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STRUCTURAL DIFFERENCE BETWEEN HETEROMERIC SOMATIC AND HOMOMERIC AXONAL GLYCINE RECEPTORS IN THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

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Abstract—Glycine receptors are ionotropic receptors formed by either the homomeric assembly of ligand-binding α subunits or the heteromeric combination of an α subunit and the auxiliary β subunit. Glycine receptors in the brain are found at either pre- or post-synaptic sites. Rat supraoptic nucleus neurons express glycine receptors on the membrane of both their soma and dendrites within the supraoptic nucleus, and their axon terminals in the neurohypophysis. Taking advantage of the well-separated cellular compartments of this system, we correlated the structural properties of the receptors to their subcellular localization. Immunohistochemical study using the generic mAb4a antibody revealed that somatodendritic receptors were clustered, whereas axonal glycine receptors showed a more diffuse distribution. This was paralleled by the presence of clusters of the glycine receptor aggregating protein gephyrin in the supraoptic nucleus and its complete absence in the neurohypophysis. Moreover, another antibody recognizing the α1/α2 subunits similarly labeled the axonal glycine receptors, but did not recognize the somatodendritic receptor clusters of supraoptic nucleus neurons, indicative of structural differences between somatic and axonal glycine receptors. Furthermore, the subunits composing the somatic and axonal receptors have different molecular weight. Functional study further differentiated the two types of glycine receptors on the basis of their sensitivity to picrotoxin, identifying somatic receptors as α/β heteromers, and axonal receptors as α homomers. These results indicate that targeting of glycine receptors to axonal or somatodendritic compartment is directly related to their subunit composition, and set the hypothalamo-neurohypophysial system as an excellent model to study the mechanisms of targeting of proteins to various neuronal cellular compartments.

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Abbreviations: EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; GlyR, glycine receptor; HNS, hypothalamo-neurohypophysial system; OT, oxytocin; PBS, phosphate-buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; SDS, sodium dodecyl sulfate; SON, supraoptic nucleus; VP, vasopressin.

Key words: hypothalamus, neurohypophysis, presynaptic receptors, receptor targeting, taurine, neuroendocrine cells.

Glycine receptors (GlyRs) are ionotropic receptors known to mediate major synaptic inhibitory transmission in spinal cord and brain stem. They are made of two types of subunits, the α subunit, which bears the ligand-binding site, and the auxiliary β subunit. Four genes encoding α subunits (α1–4) and one gene encoding the β subunit have been cloned, and further diversity is generated by alternative splicing of α subunits (Betz et al., 1999; Legendre, 2001). The mature synaptic GlyRs are believed to mainly consist of the heteropentameric assembly of three α and two β subunits. Clustering at postsynaptic densities implies an additional cytoplasmic protein, gephyrin, which interacts directly with an intracellular loop of the β subunit, as well as with cytoskeletal elements, so that both proteins appear necessary for proper targeting and stabilization at postsynaptic sites (Kneussel and Betz, 2000). GlyRs can however also exist as homomeric receptors formed with five α subunits, that functionally differ from heteromeric receptors mainly by their higher single channel conductance and higher sensitivity to blockade by picrotoxin (Bormann et al., 1993; Pribilla et al., 1992). Such receptors have been described in fetal spinal neurons, and are believed to represent the major form of GlyR during early development (Legendre, 2001). Interestingly, presynaptic GlyRs have also been described in adult brain and spinal cord (Turecek and Trussell, 2001; Jeong et al., 2003; Ye et al., 2004) and some of them have been postulated to also be homomeric receptors, based on their picrotoxin sensitivity or single channel conductance.

Aside from the spinal cord and brain stem, functional GlyRs are also found in many higher brain regions. One area where GlyRs have been well characterized is the hypothalamo-neurohypophysial system (HNS). The HNS consists of vasopressin (VP) and oxytocin (OT) neurons located in the hypothalamic supraoptic (SON) and paraventricular nuclei, which send their axon in the neurohypophysis where they release the neurohormones in the general circulation. SON neurons express high levels of functional GlyRs on their soma and dendrites (Hussy et al., 1997; Deleuze et al., 2005). Somato-dendritic receptors appear as clusters, but are localized at extrasynaptic sites closely associated with astrocytic processes (Deleuze et al., 2005). This localization is in agreement with their endogenous activation by the agonist taurine, which is abundant in SON astrocytes and released upon osmotic stim-

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ulation (Deleuze et al., 1998; Hussy et al., 2000; Hussy, 2002). Interestingly, high levels of functional GlyRs are also found in the axon terminals of these neurons, in the neurohypophysis, where they are also activated by taurine released from glial cells, and mediate the osmoregulation of SON neuron secretory activities (Hussy et al., 2001; Hussy, 2002).

We took advantage of the well-separated and identifiable subcellular compartments of SON neurons to study the functional and structural properties of somatic and axon terminal GlyRs. We here report that somato-dendritic GlyRs in the SON are clustered heteromeric α/β complexes, whereas axonal GlyRs are present as unclustered homomeric channels, most probably formed with a different α subunit than the somatic receptors. These results reinforce the idea of heteromeric somatic and homomeric presynaptic GlyRs, and indicate that the HNS is a particularly interesting structure to elucidate the mechanisms responsible for specific targeting of GlyRs.

EXPERIMENTAL PROCEDURES

Recordings of acutely isolated SON neurons

All experiments conformed to European guidelines on the ethical use of animals. Everything was done to minimize the number of animals used and their suffering.

Acutely dissociated neurons from rat SON were obtained with a method modified from that described previously (Hussy et al., 1997). After decapitation without anesthesia, the brain from young adult (4–5 weeks old) Wistar rats (Centre d'élevage Depré, St Doulchard, France) was removed and briefly chilled in Locke medium (in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; KH_2PO_4, 1.2; HEPES-Na, 10; glucose, 10; pH 7.4) at 4 °C. SON were dissected and incubated for 30–40 min in Locke containing protease X and XIV (1 mg ml^{-1} each, Sigma, St Quentin, France) and DNAse I (650 unit ml^{-1}, Sigma), at room temperature. Cells were then triturated, plated onto Petri dishes. Magnocellular neurons were recorded within a few hours after dissociation under whole-cell voltage clamp. They were constantly perfused with an external solution (in mM: NaCl, 140; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 2; HEPES-Na, 10; glucose, 10; pH 7.4; osmolarity 295–305 mosmol l^{-1}). Electrodes were filled with an internal solution (in mM: CsCl, 140; $MgCl_2$, 2; EGTA-Na, 1.1; $CaCl_2$, 0.1; HEPES-Na, 10; ATP-Mg, 2; GTP-Na, 0.5; pH, 7.2; osmolarity, 295 mosmol l^{-1}), and had a resistance of 3–6 MΩ. Currents were amplified (AxonPatch 200A amplifier, Axon Instruments, Union City, CA, USA), filtered at 60 Hz with a Cyberamp signal conditioner (Axon Instruments), and digitized at 133 Hz using pClamp software (Axon Instruments). Series resistance was systematically compensated to 80–90%. Holding potential was −50 mV. Rapid application of glycine was realized using a U-tube perfusion system. Antagonists were applied both through the general perfusion and the U-tube.

Measurements of $[Ca^{2+}]_i$

Adult male Wistar rats were decapitated and neurohypophyses were taken up and freed from the pars intermedia. They were homogenized at 37 °C (in 100 μl of a solution containing (in mM): 270 sucrose, 0.1 or 2 EGTA, and 20 HEPES, pH 7.2) and spun at 100×*g* for 1 min, and the supernatant was further spun at 2400×*g* for 4 min. The final pellet containing highly purified nerve terminals (Cazalis et al., 1987) was resuspended in Locke's solution. Isolated nerve terminals were seeded onto glass coverslips and incubated in Locke's solution containing fura-2 AM (2.5 μM; Molecular Probes, Europe, Leiden, The Netherlands) and 0.01% pluronic acid at room temperature for 1 h. After washing, nerve terminals were perfused at a rate of 100 μl min^{-1} with Locke's or high (25 mM) K^+ solution (Locke's solution with KCl replacing NaCl). Fluorescence measurements of $[Ca^{2+}]_i$ were performed with a Zeiss Microscope Photometer System (FFP; Zeiss, Oberkochen, Germany), based on an inverted microscope (Axiovert 100; Zeiss) equipped for epifluorescence (objective, Plan-Neofluar 100×/1.30 numerical aperture oil immersion). With fluorescence values corrected for background and dark current, $[Ca^{2+}]_i$ was calculated from the ratio between 340 and 380 nm recordings, after fura-2 calibration performed as described previously (Dayanithi et al., 1996).

Immunohistochemistry

After deep anesthesia with pentobarbital (300 mg/kg), animals were perfused through the ascending aorta with 100 ml phosphate-buffered saline (PBS), pH 7.4, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed and immersed in the same fixative for 8 h. Tissues were cut with a vibratome into 40-μm-thick sections and rinsed in PBS. Sections were incubated for 48 h at 4 °C with the GlyR antibodies mAb4a (monoclonal mouse IgG, that recognizes all α subunits, Alexis Corporation, Lausen, Switzerland, diluted 1:200), or a polyclonal anti $\alpha1/\alpha2$ (rabbit IgG, Chemicon, Temecula, CA, USA, diluted 1:200), or the monoclonal gephyrin antibody mAb7a (mouse IgG, Alexis, diluted 1:1000). Some sections were incubated with two primary antibodies including a rabbit IgG polyclonal antibody against OT or VP (obtained by G. Alonso, diluted 1:2000), and a monoclonal antibody against either GlyR (mAb4a) or gephyrin (mAb7a). After rinsing in PBS, sections were incubated for 4 h with corresponding secondary antibodies against rabbit or mouse IgG conjugated with either Cy3 (Jackson Laboratories, West Grove, PA, USA) or Alexa 488 (Molecular Probes), diluted 1:2000. Primary and secondary antibodies were diluted in PBS containing 2% bovine serum albumin and 0.1% Triton X-100. After rinsing, sections were mounted in Mowiol (Calbiochem, San Diego, CA, USA) and observed under a Biorad MRC 1024 confocal laser scanning microscope (Zeiss) equipped with a krypton/argon mixed gas laser. The background noise of each confocal image was reduced by averaging five image inputs. Immunostained structures were studied on single confocal images (1 μm thick) or on stacked images obtained by projecting 10–20 consecutive confocal images on the same plane. Unaltered digitized images were transferred to a PC computer and Photoshop (Adobe France, Paris, France) was used to prepare final figures. The specificity of antibodies against VP, OT, GlyRs and gephyrin was established previously (Alonso, 1988; Schröder et al., 1991; Kirsch and Betz, 1993; Geiman et al., 2002). Additional control consisted in omitting the primary antibodies and applying the secondary antibodies alone.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting

Adult male Wistar rats were decapitated and SON were microdissected, pooled and frozen in liquid nitrogen and stored at −80 °C for up to 2 weeks. Tissues were thawed and homogenized in 200 μl 50 mM Tris–HCl pH 7.4 using a Teflon Eppendorf piston connected to a mechanic drill at 1000 r.p.m. Proteins in the homogenate were quantified and the equivalent of 5 μg protein were diluted in loading buffer and loaded in 1.5 mm thick SDS minigels containing a 4% polyacrylamide stacking gel. The separating gel was at 10% (acrylamide/bisacrylamide 19:1, both for stacking and separating gel). Proteins were separated at constant current (30 mAmp) until the Bromphenol Blue front reached the end of the gel (3–3.5 h). The migration was visualized by using prestained protein markers (apparent molecular weights 6.5–175 kDa, New England Biolabs, Ipswich, MA, USA). Proteins were