



GABA and glutamate transporters: new events and function in the vertebrate retina

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Abstract

The neural retina is a highly complex tissue composed of excitatory and inhibitory neurons and glial cells. Glutamate, the main excitatory neurotransmitter, mediates information transfer from photoreceptors, bipolar cells, and ganglion cells, whereas interneurons, mainly amacrine and horizontal cells, use γ -aminobutyric acid (GABA), the main inhibitory neurotransmitter. In this review we place an emphasis on glutamate and GABA transporters as highly regulated molecules that play fundamental roles in neurotransmitter clearance, neurotransmitter release, and oxidative stress. We pharmacologically characterized glutamate transporters in chicken retina cells and identified two glutamate transporters: one Na^+ -dependent transporter and one Na^+ -independent transporter. The Na^+ -dependent uptake system presented characteristics related to the high-affinity x_{AG}^- system (EAAT1), and the Na^+ -independent uptake system presented characteristics related to the x_{CG}^- system, which highly contributes to glutamate transport in the retina. Glutamate shares the x_{CG}^- system with another amino acid, L-cysteine, suggesting the possible involvement of glutathione. Both transporter proteins are present mainly in Müller glial cells. GABA transporters (GATs) mediate high-affinity GABA uptake from the extracellular space and terminate the synaptic action of GABA in the central nervous system. GABA transporters can be modulated by molecules that act on specific sites to promote transporter phosphorylation and dephosphorylation. In addition to a role in the clearance of GABA, GATs may also release GABA through a reverse transport mechanism. In the chicken retina, a GAT-1 blocker, but not GAT2/3 blocker, was shown to inhibit GABA uptake, suggesting that GABA release from retina cells is mainly mediated by a GAT-1-like transporter. **Keywords:** neuroretina cells, culture, glutamate transporter, GABA transporter.

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Introduction

The neural retina is a highly complex tissue composed of excitatory and inhibitory neurons and glial cells. Knowledge of retinal neurochemistry has helped to understand the organization of the receptive field and a highly integrated network that is critical for spatial, temporal, and chromatic information processing in the visual system. Glutamate and γ -aminobutyric acid (GABA) are generally regarded as excitatory and inhibitory neurotransmitters, respectively. However, the outer retina is one place where glutamate also

plays an inhibitory role in a subpopulation of bipolar cells, specifically ON bipolar cells. Glutamate is the main excitatory neurotransmitter in the outer and inner plexiform layers, mediating direct information transfer from photoreceptors, bipolar cells, and ganglion cells, whereas interneurons, mainly amacrine and horizontal cells, use GABA. In this review we emphasize studies of glutamate and GABA transporters as highly regulated molecules that play fundamental roles as sensors of both GABA and glutamate uptake and important roles in neurotransmitter clearance, neurotransmitter release, and oxidative stress.

Glutamate transporter

The intense activation of glutamate receptors produces excitotoxicity and subsequent cell death. Maintaining the extracellular concentrations of glutamate at low levels (1-10 μM) is necessary. Thus, characterization of the cellular and molecular mechanisms that govern the uptake of glutamate is very important in studies related to the visual system. Glutamate transporter proteins perform this regulation because no extracellular enzymes have been found to be responsible for glutamate metabolism (O'Shea, 2002).

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Once released at glutamatergic synapses, glutamate is cleared from the extracellular space by several high-affinity transport mechanisms. Glutamate uptake by astroglia also regulates the efficiency of glutamatergic synapses by modulating the amount of glutamate within the synaptic cleft and its diffusion from the synapse (Danbolt, 2001; Robinson, 2006). Glutamate transport deficiencies often appear to be associated with neuropathologies that depend on different centers in the central nervous system (CNS) (Doble, 1999; Obrenovitch, Urenjak, Zilkha, & Jay, 2000). Thus, treatment of these diseases could be pharmacologically based on the development of compounds that modulate the activity of these transporter proteins.

In the CNS, excitatory amino acid transporters (EAATs) are the main transporters responsible for removal of the glutamate neurotransmitter, maintaining its extracellular concentration below excitotoxic levels (Pines et al., 1992; Danbolt, 2001; Balcar, 2002; Kanai & Hediger, 2004). Glutamate transport by the EAAT is electrogenic, in which it is thermodynamically coupled to the cotransport of at least two sodium ions (Na^+) and one proton (H^+) and the countertransport of a potassium ion (Tanaka et al., 1997; Amara & Fontana, 2002; Bridges & Esslinger, 2005). These transporters are known as Na^+ -dependent high-affinity glutamate transporters (Shigeri, Seal, & Shimamoto, 2004; Bridges & Esslinger, 2005). These transporters are also coupled to a Cl^- channel, but its function has not yet been described. Furthermore, numerous membrane transporters have been characterized that regulate the flux of glutamate in distinct areas of the CNS including the retina. These transporters are commonly differentiated based on their ionic dependence and include:

- Sodium-dependent x_{AG}^- system. This system is represented by EAATs (Palacin, Estevez, Bertran, & Zorzano, 1998; McBean, 2002). In vertebrates, five different isoforms of glutamate transporters have been identified: EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5. These transporters exhibit distinct regional and cellular localization and different pharmacological and molecular characteristics (Danbolt et al., 1998; Seal & Amara, 1999). The availability of inhibitors that show a high degree of selectivity for only one of the EAATs is still limited, thus hindering the characterization of these transporters isolated in different areas of the CNS. However, the appearance of new compounds with greater selectivity is intensifying with the advancement of new techniques in molecular modeling (Bridges & Esslinger, 2005).
- Sodium-independent x_{CG}^- system. This system is mainly represented by a cystine-glutamate exchanger, a member of the glycoprotein-associated amino acid transporter family, and has been described in hepatocytes, alveolar type II cells, human endothelial cells, and macrophages (Ishii, Sato, Miura, Sagara, & Bannai, 1992). In the

CNS, this system was identified in primary cultures of neurons (Sagara, Miura, & Bannai, 1993) and astrocytes (Allen, Shanker, & Aschner, 2001; Gochernauer & Robinson, 2001), C_6 glioma cells (Cho & Bannai, 1990), microglia (Piani & Fontana, 1994), and human glioma cells (Ye, Rothstein, & Sontheimer, 1999). Under physiological conditions, the x_{CG}^- system transports cystine into cells coupled to the efflux of intracellular glutamate through a Na^+ -independent mechanism (Bender, Reichelt, & Norenberg, 2000). Once taken up, cystine is rapidly and spontaneously reduced to cysteine, which is required for the synthesis of glutathione (GSH), an endogenous antioxidant essential for cellular defense (McBean, 2002; Tomi et al., 2003).

In the retina, glutamate is stored and released by photoreceptors, bipolar cells, and ganglion cells. Glutamate is the major excitatory neurotransmitter responsible for phototransduction (Kalloniatis & Napper, 1996). In the outer layer of the retina, photoreceptors continuously release glutamate. This release is modulated by a light membrane of bipolar and horizontal cells. In the inner plexiform layer, two types of bipolar cells release glutamate – ON bipolar cells and OFF bipolar cells – that release the neurotransmitter in the presence and absence of light, respectively. Amacrine and ganglion cells are targets of glutamate release from the inner plexiform layer (Copenhagen & Jahr, 1989; Rauen, Rothstein, & Wassle, 1996).

High-affinity glutamate transporters (EAATs) have been well characterized in retinal tissue. Immunocytochemical studies demonstrated the presence of these transporters in different retina cells (Eliasof, Arriza, Leighton, Kavanaugh, & Amara, 1998; Pow & Barnett, 2000). However, little is known about the activity of different EAAT subtypes in chicken retinal tissue and the contribution of other transport systems for glutamate uptake.

We characterized the involvement of different transport systems for glutamate in chicken retinal cells. The results showed that removal of the Na^+ ion changed the value of the uptake of this neurotransmitter, thus characterizing the presence of two glutamate mechanisms in the retina: Na^+ -dependent and Na^+ -independent. These components accounted for 52% and 48% of the total transport of glutamate, respectively (Table 1).

Table 1. Glutamate transporters in the central nervous system: Na^+ -dependent and -independent transport

	Glutamate uptake (%)
NaCl	100 ± 8.7%
LiCl	50.3 ± 3.4%

The activity and participation of each of the different transporter proteins (EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5) in Na^+ -dependent glutamate uptake was characterized by observing the effects of various pharmacological antagonists that are specific to each type of carrier in the presence of NaCl. The

functionality of these transporters is directly related to the presence of Na⁺ ions. The pharmacological action of specific EAAT inhibitors is summarized in Table 2.

Table 2. Pharmacology of glutamate transporters in the central nervous system: Na⁺-dependent

TRANSPORTERS		COMPETITIVE SUBSTRATE
EAAT1 (GLAST)	TBOA	L-CCG-III, THA, PDC, 4MG, MPDC, SOS, T4HG
EAAT2 (GLT-1)	MPDC, TBOA, 4MG, DHKA; 3MG, KA7	L-CCG-III, PD, THA, T4HG, SOS
EAAT3 (EAAC1)		L-CCG-III, THA, MPDC, PDC, SOS
EAAT4	TBOA	THA, PDC, αAA
EAAT5	No specific blockade	No specific blockade

DHKA (a specific antagonist of EAAT2), SOS (a specific antagonist of EAAT3), and αAA (a specific inhibitor of EAAT4) showed no inhibitory effect on total glutamate uptake. However, 4MG, a specific antagonist of EAAT1, inhibited the uptake of glutamate in chicken retina cells. EAAT5, a carrier expressed on photoreceptors and bipolar cell terminals, has glutamate-gated Cl⁻ conductance but transports substrates poorly, limiting its potential contribution to glutamate clearance in the retina. Although some overlap was found with the compounds tested, the main Na⁺-dependent glutamate transporter in chicken retina cells appears to be mediated mainly by EAAT1.

Strong evidence suggests the existence of a sodium-independent x_{CG}^- system, represented mainly by a cystine-glutamate exchanger in chicken retina cells. Under physiological conditions, the x_{CG}^- system transports cystine into cells coupled to the efflux of intracellular glutamate through a Na⁺-independent mechanism (Bender et al., 2000). Once taken up by cells, cystine is rapidly and spontaneously reduced to cysteine, which is required for the synthesis of glutathione (GSH), an endogenous antioxidant essential for cellular defense (McBean, 2002; Tomi et al., 2003). The tripeptide GSH is produced from the amino acids glutamate, cysteine, and glycine by the consecutive actions of two enzymatic reactions (Yoneyama et al., 2008), and its role as a free radical scavenger is particularly important in the retina because this tissue is extremely vulnerable to oxidation because of its high oxygen consumption, high unsaturated fatty acid content, and exposure to light (Handelman & Dratz, 1986; Ahuja, Caffé, Ahuja, Ekstrom, & Van Veen, 2005). Furthermore, the x_{CG}^- system can also mediate the influx of glutamate when its concentration outside the cell is higher (Bringmann et al., 2009). Thus, two main glutamate transporter proteins in the chicken retina and the majority of glutamate uptake occur mainly in Müller glial cells.

GABA transporter in the retina

GABA is the main inhibitory neurotransmitter in the CNS, including the retina. In this tissue, the activation

of most interneurons promotes GABA release, which binds to its postsynaptic receptors. The two main receptor types are GABA_A and GABA_C. These receptors are ionotropic ligand-activated chloride channels that promote Cl⁻ influx. GABA_B receptors are metabotropic ligand-activated K⁺ channels that cause K⁺ efflux. The activation of GABA receptors promotes inhibitory postsynaptic potentials (IPSPs), which can lead to three forms of inhibition in the cell: (1) generation of hyperpolarization, which lowers the excitation limit in the inhibited neuron and lowers excitatory postsynaptic potentials (EPSPs); (2) takes the cell at rest and leads to a membrane potential close to the potential of K⁺, preventing it from reaching the threshold, and (3) an increase in membrane conductance, which reduces the EPSP amplitude (Kandel, Schwartz, & Jessel, 1991).

GABA transport occurs through cellular transmembrane transporter proteins. These proteins have the ability to remove the neurotransmitter from the synaptic cleft, thereby influencing neuronal transmission. Transporter activity can be modulated by hormones (Cushman & Wardzala, 1980), ion channels (Cammack & Schwartz, 1993), and molecules that promote phosphorylation/dephosphorylation at specific sites (Corey, Davidson, Lester, Brecha, & Quick, 1994).

Guastella et al. (1990) used molecular biology techniques to generate the first GABA transporter (GAT) clone from rat brains. Pharmacological and kinetic studies suggested the presence of various GAT subtypes including GAT-1, GAT-2, GAT-3, and GAT-4. GAT-1 has the highest affinity for GABA in rodents and humans (Brecha & Weigmann, 1994). It is a transmembrane protein with approximately 599 amino acids that form 12 membrane domains (Guastella et al., 1990).

GABA uptake occurs via the GAT through Na⁺ and Cl⁻ ion co-transport driven by the [Na⁺] electrochemical gradient (Cammack & Schwartz, 1993; Santos, Gonçalves, & Carvalho, 1990). Over the years, several synaptic physiologists considered that GATs constantly operate in the uptake mode at their maximum rate and are able to eliminate nearly all extracellular GABA. However, this premise is not compatible with transporter thermodynamics. More current conceptualizations indicate that these transporters have the ability to reverse uptake (Attwell, Barbour, & Szatkowski, 1993; Cammack, Rakhilin, & Schwartz, 1994; Levi & Raiteri, 1993; Lu & Hilgemann, 1999; O'Malley, Sandell, & Masland, 1992; Pin & Bockaert, 1989; Schwartz, 1987). Indirect evidence indicates that GATs are near equilibrium under resting conditions and are thus relatively inactive (Richerson & Wu, 2003). A theoretical limit may also exist with regard to how much the GAT can reduce ambient GABA (Attwell et al., 1993; Cavellier, Hamann, Rossi, Mobbs, & Attwell, 2005; Richerson & Wu, 2003).

GABA can be stored and released by a classic Ca²⁺-dependent vesicular mechanism. However, GABA in the cytosol can also be released to the extracellular space through a Ca²⁺-independent, Na⁺-dependent mechanism,

reflecting membrane transporter reversal (Wu, Wang, & Richerson 2001, 2003). Transporter-mediated GABA release can be blocked by the presence of intracellular Ca^{2+} , indicating that Ca^{2+} can modulate transport activity (Gonçalves & Carvalho, 1994; Gonçalves, Carvalho, & Vale, 1997). Inhibitory postsynaptic neuron activation induced by the binding of glutamate to non-*N*-methyl-D-aspartate receptors promotes Na^+ and Ca^{2+} influx and subsequent depolarization. The activation of voltage-dependent channels also promotes the influx of cations and activation of the GAT (do Nascimento, Ventura, & Paes De Carvalho, 1998; Santos et al., 1990).

Changes in the Na^+ gradient can influence GABA transport, independent of changes in membrane potential (Do Nascimento & de Mello, 1985; Do Nascimento et al., 1998). Each GABA molecule is co-transported with two Na^+ ions in which the driving force for GAT-1 is much more strongly dependent on the Na^+ gradient than on GABA or Cl^- gradients or membrane potential. Therefore, even modest changes in neuronal activity would be expected to alter the driving force for GAT-1 and may favor GAT-1 reversal. The influence of Na^+ levels on tonic GABA inhibition may be even greater under pathological conditions.

Other forms of GAT-1 control can also be attributed to the transporter phosphorylation/dephosphorylation conformation. Protein phosphorylation is an important posttranslational modification that regulates several biological functions including the transport of neurotransmitters like GABA. Transporter phosphorylation is directly related to normal inhibitory neurotransmission, prevention of psychiatric disorders, and modulation of synaptic efficiency (Santos et al., 1990).

Tian, Knaus, & Shipston (1998) suggested that uptake regulation by the GAT occurs because of phosphorylation, which is not observed for most transporters (e.g., the dopamine transporter), demonstrating differential posttranslational regulation by the GAT.

Previous studies have provided evidence of the influence of phosphorylation and dephosphorylation in GAT activity through the use of phosphatase inhibitors and stimulators. Gonçalves, Meireles, & Vale (1999) found that okadaic acid and Caliculin A, both inhibitors of PP1 and PP2A, inhibited the uptake of GABA in synaptosomes. Cyclosporine A, a PP2B inhibitor, had a stimulatory effect on uptake. These results differ from those found in chicken retina cells in which the PP1 and PP2A inhibitors okadaic acid and Caliculin A did not influence GABA uptake. Only PP2B inhibition influenced both uptake and glutamate-induced release (Soeiro Pantoja, 2002). The stimulatory effect was much greater when the drugs were administered in the same medium, indicating that GAT activity may be related to the activity of the enzyme calcineurin (Perrino, Ng, & Soderling, 1995).

Soeiro Pantoja (2002) demonstrated possible second messengers that regulate GAT phosphorylation

and dephosphorylation. The results indicated that endogenous Ca^{2+} variations appear to partially interfere with GAT-1 activity and the participation of the Ca^{2+} /calmodulin complex, which is capable of modulating other signaling pathways such as protein kinase A (PKA) and protein kinase C (PKC). The use of PKA inhibitors and activation agents supported the hypothesis of PKA-dependent adenylate cyclase regulation of GABA uptake (Soeiro Pantoja, 2002). However, staurosporine and phorbol ester, a PKC inhibitor and activator, respectively, did not affect GAT-1 in chicken retina cells. These data are summarized in Table 3.

Table 3. Effects of PKA, PKC, and Ca^{2+} pathways on [^3H] GABA uptake from cultured chick retina cells

Drugs	Mechanism of action	Uptake (%)
PKA		
Control		100 ± 2
BrAMPc	AMPc analog	
100 μM		98.2 ± 6
200 μM		111.4 ± 5
Forskolin	Adenylyl cyclase activator	
20 μM		120.8 ± 6
PKI	Protein kinase inhibitor	
1 μM		96.3 ± 5
10 μM		60.1 ± 4
50 μM		12.4 ± 2
H89	PKA inhibitor	
1 μM		95.2 ± 7
10 μM		81.6 ± 3
50 μM		58.4 ± 2
100 μM		23.3 ± 3
PKC and Ca^{2+}		
Ca^{2+} free medium		50.2 ± 5.6
EGTA (2 μM)	Ca^{2+} chelator	20.4 ± 3.1
W7 (10 μM)	Calmodulin inhibitor	60 ± 4.2
BAPTA AM (10 μM)	Ca^{2+} chelator	20 ± 5.2
Phorbol ester (10 nM-10 μM)	PKC activator	98.2 ± 3.5
Staurosporine (10 nM-10 μM)	PKC inhibitor	97.1 ± 5.3

Ca^{2+} ions and GAT phosphorylation greatly influence GABA uptake. The presence of Ca^{2+} and absence of protein phosphatase inhibitors favor the effect of calcineurin, corresponding to uptake inhibition when dephosphorylation sites are activated. The presence of Ca^{2+} ions and PP2B inhibitors corresponds to an increase in GABA uptake via GAT-1. Phosphorylation in the absence of Ca^{2+} decreases GABA uptake. These results indicate the participation of second messengers

in both cellular phosphorylation and dephosphorylation processes (Soeiro Pantoja, 2002).

The regulation of the GAT appears to be determined by internally localized Ca^{2+} /calmodulin-dependent phosphatase activity (calcineurin). Other phosphorylation sites that are sensitive to PP1 and PP2A inhibitors either positively or negatively potentiate the effects when the GAT is in phosphorylated and dephosphorylated states, respectively (Perrino et al., 1995).

Specific sites in the GAT-1 protein allow the transporter to present two main conformations in terms of transport, substrate, and operability, thus seemingly related to its state of phosphorylation or dephosphorylation (Gonçalves et al., 1999; Corey et al., 1994; Gomeza, Casado, Giménez, & Aragón, 1991; Sitges, Dunkeley, & Chiu, 1995). When the GAT is phosphorylated by the biochemical cascade that is activated by Ca^{2+} /calmodulin or even by PKA activation accompanied by calcineurin inhibition, its conformation favors GABA influx. When the GAT is dephosphorylated by calcineurin, the conformation favors GABA efflux, suggesting that phosphorylation sites sensitive to calcineurin are required for GAT-1 activity (Soeiro Pantoja, 2002).

In summary, cumulative evidence suggests two main GABA transport regulation processes (i.e., Na^+ gradient and GAT-1 phosphorylation/dephosphorylation), although other forms of modulation exist that control GAT distribution, trafficking, and substrates. Ongoing studies are elucidating the multiple mechanisms that influence GAT-1 transporter function and expression in the chicken retina.

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