, using single-cell polymerase chain reaction (PCR) analysis, that basal forebrain cholinergic neurons contain mRNA for both choline acetyltransferase (ChAT) and for the second cloned vesicular glutamate transporter, VGLUT2 (see later for more information about vesicular glutamate transporters).[37]

Co-transmission in monoamine neurons

The examples listed here suggest that co-transmission of 2 small-molecule neurotransmitters, although not necessarily the norm, may be relatively common in the central and peripheral nervous system. In the remaining portion of this review, I will consider data suggesting that, in agreement with the generality of the concept of co-transmission, central monoamine neurons that release NE, 5-HT and DA may all, at least under some circumstances, have the capacity to use glutamate as a co-transmitter. The possible physiologic and physiopathologic implications of this fact will then be discussed.

Early physiologic and anatomical evidence for the release of 5-HT and ACh by invertebrate neurons

Early work performed on the nervous system of invertebrates provided the first demonstration that monoamine neurons can use other small-molecule neurotransmitters as co-transmitters. Investigating the neurotransmitter phenotype of the giant metacerebral neurons of *Helix aspersa*, Hanley et al[38] and Cottrell[39] found that these neurons, otherwise known to contain 5-HT, also contained ChAT and released Ach. Similar investigations performed in some of the giant neurons of *Aplysia californica* indicated that some neurons that contained 5-HT in this species also synthesized ACh and octopamine, a monoamine neurotransmitter related to NE and mainly found in invertebrates.[40]

Plasticity of neurotransmitter phenotype in sympathetic neurons

The ability of sympathetic neurons to co-release NE together with ACh or ATP is another well-known example of co-transmission in monoamine neurons. Following some early suggestions by Burn and Rand,[41] pioneering work by Patterson and Chun[42] and by Furshpan et al[43–45] first showed that, when placed in culture, sympathetic neurons isolated from the superior cervical ganglia of neonatal rats established functional synaptic connections that release NE, ACh or both transmitters simultaneously onto dissociated heart cells. In the absence of heart cells, the same neurons only displayed an adrenergic phenotype.[46] These authors[42–46] found that, at early time points, all neurons were adrenergic and that a proportion subsequently acquired a cholinergic phenotype. Heart cells could be replaced by medium conditioned by such cells, suggesting the implication of a diffusible factor.[47] It was proposed that ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) could act as possible signals to induce cholinergic differentiation.[48] It has also been shown recently that BDNF (brain-derived neurotrophic factor) can enhance the cholinergic phenotype of cultured sympathetic neurons within as short a time period as 15 minutes.[49] The physiologic relevance of this adrenergic–cholinergic phenotypic switch, first observed in culture, was subsequently demonstrated by showing that a similar phenomenon occurred in vivo during the development of the sympathetic innervation of sweat glands.[50] It was found that upon reaching the sweat gland, NE release by the adrenergic terminals induced the release of an instructive signal by the sweat gland (sweat gland factor or SGF).[51] The action of SGF on the incoming terminals then triggered a switch in neurotransmitter phenotype from adrenergic to cholinergic.

In vivo evidence for the presence of glutamate in monoamine neurons

5-HT-containing neurons in the raphe nuclei are called “serotonergic” for the obvious reason that their primary neurotransmitter is thought to be 5-HT. A similar logic holds for DA-containing neurons of the mesencephalon. However, mounting evidence points to the possibility that these neurons may release glutamate as a second small-molecule neurotransmitter. The first direct evidence suggesting that central monoaminergic neurons in vertebrate species might use glutamate as a co-transmitter was provided by Ottersen and Storm-Mathisen,[19] who found that a proportion of rat monoamine neurons, including DA-containing, 5-HT-containing and NE-containing neurons of the mesencephalon and brain stem, were immunopositive for glutamate. Subsequent work by Kaneko et al[52] showed that these same cell populations are immunopositive for PAG, the glutamate biosynthetic enzyme. The presence of glutamate-like immunoreactivity in 5-HT neurons has also been reported by another group in both the rat and monkey,[53] whereas that of glutamate in locus coeruleus noradrenergic neurons has been confirmed in 2 reports.[54,55] Finally, Sulzer et al[56] reported the co-localization of glutamate and DA in monkey brain. These intriguing findings raised the possibility that all, or a subset of, axon terminals established by monoamine neurons might contain and release glutamate in addition to DA or 5-HT. The idea that there might be a diversity of axon terminals established by any given DA neuron or 5-HT neuron in vivo is actually suggested by previous ultrastructural data. For example, it has been reported that during the development of dopaminergic projections to the striatum in the rat, 2 types of dopaminergic fibres can be recognized; the first being thin fibres with an average diameter of 0.2 µm and the second being thicker fibres with a diameter of 0.6 µm.[57] During postnatal development of these pathways, thin fibres gradually become the largest contingent in the striatum.[57] Close examination of the axon terminals belonging to DA neurons has also revealed that, although most (about 60%–70%) are devoid of postsynaptic specializations or are "asynaptic" and probably mediate "volume" DA release in the striatum, a variable proportion (about 30%–40%) form "junctional" symmetric-type synapses.[58–60] Similar observations have been made for nerve terminals established by 5-HT neurons.[61–63] Could such junctional synapses (Fig. 1) represent sites of synaptic glutamate release?

In-vitro evidence for the synaptic release of glutamate by monoamine neurons

A direct test of such a possibility would require paired recordings between, for example, an individual DA neuron and an individual target cell in a projection area such as the striatum. Such an experiment represents a formidable challenge considering the low connectivity between these cells and the distance between the cell bodies of the presynaptic and postsynaptic neurons. However, a closer examination of such a question has been performed using in-vitro primary culture models. Using a microculture system in which isolated postnatal 5-HT neurons of the rat raphe nuclei develop on small (100–500 µm) microdroplets of substrate, Johnson[64] studied the synaptic development of 5-HT neurons. Under such conditions, isolated neurons are forced to establish synaptic contacts onto a limited subset of neighbouring neurons or, if the neuron is alone, onto its own dendritic arbour. Synaptic connections established by a neuron onto itself in such a way are referred to as "autapses" instead of "synapses," a term coined by Van Der Loos and Glaser in 1972.[65] Electrophysiologic recordings from such isolated cells revealed that about 60% of 5-HT neurons established at least a subset of terminals that released a neurotransmitter that produced an excitatory postsynaptic potential (EPSP) with a rapid time course. The complete blockade of these fast autaptic EPSPs by CNQX, an antagonist of ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate glutamate receptors, suggested that these neurons indeed released glutamate as a co-transmitter together with 5-HT.[64] An ultrastructural examination of these neurons revealed that both asymmetric and symmetric-type terminals were established, compatible with the ability of these neurons to release different transmitters.[66]

Using a very similar microculture system, analogous observations were made by Sulzer et al[56] in single rat DA neurons in primary culture. Using an immunocytochemical approach, they found that about 75% of DA neurons, identified with an antibody directed against tyrosine hydroxylase (TH), were also immunopositive for glutamate. In addition, about 50% of these DA neurons were immunopositive for PAG. Arguing for heterogeneity among the terminals established by DA neurons, these authors reported that a proportion of the terminals established by isolated DA neurons, identified by the presence of the synaptic vesicle protein synaptophysin, were immunopositive for glutamate but immunonegative for TH. Considering that TH is a cytosolic enzyme, the significance of this observation is unclear. Nonetheless, a possible interpretation is that a small subset of all terminals established by DA neurons could actually be specialized for the synaptic release of glutamate (Fig. 2). In keeping with the establishment of glutamatergic synaptic terminals, patch–clamp recordings from these neurons showed that a single action potential in a DA neuron evoked an EPSC that was completely blocked by AP5 and CNQX, antagonists of *N*-methyl-D-aspartate (NMDA) and AMPA/kainate glutamate receptors.[56]

The function and regulation of these glutamatergic terminals established by DA neurons was studied recently and deserves closer attention. It is notable that all regulatory mechanisms previously investigated at DA-releasing terminals in vivo also seem to function in a similar or identical way at these glutamatergic synapses in culture. First, as initially demonstrated by Sulzer et al,[56] D_2 DA receptors inhibit glutamate release as evidenced by the ability of quinpirole, a D_2-selective agonist, to reduce the amplitude of EPSCs recorded in isolated DA neurons in culture. This regulation has been shown recently to be presynaptic in origin (Fig. 3A–C)[67] and to be dependent on the regulation of some terminal K^+ channels that are sensitive to 4-aminopyridine (Fig. 3D).[68] The ability to measure quantal glutamatergic events arising from terminals established by DA neurons has also led to the discovery that presynaptic inhibition through terminal D_2 receptors may implicate some direct negative regulation of the exocytotic process in nerve terminals.[68] Such a possibility has not previously been addressed in vivo for DA release because of our inability to measure quantal events directly. A second example of the coordinate regulation of DA and glutamate release in DA neurons

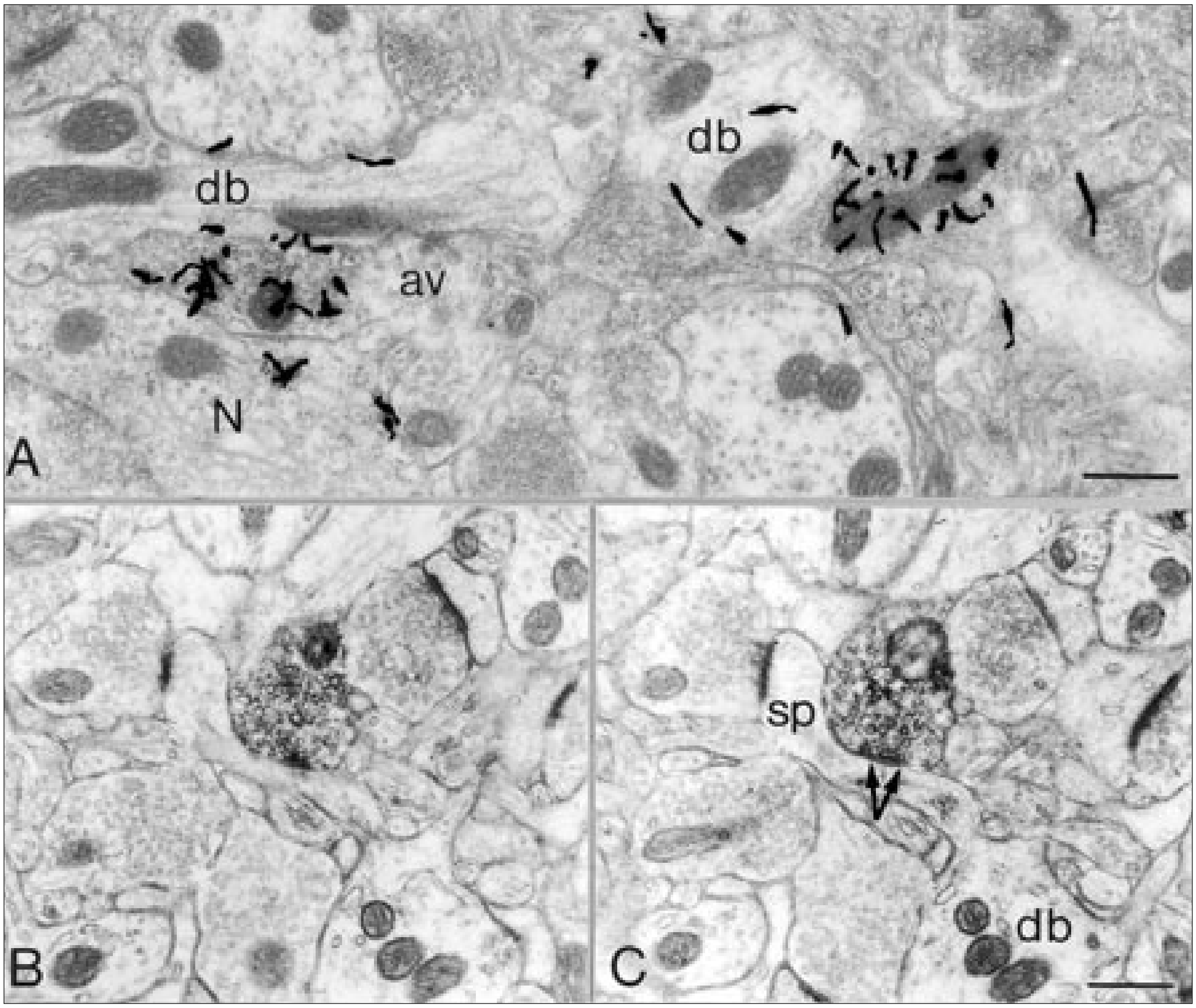

Fig. 1A: Photomicrograph obtained by combined [^{3}H]DA autoradiography (black silver grains) and electron microscopy illustrates 2 typical dopamine (DA)-containing, asynaptic free nerve endings. Note the presence of multiple small clear synaptic vesicles and the absence of any obvious synaptic specialization. The sections were obtained from the dorsal striatum of an adult rat brain (original magnification ×25 000, scale bar 0.5 µm). B and C: Serial thin sections of a neostriatal DA-immunoreactive axonal varicosity. The electron micrographs show that a proportion of nerve terminals established by DA neurons in vivo have the typical appearance of junctional synaptic contacts (arrows) (original magnification ×23 000, scale bar 0.5 µm). N = neuronal cell body, db = dendritic branch, av = axonal varicosity, sp = spine. Reproduced with permission from Wiley-Liss (*J Comp Neurol* 1996;375:167-86).[59]

is provided by the demonstration that, in accord with the trophic and protective role of glial cell line-derived neurotrophic factor (GDNF) on DA neurons, this growth factor promotes the establishment and function of glutamatergic terminals by cultured DA neurons (Fig. 4).[69] Finally, recent work investigating the regulation of cultured DA neurons by neurotensin, a peptide known to facilitate DA release, has concluded that activation of terminal neurotensin receptors decreases the ability of terminal D_2 receptors to inhibit glutamate release by isolated DA neurons in culture (Fig. 5).[70] It has been suggested that DA release in vivo may be regulated by a similar mechanism.[71] Together, these findings indicate that the regulation of glutamate and DA release by DA neurons occurs in parallel, through very similar, if not identical, mechanisms.

Considering these findings, an obvious question is whether glutamate release by DA neurons in culture occurs only under conditions of autapse formation (i.e., when the neuron is deprived of its regular postsynaptic partners). This question has been addressed recently by establishing co-cultures of ventral tegmental area DA neurons together with GABAergic medium spiny neurons of the nucleus accumbens.[72] Patch–clamp recordings from such reconstituted mesolimbic synapses showed that glutamatergic EPSCs can still be reliably detected. These findings do not prove that synaptic glutamate release occurs in the mesolimbic pathway in vivo; however, they support the view that glutamate release by DA neurons is not an artifact of isolated neuron cultures.

Indirect in-vivo electrophysiologic evidence for fast excitatory synaptic responses evoked by monoamine neurons

As described earlier, a direct demonstration of the glutamatergic nature of synapses established by 5-HT or DA neurons in vivo would require simultaneous recordings from single monoamine neurons and single synaptically connected postsynaptic target neurons in physically distant nuclei. Although this objective is currently difficult to achieve, experiments have been performed by recording from striatal neurons and stimulating extracellularly in the DA cell-body region or in the medial forebrain bundle, which carries dopaminergic axons. Early experiments showed that rapid excitatory synaptic responses could indeed be evoked by extracellular stimulation in DA cell-body areas.[73–75] However, collaterals of descending cortical fibres projecting to the midbrain could have been partly involved in these excitatory, but pharmacologically uncharacterized, responses.[76] Recent work has,

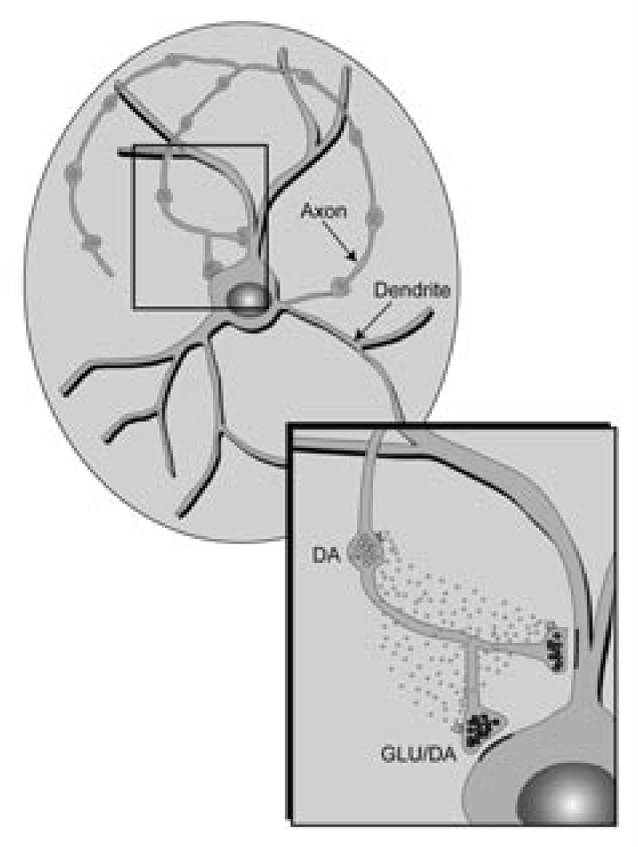

Fig. 2: Schematic representation of a single DA neuron in primary culture. A number of dendrites are represented together with a thinner axon that branches out and contacts the dendrites. The axonal processes display numerous small DA-containing varicosities that appear as free nerve endings. The inset shows the enlargement of an axonal process. Current evidence suggests that the varicosities could be the primary site of DA release. A receptor is depicted on the varicosity to represent the fact that the release of DA at these nonsynaptic terminals is controlled by dopamine D_2-type autoreceptors. In addition, a subset of terminals establish synaptic contacts onto the neuron's dendrites and cell body (autaptic contacts). Synaptic glutamate release (darker vesicles) could be restricted to these more classic synaptic contacts. It is possible that these synaptic contacts may release both DA and glutamate (GLU). Postsynaptic glutamate and DA receptors would be present at these synapses (thick blue line). Terminal D_2 autoreceptors are also present and could be activated by DA released from nearby DA-containing varicosities. Current evidence thus suggests that isolated DA neurons have the capacity to establish 2 distinct types of synaptic and nonsynaptic contacts. Modified image reproduced with permission from the Society for Neuroscience (*J Neurosci* 1998;18:4588-602).[56]

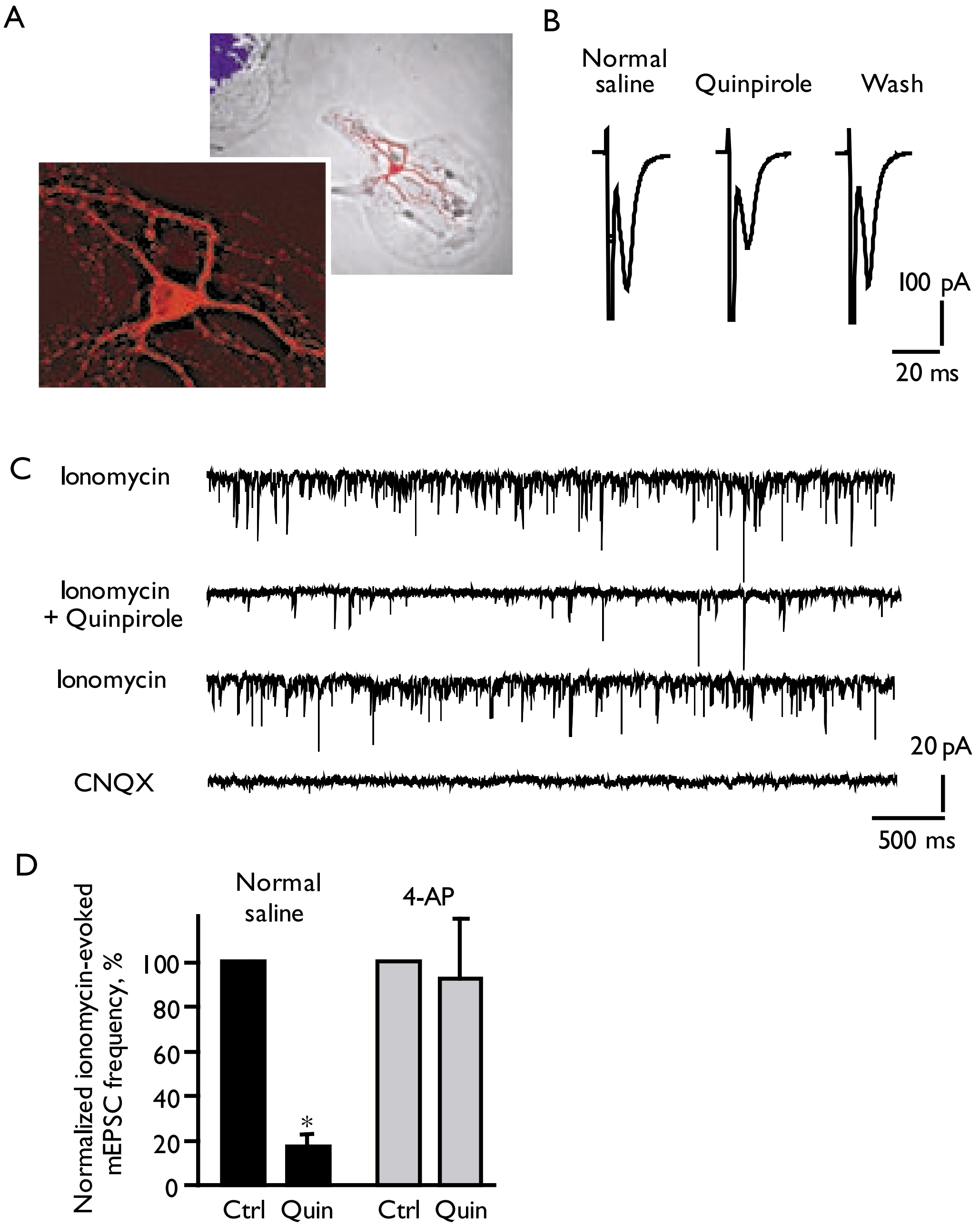

Fig. 3A: Phase contrast and immunofluorescence images of an isolated rat DA neuron in culture. The red signal identifies tyrosine hydroxylase (TH) immunoreactivity. The blue signal identifies fluorescent microspheres used to identify recorded neurons after immunocytochemical processing. B: Whole-cell patch–clamp recordings from an isolated DA neuron in culture. The first inward deflection reflects the sodium action current (clipped for clarity). The second inward deflection represents the glutamate receptor-mediated excitatory postsynaptic current (EPSC). The D_2 receptor agonist quinpirole (5 μmol/L) reduced the amplitude of the glutamate-mediated EPSC. C: Whole-cell patch–clamp recordings of action potential-independent miniature EPSCs (mEPSCs) represent the postsynaptic effect of the fusion of single glutamate-filled vesicles. The events were recorded in a single DA neuron. The basal frequency of mEPSCs was enhanced by the calcium ionophore ionomycin. Quinpirole (5 μmol/L) caused a large decrease in the frequency of occurrence of mEPSCs, reflecting a presynaptic mechanism. All events were blocked by the ionotropic glutamate receptor antagonist CNQX. D: Summary diagram shows the average effect of quinpirole on mEPSC frequency. The effect of quinpirole was completely blocked by the K^+ channel blocker 4-AP. Reproduced with permission from the American Physiological Society (*J Neurophysiol* 2002;87:1046-56).[67] Wash = washout period.

however, confirmed these initial findings, indicating that these evoked EPSPs are glutamatergic.[77] In this latter report, local application of a D_2 receptor agonist at the site of stimulation inhibited the generation of EPSPs in striatal neurons, suggesting that D_2-responsive, putative dopaminergic neurons, were indeed responsible for the glutamatergic responses. Even if these results are not as convincing as dual intracellular recordings, they provide solid additional evidence in favour of the hypothesis that DA neurons in vivo may also release glutamate through a subset of their terminals. Finally, rapid excitatory CNQX-sensitive synaptic events have also been reported to be evoked in spinal cord ventral horn motoneurones after extracellular stimulation of presumed locus coeruleus noradrenergic neurons.[55,78] Similar excitatory responses have also been found to be generated in striatal neurons and ventral horn motoneurones after extracellular stimulation of presumed 5-HT neurons in raphe nuclei.[79–81]

Localization of vesicular glutamate transporters in monoamine neurons

A renewal of interest in understanding the glutamatergic phenotype of neurons has arisen since the identification, 3 years ago and by 2 independent groups, of the first vesicular glutamate transporter. This transporter had been previously cloned in 1994 and shown to act as a brain-specific Na^+-dependent, inorganic phosphate transporter (BNPI).[82] A role in presynaptic function was first suggested by the finding that EAT-4, a *Caenorhabditis elegans* homologue of BNPI, had a critical role in glutamate-mediated neurotransmission in this organism.[83,84] BNPI was then found to be highly expressed in the synaptic vesicles of a subset of brain glutamatergic neurons.[85] Finally, overexpression studies in PC12 and BON cell lines proved that BNPI acted as a bona fide glutamate transporter depending on ATP and the vesicular proton electrochemical gradient.[86,87] Moreover, overexpression of BNPI (now called VGLUT1) in cultured GABA neurons gave these neurons the ability to co-release glutamate in addition to GABA, providing

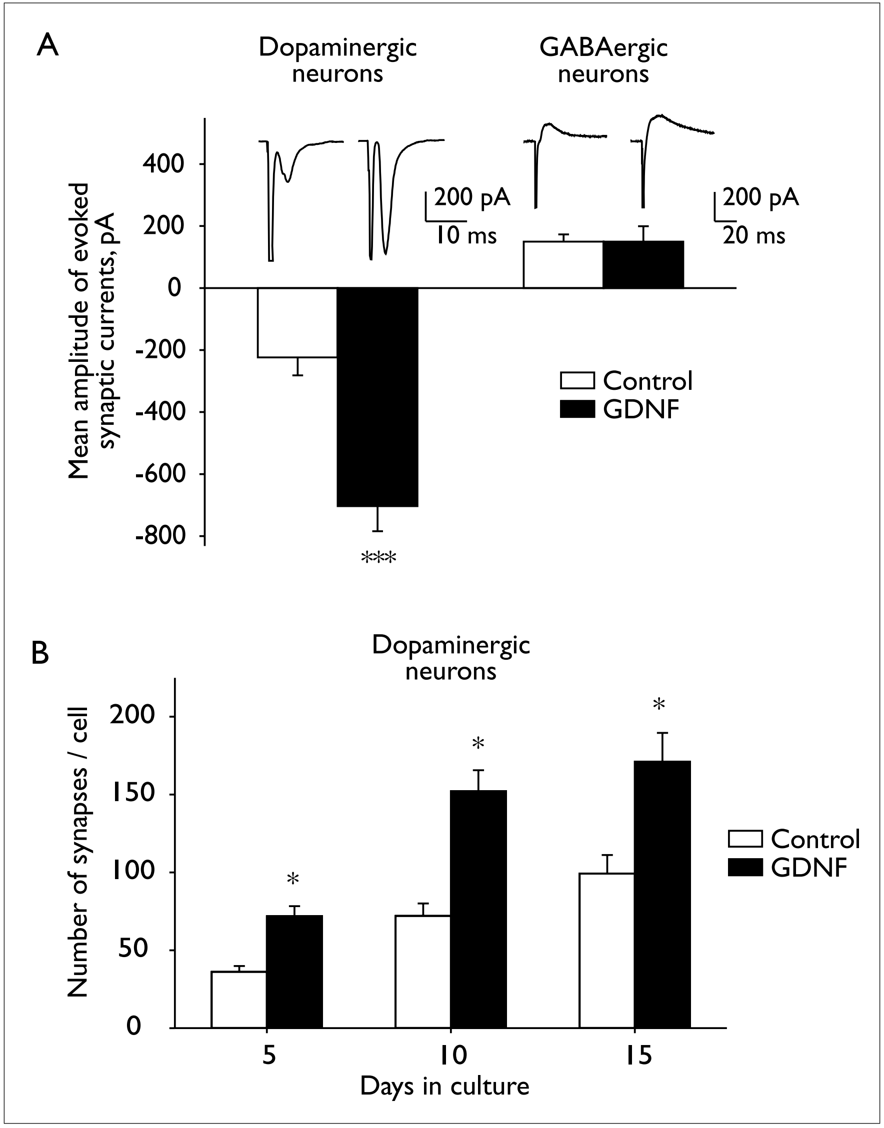

Fig. 4A: Whole-cell patch–clamp recordings of glutamate-mediated EPSCs in isolated DA neurons. Chronic application of glial cell line-derived neurotrophic factor (GDNF) (26 pmol/L) for 15 days enhanced the average amplitude of glutamate-mediated EPSCs (left traces). No such effect was observed in isolated γ-aminobutyric acid (GABA)-ergic neurons in the same cultures (right traces). The graphs illustrate the average effects.
B: Summary data illustrate the average number of morphologically identified synaptic terminals in isolated DA neurons. At 5, 10 and 15 days in culture, GDNF (26 pmol/L) caused an increase in the number of nerve terminals established by individual neurons. *$p < 0.05$, ***$p < 0.001$.
Reproduced with permission from Blackwell Publishing (*Eur J Neurosci* 2000; 12:3172-80).[69]

support for the idea that expression of a vesicular glutamate transporter may be necessary and sufficient to permit vesicular glutamate release by neurons.[87]

A close homologue of VGLUT1 was identified 2 years ago.[88] This protein, initially called differentiation-associated Na$^+$-dependent inorganic phosphate transporter (DNPI), shares 82% amino acid identity with VGLUT1, localizes to neurons in the brain[89] and is localized to a vesicular compartment.[90,91] In an exceptional convergence of research efforts, 6 groups independently reported that DNPI, now called VGLUT2, acts as the second major vesicular glutamate transporter.[92–97] 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 abundant in diencephalic and other subcortical nuclei, in deep cerebellar nuclei and in the brain stem.[89,92–94,98] Closer examination of the localization of VGLUT2 mRNA in brain-stem nuclei showed that, although this transcript is not present in brain-stem cholinergic and serotonergic neurons, it is present in most adrenergic neurons of the C1, C2 and C3 groups and in most noradrenergic neurons of the A2 group.[99,100] However, noradrenergic neurons of the locus coeruleus appear to be negative by in situ hybridization, a finding that is surprising in light of the previous demonstration of significant glutamate immunoreactivity in these neurons.[55]

Because the cloning and characterization of VGLUT1 and VGLUT2 identified patterns of expression that included most known glutamatergic neurons in the brain, the very recent identification of a third vesicular glutamate transporter (VGLUT3) came as a surprise. Even more surprising was the finding that VGLUT3, which shares 72% amino acid identity with VGLUT1 and VGLUT2, showed a more restricted expression in a limited number of neurons not classically thought of as glutamatergic. In particular, VGLUT3 mRNA was found in most 5-HT neurons of the raphe, identified by the presence of the 5-HT transporter. It was also shown

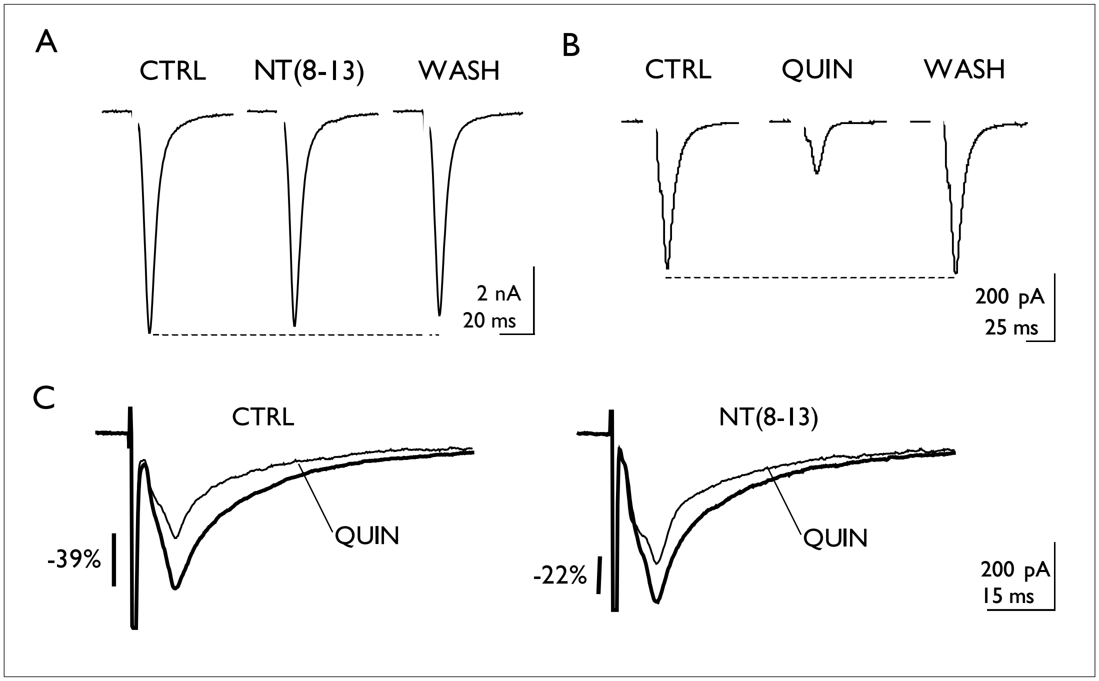

Fig. 5A: Whole-cell patch–clamp recordings from isolated DA neurons in culture. The neuropeptide neurotensin (NT) (100 nmol/L), which is known to enhance DA release in vivo, failed to enhance directly glutamate-mediated EPSCs in isolated DA neurons. B: Activation of terminal D_2 DA receptors with quinpirole strongly decreased the amplitude of action potential-evoked EPSCs in isolated DA neurons. C: Neurotensin (100 nmol/L) decreased the ability of D_2 receptor activation to decrease EPSC amplitude in isolated DA neurons, reflecting an indirect presynaptic action of neurotensin on glutamate release. Reproduced with permission from Elsevier (*Neuroscience* 2002;111:177-87).[70]

to be abundant in cholinergic interneurons of the striatum, identified by the presence of ChAT. Finally, VGLUT3 mRNA was found in some scattered populations of hippocampal, hypothalamic and cortical interneurons.[101–103] Immunocytochemical labelling further identified VGLUT3 protein immunoreactivity in cholinergic ChAT-positive terminals of the striatum[103] and in vesicular monoamine transporter-2 (VMAT2)-positive/TH-negative, presumed serotonergic terminals in the cortex and hippocampus.[101] Finally, double-labelling studies suggest that VGLUT3-positive nerve terminals do not co-express VGLUT1 or VGLUT2.[101] Taken together with the work showing that 5-HT neurons contain glutamate immunoreactivity in vivo and establish functional glutamate-releasing terminals in culture, these data provide strong support for the hypothesis that glutamate co-transmission is widespread in monoamine neurons.

Although initial Northern blot experiments provided support for the presence of VGLUT2 in the substantia nigra,[88] neither VGLUT1, nor VGLUT2, nor VGLUT3 mRNA have been conclusively detected in DA neurons by in situ hybridization in material prepared from adult animals.[101,103] These findings do not exclude low expression levels. However, they may be considered somewhat paradoxical considering the fact that DA neurons in culture clearly release glutamate at synapses. To resolve this issue, we have recently examined the presence of VGLUT1, VGLUT2 and VGLUT3 in postnatal rat mesencephalic neurons in primary culture. Using immunocytochemical labelling, we found that, although isolated DA neurons expressed neither VGLUT1 nor VGLUT3, about 80% of TH-positive neurons were immunopositive for VGLUT2.[104] The labelling was punctate in nature and particularly concentrated close to major dendrites and the cell body of DA neurons. Interestingly, although most VGLUT2-positive varicosities were TH positive, many neurons displayed long thin axon-like segments bearing multiple TH-positive/VGLUT2-negative varicosities. In addition, in a triple-labelling experiment, it was apparent that a large number of nerve terminals, identified by the presence of the synaptic protein SV2, were VGLUT2 negative. This finding is compatible with the hypothesis that DA neurons can establish distinct sets of terminals, only a proportion of which have the ability to co-release glutamate. The expression of VGLUT2 in single DA neurons was confirmed by single-cell reverse-transcriptase PCR.[104]

To determine whether the ability to express VGLUT2 was the result of delayed upregulation happening because of the cell-culture conditions, we evaluated VGLUT2 immunolabelling in DA neurons at different time points in culture. We found that as soon as 24 hours after being isolated and put in culture, more than 50% of DA neurons were VGLUT2 positive.[104] This result is compatible with the possibility that DA neurons isolated from postnatal day 0 to postnatal day 2 rat pups already have some level of basal VGLUT2 expression or, alternatively, can very rapidly upregulate its expression. If VGLUT2 can be readily detected after 24 hours in culture, then why is it that mRNA cannot be detected by in situ hybridization in vivo? Although an answer to this question is currently unavailable, it is important to point out that the developmental profile of VGLUT2 mRNA expression remains to be determined in vivo. Only adult animals have been examined by in situ hybridization. Northern blot analysis of general VGLUT2 expression during the prenatal and postnatal period has shown that, in contrast to VGLUT1, which appears mostly after birth, VGLUT2 mRNA is already abundant before birth.[88,101] This raises the hypothesis that VGLUT2 mRNA is abundant in DA neurons during the prenatal and neonatal period, but gradually declines after birth, thus explaining the absence of robust signal by in situ hybridization in adult brain.

Physiologic and pathophysiologic roles of glutamate co-transmission in aminergic neurons?

In view of the generality of co-transmission in the nervous system and the wealth of evidence that monoamine neurons are not an exception to this rule, it is worth evaluating the possible physiologic and pathophysiologic implications of glutamate co-release by monoamine neurons.

Fast synaptic action

To this day, most models of the physiologic function of monoamine neurons describe a modulatory role for DA, 5-HT and NE in the brain. Such considerations are based upon the established fact that most monoamine axon terminals in the CNS are “asynaptic” free nerve endings thought to mediate “volume transmission” of signals[58–63,105,106] and to modulate the activity of nearby fast-acting synapses releasing glutamate or GABA. It has always been clear, however, that a variable proportion of junctional contacts is always established by monoamine neurons. The possibility that these junctional or conventional-looking synapses mediate rapid glutamatergic synaptic transmission should now be seriously considered. If this turns out to be true, then perhaps information transfer through either a subset or all of the neuronal pathways implicating monoamine neurons involves both fast and slow signalling. Such information transfer could be dynamically regulated during variations in the firing rate of monoamine neurons. At low firing frequencies, where monoamines are thought to be inefficiently released, perhaps glutamate synaptic transmission through activation of ionotropic receptors plays a significant role. However, during burst firing or at higher firing frequencies, monoamine (and neuropeptide) release might take over the preponderant role through massive activation of G-protein-coupled receptors. The presence of terminal D_2 receptors on glutamatergic terminals established by DA neurons might also contribute to increasing the dopamine-to-glutamate ratio under such circumstances by mediating presynaptic inhibition of glutamate release.[56,68] It is clear that much additional research will be required to investigate such hypotheses and determine the exact physiologic role of such co-transmission in the nigrostriatal, mesolimbic and other monoamine pathways.

Possible developmental role of an early glutamatergic phenotype

Although the paucity of data preclude the construction of detailed hypotheses at this time, 2 sets of findings suggest that co-release of glutamate by monoamine neurons may play some developmental role. First, as already described, Northern blot analyses have shown that, although VGLUT1 mRNA is expressed mostly during the postnatal period, VGLUT2 mRNA appears to be expressed very early in prenatal development.[88,101] Second, although initial mappings of VGLUT2 mRNA have suggested that it might be absent from DA neurons in adults, VGLUT2 immunoreactivity can be readily detected in DA neuron cultures established from the neonatal rat brain.[104] It will have to be determined experimentally whether glutamate release by DA neurons intervenes in early synapse formation by these neurons. The surprising finding by Zhou and Palmiter[107] that DA-deficient mice undergo normal development of their nigrostriatal pathway shows quite clearly that DA release is not necessary for the early development of this pathway. In this context, it is noteworthy that recent experiments performed in nigrostriatal-cortical explant cultures have demonstrated that blocking metabotropic glutamate receptors dramatically reduces synapse formation by DA neurons.[108] It will be important to pursue such work to clarify the specific role of glutamate release by monoamine neurons in synapse formation by these neurons.

Involvement in physiopathology?

A final point to consider is the possible physiopathologic implication of glutamate co-release by monoamine neurons. This possibility must be taken seriously considering the likelihood that neurotransmitter phenotypic switches within neuronal populations may not be that unusual. For example, as described earlier, recent work in brain-slice preparations has convincingly demonstrated that glutamatergic granule neurons of the hippocampus, which initially release little, if any GABA, can be induced, within a matter of 3 hours, to co-release GABA following a kindling stimulation protocol.[26] When investigating neurotransmitter phenotype in hypothalamic neurons in culture, Belousov et al[109,110] found that chronic NMDA-receptor blockade can induce a large increase in the proportion of hypothalamic neurons releasing ACh. Although these authors have not directly determined the phenotype of the neurons before their switch to a cholinergic status, their data provide yet another example of an activity-dependent switch in neurotransmitter phenotype. Within the context of such work, it will be of interest to determine whether long-term treatment of animals with drugs of abuse, antipsychotic drugs or antidepressant drugs triggers delayed neurotransmitter phenotypic switches in monoamine neurons. In view of the possible developmental variation in VGLUT2 expression in DA neurons, it might even be envisioned that schizophrenia is associated with a perturbation of VGLUT2 expression in DA neurons.

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