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Functional plasticity of GAT-3 in avian Müller cells is regulated by neurons via a glutamatergic input

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ABSTRACT

GABA (γ-amino butyric acid) is the major inhibitory transmitter in the central nervous system and its action is terminated by specific transporters (GAT), found in neurons and glial cells. We have previously described that GAT-3 is responsible for GABA uptake activity in cultured avian Müller cells and that it operates in a Na^+ and Cl^- dependent manner. Here we show that glutamate decreases [^{3}H] GABA uptake in purified cultured glial cells up to 50%, without causing cell death. This effect is mediated by ionotropic glutamatergic receptors. Glutamate inhibition on GABA uptake is not reverted by inhibitors of protein kinase C or modified by agents that modulate cyclic AMP/PKA. Biotinylation experiments demonstrate that this reduction in GABA uptake correlates with a decrease in GAT-3 plasma membrane levels. Interestingly, both GAT-1 and GAT-3 mRNA levels are also decreased by glutamate. Conditioned media (CM) prepared from retinal neurons could also decrease GABA influx, and glutamate receptor antagonists (MK-801 + CNQX) were able to prevent this effect. However, glutamate levels in CM were not different from those found in fresh media, indicating that a glutamatergic co-agonist or modulator could be regulating GABA uptake by Müller cells in this scenario. In the whole avian retina, GAT-3 is present from embryonic day 5 (E5) increasing up to the end of embryonic development and post-hatch period exclusively in neuronal layers. However, this pattern may change in pathological conditions, which drive GAT-3 expression in Müller cells. Our data suggest that in purified cultures and upon extensive neuronal lesion *in vivo*, shown as a Brn3a reduced neuronal cells and an GFAP increased gliosis, Müller glia may change its capacity to take up GABA due to GAT-3 up regulation and suggests a regulatory interplay mediated by glutamate between neurons and glial cells in this process.

1. Introduction

Astroglial cells are in close contact with neurons in every compartment of the brain, and evidence indicates that secreted factors participate in a bidirectional communication throughout the nervous system, including the retina (de Melo Reis et al., 2008). The concept of gliotransmitters refers to active molecules that modulate several neuronal functions, such as survival, differentiation and protection, and that are released by glial cells. In this sense, GABAergic synapses within the central nervous system (CNS) are constituted by a tripartite structure composed of a presynaptic neuron filled with vesicles containing neurotransmitter, a post-synaptic component with ionotropic $GABA_A$ (and $GABA_C$ in the retina) and/or metabotropic $GABA_B$ receptors and glial cells in a complex environment involved in GABAergic signaling. The removal and/or inactivation of GABAergic inhibitory signals depend largely on membrane transporters present not only in the presynaptic neuronal terminals or in the glial compartment, but also on ependymal and arachnoid cells (Eulenburg and Gomeza, 2010; Kanner, 2006).

Thus far, several GABA transporters have been described (GAT1-4 and BGT) (Madsen et al., 2010; Wu et al., 2006). All types are expressed in glial cells, with GAT-3 being the predominant glial isoform (reviewed in Schousboe, 2003). The stoichiometry of operation of GABA transport via GAT1-4 requires $2Na^+$:$1Cl^-$:1GABA whereas for BGT1 it requires $3Na^+$:$2Cl^-$:1GABA (Karakossian et al., 2005; Loo et al., 2000).

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GAT-1 and GAT-3 transporters are found in the retina of several species, from skate (Birnbaum et al., 2005) to rodents (Brecha and Weigmann, 1994; Pow et al., 2005), cat, monkey and human (Biedermann et al., 2002; Casini et al., 2006; Pow et al., 2005; Zhao et al., 2000). It belongs to a subfamily of three high-affinity GABA transporters GAT1-3. GABA transporters expressed in neurons display a greater capacity of transport compared to glial transporters and are particularly related to the recycling of the neurotransmitter into GABAergic neurons. However, since GAT-1 is present in both neurons and glial cells, it is not possible to distinguish the exact role of glial and neuronal transporters. GABA is also taken up by astrocytes, where it can be degraded or converted into glutamine (Eulenburg and Gomeza, 2010). In addition to their contribution to controlling neurotransmission and GABA synthesis in retinal cells (Calaza et al., 2001), glial transporters can release GABA in a reversal mode of activity. This action is mediated by cell depolarization and might be important in conditions of low levels of the transmitter (Richerson and Wu, 2004). Therefore, the regulation of GABA concentration in extra-synaptic sites due to the uptake by glial and neuronal transporters might exert a modulatory action on the overall activity of GABAergic synapses and constitutes a potential therapeutic target (Borden, 1996; Madsen et al., 2010; Schousboe, 2000).

We have previously described that GAT-3 operates in avian Müller cells in culture in a Na^+ and Cl^- dependent manner, inhibited by β-alanine and Zn^{2+}, but not by NNC-711, a neuronal GAT-1 blocker. Here we have extended the previous reports showing that plastic changes in GAT-3 distribution can be observed when glia–neuron interactions are disrupted. A regulatory interplay, based on glutamatergic signaling, is presented as a possible factor able to modify GABA uptake in the context of gliosis, excitotoxicity and neuronal death.

2. Material and methods

2.1. Materials

[^{3}H] GABA (Amersham Pharmacia Biotech, Piscataway, NJ, USA), GABA, glutamic acid, PMA, Ro 31-8220 methanesulfonate salt, calphostin C, staurosporine, forskolin and TPA (Sigma, St. Louis, MO, USA) were used throughout this work. GAT-3 (AB1574) was from Millipore. EZ-Link Sulfo-NHS-LC-Biotin, No-WeighTM Format (Cat. 21335, from Thermo Scientific), CaptAvidin agarose sedimented bead suspension (Cat. 21386) and Fura-2 AM were from Molecular Probes, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and gentamycin were obtained from Gibco (USA). Glutamate (Cat. No. 0218), MK801 (Cat. No.0924), CNQX (Cat. No. 1045), and DNQX were obtained from Tocris-Cookson (St. Louis, MO, USA). OPTI-MEM was obtained from Life Technologies (Cat. No. 22600-050) and PACAP was from Bachem (Bubendorf, Switzerland). All other reagents were of analytical grade.

Fertilized white Leghorn eggs were purchased from a local hatchery. The embryos were staged according to Hamburger and Hamilton 1951, and sacrificed by decapitation on embryonic day 9 (E9). The eyes were removed and the retinas were dissected out in a Ca^{2+}- and Mg^{2+}-free Hanks' (CMF) solution. The retinas were then used for culture preparations. We also used young chicks (up to 10 days) injected with NMDA in the right eye. All experiments involving animals were approved by the Animal Care and Use Committee of the Biophysics Institute (CEUA permit number IBCCF-035). Efforts were made to minimize animal suffering.

2.2. Retinal Müller glia

Retinal Müller glial cultures were generated from E9 chick retina essentially as described before (De Sampaio Schitine et al., 2007; Kubrusly et al., 2008; Reis et al., 2002). Briefly, chick retina (0.25 retina per well) was dissociated using trypsin, and cells (neurons, glia and progenitors) were seeded over 6 well, 35 mm culture dishes in DMEM containing 10% FCS. After 10 days, cell cultures were incubated with 4 mM ascorbic acid for approximately 1.5 h to eliminate neurons, if necessary (Reis et al., 2002). After this stage, only glia cells were found, this confirmed through immunocytochemistry analysis for glial or neuronal markers (not shown). Cultures were subsequently washed with DMEM, and purified glial cultures were obtained at E9C11-15 (cultures prepared from 9-day-old embryos maintained for 11–15 days *in vitro*).

2.3. GABA uptake by Müller glia

Glial cultures were rinsed with Hank's solution (128 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 4 mM glucose, 20 mM HEPES) and subsequently incubated in Hank's solution with [^{3}H] GABA (0.25 μCi/plate) and 100 μM GABA as substrate. The specific drugs used in the experiments were added 15 min before the [^{3}H] GABA incubation: PMA (0.1–2 μM), DHPG (10, 50 and 100 μM), MK801 (10 μM), DNQX (70 μM).

2.4. Live dead assay

A fluorescence-based viability assay was performed as instructed by the manufacturer (Live-dead assay; Invitrogen, Carlsbad, CA).

2.5. L-glutamate quantitation by HPLC–ED

Levels of L-glutamate in cultured cells and media were measured by high performance liquid chromatography coupled with electrochemical detection (HPLC–ED; Shimadzu, Japan) as described elsewhere (Monge-Acuña and Fornaguera-Trías, 2009). Briefly, samples were deproteinized with trichloroacetic acid (TCA; 10% w/v) and frozen at –70 °C until further use. On the day of analysis, thawed samples were mixed with an o-phthalaldehyde-sulfite derivatizing solution (Rowley et al., 1995) and run through a C-18 reverse phase column (Sigma-Aldrich, USA) before detection.

2.6. Western blot

Western blotting was done as previously described (Pohl-Guimaraes et al., 2010). Briefly, Müller glia cultures were washed twice in phosphate buffer saline (PBS), homogenized in the presence of protease inhibitors and subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to nitrocellulose membranes, briefly stained with Ponceau red, washed with an appropriate buffer and pre-incubated with 5% casein and 1% bovine serum albumin (BSA) for 2 h at room temperature in a shaker. The membranes were rinsed with Tris-buffered saline containing Tween and incubated with the primary antibodies against the GABA transporter GAT-3 (1:500) at 4 °C overnight under agitation. Secondary horseradish peroxidase (HRP)-conjugated antibodies (Sigma) were used to develop the protein band corresponding to each of the expected molecular weights of the proteins of interest, using ECL-plus (Pharmacia-Amersham).

2.7. Eye injections

Ten days post-hatched chicken was used to perform the *in vivo* assays. The animals were injected with 5 μl of 50 mM NMDA in the right eye while the animal received an injection of sterile PBS at the same amount in the left eye. The animals were kept under normal light cycle for 15 days and then sacrificed, by decapitation and the eyes were removed.