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SPECIAL REPORT

Activation of a glycine transporter on spinal cord neurons causes enhanced glutamate release in a mouse model of amyotrophic lateral sclerosis

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The release of [^{3}H]D-aspartate ([^{3}H]D-ASP) or [^{3}H]GABA evoked by glycine from spinal cord synaptosomes was compared in mice expressing mutant human SOD1 with a Gly^{93} Ala substitution ([SOD1-G93A(+)]), a transgenic model of amyotrophic lateral sclerosis, and in control mice. Mice expressing mutated SOD1 were killed at the advanced phase of the pathology, when they showed signs of ingestion disability, because of paralysis of the posterior limbs. In control mice glycine concentration-dependently evoked [^{3}H]D-ASP and [^{3}H]GABA release. Potentiation of the spontaneous release of both amino acids is likely to be mediated by activation of a glycine transporter, since the effects of glycine were counteracted by the glycine transporter blocker glycyldodecylamide but not by the glycine receptor antagonists strychnine and 5,7-dichlorokynurenate. The glycine-evoked release of [^{3}H]D-ASP, but not that of [^{3}H]GABA, was significantly more pronounced in SOD1-G93A(+) than in control animals.
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Abbreviations: ALS, amyotrophic lateral sclerosis; [^{3}H]D-ASP, [^{3}H]D-aspartate; SOD1, superoxide dismutase

Introduction Glutamate is the major excitatory transmitter in the mammalian CNS. Abnormally elevated glutamatergic transmission in discrete CNS regions can lead to excitotoxicity phenomena that are believed to play crucial roles in a number of neurodegenerative conditions. Amyotrophic lateral sclerosis (ALS) is a progressive neurological disorder characterized by degeneration of upper and lower motor neurons. There is increasing evidence that glutamate-mediated excitotoxicity is implicated in ALS (see, for reviews: Morrison & Morrison, 1999; Cluskey & Ramsden, 2001). An abnormality in the function of the glial glutamate transporter GLT-1 (EAAT-2), which is largely responsible for removing extracellular glutamate, has been proposed as one major cause of excessive glutamate receptor activity in both sporadic and familiar ALS (Rothstein *et al.*, 1995). On the other hand, alterations in a glial glutamate transporter may not entirely explain the excitotoxic degeneration (see Morrison & Morrison, 1999). Other reasons for the gains of glutamatergic function present in mutant forms of Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1), including increase of glutamate release, should therefore be considered. We describe here a novel way by which extracellular glutamate can be enhanced in a transgenic mouse model of familiar ALS carrying a human SOD1 cDNA with the G93A-associated mutation and which exhibits neuromuscular impairments very similar to those in human ALS (Gurney *et al.*, 1994).

During the last decade, our laboratory has found that transporters for different neurotransmitters often coexist on the same axon terminal; that is, a transporter for the reuptake of the endogenous transmitter just released (homotransporter) and transporters that recognize and take up transmitters coming from neighboring structures (heterotransporters). We also observed that the activation of a heterotransporter invariably elicits release of the transmitter taken up previously through the coexisting homotransporter or endogenously synthesized (see, for reviews, Bonanno & Raiteri, 1994; Raiteri *et al.*, 2002). More recently, heterotransporters have been reported by other authors to exist also on neuronal cell bodies/dendrites (Augood *et al.*, 999) as well as on axon terminals (Sepkuty *et al.*, 2002). In the present work, we studied the release of [^{3}H]D-aspartate ([^{3}H]D-ASP) and of [^{3}H]GABA elicited by glycine from synaptosomes prepared from the spinal cord of transgenic mice overexpressing the mutated form of human SOD1 and compared it with the releases from the control synaptosomes. It was found that activation of glycine transporters on glutamatergic terminals of transgenic animals increased glutamate release more actively than the activation of glycine transporters on glutamatergic terminals of control mice. Conversely, the glycine-evoked GABA release did not differ significantly.

Methods B6SJL-TgN (SOD1-G93A)1Gur mice expressing high copy number of mutant human SOD1 with a Gly^{93} Ala substitution [SOD1-G93A(+)] and B6SJL-TgN (SOD1)2Gur mice expressing wild-type human SOD1 [SOD1(+)] were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Transgenic mice were originally produced by microinjection of the transgene in fertilized eggs obtained from

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hybrid mice: the G1 line was used for G93A and the N1029 line for SOD1 (Gurney *et al*., 1994). The transgene number is different in the two lines, with G1 having more than double the number of gene copies. As estimated by Gurney *et al.* (1994), G1 mice have a gene copy (per diploid gene) = 18, while N1029 mice have a gene copy (per diploid gene) = 7.2; however, it was reported that the N1029 line expressed comparable or even greater amounts of total brain human SOD (Dal Canto & Gurney, 1994; Gurney *et al*., 1994). Selective breeding maintained each transgene in the hemizygous state on an F1 hybrid C57Bl6 × SJL genetic background (Gurney *et al*., 1994). Nontransgenic littermates of SOD1-G93A(+) and SOD1(+) mice were used as controls [G93A(−)/SOD1(−)]. All transgenic mice were identified by analyzing extracts from tail tips (homogenized in phosphate-buffered saline, freeze/thawed twice and centrifuged at 14,000 rpm for 15 min at +4°C) by staining for SOD1 activity after Laemmli's polyacrylamide gel electrophoresis (10% resolving and 4% stacking). Animals were bred and maintained at the Fondazione Santa Lucia IRCCS in Rome, for about 2 months, then sent to the animal facility of the Pharmacology and Toxicology Section, Department of Experimental Medicine in Genoa, where they remained until the experiments of release. In the SOD1-G93A(+) strain, the first signs of the disease appeared between 120 and 140 days of life. Progression of the disease was then rapid so that after 7–9 days the animals were killed because of their ingestion disability, due to paralysis of the posterior limbs. In some experiments, 4–5-month-old female Swiss mice (Charles River, Calco, Italy) were also used. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular dark–light schedule (light on 7 a.m.–7 p.m.). Food and water were freely available. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC). Animals were killed by cervical dislocation and the spinal cord rapidly removed after exposure of the spinal column. Synaptosomes were prepared and resuspended in a physiological medium having the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aeration with 95% O_2 and 5% CO_2); pH 7.2–7.4. Synaptosomes were incubated at 37°C for 15 min in the presence of 0.02 μM [^{3}H]GABA and 50 μM of the GABA transaminase inhibitor aminooxyacetic acid, to avoid [^{3}H]GABA metabolism, or 0.02 μM [^{3}H]D-ASP, a nonmetabolized compound often used as a marker to mimic glutamate in release studies. Aliquots (about 0.05 mg of protein) of the synaptosomal suspension were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri & Raiteri, 2000). Superfusion was then started with standard medium (containing aminooxyacetic acid in experiments of [^{3}H]GABA release) at a rate of 0.5 ml min^{-1} and continued for 48 min. After 33 min of superfusion to equilibrate the system, five 3-min fractions were collected. Synaptosomes were exposed to various concentrations (0.01–3 mM) of glycine at the end of the second fraction collected (t = 39 min). Glycyldodecylamide, strychnine or 5,7-dichlorokynurenate was introduced at t = 30 min. Radioactivity was measured in each fraction collected and in the superfused filters. In some experiments, fractions were analyzed for their endogenous glutamate and GABA content. Glutamate and GABA were determined by h.p.l.c. analysis after precolumn derivatization with *o*-phthalaldehyde and fluorimetric detection. Neurotransmitter efflux into the samples collected was calculated as fractional rate. The effects of drugs were evaluated by performing the ratio between the efflux in the fourth fraction collected (in which the maximum effect of glycine was generally reached) and that in the second fraction. This ratio was compared to the corresponding one obtained under control conditions. Appropriate controls were always run in parallel. The two-tailed Student's t-test was used for statistical comparison of the data.

Results Figure 1 (upper panel) illustrates the effects of glycine, added to the superfusion medium at varying concentrations (0.01–3 mM), on the basal release of [^{3}H]D-ASP (left) or [^{3}H]GABA (right) previously taken up into spinal cord synaptosomes of Swiss mice. Glycine elicited concentration-dependent release of both [^{3}H]amino acids over basal. The maximal effects amounted to ≈170% ([^{3}H]D-ASP release) and to ≈200% ([^{3}H]GABA release) and were reached in the presence of 1 mM glycine.

The lower panel of Figure 1 shows that the glycine (100 μM)-evoked releases of [^{3}H]D-ASP (left) and [^{3}H]GABA (right) were strongly prevented by the GLYT-1/GLYT-2 glycine transporter blocker glycyldodecylamide (Javitt & Frusciante, 1997), but were unaffected by the glycine receptor antagonists strychnine and 5,7-dichlorokynurenate.

These results are in line with those previously observed with several neurotransmitter systems and which show that transporters for different transmitters may coexist on the same axon terminal (see Bonanno & Raiteri, 1994; Raiteri *et al*., 2002). In the present work: (i) heterotransporters for glycine coexist with homotransporters mediating the uptake of glutamate or GABA and (ii) activation of glycine transporters under basal conditions elicits the release of glutamate and GABA.

Figure 2 describes the results obtained when synaptosomes prepared from the spinal cord of control [(SOD1(−)/G93A(−)], SOD1(+) or SOD1-G93A(+) mice were labelled with [^{3}H]D-ASP or [^{3}H]GABA and exposed in superfusion to varying concentrations of glycine.

Glycine, tested at 0.03, 0.1 or 1 mM, increased the basal release of [^{3}H]D-ASP to the same extent in synaptosomes from control and SOD1(+) mice (Figure 2, left). When comparing these values with those obtained with spinal cord synaptosomes from Swiss mice (cf. Figure 2, left with Figure 1, upper left panel), no significant differences emerged either. The effects of glycine on [^{3}H]D-ASP release are clearly more pronounced in synaptosomes prepared from the spinal cord of SOD1-G93A (+) mice (Figure 2; left). In the presence of 0.03 or 0.1 mM glycine, the [^{3}H]D-ASP released from spinal cord synaptosomes of SOD1-G93A(+) mice was doubled with respect to that released from synaptosomes of control, SOD1(+) and Swiss mice. Furthermore, also in transgenic animals, the effect of glycine was sensitive to the transporter blocker glycyldodecylamide (not shown).

Figure 2 (right) shows that the [^{3}H]GABA released by 0.03, 0.1 or 1 mM glycine did not differ significantly among synaptosomes from Swiss, control [(SOD1(−)/G93A(−)] and SOD1(+) mice (cf. Figure 2 right, with Figure 1, upper right panel). Interestingly, no differences also were found when the

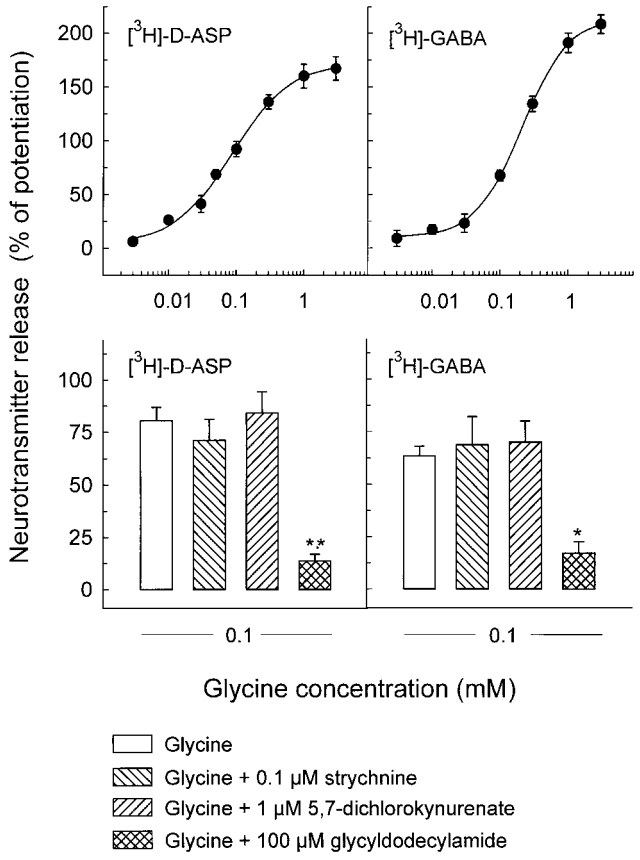

Figure 1 Effect of glycine on the release of [^{3}H]D-ASP or [^{3}H]GABA from mouse spinal cord synaptosomes. Upper panel: Concentration–response curves of the effect of glycine on the release of preaccumulated [^{3}H]D-ASP (left) and [^{3}H]GABA (right) from superfused synaptosomes of Swiss mice. Synaptosomes were labelled with the radioactive tracers and exposed to glycine in superfusion. Fractions were collected and counted for radioactivity. The tritium released in the first fraction collected (control basal release, expressed as a percentage of the total tritium present in the synaptosomes at the onset of the fraction considered) was $3.3\pm0.2\%$ ($n=11$) and $3.6\pm0.3\%$ ($n=10$) for [^{3}H]D-ASP and [^{3}H]GABA release experiments, respectively. The data presented are means $\pm$s.e.m. of 5–11 experiments in triplicate (three superfusion chambers for each experimental condition). Lower panel: Effects of the glycine receptor antagonists strychnine and 5,7-dichlorokynurenate and of the glycine uptake inhibitor glycyldodecylamide on the release of [^{3}H]D-ASP (left) or [^{3}H]GABA (right) induced by 0.1 mM glycine in Swiss mouse spinal cord synaptosomes. Strychnine, 5,7-dichlorokynurenate or glycyldodecylamide was added to the superfusion medium 8 min before glycine. Means $\pm$ s.e.m. of 4–7 experiments in triplicate are reported. Each experiment was performed using a single mouse. $^*P<0.01$, $^{**}P<0.001$ when compared to the effect of glycine alone.

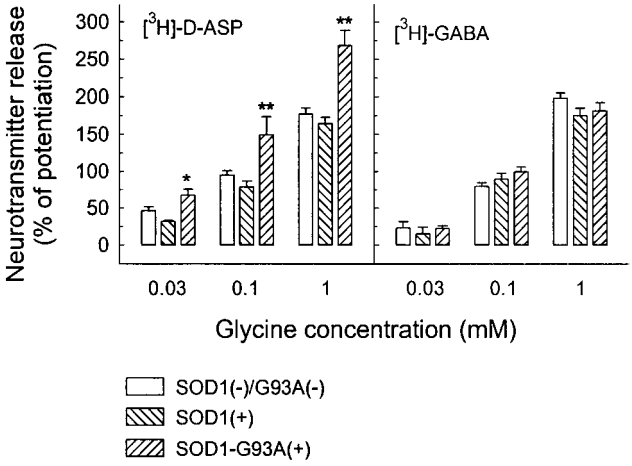

Figure 2 Effect of glycine on the release of [^{3}H]D-ASP (left) and [^{3}H]GABA (right) in synaptosomes prepared from the spinal cord of SOD1(−)/SOD1-G93A(−) (nontransgenic littermates), SOD1(+) or SOD1-G93A(+) mice. Experiments were performed in 125–150-day-old animals, when mice expressing mutated SOD1 showed signs of ingestion disability, because of paralysis of the posterior limbs. Synaptosomes were labelled with the radioactive tracers and exposed to glycine in superfusion. Fractions were collected and counted for radioactivity. Means $\pm$ s.e.m. of 5–7 experiments in triplicate are reported. Each experiment was performed using a single mouse for each group. $^*P<0.05$, $^{**}P<0.01$ when compared to the effect of glycine in SOD1(+) mice.

above values were compared with those obtained in SOD1-G93A(+) mice.

In order to rule out the possibility that the increase of [^{3}H]D-ASP release in pathological mutants is related to modifications other than the mutation itself, that is, secondary to the loss of motor neurones in the advanced stages of the disease, we investigated on the glycine effects in pre-symptomatic animals. Experiments were performed on 2-month-old mice and the results obtained seem to confirm those from symptomatic mice: the percentage of potentiation of [^{3}H]D-ASP release by 100 μM glycine amounted to 110.7 ± 6.3 and 66.8 ± 3.5 ($n=4$; $P<0.001$) in SOD1(+)-G93A(+) and in SOD1(+) mice, respectively.

Finally, in order to verify if release of preaccumulated radioactive amino acids reflects release of endogenous glutamate and GABA, spinal cord synaptosomes from 2-month-old transgenic mice were exposed to glycine, and the release of endogenous glutamate and GABA was monitored by h.p.l.c. Glycine (100 μM) increased the spontaneous release of both amino acids in SOD1(+)-G93A(+) and SOD1(+) mice; similar to the results obtained with [^{3}H]GABA and [^{3}H]D-ASP, the augmentation of endogenous GABA release was comparable ($\sim$140% over basal) in the two groups of animals, while the glycine-evoked release of endogenous glutamate was higher ($\sim$120%) in SOD1(+)-G93A(+) than in SOD1(+) mice ($\sim$70%).

Discussion Glycine and GABA are the two major inhibitory neurotransmitters and glutamate is the major excitatory neurotransmitter in the mammalian spinal cord. The reciprocal interactions among these transmitters at the level of presynaptic terminals have only in part been investigated. We here show that, while the ability of glycine to penetrate through selective transporters into GABA-releasing terminals and to enhance the basal release of GABA from spinal cord synaptosomes appears to be identical in SOD1-G93A(+) mice and control animals, activation of glycinergic transporters on glutamatergic terminals increases the release of glutamate much more efficiently in SOD1-G93A(+) than in control mice. If it occurs *in vivo*, this effect could induce an unbalance between inhibitory and excitatory transmitters. It is accepted that the mechanism of degeneration in ALS involves the

mutation in SOD-1 and includes an increase in glutamate receptor activity. According to some authors, the mutant forms of SOD-1, which may be numerous (see de Belleroche *et al*., 1998), appear to exhibit gains-of-function in their glutamatergic transmission, although the step(s) involved have not yet been entirely identified (Lee *et al*., 2001; see, for a review, Morrison & Morrison, 1999). Clearly, the activity of glutamate receptors can be increased when the levels of extracellular glutamate are elevated, by increased synaptic release or/and decreased uptake. If a disruption of glial GLT-1 transport function has received abundant experimental support, thus far there has been no evidence for increased release of glutamate in ALS. Our results show for the first time that glutamate release can be enhanced in a model of familial ALS. The increased release of glutamate following glycine transporter activation may have various origins that include trafficking of glycine transporters, changes of external calcium- or/and internal calcium-dependent glutamate exocytosis, glutamate transporter reversal or other events, the understanding of which requires detailed investigation.

The processes listed above can be investigated with the tools available and their respective involvement in the potentiation of the glycine-evoked release observed in mutant mice will probably be at least in part clarified. In contrast, the relation between SOD1 mutation and enhanced release of glutamate by glycine remains at present only a matter of speculation, although mutant SOD1 may catalyze several aberrant biochemical reactions (see, for a review, Cluskey & Ramsden, 2001). Reactive astrocytosis, which has been found to occur in the spinal cord of SOD1 transgenic mice (see, for instance, Bruijn *et al*., 1997), seems unlikely to explain our findings, in as much as our work was carried with isolated nerve terminals. In any case, the finding that the release of glutamate evoked by glycine was higher in the mutant animals than in control mice also in experiments with presymptomatic animals gives support to the idea that the effect is related to mutation, and it is not an aspecific consequence of the loss of motoneurones. Since we used isolated nerve endings in a superfusion system which was shown to exclude or minimize indirect effects (see Raiteri & Raiteri, 2000), one would expect SOD1 mutation to cause modification(s) of processes localized in glutamatergic axon terminals. If this is the case, a thorough examination of the functional characteristics of spinal cord glutamatergic axon terminals, in particular of the mechanisms involved in transmitter release, should shed some light on the relation between SOD mutation and enhanced release of glutamate.

It should be noted that the increase of glutamate release in mutant mice requires the presence of relatively high concentrations of glycine in the extracellular space. On the other hand, although the glycine concentrations in the synaptic cleft remain unknown, they could be inferred from the K_m values of the glycine uptake processes. These values were calculated from experiments with various systems, including transiently or stably expressed GLYT1 and GLYT2 transporters (López-Corcuera *et al.,* 1998; Morrow *et al*., 1998). Interestingly, the glycine concentrations found to release glutamate in the present work are well in the range of the K_m values reported in the literature, which leaves open the possibility that the glycine-evoked glutamate release may have pathophysiological significance.

Finally, it has been reported that glycine transporters exist as two subtypes, termed GLYT1 and GLYT2 with different cellular and anatomical distribution throughout the spinal cord (Zafra *et al.*, 1995; for a review, see López-Corcuera *et al.*, 2001). Interestingly, selective blockers of these transporter isoforms are being developed (Núñez *et al.*, 2000; Caulfield *et al*., 2001). These molecules could be useful to limit the glutamate release evoked by glycine heterotransporter activation.

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