Abstract
The regulation of neurotransmitter transporters is a central aspect of their physiology. Recent studies that focused on syntaxin-1 transporter interactions led to the postulation that syntaxin-1 is somehow implicated in protein trafficking. Because syntaxin-1 is involved in the exocytosis of neurotransmitters and it interacts with glycine transporter 2 (GLYT2), we stimulated exocytosis in synaptosomes and examined its effect on GLYT2 surface-expression and transport activity. We found that GLYT2 is rapidly trafficked first towards the plasma membrane and then internalized under conditions that stimulate vesicular glycine release. However, when syntaxin-1 was inactivated by pre-treatment of synaptosomes with the botulinum neurotoxin C, GLYT2 was unable to reach the plasma membrane but still was able to leave it. These results indicate the existence of a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-mediated regulatory mechanism that controls the surface expression of GLYT2. Syntaxin-1 is involved in the transport of GLYT2 to, but not its retrieval from, the plasma membrane. Immunogold-labelling on purified vesicular preparations from synaptosomes showed that GLYT2 is present in small synaptic-like vesicles. This may represent neurotransmitter transporter that is being trafficked. The subcellular distribution of the glycine transporters was further examined in PC12 cells that were stably transfected with the fusions of GLYT1 and GLYT2 with green fluorescent protein. There was a clear difference in their intracellular distribution, GLYT1 being present mainly on the plasma membrane and GLYT2 being localized mainly on large, dense-core vesicles. We are trying to find signal sequences responsible for this differential localization.

Introduction
Glycine neurotransmitter transporters are plasma membrane proteins that are responsible for the clearance of extracellular glycine during glycine-mediated neurotransmission. The cloning of the glycine transporters revealed that they are encoded by two different genes (glyt1 and glyt2) both of which belong to the Na⁺- and Cl⁻-coupled neurotransmitter transporter family that includes transporters for γ-aminobutyric acid and monoamines [1–7]. Glycine transporters 1 and 2 (GLYT1 and GLYT2) differ in their brain and cellular distribution. The glial GLYT1 and neuronal GLYT2 are also unique in their functional properties, including their pharmacological profile, stoichiometry and electrical behaviour [8–12]. GLYT1 and GLYT2 play an important role after the release of glycine by the presynaptic terminals, by terminating the neurotransmission signal. Therefore, the action of these transporters has to be properly regulated by a fine-tuning mechanism to control the duration of the synaptic action [13].

Exocytosis of neurotransmitters is a specialized process that requires the fusion of synaptic vesicles containing the transmitter, with the plasma membrane in a calcium-dependent manner [14,15]. In this process, three membrane proteins are known to be involved: synaptobrevin (VAMP), synaptosome-associated protein 25 (SNAP-25) and syntaxin-1 [16–18]. SNAP-25 and syntaxin-1 form a complex on the plasma membrane, which functions as a receptor for VAMP, which is present on synaptic vesicles. The formation of a heteromeric complex between VAMP, syntaxin-1 and SNAP-25 allows synaptic vesicles to come in close contact with the plasma membrane and fusion between the two membranes may occur [19]. A co-ordinated regulation of the neurotransmitter release and its re-uptake by neurotransmitter transporters is expected. By controlling protein trafficking, a close correlation between release and uptake of neurotransmitters may be achieved. Indeed, many aspects of neuronal function are regulated by this
mechanism and neurotransmitter transporters seem also to be subjected to protein redistribution processes [20,21]. The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein syntaxin-1A (an isoform of syntaxin 1) is involved in the regulation of intracellular membrane trafficking [22]. The interaction between syntaxin-1 and several plasma membrane proteins, including the γ-aminobutyric acid transporter, GAT1, has been reported to decrease the activity of these proteins and also to interfere with their trafficking [23–25]. Protein redistribution mechanisms might also direct transporters to different subcellular locations. This is an important determinant of the impact of transport activity on synaptic function (see Zafra and Giménez, this issue, pp. 746–750).

Initial studies on the regulation of GLYTs

Time- and concentration-dependent decrease in high-affinity Na+-dependent glycine transport by C6 glioma cells caused by incubation with phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC), was reported. This inhibition could be completely suppressed by the addition of the PKC inhibitor staurosporine. The PMA effects could be mimicked by oleoyl-acetylglycerol and exogenous phospholipase C. Northern and Western analysis indicated that C6 cells express GLYT1. Glycine uptake decreased in COS cells that transiently express GLYT1, when incubated with PMA [26].

The same inhibitory effect on glycine uptake by human embryonic kidney (HEK 293) cells expressing the mouse GLYT1 was shown after treatment with PMA. Interestingly, this inhibition was also observed after removing all five predicted phosphorylation sites for PKC in GLYT1 by site-directed mutagenesis, indicating that glycine transport by GLYT1 is modulated by PKC through indirect phosphorylation mechanisms or through phosphorylation on non-canonical consensus sequences [27].

Interaction of GLYTs with syntaxin-1A

To investigate whether syntaxin-1A regulates the GLYTs, both syntaxin-1A and GLYTs were transiently co-expressed in COS cells [28]. When compared with a control where syntaxin-1A was not expressed, glycine transport, either by GLYT1 or by GLYT2, decreased by approx. 40%. When syntaxin-1A was co-expressed with the syntaxin-1A-binding protein Munc18, the inhibition caused by syntaxin-1A on glycine transport by GLYTs was reversed. This result can be explained by the fact that Munc18 binds tightly to syntaxin-1A and prevents its association with other syntaxin-1A partners (i.e. GLYTs). The co-expression with syntaxin-1A also leads to a reduction in the number of transporters in the plasma membrane, as assessed by cell surface biotinylation of co-transfected COS cells. Furthermore, it was found, by using co-immunoprecipitation experiments, that a physical interaction existed between syntaxin-1A and GLYTs, both in transfected COS cells and in brain extract. These experiments demonstrated that GLYTs were regulated by syntaxin-1A. Finally, SNAP-25 does not seem to participate in the regulation, since its co-expression with GLYTs, with or without syntaxin-1A, had no functional effect, and its presence was not observed in GLYT immunocomplexes. In addition, the interaction between syntaxin-1A and the GLYTs is not affected by glycine under the conditions used [28].

Calcium- and syntaxin-1-mediated trafficking of GLYT2

On the basis of the functional and physical interaction detected for GLYTs and syntaxin-1, we tried to delineate the physiological role of the syntaxin-1 regulation. Because syntaxin-1 is an important protein in the exocytosis of neurotransmitters and in the regulation of their re-uptake by transporters, we decided to stimulate exocytosis and, subsequently, to study its effect on GLYT2, both in the presence and in the absence of functional syntaxin-1 [29]. In these studies, purified sealed synaptic terminals (synaptosomes) were used because they contain the complete functional machinery of exocytosis, including syntaxin-1, and they express GLYTs. Synaptosomes can also be treated with the botulinum neurotoxins C (Bont-C) or B (Bont-B) that inactivate syntaxin-1 and VAMP, respectively [30,31].

Calcium-dependent exocytosis was provoked by plasma membrane depolarization using 4-aminopyridine in the presence of extracellular Ca2+ to a preparation of synaptosomes preloaded with [3H]glycine [32]. This release was mainly due to exocytosis of [3H]glycine-filled synaptic vesicles, since preincubation of the synaptosomes with bafilomycin, an inhibitor of the V (vacuolar)-ATPase that prevents the entrance of neurotransmitter into synaptic vesicles [33], strongly reduced the release. In a similar way, treatment of
synaptosomes with Bont-C, which cleaves syntaxin-1, or Bont-B, which cleaves VAMP, strongly reduced both the release of \(^{3}H\)glycine and the uptake by GLYT2. This decrease in glycine transport was due to a decrease in the number of plasma-resident transporters, as proved by the decrease of GLYT2 surface immunoreactivity after cell biotinylation. These results show that two SNARE proteins (syntaxin-1 and VAMP) somehow control the number of GLYT2 proteins on the plasma membrane. The time course of GLYT2 disappearance from the plasma membrane under conditions of sustained exocytosis was established by measuring the GLYT2-surface immunoreactivity at different time points after 4-aminopyridine and \(\text{Ca}^{2+}\) addition. The effect peaked after 15 min. The removal of extracellular \(\text{Ca}^{2+}\) by washing with EGTA, reverses this effect. When this experiment was performed with synaptosomes that were pre-treated with Bont-C, GLYT2 was still able to leave the plasma membrane after depolarization, but it lost its capacity to return after EGTA washing, thereby demonstrating that syntaxin-1 is necessary for the arrival of GLYT2 at the plasma membrane, but is not involved in its removal from the plasma membrane. This long-term effect of \(\text{Ca}^{2+}\) on the regulation of neurotransmitter transporters might well be modulated by protein kinases [34], an idea that is strengthened by the previously reported inhibition of glycine transport upon PKC activation (see above) [26,27].

A rapid effect on GLYT2 trafficking exerted by \(\text{Ca}^{2+}\) was also detected by performing depolarization in the presence of extracellular calcium of synaptosomes at 25 °C and measuring GLYT2-surface density during the first few seconds. An immediate increase in the number of GLYT2 molecules on the plasma membrane after depolarization, followed by the demonstrated retrieval of GLYT2 from the plasma membrane, was detected. However, synaptosomes that had been previously treated with Bont-C did not show this rapid increase, again demonstrating that syntaxin-1 is necessary for transport of GLYT2 to the plasma membrane [29].

Consistent with this active trafficking of GLYT2 towards and from the plasma membrane, we detected GLYT2 in small synaptic-like vesicles by immunogold-labelling on purified vesicular preparations from synaptosomes. These vesicles may represent neurotransmitter transporters that are being trafficked [29].

**Subcellular distribution of GLYTs in PC12 cells**

Upon the finding that GLYT2 is present in synaptic-like vesicles, GLYT1 and GLYT2 were stably transfected into PC12 cells in order to study their subcellular localization. These rat pheochromocytoma cells secrete acetylcholine and catecholamines and, upon treatment with nerve growth factors, develop a neuronal-like phenotype [35]. The GLYT1 and GLYT2 were fused with green fluorescent protein (GFP) to facilitate visualization (A. Geerlings, E. Núñez, B. López-Corcuera and C. Aragón, unpublished observations). As demonstrated by confocal microscopy and surface biotinylation experiments, a clear difference was found in the subcellular distri-

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**Figure 1**

GLYT2 regulation by calcium and syntaxin-1

Membrane depolarization followed by a calcium influx triggers exocytosis of GLYT2, and is immediately followed by its endocytosis. Syntaxin-1 is not only involved in the fusion of synaptic vesicles containing neurotransmitter to the plasma membrane, but also in the delivery of GLYT2 to this membrane, possibly by a very similar mechanism.

\[ \text{Ca}^{2+}, \text{slow effect} \]

\( \text{endocytosis} \)

GLYT2 (intra-cellular) \( \leftrightarrow \) GLYT2 (plasma memb.) \( \text{exocytosis} \)

\[ \text{Ca}^{2+}, \text{fast effect} \]

Bont/C

syntaxin1
bution of GLYT1 and GLYT2. Approx. 80% of GLYT1 was present on the plasma membrane, compared with 30% of GLYT2. Intracellular GLYT2 was localized mainly on large dense-core vesicles, as was shown after subcellular fractionation of the cells on a glycerol gradient. Additionally, in the absence of detergent, GLYT1 and GLYT2 were shown to co-precipitate with chromogranin B, a large dense-core vesicle marker. Finally, when the stable cell lines were treated with nerve growth factor, GLYT2-GFP was exclusively present in large vesicular structures in the terminals, whereas GLYT1-GFP was localized mainly on the plasma membrane throughout the whole cell. The signal sequences responsible for the differential localization of the GLYT1s are not contained in the hydrophilic terminal ends, since truncated or chimeric transporters of the N- and C-terminal portions exhibit the same localization as the wild types (A. Geerlings E. Nuñez, B. López-Corcuera and C. Aragón, unpublished results).

Conclusions
GLYT1 and GLYT2 interact functionally and physically with syntaxin-1 and this interaction modulates the amount of transporter at the plasma membrane. The trafficking of GLYT2 to the plasma membrane is up-regulated during conditions that trigger exocytosis in a calcium- and syntaxin-1-dependent manner (see Figure 1). The sustained exocytosis down-regulates plasma membrane GLYT2 by a mechanism that is syntaxin-1-independent. The trafficking of GLYT2 to and from the plasma membrane seems to be mediated by small synaptic-like vesicles in synaptosomes. GLYT1 and GLYT2 show different subcellular localizations in PC12 cells; GLYT1 is present mainly on the plasma membrane and GLYT2 is localized intracellularly in large dense-core vesicles.

References

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