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Glycine neurons in the brain and spinal cord. Antibody production and immunocytochemical localization

GHISLAINE CAMPISTRON¹, RUUD M. BUIJS² and MICHEL GEFFARD¹¹Laboratoire de Neuroimmunologie, Institut de Biochimie Cellulaire et Neurochimie du CNRS, Bordeaux (France) and²Netherlands Institute for Brain Research, Amsterdam (The Netherlands)

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Antibodies were raised against glycine and they were specific for immunocytochemistry. Obtained from rabbits immunized with glycine conjugated to glutaraldehyde-protein-carriers, antisera were then purified by adsorption on the various glutaraldehyde-conjugated protein-carriers. Using a modified ELISA method, their specificity was determined in competition experiments between conjugated glycine and either non-conjugated glycine or other conjugated amino acids or derivatives, preincubated with anti-glycine antibodies. Calculated at half-displacement, the resulting cross-reactivity ratios showed conjugated glycine to be the best recognized compound. By revealing the presence of the majority of the glycine-containing cell bodies in the brainstem and spinal cord, immunocytochemical applications of glycine antibodies confirmed their use as specific tools for a better understanding of the role of glycine in the central nervous system.

From neurophysiological studies^{5,19} it has been suggested that glycine, an amino acid, could function as a major inhibitory neurotransmitter in the central nervous system of mammals. More precisely, glycine was observed to behave very much like the natural inhibitory transmitter in the spinal cord and in several regions of rat brain⁸. By using brain slices or synaptosomal preparations, some authors have observed a depolarization-induced release of glycine from central nervous tissues as well as high levels of glycine in the spinal cord, suggesting it may have a function for spinal interneurons². Other investigations performed upon the uptake of [³H]glycine in rat central nervous system slices and homogenates, coupled or not with autoradiography, have contributed to support the idea that glycine was an important inhibitory substance^{9,10,12,13,15–17}. The characterization of the glycine receptor has reinforced the physiological action of glycine as an inhibitory neurotransmitter^{14,15}.

However, glycine was neither demonstrated directly nor specifically because it is difficult to raise specific antibodies against small-sized haptens. The

development of antibodies against other amino acid transmitters and their derivatives encouraged us to attempt to obtain antibodies to glycine, using experimental conditions similar to those previously described for small neurotransmitter-like molecules^{3,4,6,7,18}.

The present report describes the results of our studies, including the characterization of glycine antibodies as well as their utilization for immunocytochemically localizing glycine in rat brain.

To make it immunogenic, glycine (Gly, Merck) was conjugated to protein-carriers treated with glutaraldehyde^{3,4}. Gly (5 mg) was dissolved in 1 ml of a 1.5 M acetate solution, pH 8. The pH was adjusted to 7.6. Two μ l of [³H]Gly (2.26×10^{-5} M, spec. act. 44.2 Ci/mmol, NEN) was added in order to control each step of the immunological procedure. Then, 500 μ l of a 0.5 M glutaraldehyde solution (Merck) was added for 5 min. The mixture containing activated Gly was rapidly mixed with 1 ml of a 1.5 M acetate solution, pH 8, containing 15 mg of either bovine serum albumin (BSA, Sigma), or human serum albumin (HSA,

Correspondence: M. Geffard, Laboratoire de Neuroimmunologie, Institut de Biochimie Cellulaire et Neurochimie du CNRS, 1 rue Camille Saint-Saëns, 33077 Bordeaux CEDEX, France.

Centre de Transfusion Sanguine, Bordeaux), or poly-L-lysine (PL, Sigma), or thyroglobulin (TH, Sigma). The reaction-time lasted 10 min at room temperature. The coupling reaction was over when the mixture became yellow in colour. Then, 500 μ l of a 0.1 M sodium borohydride solution (Fluka) was put into the mixture, which became translucent, indicating that the saturation process was over. After one day dialysis at 4 °C, insoluble material was removed by centrifugation at 10,000 g for 15 min. Beta-counting was performed on a 100 μ l aliquot, both before and after dialysis, in order to calculate the concentration of conjugated Gly in conjugates. After weighing the lyophilized immunogen from 1 ml of the solution, the molar coupling ratios were calculated. According to the protein-carrier used, the coupling ratios were as follows: Gly-G-BSA = 60, Gly-G-HSA = 60, Gly-G-PL = 190, Gly-G-TH = 320.

Two rabbits were immunized according to our protocol^{3,6}. Every 10 days, they were alternately injected, either s.c. or i.m., with 500 μ g of immunogen emulsified in 500 μ l of phosphate-buffered-saline (PBS) and 500 μ l of Freund's complete adjuvant (Difco). Rabbits received in rotation: Gly-G-HSA, Gly-G-PL, Gly-G-TH. The sera were collected 7 days after each injection and tested in vitro in order to evaluate the antibody affinity and specificity.

Using a modified ELISA method⁶, competition experiments were performed between a Gly-G-BSA solution coated on polystyrene well-plates (Nunc) and either conjugated amino acids and derivatives or non-conjugated Gly, preincubated with antisera purified on glutariled protein-carriers. These latter were used as immunogen-carriers. A solution containing 5 mg of lyophilized proteins conjugated via glutaraldehyde was mixed with 1 ml of crude antiserum for 18 h at 4 °C. Then, the serum was centrifuged at 10,000 g for 10 min to discard the pellet containing the immune complexes bearing immunoglobulins plus protein-carriers. The purified supernatant was used both for ELISA and immunocytochemical studies. GABA, glutamate (Glu), aspartate (Asp), beta-alanine (beta-Ala), taurine (Tau) were previously conjugated via glutaraldehyde to TH under the same experimental conditions as those used during the immunogen synthesis. The use of tritiated GABA, Glu, Asp, beta-Ala and Tau allowed to calculate the concentration of the conjugated amino acids and deriva-

tives after purification by dialysis. All these conjugates were reduced by a sodium borohydride solution. Part of conjugated Gly that bore TH as a polypeptidic carrier was not reduced and is referred to as Gly=G=TH. The displacement curves derived from competition experiments that were performed with an adapted ELISA test⁶. Polystyrene wells (Nunc) were coated with a solution containing either a Gly conjugate, or a glutariled protein, preferentially conjugated-BSA. Thus, the wells were coated with 200 μ l of either a Gly-G-BSA solution or a G-BSA solution at 1 μ g/ml in 0.05 M carbonate buffer, pH 9.6, for 16 h at 4 °C. Then, they were filled up with PBS containing 0.05% Tween 20, plus 1% bovine serum (BS) and left for 1 h at 37 °C, in order to prevent the non-specific binding of the antisera by saturation of the wells. After 3 rinses, antisera purified on glutariled proteins, preincubated or not, and diluted at 1/15,000 in PBS-Tween plus 1% BS were applied onto wells coated with either Gly-G-BSA, or G-BSA, for 2 h at 37 °C. After 3 more rinses, the wells were filled up with 200 μ l of goat anti-rabbit labelled with horseradish peroxidase (HRP, Miles) diluted (1/7500) in PBS-Tween containing 1% BS and incubated at 37 °C for 1 h. After this time, the wells were rewashed 3 times with PBS-Tween. Peroxidase was assayed by incubating with an orthophenylene diamine solution containing 1% H₂O₂ (Merck), for 10 min in darkness. The reaction was stopped by addition of 50 μ l of a 4 M H₂SO₄ solution per well. The

TABLE I

Specificity of anti-glycine antibodies

<i>Compounds</i>	<i>Cross-reactivity ratios*</i>
Glycine-G-TH (Gly-G-TH)	1
Beta-Alanine-G-TH (beta-Ala-G-TH)	1/107
GABA-G-TH	1/501
Taurine-G-TH (Tau-G-TH)	1/1000
Aspartate-G-TH (Asp-G-TH)	1/20,000
Glutamate-G-TH (Glu-G-TH)	1/>20,000
Glycine=G=TH (Glycine=G=TH)	1/>100,000
Non-conjugated glycine (Gly)	1/>100,000

* Cross-reactivity ratios were calculated from the displacement curves at half displacement ($B/B_0 = 0.5$) as follows: concentration of the best recognized conjugate (i.e. Gly-G-TH) divided by the concentration of each other conjugate. When the displacement curves had a B/B_0 ratio superior to 0.5, the cross-reactivity ratios were calculated after extrapolation of the curves. N.B. the non-reduced conjugate is written as Gly=G=TH.

Fig. 1. Displacement curves established from competition experiments with a modified ELISA method⁶. Competitions were performed between the conjugate Gly-G-TH coated on wells and either each of the conjugated amino acids and derivatives or non-conjugated Gly, previously incubated with anti-Gly antibodies purified on protein-carriers for 16 h at 4 °C: curve 1, Gly-G-TH; curve 2, beta-Ala-G-TH; curve 3, GABA-G-TH; curve 4, Tau-G-TH; curve 5, Asp-G-TH; curve 6, Glu-G-TH; curve 7, Gly=G-TH; curve 8, non-conjugated Gly. B/B_0 is the ratio between the absorbances with competition (B) and without (B_0) optical density for a 1/15,000 antibody dilution. Each point drawn represents the average of 3 experiments.

measurement of the optical density at 492 nm was read with a Multiskan Titertek apparatus. Experimental values were corrected by deducting blank values from well-plates coated with G-BSA.

The most immunoreactive conjugate was Gly-G-TH (Fig. 1, curve 1) which showed a self-displacement between 10^{-9} and 10^{-6} M, indicating a rather high affinity, slightly inferior, however, to that obtained for GABA antisera¹⁸. The most immunoreactive conjugated analogues, after Gly-G-TH, were as follows: beta-Ala-G-TH (curve 2), GABA-G-TH (curve 3) and Tau-G-TH (curve 4), 107, 501 and 1000 times less recognized than Gly-G-TH, respectively.

Fig. 2. Transverse section of the caudal rat brain in the regions of the central grey and raphe dorsalis. The arrows point to Gly-positive cell bodies in the central grey and in the deep mesencephalic nucleus (v, central aqueduct; bar = 100 μ m).

Conjugated Asp (curve 5) and conjugated Glu (curve 6) were not recognized at all. Gly=G-TH, the non-reduced conjugate (Fig. 1, curve 7) and non-conjugated Gly (curve 8), exhibited no immunoreactivity at all. These results demonstrated the high specificity of anti-Gly antibodies. Though slight chemical differences, such as a CH_2 , between beta-Ala and Gly, and two CH_2 between GABA and Gly, provoked a decrease in antibody affinity of factors 107 and 501 respectively, a perfect discrimination existed between conjugated molecules of Gly, beta-Ala and GABA.

For the immunocytochemical localization, rat brains were perfused transcardially by 5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.5. Transverse 50 μ m sections were obtained with a Lancer vibratome and since the non-reduced conjugate was not recognized at all, it became necessary to treat the sections with 5 mg/ml sodium borohydride for 5 min. Immunocytochemical localization was accomplished

Figs. 3–5. Transverse sections of rat brain stained with the glycine antibody diluted at 1/4000.

Fig. 3. Low (A) and higher (B) magnification of the cerebellum with many positive cell bodies in the granular layer, G, presumably Golgi cells; but some basket cells are also positively stained because positive cell bodies can also be seen in and above the Purkinje cell layer to innervate the Purkinje cells (P). Note the presence (in B) of many positive Gly fibers in the granular and molecular layer and around the Purkinje cells (P). A, bar = 100 μ m, B, bar = 50 μ m.

Fig. 4. Gly-positive cell bodies in the brainstem spread over the reticular formation (single arrow). Note the presence of many positive cell bodies in the medial nucleus of the trapezoid body arrows (bar = 100 μ m).

Fig. 5. Transverse section of the cranial part of the spinal cord. Large Gly-positive cell bodies can be seen in the ventral horn of the spinal cord (double arrows). Many small Gly cell bodies can be seen in layer 3 of Rexed (single arrows). Bar = 100 μ m.

as described previously³. Without reduction of the sections, no positive staining was observed with the anti-Gly antibodies at a 1/1000 dilution. The reduction of the sections resulted in the appearance of numerous positively stained neurons with an antibody dilution of 1/4000. This positive staining disappeared only when the antiserum was adsorbed with Gly-G-TH and was unaffected when the antibody was adsorbed with either beta-Ala-G-TH, GABA-G-TH, or Tau-G-TH.

Cell bodies were found in the intermediodorsal nucleus of the thalamus and were unevenly distributed in the central grey spreading into the deep mesencephalic nucleus (Fig. 2). In the cerebellum, positive cell bodies were seen in the granular layer, the majority of which seemed to be Golgi cells, but basket cells were also positively stained (Fig. 3A,B). The fact that there was no cross-reaction with GABA can be concluded from the observation that after adsorption of the antibody with GABA-G-TH, the positive staining remained, and that with a GABA antibody, apart from Golgi and basket cell bodies, many stellate cell bodies were also stained¹⁸.

The majority of the positive neurons was seen in the brainstem, spread over the reticular formation, or sometimes concentrated in cell groups such as in the medial nucleus of the trapezoid body (Fig. 4). Gly fibers could be seen in many brain regions but glycine-containing cell bodies were preferentially found in caudal brain areas.

In the whole spinal cord, many positive Gly neurons were found, the cell bodies of which were lo-

cated in some large motor neurons in the ventral horn, but also in small interneurons in the second layer of Rexed (Fig. 5). Especially in the funiculus of the spinal cord and in several large myelinated fiber bundles in the brainstem, transversely sectioned material revealed a positive staining of the axon. After immunoadsorption with conjugated-Gly, the neurons in the spinal cord were no more stained, as already observed for those from other brain regions. As noted for Asp³, the Gly metabolic pool was too weakly concentrated to hamper the Gly transmitter localization in immunocytochemistry. A possible explanation could be that, at the actual state of experiment, the Gly antibodies cannot discriminate between Gly as a neurotransmitter or present in the metabolic pool.

These observations are in good agreement with the results that describe the possible function of Gly as a neurotransmitter in spinal cord and other brain regions^{2,5,8-17,19}. The visualization of Gly by means of a Gly antibody will be a valuable tool to reveal the exact localization and distribution of Gly in the brain and will enable further studies of the role of Gly in neurotransmission.

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