Coexistence and function of different neurotransmitter transporters in the plasma membrane of CNS neurons

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Abstract

Transporters able to recapture released neurotransmitters into neurons can no longer be considered as cell-specific neuronal markers. In fact, colocalization on one nerve terminal of transporters able to selectively recapture the released endogenously synthesized transmitter (homotransporters) and of transporters that can selectively take up transmitters/modulators originating from neighboring structures (heterotransporters) has been demonstrated to occur on several families of nerve terminals. Activation of heterotransporters often increases the release of the transmitter stored in the terminals on which the heterotransporters are localized. The release caused by heterotransporter activation takes place through multiple mechanisms including exocytosis, either dependent on external Ca2+ or on Ca2+ mobilized from intraterminal stores, and homotransporter reversal. Homocarrier-mediated release elicited by heterocarrier activation represents a clear case of transporter–transporter interaction. Although the functional significance of transporter coexpression on one nerve terminal remains to be established, it may in some instances reflect cotransmission. In other cases, heterotransporters may mediate modulation of basal transmitter release in addition to the modulation of the evoked release brought about by presynaptic heteroreceptors. Heterotransporters are also increasingly reported to exist on neuronal soma/dendrites. With the exception of EAA T4, the glutamate transporter/chloride channel situated on GABAergic Purkinje cells in the cerebellum, the functions of somatodendritic heterocarriers is not understood.

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Sub. Class.: Neurotransmitter uptake transporters; Transporter coexistence; Heterotransporters; Transporter–transporter interactions; Neurotransmitter release; Exocytosis; Carrier-mediated release

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Abbreviations: ACh, acetylcholine; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; BoNT/F, botulinum toxin type F; CNS, central nervous system; DA, dopamine; EAA, excitatory amino acid transporter; GABA, /-aminobutyric acid; NE, norepinephrine; SKF 100330A, N-(4,4-diphenyl-3-butenyl)-guvacine; SNARE, SNAP receptor; TeTx, tetanus toxin; VAMP, synaptobrevin; VSCC, voltage sensitive Ca2+ channels

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1. Introduction

The physiological release of neurotransmitters from depolarized nerve endings occurs by a process of vesicular exocytosis (Greenard et al., 1993; Südhof et al., 1993; Bennett and Scheller, 1994; Sollner and Rothman, 1994; Schweizer et al., 1995; Stanley, 1997). Transmitter release occurs predominantly and therefore they can carry neurotransmitters not only from the extracellular fluid into the nerve endings but also vice-versa. Such an inside-out transport is known as carrier-mediated release or transporter reversal. The process has been clearly shown to occur in the presence of certain drugs or following ionic changes characteristic of some pathological conditions such as ischemia, although a physiological role for the carrier-mediated release cannot at present be ruled out (Levi and Raiteri, 1974; Bernath and Zigmond, 1988; see for reviews, Attwell et al., 1993; Levi and Raiteri, 1993). Independently of its functional significance, the existence of the carrier-mediated release indicates that membrane transporters can subserve functions other than neurotransmitter reuptake. Clearly, while reuptake is aimed at limiting neurotransmission by removing neurotransmitters from the receptor biophase, the activation of carrier-mediated release is expected to increase the availability of neurotransmitters at their receptors.

Results from several investigations appear to further amplify the roles of neurotransmitter transporters. As shown in this review, evidence exists that several families of nerve terminals, neurotransmitters are inactivated by either enzymatic degradation or active recapture into nerve endings and uptake into glial cells. In fact, it has been known for many years that neuronal axon terminals are endowed with membrane transport mechanisms able to selectively recognize the endogenously synthesized neurotransmitter just released and to remove it from the extracellular space through a reuptake process (Iversen, 1970; Snyder et al., 1973; Masson et al., 1999). Essential to this mechanism of reuptake are selective membrane carriers or transporters, macromolecular entities of protein nature most of which have been cloned and recently shown to exist as multiple isoforms (Guastella et al., 1990; Shimada et al., 1990; Fischolzcyk et al., 1991; Amara and Kuhar, 1993; Kanai et al., 1993; Gegebashvili and Schousboe, 1997; Masson et al., 1999; Seal and Amara, 1999).

Based on in vitro and in vivo findings, it is now generally recognized that neurotransmitters can, in some conditions, be released from axon terminals by a mechanism different from exocytosis. Membrane transporters can work bidirectionally and therefore they can carry neurotransmitters not only from the extracellular fluid into the nerve endings but also vice-versa. Such an inside-out transport is known as carrier-mediated release or transporter reversal.
2. Heterotransporter-induced neurotransmitter release

It was during a study of the effects of γ-aminobutyric acid (GABA) on the release of acetylcholine (ACh) that the possibility of a novel function for neurotransmitter transporters emerged. The amino acid provoked release of ACh from resting hippocampus synaptosomes; however, contrary to the expectation, the effect of GABA occurred independently of release-regulating presynaptic receptors, being instead mediated by a GABA transporter sited on cholinergic nerve terminals. The finding suggested the existence of nerve terminals which not only possess a choline transporter but also a GABA heterocarrier, the activation of which brings about release of ACh (Bonanno and Raiteri, 1986, 1987a). Subsequent studies of the above interaction between GABA and ACh showed it to be heterogeneously distributed in different areas of the rat brain (Bonanno and Raiteri, 1987b) and to occur also in human neocortex (Bonanno et al., 1991).

These observations lent themselves to various interpretations: (i) although neuronal cotransmission generally involves one classic neurotransmitter plus one or more neuromodulators (see Hökfelt et al., 1980), some neurons may costore ACh and GABA (Brashear et al., 1986; Caffe et al., 1996; Safieddine et al., 1997) and therefore possess two reuptake carriers, one for choline and the other for GABA; moreover, GABA might regulate ACh release through GABA transporters; (ii) independently of cotransmission, some neurons might express release-regulating presynaptic heterotransporters, in analogy to the classic release-regulating presynaptic heteroreceptors generally present on axon terminals (Vizi, 1979; Langer, 1981; Starke, 1981; Chessette, 1984; Raiteri et al., 1984; Raiteri, 2001).

2.1. Heterotransporter-induced and carrier-mediated neurotransmitter release

In addition to the GABA heterotransporters on cholinergic neurons, other neurotransmitter systems were investigated. In particular, in order to establish whether transporter coexistence on one nerve terminal, or the presence of heterotransporters, reflects cotransmission, neuronal systems known to costore neurotransmitters were compared to others in which cotransmission had not been reported to occur. During these studies, many examples of terminal heterocarrier-mediated release involving different neurotransmitters have been identified (see Bonanno and Raiteri, 1994). As to the mechanisms underlying the function of heterotransporters, in particular the stimulation of neurotransmitter release, these have been deeply investigated only in a limited number of systems and appear quite heterogeneous.

It should be noted in this regard that activation of heterocarriers implies uptake of the transmitter/modulator into the host neuron (i.e. GABA into cholinergic axon terminals, in the above mentioned example). This uptake can trigger the activation of intraterminal pathways leading to the release of the transmitter synthesized in that neuron. The latter process may occur by different mechanisms (see Chapter 3), including carrier-mediated release due to reversal of transporters (Attwell et al., 1993; Levi and Raiteri, 1993). Thus, in the text, a sentence like “heterotransporter-mediated transmitter release” refers to the complex cascade of events starting with uptake of the transmitter/modulator through its specific heterotransporter into the releasing terminals. The term “carrier-mediated release” refers to a process exclusively involving the homotransporter that mediates the exit of the neurotransmitter characteristic of that neuronal phenotype from the cytosol to the extracellular space by transporter reversal.

2.2. Methodological aspects

To demonstrate that two transporters coexist on the same nerve ending, one has to use experimental approaches able to minimize indirect effects and to provide results that can be easily and unequivocally interpreted. Most of the works discussed in this article have been carried out using isolated brain nerve endings (synaptosomes) prepared from various CNS regions, distributed as very thin layers on microporous filters and superfused in parallel superfusion chambers according to a well-established technique introduced several years ago (Raiteri et al., 1974) and recently revisited in detail in a review article (Raiteri and Raiteri, 2000). Some of the properties of this experimental set-up make it the method of choice to functionally identify and characterize constituents belonging to a particular family of nerve endings. Data from several laboratories demonstrate that, in this system, synaptosomal reuptake of released neurotransmitters is prevented or minimized during superfusion. The continuous and quick removal by the superfusion fluid of the compounds released prevents not only their reuptake but also their presence in significant concentrations in the biophase of transporters and receptors, which, therefore, remain fully available to ligands added at the desired concentrations to the superfusion medium. Indirect effects (i.e. those that are due to released compounds acting on adjacent structures or to compounds released from adjacent structures) also appear minimized. Therefore, if the release of neurotransmitter A is increased or decreased upon addition of compound B to the superfusion fluid, these effects can be assumed to be due to B acting directly on the nerve ending which releases A. In our case, if B releases A following penetration through a transporter selective for B and the released form of A is a radioactive transmitter previously taken up by a transporter selective for A, it can be concluded that the axon terminal releasing transmitter A possesses two transporters, one selective for A and one selective for B.

To exemplify this conclusion, we will briefly describe the experimental procedure followed in the work, which led...
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* peripheral nervous system, n.d.: not determined. A: Pittaluga and Raiteri (1987); B: Bonanno and Raiteri (1994); C: Bernath and Zigmond (1989); D: Bonanno and Raiteri (1987b); E: Bonanno et al. (1991); F: Bonanno and Raiteri (1986); G: Bonanno and Raiteri (1987a); H: Snow et al. (1992); I: Vaney and Young (1988); J: Reinmann et al. (1982); K: Bonanno and Raiteri (1987c); L: Raiteri et al. (1992); M: unpublished observations; N: Bonanno et al. (1993); O: Avgousti et al. (1995); P: Frahm et al. (2000); Q: Bonanno and Raiteri (1987d); R: Bonanno and Raiteri (1987e); S: Fassio et al. (1996); T: Fassio et al. (1999a); U: Raiteri et al. (1991); V: Minelli et al. (1995); W: Raiteri et al. (2001); X: Bonanno et al. (1994); Y: Tseri et al. (2000); Z: Litchfield and White (1990); AA: Poli et al. (1991); B: do Nascimento and de Mello (1985); CC: Coco et al. (1997); DD: Funa et al. (1997); EE: Rothstein et al. (1994); FF: Sepulski et al. (1997).
3. Mechanisms of heterotransporter-induced neurotransmitter release

The mechanisms involved in the neurotransmitter release elicited by heterotransporter activation have so far been investigated in detail in only a very limited number of cases. In the following sections of the article, we will describe and discuss the mechanisms that have been best elucidated. The results clearly indicate that the mechanisms involved are far from being simple and predictable. They are rather complex and heterogeneous. A paradigmatic example is represented by the heterotransporters present on catecholaminergic axon terminals: activation of the GABA heterotransporter sited on noradrenergic nerve endings elicits NE release by a mechanism that differs from that involved in the heterotransporter-induced release of dopamine (DA).

3.1. Mechanisms of transmitter release induced by activation of GABA heterotransporters sited on noradrenergic, dopaminergic and cholinergic nerve terminals

Trying to understand the mechanisms by which activation of a GABA heterotransporter can elicit transmitter release, we first compared the characteristics of the GABA heterocarrier-evoked release of NE from hippocampal nerve endings with those of two previously identified systems, the GABA heterocarrier-evoked release of DA in rat striatum (Bonanno and Raiteri, 1987c) and the GABA heterocarrier-evoked release of ACh in rat hippocampus (Bonanno and Raiteri, 1987a,b).

It may be helpful to remember that GABA had been found able to increase the basal release of [3H]DA from rat striatum and cortical synaptosomes (Bonanno and Raiteri, 1987c). The effect of GABA was mimicked neither by muscimol nor by [−]baclofen, but it was blocked when GABA uptake was prevented, supporting the idea that striatal and cortical dopaminergic axon terminals possess at least two transporters, one homotransporter for the reuptake of DA and a heterotransporter that can take up GABA, causing release of DA. Similarly, when the effect of GABA on the basal release of [3H]ACh was investigated using hippocampal synaptosomes prelabeled with [3H]choline, it was found that the amino acid added to the superfusion medium caused enhancement of the basal efflux of [3H]ACh. Also in this case the effect of GABA was receptor independent but was blocked by selective GABA uptake inhibitors (Bonanno and Raiteri, 1987a). Interestingly, the affinity values calculated from the concentration-response curves of the GABA-evoked NE, DA and ACh release were well in the range of those previously reported for the high-affinity uptake of GABA into CNS synaptosomes (see Fassio et al., 1996).

As the reader will see, the results indicate that the mechanisms involved differ among the three neurotransmitter systems investigated: while activation of GABA heterotransporters seems to elicit release of [3H]NE by classical exocytosis, the GABA-evoked releases of [3H]DA and [3H]ACh, although of vesicular origin, appear different from conventional exocytotic release.

Exocytotic release of neurotransmitters is a complex process which possesses a number of characteristics: conventional exocytosis occurs directly from synaptic vesicles; it is dependent on extracellular Ca^{2+} and on the activation of voltage-sensitive Ca^{2+} channels (VSCCs) triggered by depolarization; it involves several nerve terminal-associated proteins; it can be blocked by clostridial toxins; it is insensitive to neurotransmitter transporter inhibitors (which instead block the carrier-mediated release occurring through reversal of the transporter); it can be modulated by presynaptic receptors (Greengard et al., 1993; Levi and Raiteri, 1993; Stühler et al., 1993; Bennett and Scheller, 1994; Stühler and Rothman, 1994; Schweizer et al., 1995; Stanley, 1997).

Because of this remarkable complexity, the possibility that...
slightly different versions of exocytosis exist may not be remote. The process might exhibit differences between neuronal families or within the same neuronal system, depending on the releasing stimulus. For instance, release occurring in the absence of external Ca²⁺ may still be exocytic and Ca²⁺-dependent because of the mobilization and utilization of internal Ca²⁺ pools (Blaustein, 1988; Bernath, 1991; Adam-Vizi, 1992).

Apart from vesicular exocytosis, transmitter release may sometimes occur by a Ca²⁺-independent process sensitive to transporter blockers, i.e. by carrier-mediated release (Artwell et al., 1993; Levi and Raiteri, 1993). Selective blockers of the NE, DA, or choline transporters were unable to prevent the release of [³H]NE, [³H]DA, or [³H]ACh caused by activation of GABA heterocarriers; thus, while the releasing agent (GABA) enters the nerve terminals through a transporter (heterotransporter), the transmitters released do not utilize their homotrainers to exit from the nerve terminals. On the other hand, exchange between GABA and the released transmitters through the GABA heterocarrier should be excluded because of the selectivity of the transporters involved. To conclude, the release of NE, DA, or ACh evoked by GABA heterotransporter activation does not seem to be carrier-mediated.

3.1.1. GABA-evoked norepinephrine release

The GABA-evoked release of [³H]NE was strongly external Ca²⁺-dependent. It is known that the Na⁺-dependent uptake of GABA is electrogenic, giving rise to depolarization of presynaptic membranes (Martin, 1976; Johnstone, 1979; Finger, 1985; Kanner and Schuldiner, 1987; Cammack and Schwartz, 1993; Kanner, 1994). Since the release that is due to heterocarrier activation is also Na⁺-dependent, one could hypothesize that depolarization of nerve terminals caused by the Na⁺ cotransported with GABA triggers the opening of VSCCs leading to an exocytotic release of [³H]NE. The sensitivity to tetrodotoxin of the GABA-induced [³H]NE release also suggests involvement of Na⁺ ions entering through voltage-dependent Na⁺ channels (Catterall, 1984). Both dependence on external Ca²⁺ and sensitivity to tetrodotoxin are characteristics of the electrically or the high-K⁺-evoked release of NE; processes generally thought to occur by exocytosis (Scholfeimeyer et al., 1981; Starke et al., 1989; Raiteri et al., 1990).

If GABA heterocarrier activation provokes release of NE by conventional exocytosis, VSCCs should open consequently to transporter activation. Indeed the process was found to be sensitive to Cd²⁺ ions (Fassio et al., 1999b) and this prompted us to characterize the Ca²⁺ channels involved. Nifedipine, the L-type Ca²⁺ channel blocker, failed to inhibit the release of NE provoked by GABA (Fassio et al., 1999a), consistent with the observation that the L-type Ca²⁺ channels are not generally involved in neurotransmitter exocytosis (Takahashi and Momiyama, 1993; Carvalho et al., 1995), including the K⁺-induced exocytotic NE release from hippocampal nerve terminals (Sabrià et al., 1995).

Opening of N-type Ca²⁺ channels seems to occur following GABA heterocarrier activation since the selective neurotoxin α-conotoxin GVIA (Olivera et al., 1994) inhibited NE release evoked by GABA. Activation of N-type channels accounts for a relatively modest percentage (~30%) of the GABA heterocarrier-induced release. Involvement of N-type Ca²⁺ channels had previously been reported in both KCl- and electrically-induced NE release in rat hippocampus (Gaur et al., 1994; Sabrià et al., 1995). The findings that the heterocarrier-induced NE release was inhibited by nanomolar concentrations of α-conotoxin MVIIIC and that this effect and that of α-conotoxin GVIA are additive suggest that activation of GABA transporters on hippocampal noradrenergic nerve terminals induces NE release also by opening channels of the P/Q type. It has to be added that, in spite of the complete inhibition observed with the non-selective VSCC blocker Cd²⁺, the effect of the conotoxins was clearly incomplete, suggesting that a non-N, non-P/Q component may still be present. Similar observations were made in previous studies of neurotransmitter release from isolated nerve terminals (Turner et al., 1995; Turner and Dunlap, 1995).

Finally, the GABA heterocarrier-induced [³H]NE release was strongly inhibited by clonidine, an agonist at the α₂-autoreceptors localized on noradrenergic axon terminals. Sensitivity to presynaptic α₂-autoreceptor activation seems to be an important characteristic of NE exocytosis (Langer, 1981; Starke, 1981, 2001; Raiteri, 2001). Altogether the results available suggest that activation of GABA heterotransporters on hippocampal noradrenergic terminals provokes release of NE by conventional vesicular exocytosis (see Fig. 1).

3.1.2. GABA-evoked dopamine or acetylcholine release

Whether an exocytotic process is also involved in the GABA heterotransporter-induced release of ACh or DA is less clear. In neither system did the effect of GABA require external Ca²⁺, whereas synaptosomes depolarized with high-K⁺ have been reported to exocytotically release DA and ACh in a manner largely dependent on external Ca²⁺ and on the activation of VSCCs (McMahon and Nicholls, 1991). Moreover, the GABA-evoked releases of [³H]ACh and [³H]DA were insensitive to tetrodotoxin. It would therefore seem that, while GABA uptake into noradrenergic terminals depolarizes the presynaptic membrane sufficiently to activate VSCCs, ACh and DA terminals are not depolarized enough for external Ca²⁺ (and Na⁺) to penetrate into the terminals (see Blaustein and King, 1976). If this is the case, how can ACh and DA exit from their terminals following activation of GABA heterocarriers?

It is now well accepted that neurotransmitter release occurring in the absence of external Ca²⁺ may still be Ca²⁺-dependent because internal Ca²⁺ pools can be mobilized (see reviews by Blaustein, 1988; Bernath, 1991; Adam-Vizi, 1992 and references therein). One could therefore hypothesize that the entry of GABA and Na⁺ into cholinergic and dopaminergic terminals triggers Ca²⁺-
Fig. 1. Release of norepinephrine (NE) evoked by activation of GABA heterotransporters (GAT-1) in rat hippocampal nerve terminals prelabeled with the radioactive catecholamine through the noradrenergic homotransporter NET. Co-transport of GABA and Na\(^{+}\) (1, 2) depolarizes the plasma membrane, leading to entry of Na\(^{+}\) (3) and Ca\(^{2+}\) (4), through the respective voltage-sensitive ion channels. The increase of cytosolic Ca\(^{2+}\), possibly involving mobilization from internal inositol triphosphate receptor (InsP\(_3\)-R)- or ryanodine receptor (RyR)-sensitive stores, elicits NE exocytosis (5).
not require activation of terminal VSCCs, but depend on 
\([Ca^{2+}]_i\).

### 3.1.3. Autoreceptor-mediated modulation of the hetero

transporter-induced neurotransmitter release

As mentioned above, there is substantial evidence that the exocytotic release, contrary to the non-vesicular carrier-mediated release, can be modulated through presynaptic autoreceptors. Accordingly, since exocytosis is Ca\(^{2+}\)-dependent, while carrier-mediated release occurs independently of Ca\(^{2+}\) ions, it is believed that presynaptic autoreceptors selectively modulate Ca\(^{2+}\)-dependent exocytosis of neurotransmitters independently of the origin (extracellular, through VSCCs, or intracellular) of cytoplasmic Ca\(^{2+}\) (Schoffelmeer and Mulder, 1983; Starke et al., 1989). Our results suggest that this view should in part be reconsidered. In fact, while the GABA-evoked release of \([^{3}H]NE\) could be modulated by activating \(\gamma\)-autoreceptors, neither oxotremorine nor quinpirole, agonists at muscarinic and dopaminergic autoreceptors, respectively, affected the release of \([^{3}H]ACh\) or \([^{3}H]DA\) provoked by GABA heteroreceptor activation. Thus it seems that \([Ca^{2+}]_i\)-dependent vesicular release may, in some conditions, be insensitive to autoreceptor activation.

### 3.1.4. Sensitivity to clostridial toxin of the GABA-evoked neurotransmitter release

If GABA, acting at GABA heteroceptors, elicits vesicular exocytosis of NE, ACh, and DA by mechanisms that exhibit clear differences, it was important to further investigate the characteristics of these exocytotic processes. Since synaptosomes have previously been used to correlate cleavage of presynaptic proteins by tetanus or botulinum neurotoxins and inhibition of transmitter exocytotic secretion (Link et al., 1992; Blasi et al., 1993a,b, 1994), we analyzed the effects of tetanus toxin (TeTx) and botulinum toxin type F (BoNT/F), two clostridial toxins able to cleave VAMP/synaptobrevin (Link et al., 1992; Schiavo et al., 1992, 1993), on the release of NE, ACh, and DA.

Depolarization of nerve terminals with high-K\(^+\) is known to evoke transmitter release by classical exocytosis. Accordingly, TeTx and BoNT/F were found to decrease the K\(^+\)-evoked release of NE, ACh, and DA, in line with the idea that cleavage of synaptobrevin affects exocytosis induced by depolarization and consequent entry of external Ca\(^{2+}\) through VSCCs.

When we compared the effects of TeTx and BoNT/F on the vesicular releases of the three transmitters evoked by GABA heteroreceptor activation, we found that only the release of NE was sensitive to the toxins, whereas neither TeTx nor BoNT/F affected the GABA-induced overflow of ACh or DA. Thus the GABA-evoked release of NE resembles that elicited by depolarizing concentrations of K\(^+\), strengthening the idea that it occurs by conventional exocytosis involving the SNARE component synaptobrevin and activation of VSCCs, the latter being triggered by the electrogenic GABA\(Na^+\) cotransport into noradrenergic terminals (Leveque et al., 1994; Lester et al., 1996). On the other hand, the releases of ACh and DA evoked by GABA uptake may occur by an exocytotic process that is independent of external Ca\(^{2+}\) and VSCC activation and seems not to require VAMP. Experiments carried out with Cd\(^{2+}\) ions, which are blockers of VSCCs, support this view: while the K\(^+\)-evoked release of all the three transmitters studied was abolished by Cd\(^{2+}\) ions, only the GABA-evoked release of NE was prevented, whereas the release of ACh or DA was Cd\(^{2+}\)-resistant (Fassio et al., 1999b). There may therefore exist a parallelism between sensitivity of the exocytotic release to TeTx and BoNT/F and involvement of VSCCs. Interactions of components of the SNARE complex with presynaptic N- and P/Q-types Ca\(^{2+}\) channels have indeed been reported (Leveque et al., 1994; El Far et al., 1995; Martin Moutot et al., 1996).

In order to shed light on the possible relation between toxin sensitivity and involvement of VSCCs, we tested the effect of TeTx and BoNT/F on the releases evoked by the Ca\(^{2+}\) ionophore ionomycin. The aim was to increase cytosolic Ca\(^{2+}\) without activating VSCCs, similar to what apparently occurs during the GABA-induced release of \([^{3}H]ACh\) and \([^{3}H]DA\) (Fassio et al., 1996). It is thought that Ca\(^{2+}\) ionophores elicit exocytotic release (Sanchez Prieto et al., 1987; Verhage et al., 1991; Meffert et al., 1994; Von Gersdorff and Matthews, 1994). Our findings that ionomycin released unmetabolized transmitters and that the ionomycin-evoked overflows were external Ca\(^{2+}\)-dependent and insensitive to blockers of non-vesicular carrier-mediated release are consistent with an exocytotic-like process. If TeTx or BoNT/F-insensitive exocytosis occurs when intraterminal \([Ca^{2+}]_i\) increases independently of VSCC activation, one would expect the ionomycin-induced release to be toxin-insensitive. Indeed, under our experimental conditions, pretreatment with TeTx or BoNT/F had no significant effect on the ionomycin-induced release of \([^{3}H]NE\), \([^{3}H]ACh\) or \([^{3}H]DA\) (see Fassio et al., 1999b for a discussion of these results).

### 3.1.5. Concluding remarks

In conclusion, GABA heteroreceptors present on noradrenergic and cholinergic nerve terminals in hippocampus and on dopaminergic terminals in the corpus striatum represent clear examples of transporters that permit high-affinity GABA uptake into non-GABAergic terminals. Since the cotransport of GABA and Na\(^+\) is electrogenic, the membrane of noradrenergic terminals can be depolarized, during GABA heteroceptor activation, up to a point that VSCCs are activated and the entry of Ca\(^{2+}\) triggers conventional exocytosis. Differently from noradrenergic terminals, activation of GABA heteroreceptors on cholinergic and dopaminergic terminals leads to ACh and DA release endowed with the following characteristics: (i) it is external Ca\(^{2+}\)-independent, but originates from synaptic vesicles, possibly through the involvement of internal Ca\(^{2+}\); (ii) it
is insensitive to presynaptic autoreceptor activation; (iii) it is not affected by synaptobrevin cleavage. The process may represent a form of atypical exocytosis that occurs in the absence of VSCC activation, but requires the intervention of strategic pools of internal Ca\(^{2+}\). Also, considering the different mechanisms involved, heterocarrier activation may represent an additional stimulus able to trigger exocytotic or exocytotic-like release which may lead to better understanding of the exocytotic process.

3.2. Mechanisms of GABA release stimulated by activation of glycine heteroreceptors sited on GABAergic axonal terminals in the spinal cord

Glycine (GLY) and GABA are the two major inhibitory neurotransmitters in the mammalian spinal cord. Their inhibitory functions mainly involve activation of ligand-gated chloride channels, the GLY receptors and GABA\(_A\) receptors (see, for reviews, Betz, 1992; Todd and Spike, 1993).

Glycine- and GABA-like immunoreactivities coexist in the terminal boutons of subpopulations of spinal interneurons (Todd and Sullivan, 1990; Orum et al., 1994; Taal and Holstege, 1994; Todd et al., 1995, 1996). Ultrastructural evidence has been provided that GLY, GABA, and their receptors are colocalized at the same synapse in the spinal cord (Todd et al., 1996), strongly supporting the idea that GABA and GLY can act as cotransmitters within this region. In a recent functional study (Jonas et al., 1998), it was found that some spinal interneurons release both GLY and GABA to activate strychnine-sensitive GLY receptors and bicuculline-sensitive GABA\(_A\) receptors located on motoneurons. Moreover, a subset of miniature synaptic currents showed both components, consistent with corelease from individual synaptic vesicles. Previous works indicated the possible costorage of GLY and GABA within the same vesicles through a common vesicular transporter (Christensen and Pfromm, 1991; McIntyre et al., 1997; Sagué et al., 1997; Chaudhry et al., 1998).

If the idea that GLY and GABA are cotransmitters in the spinal cord appears well supported, very little is known on the reciprocal interactions that may occur between these cotransmitter systems, particularly at the presynaptic level. Interactions can obviously occur at the proposed common terminal boutons of subpopulations of spinal interneurons. Moreover, a subset of miniature synaptic currents showed both components, consistent with corelease from individual synaptic vesicles. Previous works indicated the possible costorage of GLY and GABA within the same vesicles through a common vesicular transporter (Christensen and Pfromm, 1991; McIntyre et al., 1997; Sagué et al., 1997; Chaudhry et al., 1998).

3.2.1. Glycine-evoked GABA release

Previous results from experiments with synaptosomes of rat spinal cord showed that GLY can directly evoke release of preaccumulated \(^{3}H\)GABA (Raiteri et al., 1992). The GLY-induced GABA overflow was unaffected by the GLY receptor antagonist strychnine, but it was highly dependent on Na\(^{+}\). Thus, (in the absence of selective GLY transport blockers) the possibility was proposed that the effect of GLY in increasing \(^{3}H\)GABA release involves transport of GLY into GABA-releasing terminals. Of note, as in the case of GABA heteroreceptors, the affinity values for the GLY-evoked GABA release were close to those of the GLY transporters (Raiteri et al., 1992, 2001).

The increasing evidence that GLY and GABA are cotransmitters in the spinal cord, the recent findings that GLY transporters exist as isoforms for which some selective blockers are now available, together with the potential pathophysiological relevance of the coexistence of the two inhibitory agents, prompted us to extend the above previous observations. In addition, the new experiments have been performed using spinal cord synaptosomes from mice, in view of the possible future utilization of genetically modified animals (Raiteri et al., 2001).

Based on the methodological considerations previously reported, the elevation of GABA release observed when synaptosomes are exposed to GLY indicates that certain GABA-releasing axon terminals in the mouse spinal cord possess targets for GLY. Since the effect of GLY on the release of GABA is stimulatory, it would seem reasonable to suspect that GLY acts at excitatory NMDA receptors as a glutamate coagonist. The existence on GABAergic neurons of NMDA receptors mediating enhancement of GABA release has been reported (see, for instance, Dreger et al., 1987). However, the idea is unlikely for two reasons: (i) the effect of GLY is insensitive to 5,7-dichlorokynurenic acid, a selective antagonist at the GLY site of the NMDA receptor; (ii) as discussed above, endogenous compounds (including the GLY coagonists glutamate/aspartate) released from superfused synaptosomes are quickly removed from their targets.

Besides the stimulatory role as a glutamate coagonist at NMDA receptors, GLY has been reported to enhance neurotransmitter release directly via strychnine-sensitive receptors (Raiteri et al., 1990; Darstein et al., 2000). However, in our experiments, the GLY-evoked GABA release was not sensitive to strychnine, excluding the involvement of a GLY receptor.

If GLY and GABA are cotransmitters in some terminals of the spinal cord, one would expect these terminals to possess, on their plasma membrane, reuptake transporters selective for GABA and reuptake transporters selective for GLY. Certainly the terminals under study possess GABA transporters, inasmuch as the \(^{3}H\)GABA release by GLY had previously been taken up. On the other hand, the GLY-induced GABA release was strictly Na\(^{+}\)-dependent, compatible with the involvement of GLY transporters; also, the idea is supported by the finding that the GLY effect can be blocked by various GLY transport inhibitors, including glycyldodecylamide (Raiteri et al., 2001), a compound found to inhibit the uptake of GLY into rat cortex synaptosomes (Javitt and Frisvani, 1997; Javitt et al., 1997). Thus, in the mouse spinal cord, axon terminals able to take up GLY also can take up GLY. Most likely, these axon terminals are those that store and release GABA and GLY as cotransmitters.
Two types of GLY transporters have been identified and termed GLYT1 (Guastella et al., 1992; Liu et al., 1992; Smith et al., 1992) and GLYT2 (Liu et al., 1993). GLYT2 is thought to be specifically associated with inhibitory glycineergic neurotransmission, being localized on GLY-immunoreactive neurons, at the presynaptic side of glycineergic synapses in the spinal cord (Zafra et al., 1993a; Poyatos et al., 1997). The cellular and subcellular localizations of GLYT1 transporters are somewhat less well defined. Although GLYT1 was shown to be expressed in glia (Adams et al., 1995; Zafra et al., 1995a), its presence also in neurons has been proposed (Guastella et al., 1992; Borowsky et al., 1993; Zafra et al., 1995a).

Our results clearly indicate that the synaptosomal preparations used contain both GLYT1 and GLYT2; indeed the effect of GLY on the release of GABA consists of two quantitatively similar components, one sensitive to the GLYT1 substrate/inhibitor sarcosine and the other sensitive to amoxapine, a compound very recently reported to selectively inhibit GLYT1 transporters (Núñez et al., 1995b). The sarcoptine- and amoxapine-sensitive components are additive (Raiteri et al., 2001). If GLYT2 is on axon terminals, where are GLYT1 transporters located?

Synaptosomal preparations are known to contain contaminating gliaosomes. These are fragments of glial cells produced during tissue homogenization and then resealed (Henn et al., 1976). The viability of these particles is controversial. Gliasomes from adult glia have been described as metabolically inactive and vesicle-free (Henn et al., 1976; see also Ashton and Dolly, 2000). However, a gliaosome fraction isolated from adult rat brain was able to accumulate [3H]GABA (Nakamura et al., 1993). In any case, the results of our experiments (see Raiteri et al., 2001, for details) support the idea that GLYT1 transporters enhancing GABA release are located on neuronal terminals.

Thus, based on the characteristics of the technique used here, both GLYT1 and GLYT2 appear to be present on axon terminals that also possess GABA transporters. We favor the view that GLYT1 and GABA transporters coexist on some axon terminals, while GLYT2 and GABA coexist on others. The colocalization of GLYT1, GLYT2, and GABA transporters on the same axon terminals cannot be entirely excluded, although it appears less likely, considering that the two GLYT transporters display a completely different expression pattern (Adams et al., 1995; Zafra et al., 1995a,b).

3.2.2. Carrier-mediated release of GABA induced by glycine

As to the mechanisms that underlie the effects of GABA stimulated by activation of GLYT1 and GLYT2 heteroearciers, it should be considered that GLY and GABA share the vesicular transporter (Christensen and Fonnum, 1991; Sagüé et al., 1991; Chaudhry et al., 1998). Thus, the GLY taken up into the cytoplasm by the plasma membrane GLY transporter could enter the vesicles and cause exit of GABA into the cytoplasm by heteroexchange; cytosolic GABA could in turn be released through reversal of the plasmalemmal GABA transporter possibly facilitated by the Na+ ions cotransported with GABA by the plasma membrane GABA carriers. Carrier-mediated release is known to be blocked by transporter inhibitors (Levi and Raiteri, 1993). Our results show that the release of GABA elicited by GLY was indeed sensitive, although in part, to the selective GABA transporter inhibitor SKF 100330A, indicating that carrier-mediated release is one of the mechanisms by which GABA exits when GLY transporters are activated (Raiteri et al., 2001) (see Fig. 2).

3.2.3. Vesicular exocytosis release of GABA induced by glycine

Since only a fraction of the GABA released by GLY exits through transporter reversal, other mechanisms must be involved. Classical exocytosis seems to be excluded, however. In fact, the GLY-evoked release of GABA is independent of external Ca2+ and occurs therefore in the absence of VSCC activation. Inasmuch as vesicular exocytosis can take place following stimuli that cause mobilization of Ca2+ from intraterminal stores (see Berridge, 1998), we tested this possibility. When the Ca2+ chelator BAPTA was entrapped inside the synaptosomes, the effect of GLY on GABA release was prevented, suggesting that uptake of GLY can cause release of Ca2+ into the cytosol from intraterminal stores. Pretreatment with thapsigargin, known to cause depletion of intracellular Ca2+ ions by blocking Ca2+-uptake into the endoplasmic reticulum, led to significant decrease of the effects of GLY on GABA release (Raiteri et al., 2001). The exact pool of the Ca2+-ions depleted in the endoplasmic reticulum, i.e. ryanodine-sensitive or/and inositol trisphosphate-sensitive, remains to be established. As to the mechanism by which GLY uptake causes Ca2+-mobilization, recent data suggest that Na+ cotransported with GLY can cause Ca2+-release from mitochondria through a Na+/Ca2+-exchanger. This mitochondrial Ca2+-ion could trigger a CICR with the endoplasmic reticulum, thus providing additional cytosolic Ca2+ (see Fig. 2). The involvement of mitochondria is supported by the finding that the GLY-induced GABA release was sensitive to CpG 37157, a selective blocker of the mitochondrial Na+/Ca2+-exchanger (unpublished results).

In any case, it seems that the Ca2+-ions made available during GLY uptake elicit release of GABA from vesicles, as suggested by the experiments with bafilomycin A1. This compound is known to inhibit the V-type ATPase present on synaptic vesicles and to cause depletion of the vesicular neurotransmitter pool (Bowman et al., 1988; Mortyama and Futai, 1990; Cousin et al., 1997). When mouse spinal cord synaptosomes were pretreated with bafilomycin before incubation with [3H]GABA, in order to decrease vesicular labelling, the subsequent exposure to GLY caused much lower release than in control synaptosomes, strengthening the view that GLY uptake triggers internal Ca2+-dependent exocytosis of GABA. As expected, bafilomycin no longer inhibited...
Fig. 2. Release of GABA evoked by activation of glycine (GLY) heteroceptors (GLYT1 and GLYT2) in mouse spinal cord nerve terminals prelabeled with $[3^H]$GABA through the GABAergic homotreporter (GAT-1). A portion of the release occurs from the cytoplasm by reversal of GAT-1 (5). Possibly, GLY entering the nerve terminal through its specific heteroceptors (1) displaces GABA from GLY/GABA-containing vesicles (see bicoloured circles) since the two amino acids share the same vesicular carrier, thus increasing the cytoplasmic concentration of GABA. The remainder of the release seems to occur by vesicular exocytosis, probably as a GABA/GLY corelease, exploiting Ca$^{2+}$ from various intraterminal pools. In our model, mitochondrial Ca$^{2+}$ can be released by Na$^+$ which enters with GLY and can activate the mitochondrial Na$^+$/Ca$^{2+}$ exchanger (2). This Ca$^{2+}$ can trigger Ca$^{2+}$ release from the endoplasmic reticulum (3) leading to GABA release by vesicular exocytosis (4).

the GLY effect in synaptosomes that had been staffed with BAPTA (Raiteri et al., 2001).

3.2.4. Concluding remarks
Motoneurons receive inhibitory glycinergic innervation from spinal cord interneurons. According to Jonas et al. (1998), a single interneuron can inhibit spinal motoneurons through GABA-only synapses, representing a small (~15%) fraction of the inhibitory input, GLY-only synapses and GLY–GABA mixed synapses which contribute equally (~45%) to the input to motoneurons. In the scheme proposed by Nicoll and Malenka (1998), GLYT2 is present on glycinergic terminals making GLY-only synapses, in line with the report by Poyatos et al. (1997); the scheme leaves undefined the GLY transporters expressed on the terminals making mixed synapses. Interestingly, Adams et al. (1995) found both GLYT2 and the GLY receptor subunit GlyRα2 to be expressed preferentially throughout the ventral horns of the spinal cord, where motoneurons are located. Accordingly, our results are compatible with the view that GLYT2 and GABA transporters coexist on the terminals making mixed synapses on motoneurons bearing GLY and GABA receptors. The targets of the axon terminals carrying GLYT1 and GABA transporters remain a matter of speculation; several hypotheses could be proposed, however, considering that our experiments were carried out with synaptosomal preparation from the whole spinal cord. Of note, GLY and GABA were found to coexist also in boutons making axo-axonic synapses in the spinal cord (Todd et al., 1995); these boutons may differ in some aspects, including their GLY transporter, from those forming interneuron–motoneuron synapses.
It is well accepted that, like other transporters, GLY transporters are crucial for the termination of synaptic neurotransmission. Our results suggest that GLY transporters, both GLY1 and GLY2, may have an additional function, i.e. to mediate fine regulation of the release of GABA from the mixed GLY–GABA terminals. The GLY-induced release of GABA, which appears in part exocytotic and in part carrier-mediated, may represent an important aspect of the interactions occurring at the spinal cord synapses where the two transmitters and their receptors are colocalized (see Todd et al., 1996; Jonas et al., 1998).

Precise knowledge of the mechanisms involved in the inhibitory control of glutamate-mediated excitation of spinal motoneurons is of great importance in the development of therapeutic strategies aimed at limiting abnormal excitatory neurotransmission likely to be present in some neurodegenerative conditions, including amyotrophic lateral sclerosis.

4. Heterotransporters are not exclusively localized on nerve endings and are not confined to CNS

4.1. Somatodendritic heterotransporters

The experimental approach we have utilized since 1986 to identify and characterize heterotransporters permits the study of heterotransporters that are localized on particular families of nerve endings and whose activation elicits transmitter release. However, evidence has been provided that the heterotransporter phenomenon is not limited to axon terminals. In fact, heterotransporters have been reported to exist at the soma/dendritic level in discrete CNS regions where they are present on neurons that, in a few instances, have been well identified.

4.1.1. Glutamate heterotransporters

Several types of excitatory amino acid transporters exist in the plasma membrane of CNS cells. Five such glutamate (GLU) transporters have been cloned so far and termed EAAT1 to EAAT5 (for reviews see: Gogelashvili and Schousboe, 1997; Saer, 1999; Slothboom et al., 1999; Dausbolt, 2001). Glutamate transporters of the EAAT3 type were found on GABAergic neurons, including Purkinje cells in the cerebellum and medium spiny neurons in the striatum. EAAT3 immunoreactivity could be observed in the soma/dendritic plasma membrane of these neurons (Coco et al., 1997). Some authors have proposed that EAAT3 also exist on the axon terminals of GABAergic Purkinje cells, in the deep cerebellar nuclei (Rothstein et al., 1994; Furuta et al., 1997). It would be of interest to isolate synaptosomes from deep cerebellar nuclei and to investigate the possible relations between the GABA homotransporters that should be present on the Purkinje axon terminals and the EAAT3 heterotransporters proposed to exist on these terminals. By the way, the presence of glutamatergic transporters on GABAergic terminals in the deep cerebellar nuclei would not represent an exception. Several years ago glutamate transporters of unidentified type were found to exist on GABAergic terminals of rat cerebral cortex, hippocampus, and cerebellum (Bonanno et al., 1993). Subsequently, Rothstein’s group (Sepuku et al., 1997) reported the existence of EAAT3 heterotransporters on GABAergic terminals of the hippocampus. What remains surprising is that some GABAergic axon terminals possess EAAT3 transporters for GLU, whereas this type of transporter can apparently not be found on glutamatergic axon terminals, in fact, axotomy of the corticostriatal glutamatergic pathway or of other glutamatergic pathways was found not to reduce EAAT3 in the innervated regions (Ginsberg et al., 1995, 1996). Actually, the reuptake homotransporter probably present on glutamatergic axon terminals has not yet been unequivocally classified.

As to EAAT4, the distribution of this type of GLU transporter is largely restricted to the cerebellar Purkinje cells, which are GABAergic neurons (Furuta et al., 1997). Based on electron microscopic observations, the EAAT4 heterotransporter seems not to be associated with axon terminals, being exclusively confined to the plasma membrane of Purkinje cell soma and dendrites (Yamada et al., 1996; Furuta et al., 1997; Nagao et al., 1997). Thus, while EAAT3 and EAAT4 are colocalized on Purkinje cell soma/dendrites spines (Furuta et al., 1997), representing one example of a neuron possessing two heterotransporters, the GABAergic axon terminals of Purkinje cells would only be endowed with GLU transporters of the EAAT3 type, probably coexisting with the GABA homotransporter.

4.1.2. GABA heterotransporters

In a study on the localization of the GABA transporter GAT-1, Minelli et al. (1995) report that, although the majority of rat cerebrocortical neurons expressing GAT-1 mRNA also contain glutamate decarboxylase immunoreactivity, i.e. they are sensu stricto GABAergic cells, GAT-1 mRNA was also seen in a few non-GABAergic pyramidal neurons. Augood et al. (1999) found that glutamatergic parvalbumin- and calretinin-positive neurons in the human subthalamic nucleus are GAT-1 messenger RNA-positive, suggesting the possibility that they actively accumulate GABA. In a very recent work, Frahm et al. (2000) analyzed the distribution of mRNA for GAT-1 in the rat hippocampus. As expected, the authors found that most GABAergic interneurons were stained for GAT-1 mRNA, suggesting the presence of transporters for GABA reuptake into the releasing cells. However, prominent signals for GAT-1 mRNA were also found in glutamatergic granule and pyramidal cells, compatible with GABA entry into glutamatergic neurons through heterocarriers. Likewise, the glutamic acid decarboxylase mRNA signal seen in hippocampal granule cells would reflect production of GABA in these glutamatergic neurons. GAT-1 mRNA does not necessarily mean translation into functional protein. However, our previous results showing that hippocampal glutamate-releasing synaptosomes possess...
GABA transporters on their plasma membrane (Bonanno et al., 1993) are consistent with the existence of functional GABA transport proteins on the axon terminals of cholinergic neurons.

Using in situ hybridization histochemistry, it was observed that rat spinal cholinergic motoneurons also express GABA transporters of the GAT-1 type. However, these neurons do not express glutamate decarboxylase, the synthetic enzyme for GABA (Snow et al., 1992). On the other hand, GABA synthesis is not necessarily required to perform corelease in neurons bearing GABA heterotransporters. In the rabbit retina only half the neurons in the ganglion cell layer that are GABA-immunoreactive are also immunoreactive for glutamate decarboxylase. All neurons that are GABA-immunoreactive can accumulate GABA, however, suggesting that the GABA positive/glutamate decarboxylase-negative amacrine cells (which are cholinergic neurons) obtain their GABA by uptake rather than synthesis (Vance and Young, 1988). Our results with synaptosomes had shown that GABA heterotransporters localized on cholinergic terminals/varicosities can be activated by exogenously added GABA which, independently of GABA synthesis, penetrates into the terminals and elicits ACh release (Bonanno and Raiteri, 1987a,b). Moreover, heterotransporter substrates need not be produced in strict proximity of the transporter. Indeed, as recently suggested (Vizi, 2000; Kiss and Vizi, 2001), transporters sited on terminals/varicosities can also be activated in a non-synaptic mode by ligands originating from distant sites.

In this context, a special case is represented by GLY/GABA cotransmission. It has been proposed that GLY and GABA share the same vesicular transporter (Christensen and Fonnum, 1991; Chaudhry et al., 1998). This opens the possibility for a GABAergic neuron to co-store and corelease GABA and GLY without synthesizing the latter; GLY, originating from unknown sites, can be taken up from the extracellular space through GLY plasma membrane heterotransporters (Nicoll and Malenka, 1998; Raiteri et al., 2001).

4.2. Heterotransporters in the peripheral nervous system

The presence of heterotransporters does not seem to be confined to the CNS. In chich parasympathetic ciliary ganglia, GLY transporters of the GLYT1 type were reported to be localized to the cholinergic presynaptic terminal membrane (Tsen et al., 2000). According to the authors, GLY can be taken up into cholinergic terminals and subsequently coreleased with ACh upon depolarization. Interestingly, while the evoked release of ACh onto nicotinic postsynaptic receptors occurs by vesicular exocytosis, depolarization elicits GLY release by GLY transporter reversal (Tsen et al., 2000).

It is at present unknown if GLY uptake into cholinergic terminals can trigger release of ACh in ciliary ganglia as it happens in the spinal cord where GLY uptake elicits release of GABA (Raiteri et al., 2001). Also unknown are the possible effects of the activation of the choline transporter present on cholinergic terminals of the ciliary ganglia on the release of GLY. Using rat hippocampus synaptosomes, it was previously found that choline could increase the release of endogenous GABA. Choline did not act at octanitric cholinergic receptors, where it can behave as a selective agonist, but through activation of transporters sensitive to hemicholinium-3 and located on GABA-releasing terminals (Pittaluga and Raiteri, 1987).

To conclude, the heterocarrier phenomenon appears quite diffuse in the nervous system. It can be observed in the CNS and in the peripheral nervous system, at nerve terminals and at the soma/dendritic level, in mammals, including humans, and outside of the mammalian world.

5. Area and phenotype specificity of heterotransporters

Heterocarriers located on presynaptic nerve terminals exhibit clear regional heterogeneity. Transporters for the uptake of NE and GABA coexist on nerve terminals of rat hippocampus and cerebral cortex, where GABA can enhance the release of NE via uptake into noradrenergic nerve endings. GABA heterocarriers have, however, not been found on NE terminals of the hypothalamus (Bonanno and Raiteri, 1987e). It has been reported that hypothalamic noradrenergic terminals, differently from those in neocortex and hippocampus, originate from neurons located in subcoeruleus nuclei of the pons-medulla (Foote et al., 1983). Interestingly, the absence of GABA heteroreceptors is not the only characteristic that distinguishes hypothalamic from cortical and hippocampal NE terminals. The noradrenergic neurotoxin DSP-4 damages NE terminals in cortex and hippocampus but does not affect significantly those in the hypothalamus (Fritschy and Grzanna, 1989). Furthermore, NE nerve endings in cortex and hippocampus, but not in the hypothalamus, possess ‘depolarizing’ heteroreceptors of the GABA_A type, activation of which brings about enhancement of basal NE release (Fung and Fillenz, 1983; Bonanno and Raiteri, 1987e).

Dopaminergic nerve terminals in cerebrocortex and striatum possess, besides the homocarrier that recaptures DA, GABA heterocarriers. Their presence is, however, barely detectable in dopaminergic nerve endings of the hypothalamus (Bonanno and Raiteri, 1987c). Also in this case, the difference observed most likely originates from differences among the brain dopaminergic pathways. In particular, DA terminals in the hypothalamus have been reported to differ from mesostriatal and mesocortical terminals because they possess only a low-affinity DA uptake system (Amunziato et al., 1986) and lack DA autoreceptors (Rackè et al., 1987).

Transporters for GABA coexist with choline carriers on cholinergic terminals of rat hippocampus, neocortex, and hypothalamus, but not on cholinergic nerve endings of the
corpus striatum (Bonanno and Raiteri, 1987b). These regional differences may be due to the fact that neocortex, hippocampus, and hypothalamus are cholinergic projection areas, whereas striatal cholinergic terminals originate from interneurons. Terminals of striatal cholinergic interneurons have indeed been reported to differ in some properties from those of projection neurons (Marchi et al., 1983).

The heterotransporter-mediated transmitter release may also display phenotypic specificity. Release-enhancing transporters for GABA can be found on cholinergic, noradrenergic, and dopaminergic axon terminals (with the reported regional heterogeneities), as well as on terminals releasing other transmitters (see Table 1), but could not be detected, by our technical approach, on serotonergic axon terminals, in several CNS areas. Indeed, in experiments performed with nerve endings isolated from cerebral cortex, hippocampus, hypothalamus, midbrain, and cerebellum and prelabeled with \(^{3}H\)-hydroxytryptamine, GABA was unable to affect the basal release of the indoleamine (Bonanno and Raiteri, 1987e). This suggests that release-regulating GABA heterocarriers are not present on serotonergic nerve endings, although the presence of GABA heterotransporters whose activation produces effects other than release stimulation cannot be ruled out.

To conclude, although heterotransporters seem to be widely distributed on axon terminals of the central nervous system, the phenomenon bears characteristics of selectivity. In particular, (i) heterotransporters are substrate-selective and display affinities for their substrates that are very similar to those of the corresponding homotransporters; (ii) a given heterotransporter is present on a given nerve ending population in some brain regions but not in others; (iii) this regional heterogeneity differs among different nerve ending populations carrying the same heterotransporters; (iv) heterocarriers for major and widespread neurotransmitters (i.e., GABA) are present on many nerve ending populations, although there may be populations lacking entirely such heterotransporters.

As to somatodendritic heterotransporters, the glutamate carrier EAAT4 has only been detected in one cell type in the adult rat CNS, namely the GABAergic Purkinje neurons of the cerebellum (Yamada et al., 1996; Nagao et al., 1997; Dehnes et al., 1998). The localization is the same in man (Ish et al., 1997; Inage et al., 1998). Based on electron microscopic observations, EAAT4 does not seem to be associated with axon terminals or Bergman glia, but is exclusively confined to the plasma membrane of Purkinje cell soma and dendritic spines (Furuta et al., 1997; Nagao et al., 1997). The levels of EAAT4 outside the cerebellum and the Purkinje neurons are very low, making EAAT4 a good example of non-terminal heterotransporter with phenotypic selectivity. Northern blotting carried out in mammals shows a strong signal of the glutamate transporter EAAT5 in retina but no detectable signals in the brain (Arriza et al., 1997).

6. Functional significance of heterotransporters
6.1. Heterotransporters as an index of transmitter coexistence

Coexistence of transporters on one nerve terminal could be expected in case of cotransmission when both cotransmitters require reuptake systems to be removed from the synaptic cleft. Neuronal colocalization has been reported for some classic neurotransmitters that not only are coreleased from the same nerve ending but also can be recaptured through coexisting selective transporters.

On the other hand, as previously mentioned, the presence of a heterotransporter on a neuron does not necessarily imply that the neuron synthesizes the corresponding transmitter. Thus, one function of heterotransporters could be that of importing cotransmitters from the extracellular space. In the spinal cord, terminals of GABAAergic interneurons are believed to take up GLY through selective plasma membrane heterotransporters because these terminals cannot synthesize GLY (Nicoll and Malenka, 1998; Raiteri et al., 2001); interestingly, the GABA vesicular transporter can also accumulate GLY, so that GABA and GLY could be exocytotically coreleased from the same vesicle (Jonas et al., 1998). In chick parasympathetic ciliary ganglia, GLY heterotransporters of the GLY T1 type are localized to the cholinergic presynaptic terminal membrane; also in this case, extracellular GLY is taken up to be coreleased with ACh. However, the vesicular ACh transporter does not recognize GLY, and glycineergic vesicles are not available; therefore, GLY remains in the cytoplasm. According to Tsen et al. (2000), depolarization would release ACh by exocytosis and GLY by carrier-mediated release through the GLY T1 heterotransporters, which would therefore perform both uptake and release of GLY.

Coexistence of glutamic acid and GABA can apparently be induced during epileptic seizures. Rat hippocampal granule cells were found to express glutamic acid decarboxylase after limbic seizures provoked in models of human temporal-lobe epilepsy (Schwarzer and Sperr, 1995; Slavkovic et al., 1996). Thus, constitutively glutamatergic nerve terminals may become capable of synthesizing and utilizing GABA in limbic epilepsy. The mode of exit of GABA from glutamatergic axon terminals is not known: release by exocytosis appears unlikely since glutamatergic vesicles should not take up GABA; instead, GABA may be released during seizure by transporter reversal (During et al., 1995), a process that would require the presence of GABA heterotransporters on the plasma membrane of glutamatergic axon terminals. Indeed, Bonanno et al. (1993) had shown that GLU-releasing hippocampal synaptosomes are endowed with high-affinity transporters for GABA, which are sited on their plasma membrane. If this is the case, GABA heterotransporters would exist on glutamatergic terminals even in the absence of seizures. The GABA...
produced in glutamatergic terminals and released through GABA heteroceptors under epileptic conditions may mediate endogenous anticonvulsive mechanisms counteracting seizure generation (Schwarzer and Sperk, 1995). Rothstein’s group in Baltimore utilized selective antisense oligonucleotides, infused intraventricularly to knockout the synthesis of glutamate transporters of the EAAT3 type. By the 10th day of treatment, corresponding to 70–90% knock-out of the EAAT3 protein, antisense animals developed epileptic signs when examined by EEG. Moreover, there was a significant loss of hippocampal GABA associated with antisense knockout of EAAT3, while no changes were seen in sense oligonucleotide-treated animals. According to the authors (Sepukti et al., 1997), their study suggests that dysfunction of EAAT3 heteroceptors on GABAergic terminals may be related to epileptogenesis and that EAAT3 may play a role in regulating GABA metabolism, i.e. GLU is taken up through EAAT3 heteroceptors and then transformed into GABA.

The results of Bonanno et al. (1993) agree with the above view. In addition, we observed that GLU uptake into GABAergic terminals of rat hippocampus could elicit release not only of endogenous GABA but also of previously accumulated [3H]GABA. These findings permit us to extend the interpretation given by Sepukti et al. (1997). In particular, when excess GLU is present in the extracellular space (for instance during epileptic seizures), the excitatory amino acid can be captured through EAAT3 into GABAergic nerve endings. This uptake not only removes excess extracellular GLU but can also elicit compensatory GABA release, exploiting various sources of GABA including recaptured GABA, stored GABA, and GABA newly synthesized from GLU originating through the heteroceptor.

6.2. Modulation of transmitter release

It should be noted that coexistence of classic neurotransmitters in the CNS probably occurs in a relatively limited percentage of neurons. Cotransmission more often involves only one classic transmitter plus one or more neuropeptides whose inactivation does not seem to occur through reuptake. Thus, the presence of heteroceptors on one nerve terminal should reflect cotransmission only in a relatively limited number of cases.

If this is the case, heteroceptors on axon terminals must be attributed other functions. In our experimental model (see Section 2.2), heteroceptors are identified because, in a technical set-up that excludes indirect actions, their activation by the corresponding heteroceptor/heteromodulator elicits release of the homotransmitter. Thus, the very way the presence of heteroceptors is established, suggests a possible function. In particular, in the case of cotransmission with two classic mediators, the coexisting transporters not only can recapture the released cotransmitters but also may allow cross-talk between the cotransmitters leading to reciprocal regulation of release.

The uniqueness of glutamatergic receptors of the NMDA type, the activation of which requires the concerted action of two coagonists, GLU and GLY, may explain why GLU and GLY can reciprocally interact through heteroceptors. Bonanno et al. (1994) found that, in rat cerebrocortical synaptosomes, GLU caused release of previously taken up [3H]GLY and, reciprocally, GLY enhanced the release of previously taken up [3H]-aspartate. These effects occur through heteroceptors and suggest colocalization of glutamatergic and glycinergic transporters on the same axon terminals. The paucity of reports showing GLU/GLY cotransmission militates against costorage, but transporter coexistence is compatible with the idea that GLU-releasing terminals are apposed to GLY-releasing structures (glia?) or can take up GLY originating from distant sources (Vizi, 2000; Kiss and Vizi, 2001). Reciprocal heterocarrier-mediated interactions may be critical for the delicate cooperation between GLU and GLY acting at NMDA receptors.

6.3. Postsynaptic removal of a presynaptically released neurotransmitter

Heteroceptors localized on neuronal somata/dendrites are, in our opinion, less likely to reflect cotransmission. Also, it is less reasonable to think that these heteroceptors are involved in transmitter release regulation, as in the case of terminal heteroceptors. One function that can be attributed to these transporters is that of removing through uptake transmitters that, for some reason, have not been recaptured by the presynaptic terminals from which they have been released or have not been taken up into glial cells. Such a function has been attributed to EAAT4, the GLU heteroceptor present on the soma/dendrites of GABAergic Purkinje cells in the cerebellum. As schematized in Fig. 3, GLU released from parallel fibers and climbing fibers activates depolarizing receptors of the AMPA type sited on Purkinje neurons. It has been proposed that EAAT4 heteroceptors not only capture extracellular GLU but can
Fig. 3. Glutamate EAAT4 heterotransporters on GABAergic cerebellar Purkinje cells. Terminals of parallel/climbing fibers release glutamate onto Na$^+$-permeable AMPA receptors (EAAR) leading to Purkinje cell membrane depolarization. Released glutamate is (in part) removed by glutamate heterotransporters of the EAAT4 type sited on Purkinje cells. Glutamate thus taken up can be transformed into GABA. Chloride channels coupled to EAAT4 permit Cl$^-$ entry and help membrane repolarization.

also work as chloride channels (Sonders and Amara, 1996; Seal and Amara, 1999). More precisely, EAAT4 heterotransporters would play the following functional roles: (i) they decrease the total extracellular concentration of the excitatory amino acid transmitter; (ii) inasmuch as their activation elicits chloride influx and consequent local hyperpolarization, they prevent excessive excitation and help membrane repolarization; (iii) they can provide GLU for the neosynthesis of GABA (Furuta et al., 1997; Seal and Amara, 1999).

Maragakis et al. (1997) prepared antisense oligonucleotides to rat EAAT4 and infused them intracisternally into rats for several days to inhibit the synthesis of the transporter protein. The synthesis of EAAT4 was blocked by $\sim$90% after 7 days of treatment. Rats treated with the antisense oligonucleotide developed a progressive ataxic gait without other clear behavioral manifestations. Ataxia was not seen after treatment with antisense oligonucleotides to EAAT1, EAAT2 or EAAT3. The authors suggest that dysfunction of EAAT4 could be a significant factor in ataxic diseases. If EAAT4 is implicated in the removal of GLU, ataxias could be related to excessive glutamatergic transmission and could benefit from treatments with drugs able to reduce glutamatergic transmission in the cerebellum. Activation of a number of serotonin receptor subtypes was shown to drastically reduce cerebellar glutamatergic transmission at pre- and postsynaptic levels (Maura et al., 1995; Marcoli et al., 1997, 1998), and administration of some serotonergic drugs was reported to be beneficial to some ataxic patients (Lou et al., 1995; Trouillas et al., 1996).

The exact localization of heterotransporters, whether synaptic or non-synaptic, cannot be easily determined at present. Several lines of evidence indicate the existence of extrasynaptic receptors and transporters in different CNS regions (Somogyi et al., 1989; Baude et al., 1995; Descarries et al., 1997; Nusser et al., 1998; for reviews see Vizi, 2000; Kiss and Vizi, 2001). These sites, involved in the so-called non-synaptic (Vizi, 1984) or volume (Agnati et al., 1986) transmission, may play a physiological role in accepting chemical messages that originate from distant neurons and that may heteroregulate non-synaptic release. According to
Vizi (2000), there are heteroreceptors and heterotransporters that cannot be easily reached by endogenous transmitters, under physiological conditions. These sites are of pharmacological or pathological importance because they could be targets for drugs or be activated by endogenous ligands released in toxic concentrations.

6.4. Concluding remarks

To conclude, it seems clear that, in general, the roles of neurotransmitter transporters in brain physiology and pathology are only in part understood. These dynamic proteins represent highly sophisticated systems able to play more refined roles than simple transmitter reuptake. All known transmitter transporters can, under some conditions, mediate transmitter release directly from the cytosol (Attwell et al., 1993; Levi and Raiteri, 1993). The transporters may regulate the time course of synaptic events by modifying the extent of activation of receptors and the level of their desensitization (Seal and Amara, 1999; Sims and Robinson, 1999). The presynaptic heterotransporters described in this article can elicit transmitter release through multiple, largely unexplored, mechanisms. Some somatodendritic heterotransporters, such as the EAAT4 of the cerebellar Purkinje cells, contain an intrinsic chloride conductance gated by GLU so that they can both remove the excitatory amino acid and reset membrane polarization. Some heterotransporters have been proposed to be involved in important pathological conditions.

7. Future perspectives

Looking at Table 1, the reader will notice that, of the heterotransporter systems listed, most involve carriers for the amino acid transmitters GABA, glutamate, and glycine. It is now well known that the transporters for these three transmitters are heterogeneous. Five transporter subtypes for GABA (Borden et al., 1992; Masson et al., 1999), five for glutamic acid (Gegelashvili and Schousboe, 1997; Saier, 1999; Slotboom et al., 1999), and two for glycine (Guastella et al., 1992; Liu et al., 1992, 1993) have been identified. With the exception of the glycine heterotransporters mediating GABA release in the spinal cord and a few somatodendritic heterotransporters, which have in part been characterized pharmacologically, it is almost unknown to what subtype the heterocarriers listed in Table 1 belong. As selective blockers for the different transporter subtypes are becoming available, it will be important to carry on a precise homo- and heterotransporter classification.

Because of the heterogeneity and the complexity of the mechanisms involved in the heterotransporter-evoked neurotransmitter release, much effort will be required to understand the mechanisms operative in the different systems. Based on the data regarding the few systems so far examined in detail, we already know that heterotransporter activation can elicit, in variable proportions, release by homotransporter reversal, by classical exocytosis involving VSCCs, and by exocytosis exploiting Ca\(^{2+}\) mobilized from different intraterminal sources. Such stores are multiple and a number of tools now permit their investigation.

The functional significance of most heterotransporters remains poorly understood. Enhancement of basal transmitter release is the only effect linked to heterotransporter activation so far investigated; however, other effects may be produced in the various neuronal systems. Although it is now clear that heterotransporters are present on the entire surface of neurons, neuronal cells in culture have not been exploited as a model to study heterotransporters. This model could provide, inter alia, information regarding the maturation of these systems and permit us to determine precisely their cellular localization. Also totally unexplored remains the possibility that heterotransporters play any role in vivo; in this case, the use of transcerebral microdialysis could provide some information. The strategies of molecular biology need to be applied to help in understanding heterotransporters. In vitro, cells could be manipulated in various ways in order to reproduce homo- and heterotransporter systems. Knockout animals lacking a given carrier or animals treated with appropriate antisense oligonucleotides would represent useful models in exploring heterotransporter function.

One aspect that has not yet been considered concerns the possible existence of transporter–transporter interactions in glial cells. Neurotransmitter transporters are certainly colocalized on glia; being generally bidirectional, these transporters may both take up and release transmitters. It would be important to see whether activation of transporter A by transporter A can cause release of transmitter B through the transporter B. If so, such a process would represent an additional way by which glia contribute to synaptic transmission.

Dopaminergic neurons in the ventral tegmental area of rats and monkeys were shown to be immunoreactive for GLU (Sulzer et al., 1998). Interestingly, these cells seem to have synaptic varicosities that are essentially dopaminergic and others that are essentially glutamatergic (Joyce and Raupp, 2000), indicating that these neurons can mediate both dopaminergic and glutamatergic signals via separate sets of synapses. In keeping with this possibility are the findings by Docherty et al. (1991) with rat brain synaptosomes purified by immunomagnetophoresis: the authors observed that dopaminergic (but also noradrenergic and serotonergic) synaptosomes exhibited Ca\(^{2+}\)-dependent amine release, whereas no evidence for GLU corelease was obtained. However, the same authors had previously found that cholinergic and GABAergic synaptosomes did release GLU together with ACh or GABA (Docherty et al., 1987). Based on the above observations, one would not expect to find transporters for DA and GLU coexisting on the same axon terminals in tangential projection areas. In line with this view, basal DA release could be enhanced by activating GLU ionotropic receptors in rat striatal synaptosomes (Desce et al., 1992). However, involvement of GLU transporters was not carefully verified. In fact, when the effect of GLU on the release
different transporters can vary dramatically. This regulated neurotransmitter transporters, the effects of these agents on factor-activated pathways and substrates regulate several at the plasma membrane. While protein kinase C, growth neurology that should be considered in future studies of hetero-

terminals through an EAAT heterotransporter (Lonart and Zigmond, 1991).

There are cotransporter systems that, for some rea-
sions, deserve priority of investigation. One of these is the GLY/GABA system. One reason for this particular interest is that the terminals on which GLY and GABA transporters have been found to coexist in the spinal cord (Raiteri et al., 2001) are probably the terminals that corelease GLY and GABA on motoneurons; it is therefore important to further understand the roles of two cotransporters which not only can recapture the cotransmitters released onto motone-

rons, but may influence their respective synaptic concen-
trations through heterocarrier-mediated regulations of their release. Another reason that makes the system attractive is its possible involvement in the inhibitory cotransmission of cerebellar Golgi cells which corelease GABA and GLY onto granules (Ottersen et al., 1990). Nerve terminals coex-
pressing GABA and GLY transporters were shown to exist using synaptosomal preparations of rat cerebellum (Raiteri et al., 1992). These nerve terminals most likely originate from Golgi cell axons. The mechanisms by which GABA and GLY induce, respectively release of GLY and GABA in the cerebellum are completely unknown, while several aspects of the GLY heterocarrier-induced release of GABA in the spinal cord have already been elucidated (Raiteri et al., 2001). It would be important to extend the study to cerebellar axon terminals, taking advantage of the recent availability of blockers selective for the GLY1 and GLY2 transporter subtypes. Interestingly, Herdón et al. (2001) have just reported that cerebellar synaptosomes are able to take up GLY through both GLY1 and GLY2 transporters. Cotransporter systems involving GLY transport are of particular interest also because of the dual function of GLY as an inhibitory transmitter at strychnine-sensitive recep-
tors and as an excitatory coagonist at glutamatergic NMDA receptors. In this context, the heterotransporter-mediated reciprocal modulation of GLU and GLY release found to exist in cerebrocortex and spinal cord (Bonanno et al., 1994) would deserve further investigation. The availability of selective blockers of glutamatergic and glycnergic trans-
transporters subtypes should be of great help in understanding the function of GLY and GLU transporter coexistence on some nerve terminals. Of note, the two amino acids have only rarely been proposed to be cotransmitters.

Finally, there is a novel aspect of transporter pathophysi-
ology that should be considered in future studies of hetero-

transporters. Evidence is accumulating that neurotransmitter transporters can be regulated by changing their expression at the plasma membrane. While protein kinase C, growth factor-activated pathways and substrates regulate several neurotransmitter transporters, the effects of these agents on different transporters can vary dramatically. This regulated trafficking of transporters was the object of a very recent review (Robinson, 2002). According to the author, this type of regulation can occur within minutes and does not require RNA transcription and translation. It should therefore be possible to investigate regulation of heterotransporter func-
tion by monitoring transmitter release from isolated nerve endings.

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