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COMPARTMENTAL LOCALIZATION OF GABA_B RECEPTORS IN THE CHOLINERGIC CIRCUITRY OF THE RABBIT RETINA

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Abstract

Although many effects of GABA on retinal function have been attributed to GABA_A and GABA_C receptors, specific retinal functions have also been shown to be mediated by GABA_B receptors, including facilitation of light-evoked acetylcholine release from the rabbit retina (Neal and Cunningham, 1995). To explain the results of a rich set of experiments, Neal and Cunningham proposed a model for this facilitation. In this model, GABA_B-receptor-mediated inhibition of glycinergic cells would reduce their own inhibition of cholinergic cells. In turn, muscarinic input from the latter to the glycinergic cells would complete a negative-feedback circuitry. In this paper, we use immunohistochemical techniques to test elements of this model. We report that glycinergic amacrine cells are GABA_B-receptor negative. In contrast, our data reveal the localization of GABA_B receptors on cholinergic/GABAergic starburst amacrine cells. High-resolution localization of GABA_B receptors on starburst amacrine cells shows that they are discretely localized to a limited population of its varicosities, with the majority of likely synaptic-release sites being devoid of detectable levels of GABA_B receptors. Finally, we identify a glycinergic cell that is a potential muscarinic-receptor-bearing target of GABA_B-modulated acetylcholine release. This target is the DAPI-3 cell.

Based on these data, we propose a modification of the Neal-and-Cunningham model in which GABA_B receptors are on starburst not glycinergic amacrine cells.

Indexing terms

Acetylcholine; glycine; amacrine cell; ganglion cell; muscarinic receptor; directional selectivity; receptor targeting

Introduction

GABA (γ -aminobutyric acid) is one of the major inhibitory retinal neurotransmitters and its localization has been established in nearly 40% of amacrine cells in all vertebrate species examined (Pourcho, 1981; Wässle and Chun, 1989; Vaney, 1990; Wässle and Boycott, 1991; Freed, 1992; Marc, 1992; Redburn, 1992; Barnstable, 1993). GABAergic effects, which were first characterized with pharmacological methods, are mediated by receptors whose structures are now beginning to be understood through molecular biological

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techniques (Brecha, 1992; DeLorey and Olsen, 1992; Macdonald and Olsen, 1994; Slaughter, 1995; McKernan and Whiting 1996). There are at least three pharmacologically distinct classes of GABA receptors -- GABA_A, GABA_B and GABA_C (GABAp) -- and all have been shown in the retina (Brecha, 1992; Cutting et al., 1991; Grünert and Hughes, 1993; Qian and Dowling, 1994; Lukasiewicz and Werblin, 1994; Lukasiewicz et al., 1994; Matthews et al., 1994; Slaughter, 1995; Koulen et al., 1998). In particular, GABA_B receptors have been localized to presynaptic membranes of amacrine cells, as well as postsynaptically in both amacrine and ganglion cells of the rat retina (Koulen et al., 1998). Although amacrine cells immunoreactive for GABA_B receptors have also been shown to double-label for GABA but not glycine (Koulen et al., 1998), the specific identities of these cells have not yet been elucidated. In the rabbit retina, both OFF- and ON-alpha ganglion cells have been shown to express GABA_B receptors, with the OFF-alpha cells exhibiting a distribution of these receptors limited to cell bodies and proximal dendrites (Rotolo and Dacheux, 2003a, b). These same cells were also shown to receive input from glycinergic amacrine cells.

The GABA_B receptor is metabotropic, being bicuculline insensitive but baclofen sensitive (Slaughter, 1995; Kaupmann et al., 1997). Unlike most G-protein coupled receptors, GABA_B receptors require heteromeric assembly of both a GABA_BR1 and a GABA_BR2 subunit in order to function (Filippov et al., 2000). GABA_B receptors typically act by modulating voltage-sensitive Ca²⁺ channels through a G-protein-coupled mechanism (Zhang et al., 1997; Takahashi et al., 1998). Inhibition of such Ca²⁺ channels in a presynaptic membrane may implement a negative feedback loop at GABAergic synapses. In other words, the GABA_B receptors may function as auto-receptors (Anderson and Mitchell, 1985; Langer, 1997; Phelan, 1999). Related to this, GABA_B receptors may be suppressed by Ca²⁺ ions within the presynaptic terminal and are thus subject to auto-regulatory feedback (Shen and Slaughter, 1999).

In rabbit retina, it has been shown by Neal and Cunningham (1995; see also Cunningham et al., 1983) that application of the GABA_B agonist baclofen, facilitates the light-evoked release of acetylcholine by inhibiting glycine release from a subtype of glycinergic amacrine cell. They have also shown that this cell is itself stimulated by acetylcholine, acting on a muscarinic receptor, thus forming an inhibitory feedback loop onto starburst cells. The effects of acetylcholine are mimicked by muscarine and blocked by atropine, while the glycinergic effects of this circuit are blocked by strychnine. They suggest that a bistratified glycinergic amacrine cell, which receives both (muscarinic) cholinergic and GABAergic input, feeds back onto the cholinergic cells either directly or indirectly through bipolar-cell terminals. In their model, Neal and Cunningham suggest that the GABA_B receptors in this circuit could be on the glycinergic interneuron. However, since their data do not provide any preference as to which specific cell type GABA_B receptors would be located (unlike other highly specific predictions based on their data), alteration of their model through alternative GABA_B-receptor localization is consistent with that data (personal communication - Neal).

Zucker and Ehinger (1998) have shown that the two $\alpha_1/\beta_{2/3}$ -subunit-containing GABA_A-receptor bands in rabbit retina come from processes of glycinergic DAPI-3 cells (Vaney, 1990; Wright et al., 1997). These cells have perikarya in the proximal rows of the inner nuclear layer and send their processes into two sublayers in the inner plexiform layer (IPL). DAPI-3 cells are bistratified with their processes in juxtaposition with those of the two mirror symmetric populations of starburst amacrine cells (Zucker and Ehinger, 1998; Zucker et al., 2000). MacNeil and Masland (1998) have also shown that the overall lamination of DAPI-3 cells is just medial to the two highly planar plexes of the starburst amacrine cells. Although limited in extent, the processes of DAPI-3 cells meander sufficiently to permit direct synaptic interactions with starburst amacrine cells. However, although ultrastructural studies of choline acetyltransferase labeled rabbit retina have shown extensive interactions

between starburst amacrine cell processes, they have failed to identify synapses with non-cholinergic amacrine cells (Brandon, 1987; Millar and Morgan, 1987). DAPI-3 cells occur with roughly the same density as OFF starburst amacrine cells and exhibit a five-fold dendritic field overlap (Wright et al., 1997). The dendritic trees of DAPI-3 cells, which range from about 150 μm up to about 300 μm , exhibit recurved looping processes, reminiscent of those described for directionally selective ganglion cells (Amthor et al., 1984; Oyster et al., 1993). These bistratified GABA_A-receptive amacrine cells express nicotinic acetylcholine receptors (Zucker et al., 2000; Dmitrieva et al., 2003), and both accumulate glycine (Vaney, 1990; Wright et al., 1997) and are immunoreactive for glycine-transporter proteins (Zucker et al., 1996; Dmitrieva et al., 2003).

The present study seeks to explore how the localization of GABA_B and muscarinic receptors relates to the cell types likely involved in the cholinergic circuitry of the rabbit's retina. In particular, we investigate the localization of GABA_B receptors in starburst and glycinergic amacrine cells. We also investigate the possible role of DAPI-3 cells, by testing whether they contain muscarinic receptors. Localization of these and GABA_B receptors provides a test of the Neal-and-Cunningham model. Because it did not pass the test in detail, we propose a modification of this model. The modified model is consistent with our results, and with previously known physiological and pharmacological data.

Methods

Young adult Dutch belted rabbits were used in this study. All experimental procedures using animals and their care conform to the rules and guidelines adopted by the National Institutes of Health, the Society for Neuroscience and the Boston University animal care and use guidelines. The animals were euthanized by injection of intravenous sodium pentobarbital (100mg/Kg) following anesthesia with an intra-muscular injection of ketamine (100mg/Kg) plus xylazine (20mg/Kg).

Antibodies

The GABA_B receptor was localized with a commercially available antibody (Chemicon International, Ab1531 – raised in guinea pig) known to recognize both the R1a and R1b forms of the GABA_B receptor (Kaupmann et al., 1997). The antibody is directed to a twenty-amino-acid sequence (PSEPPDRLSCDGSRVHLLYK) common to both R1a and R1b isoforms. With this antibody, the isoforms appear as two bands in western-blot at 100kDa and 130kDa respectively. Staining obtained with this antibody in rabbit retina colocalizes with that obtained using a mouse monoclonal antiserum directed to GABA_B receptor-bound L-baclofen (Martinelli et al., 1992). Moreover, pre-adsorption of the antiserum with a ten-fold excess of immunogen peptide eliminated all staining (data not shown). The antibody was used at a dilution of 1:2,500 – 1:8,000; the higher concentration being used for whole mount tissue with filled cells. Due to the functional requirement of heteromeric assembly that includes a GABA_BR1 subunit (Filippov et al., 2000), this antiserum is thought to label all known GABA_B receptors. This antiserum has also been used and characterized extensively in retinas from a variety of mammalian species including rabbit (Rotolo and Dacheux, 2003a,b).

To localize m2 type muscarinic acetylcholine receptors, a rat monoclonal antiserum (Mab367, Chemicon International) directed to the i3 loop of the m2 subtype muscarinic receptor (i3 loop of m2 receptor fusion protein (225–359), fused to Glutathione S-transferase) was used. Immunoprecipitation with native muscarinic receptor subtypes shows complete m2 subtype specificity (Dorje et al., 1991; Hersch et al., 1994). Immunoprecipitation assays have shown that nearly all of the muscarinic acetylcholine receptors in rabbit retina are of either the m2 or m4 types with about 80% being m2

(Williams et al., 1996). Staining and characterization of this antibody has been previously reported in rabbit central nervous system and retina (Dorje et al., 1991; Zucker and Ehinger, 2001; see also, Wassélius et al., 1998).

The GABA_A receptor subunits were localized with a commercially available mouse monoclonal antibody raised against purified GABA/benzodiazepine receptors isolated from bovine cortex (Boehringer Mannheim Biochemica, clone bd24). This antibody recognizes a 50kDa polypeptide corresponding to the α_1 subunit and its specificity has been demonstrated on cultured cells transiently expressing this subunit (Richards et al., 1987; Ewert et al., 1990, 1992). The antibody was used at a dilution of 1:25 – 1:40 as previously described for rabbit retina (Zucker and Ehinger, 1998).

Choline acetyltransferase (ChAT) was localized with a goat polyclonal antibody raised against the human placental enzyme (AB144P Chemicon, International). This antiserum is widely used by many laboratories to visualize starburst amacrine cells in rabbit retina as well as many other mammalian and non-mammalian vertebrate species. The antibody was used at a dilution of 1:200.

Glycine transporter was localized using a rabbit polyclonal antibody, generously provided by Dr. Jon Storm-Mathisen, against a synthetic peptide sequence from the C-terminus (IVGSNGSSRLQDSRI, corresponding to amino acids 623–638 of GLYT1) of the glycine transporter, GLYT1. Western blots using the affinity-purified antiserum give single bands between 50kDa and 70kDa with extracts of various parts of the CNS including retina (Zafra et al., 1995). These bands, as well as tissue staining, were blocked by pre-adsorption of the antiserum with the immunogen peptide. The antibody was used at a dilution of 1:2,500.

Intracellular filling

Twenty-four hours prior to euthanasia, animals received eye injections containing 10ng 4,6-diamidino-2-phenylindole (DAPI) while under light sedation 0.2 cc ketamine (100mg/ml) and 0.2cc xyazine (20mg/ml). Animals were euthanized by a lethal dose of barbiturates 24 hours after the eye injection. Retinal tissue was isolated, mounted and placed in a continuous superfusion chamber with physiological AMES with 95% O₂ and 5% CO₂. Using a Zeiss UEM fluorescence microscope, DAPI labeled cells were selected for cell filling with Alexa 488 (Alexa Fluor 488; Molecular Probes A-10440).

Immunohistochemical staining procedures

For whole-mount tissue containing Alexa-488-filled starburst amacrine cells, tissue was fixed with 4% paraformaldehyde for 3 hours at 4°C. Then, we processed the tissue for whole-mount immunohistochemical staining for the GABA_B receptor. In brief, following fixation, we used 5% normal serum with 0.5% Triton X-100 in PBS overnight at 4°C to block nonspecific binding and facilitate antibody penetration. The tissue was then incubated with primary antiserum at 4°C for 7 days in the same solution. Following washout of the primary antiserum, incubation with the labeled secondary antiserum (Alexa 555 or Alexa 647) took place for 48 hours at 4°C.

For tissue destined for cryostat sectioning, 6ng of DAPI was injected 24 hours before sacrifice (with an overdose of barbiturates) and enucleation. We then fixed the tissue for 4 hours with 4% freshly prepared formaldehyde. Finally, we cryoprotected the tissue overnight and cryosectioned at 10 μ m along the dorsal/ventral plane. Sections were mounted on gelatin coated (300 bloom) slides, air dried and stored at –70°C. Following co-incubation for 36 hours at 4°C in the primary antibodies, sections were incubated with species-specific (*i.e.*, preabsorbed against each opposing species used for double-labeling) secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or indodicarbocyanine

(Cy5TM; Jackson Immuno, Inc., West Grove, Pennsylvania). Controls were obtained by omitting one or both primary antibodies and by inverting the secondary reagents.

Microscopy and data analysis

The retinas were examined with a Leitz Leica TCS 4D and a Zeiss confocal laser scanning instrument equipped with krypton-argon and HeNe lasers as well as a Zeiss UEM microscope. In the confocal microscope, FITC and Alexa 488 were excited with the 488 nm emission line (blue-green) of the laser, Alexa 555 with the 547 nm line and Cy5 and Alexa 647 with the 633 nm line (red). A band pass filter was used as barrier filter for FITC, and a long-pass filter (LP665) for Cy5. Double-labeled specimens were analyzed with sequential scans. Checks in various retina preparations demonstrated no detectable crosstalk between channels.

We performed image and data acquisition from filled cells using a Plan-Apochromat 63×/1.4 oil immersion objective. Z-stacks of 0.7 μm optical sections were collected from slightly overlapping fields to encompass the entire proximal-to-distal extent of the filled starburst amacrine-cells dendrites. We collected data from nine filled cells.

Co-localization was based on the red and green channel ratio in individual 0.7- μm optical slices using the ImageJ RGB co-localization plug-in. Co-localized pixels were stored, per optical slice, in a third blue channel. A Z-projection of each confocal stack was created using the ImageJ RGB projection tool, which averaged the pixel values for each slice while maintaining color separation among the three channels. The z-projection was then analyzed in Adobe Photoshop 7.0. For analysis of varicosities, each optical field represented the outer one third of a filled starburst amacrine-cell dendritic arborization. The distal one third, considered the synaptic output region of the starburst amacrine cell, was determined based on morphologically distinct characteristics previously described by Famiglietti (1983). The RGB file was then isolated into two separate layers, one representing the green-filled cell, the other representing the blue co-localized pixels. With the green channel isolated, we determined and marked every varicosity in our sampling. Determination of a varicosity was based on dendritic diameter swelling relative to the appearance of the juxtaposed inter-varicose dendritic segments. Two individuals having no knowledge of the staining pattern of co-localized pixels independently determined the putative identity of varicosities. Upon determining the varicosity population, the co-localized channel was re-incorporated. We considered only pre-marked varicosities for the presence of GABA_B expression. Those varicosities with GABA_B expression were marked with a second discrete flag for counting relative to the total population. In total, 2,682 varicosities (from within 29, 100 μm^2 optical fields) were analyzed for co-localization with GABA_B receptors.

Likewise, we naively selected inter-varicose segments from both proximal and distal dendritic regions as to the channel representing GABA_B-receptor staining. Degree of co-localization on the inter-varicose segments was then compared to that seen over labeled varicosities. (This degree was the ratio of blue, co-localized pixels, to total green pixels within the same circumscribed areas.) In total, 83 inter-varicose segments and 368-labeled varicosities were analyzed. They came from within eighteen, 100- μm^2 optical fields obtained from six filled starburst amacrine cells).

Results

GABA_B Receptors in the Rabbit's Retina

In the present study, our main goal was to determine how GABA_B and muscarinic-receptor localization relates to the circuitry that likely plays a role in the regulation of cholinergic function in the rabbit retina. We have found that GABA_B receptors are localized to several

classes of amacrine cells, a significant proportion of ganglion cells and is widely distributed throughout the IPL with only poorly defined lamination (figure 1B). Amacrine-cell labeling appears as a fine speckling over the surface of the cell whereas the staining on ganglion-cell bodies is qualitatively different. Ganglion-cell staining seems to be mostly intracellular and to be confined to many large, extremely bright, punctae scattered throughout the cytoplasm (figure 1 B, D). Only weak GABA_B staining is seen on the membrane surface of ganglion cells.

GABA_B Receptors on Starburst Amacrine Cells

In order to determine if starburst amacrine cells express GABA_B receptors, we performed double-labeling for the GABA_B receptor and ChAT. As shown in Figure 1, all ChAT-positive cells, that is, both OFF and ON populations of starburst amacrine cells are GABA_B-receptor immunoreactive. In total, 244 ChAT-labeled starburst amacrine cells (114 – OFF; 130 – ON) were analyzed. Due to the extensive GABA_B receptor immunoreactivity throughout the inner plexiform layer, colocalization on starburst amacrine cell processes could not be resolved using vertical cross-sections.

To investigate the precise pattern of GABA_B receptors on the dendrites of starburst amacrine cells, receptor localization on intracellularly filled cells was performed. GABA_B-receptor localization was highly correlated with a subset of dendritic varicosities, spines, and boutons (collectively called varicosities hence). These processes are found predominantly along the distal third of filled starburst-cell dendrites. However, many varicosities showed no detectable GABA_B-receptor immunoreactivity (Figure 2).

Quantitatively, of 2,682 varicosities analyzed, 714 or 26% (SEM = 1.6% across twenty-nine optical fields) were found to be GABA_B-receptor positive (Figure 3A). On average, 25% of pixels comprising GABA_B receptor positive varicosities showed receptor co-localization. In contrast, intervening connecting processes in the distal third of the dendritic arbor, non-varicose proximal dendritic sections as well as unlabeled varicosities showed only about 1% pixel co-localization ($n = 368/\text{SEM} = 0.5\%$ and $n = 83/\text{SEM} = 0.1\%$ respectively; Figure 3B). Although the GABA_B receptor antiserum used in the present study provides clear staining at dilutions of 1:8,000 and above, whole-mount tissue containing filled starburst amacrine cells was incubated with the antiserum diluted to 1:2500 to assure complete saturation of antigenic sites. Rotolo and Dacheux (2003a,b) also used a dilution of 1:2,500 with this same antiserum to localize GABA_B receptors on intracellularly filled rabbit retinal ganglion cells.

Lack of GABA_B Receptors on Glycinergic Amacrine Cells

DAPI-3 cells are a subset of glycinergic amacrine cells that show strong labeling for the α_1 subunit of the GABA_A receptor and whose processes stratify near those of the starburst amacrine cells. Analysis of sections double-labeled for the GABA_A receptor α_1 subunit and GABA_B receptors shows that DAPI-3 cells do not express a detectable level of GABA_B-receptor immunoreactivity (figure 4 A-D). We analyzed 174 GABA_A-receptor-labeled DAPI-3 cells.

We also tested whether glycinergic amacrine cells, in general, contain GABA_B receptors by double labeling for glycine transporter-1 (which labels the entire glycinergic population) and GABA_B receptors. As shown in Figure 4E, F, each glycine-transporter-1-positive amacrine cell is GABA_B-receptor negative. In addition, the pattern of glycine transporter-1 staining within the inner plexiform layer is qualitatively different from that seen for GABA_B receptors. This suggests that the dendritic processes of glycinergic amacrine cells are also GABA_B-receptor negative.

Muscarinic Receptors on Glycinergic DAPI-3 Cells

Muscarinic-receptor staining was seen on the cell membrane of several classes of amacrine-cell bodies, as well as some bipolar and ganglion cells. In the IPL, strong staining was diffusely distributed between the confines of the two starburst-amacrine cell strata with a peak density at about the 50% level (Figure 5A, B). Starburst amacrine cell bodies labeled with ChAT were invariably muscarinic-receptor immunonegative (Figure 5A, B). Double-label experiments also showed that all strongly GABA_A-receptor-subunit positive amacrine cells (DAPI-3 cells) were also immunoreactive for muscarinic receptors (Figures 5C, D). In total, nearly half of the amacrine cell bodies that were muscarinic receptor immunopositive (88 of 183) were found to be DAPI-3 cells.

Discussion

Inhibitory Feedback Loop in the Cholinergic Circuitry

Over the last several decades, there has been considerable interest in the role played by both GABA and acetylcholine in visual processing. Although the details have led to significant controversy, it is widely accepted that starburst amacrine cells, which release both GABA and acetylcholine, play a central role in the generation and modulation of retinal responses to motion and especially, the response properties of directionally selective cells (Ariel and Daw, 1982a,b; Masland et al., 1984; Grzywacz et al., 1997, 1998a,b; He and Masland, 1997; Kittila and Massey, 1997; Yoshida et al., 2001; Amthor et al., 2002; Chiao and Masland, 2002; Euler et al., 2002; Fried et al., 2002; Taylor and Vaney, 2002; Amthor et al., 2003). Recent studies have shown that starburst amacrine cells provide direction specific, GABA mediated, null inhibition to directionally selective ganglion cells (Fried et al., 2002). Although a key GABAergic aspect of starburst amacrine cell function is now well defined, the cholinergic function of these cells is less clear. Consequently, it is important to understand mechanisms modulating cholinergic function.

Neal and Cunningham (1995) showed that glycinergic processes modulate acetylcholine release. They also showed that the underlying glycinergic cell is itself stimulated by acetylcholine, acting on a muscarinic receptor. Therefore, this cell forms an inhibitory feedback loop onto starburst cells. Further contribution to this loop may come from nicotinic receptors, which have been localized to many classes of glycinergic amacrine cells, including DAPI-3 cells (Zucker and Ehinger, 2001; Dmitrieva, et al., 2003). Neal and Cunningham also found that this loop depends on GABA through GABA_B receptors.

In this paper, we identified potential candidate neuronal loci that may contribute to the glycinergic-cholinergic loop and ruled out other candidates. For instance, we showed that the glycinergic DAPI-3 cell expresses significant levels of muscarinic receptors. Because of the proximity of its dendrites to those of the starburst cells, the DAPI-3 cell is a good candidate to be the glycinergic branch of the loop. Furthermore, we demonstrated that glycinergic amacrine cells, including DAPI-3 cells, are GABA_B-receptor negative. Consequently, the GABA_B receptors contributing to the loop must be elsewhere, with the starburst cells being the main candidate. We found that in the rabbit retina, GABA_B receptors are localized to cholinergic starburst amacrine cells (besides other unidentified amacrine and ganglion cells). Interestingly, these GABA_B receptors are exclusively localized to a limited proportion of the synaptic endings of any individual starburst amacrine cell. The remaining synaptic endings do not express detectable levels of GABA_B receptors.

GABA_B Receptors in Starburst Cells

Because cholinergic amacrine cells are also GABAergic (Brecha et al., 1988; Chun et al., 1988; Kosaka et al., 1988; Vaney and Young, 1988; O'Malley and Masland, 1989), our

results suggest that GABA_B receptors on starburst amacrine cells function as presynaptic auto-receptors. Moreover, such a localization of GABA_B receptors suggests a role for presynaptic inhibitory modulation of acetylcholine release in the retina. This role is consistent with the ultrastructural findings of Koulen et al. (1998). They showed that GABA_B receptors in the rat retina are frequently (but not always) presynaptic in location. They also showed that such receptors are often found in cells that also double-label for GABA, thus suggesting that these receptors function as auto-receptors. However, several factors could suggest that the classical definition of “auto-receptors” may represent an oversimplification in the case of starburst amacrine cells. Recent evidence has shown that starburst amacrine cells not only release GABA via a Ca⁺⁺ independent mechanism (O'Malley et al., 1992) but can also release GABA in a Ca⁺⁺ dependent fashion from their distal tips (Zheng et al., 2004). In addition, starburst amacrine cell dendrites extensively co-fasciculate and thus juxtapose with each other. Therefore, GABA_B receptors on a starburst amacrine-cell varicosity could be subject to influence by GABA released by the varicosity itself. Other GABAergic influences could come from neighboring varicosities and neighboring starburst amacrine-cell processes. Such GABAergic influences would be different from those of conventional synapses, with a well-defined polarity of release and reception. Here, the same patch of membrane could serve as pre- and postsynaptic in the same GABAergic action. In the present context however, the cholinceptive target is a glycinergic amacrine cell, which would define locally, any GABA released by the starburst terminal, relative to the glycinergic terminal, as presynaptic. By extension, GABA_B receptors on that terminal would be subject to GABA released from it, thus the GABA_B receptors would be auto-receptors. These GABA_B receptors may, at the same time, respond to other sources of GABA, be they synaptic or perisynaptic.

Physiologically, the GABA_B receptor agonist baclofen does not result in a detectable conductance change in starburst amacrine cells (Zhou and Fain, 1995). GABA_B receptors act typically by modulating voltage-sensitive Ca²⁺ channels through a G-protein-coupled mechanism (Zhang et al., 1997; Takahashi et al., 1998). If these receptors are auto-receptors, then inhibition of such Ca²⁺ channels implements a negative-feedback loop at GABAergic synapses. Although the scheme of a GABA_B auto-receptor inhibiting further GABA release has been confirmed by Neal and Shah (1989) in rat cortex, they have also shown that baclofen does not detectably suppress GABA release in the retina. There is growing evidence that retinal GABA_B receptors may be involved in the inhibition of glycine release rather than GABA release (Maguire et al., 1989; Müller et al., 1992; Neal and Cunningham, 1995). Neal and Cunningham proposed a model in which this inhibition is due to GABA_B receptors on glycinergic cells. However, our current findings that starburst amacrine cells are immunoreactive for GABA_B receptors and that glycinergic amacrine cells do not show detectable GABA_B-receptor immunoreactivity, would suggest an alternative. Because the majority of the GABA released by starburst amacrine cells is thought to be through a Ca²⁺-independent mechanism (O'Malley and Masland, 1989), presynaptic binding of GABA to a GABA_B auto-receptor would not be expected to affect further release of GABA. This prediction is supported by the data showing that GABA release is not suppressed by baclofen. If the GABA_B receptors were targeted to terminals whose GABA is released by a Ca²⁺ dependent mechanism, suppression by baclofen would be expected. On the other hand, the release of acetylcholine from these same cells is Ca²⁺ dependent. Therefore, modulation of Ca²⁺ influx at individual starburst-cell synaptic terminals may provide an effective mechanism to truncate (make transient) acetylcholine release at these GABA_B-receptor-positive terminals, thus possibly reducing the excitation of glycinergic cells.

We show that a glycinergic amacrine cell, the DAPI-3 cell, expresses muscarinic acetylcholine receptors (in addition to nicotinic-type receptors) and is therefore subject to starburst-cell activity. As explained above, the DAPI-3 cell may mediate the glycinergic

branch of the cholinergic circuitry. Hence, a reduction in this cell's activity (or of another glycinergic cell) may disinhibit starburst cells and thus enhance acetylcholine release from its non-GABA_B-receptor terminals. This hypothesis is consistent with a common theme that has evolved regarding the role of GABA_B receptors in the retina. They may be involved in an enhancement of transient responses (Ikeda et al., 1990; Müller et al., 1992; Slaughter, 1995 for review).

Functional Compartmentalization on Individual Neurons

Although compartmental localization of receptors on individual neurons is only recently being recognized, it is not without precedence. Rotolo and Dacheux (2003) have shown that GABA_B receptors are differentially localized to the cell bodies and proximal dendrites of OFF-alpha ganglion cells of the rabbit retina. They found little labeling on the more distal processes. Also on alpha ganglion cells, it has been shown that different isoforms of GABA_A receptors (with differing pharmacological properties) are selectively targeted to different synapses on the same dendrite (Koulen et al., 1996). Thus, alpha ganglion cells possess at least two forms of GABA_A receptors as well as GABA_B receptors, each of which is targeted to discrete and different locations on the cells' dendrites. Similar differential localization of GABA_A receptors has been described in hippocampal pyramidal cells (Nusser et al., 1996a) and cerebellar granule cells (Nusser et al., 1996b). In addition to receptors, it has recently been shown that mouse starburst amacrine cells express strongly Kv3 voltage-gated potassium channels on their cell bodies and proximal dendrites, with the intermediate and distal dendrites showing significantly lower levels of expression (Ozaita et al., 2004). Also on rabbit starburst amacrine cells, there is evidence that chloride gradients generated by two different cation-chloride co-transporters contribute to differences in biophysical properties seen on the proximal and distal dendrites of these cells (Gavrikov et al., 2003). As already discussed, starburst amacrine cells can release GABA via both Ca²⁺-independent and dependent mechanisms. Taken together, a complex set of functional compartments appears to be important to starburst amacrine cell coding in the retina. The limitation of GABA_B receptors to isolated varicosities as presently demonstrated could thus have profound implications for starburst cells.

Compartmental restriction of functionally important molecules would require a mechanism for specific targeting. GABA_B receptors belong to the so-called Family 3 of G-protein-coupled receptors (Bockaert and Pin, 1999) in which the two receptor-subunit-cytoplasmic-tail domains have been shown to interact with two isoforms of a dimeric scaffolding protein known as 14-3-3 (Couve et al., 2001). Other portions of the GABA_B receptors c-terminal tail, along with 14-3-3 are also known to interact in complex ways with a diverse array of clustering and effector proteins. It is suggested that such diversity and complexity centered on the intracellular domain of the metabotropic GABA_B receptor may play a central role in its ability to target precisely highly defined sites of action (see El Far and Betz, 2002 for review). Such targeting mechanisms may provide a functional explanation for our finding that GABA_B receptor clusters are localized to a limited population of starburst amacrine cell synaptic terminals. Inhibitory action in each terminal may not spread outside it.

Model for the Glycinergic-Cholinergic Loop

The model in Figure 6 condenses schematically the ideas that we have been discussing on the role of GABA_B receptors. This model is similar to that of Neal and Cunningham, but has modifications necessitated by our data. As in Neal and Cunningham's model, a glycinergic cell would implement a negative feedback onto the starburst amacrine cell. However, different from their model, GABA_B receptors would be on the starburst amacrine cell rather than on the glycinergic cell. These receptors would be present on some but not all varicosities (illustrated as the right varicosity in Figure 6). (There is no restriction, however,

on the location of GABAergic release.) We propose that the inhibition through GABA_B receptors does not spread across varicosities. This proposal is necessary to prevent baclofen from inhibiting acetylcholine release, contrary to the Neal-and-Cunningham data. A mechanistic justification for this proposal is the 14-3-3-protein complex described in the preceding section. We also propose that only synaptic terminals at the GABA_B containing varicosities contact the glycinergic cell in the glycinergic-cholinergic loop. This is necessary because if the other terminals contacted these cells, increased cholinergic released with baclofen would cause more glycinergic inhibition not less as observed experimentally. The GABA_B-modulated synaptic terminals would use muscarinic and nicotinic receptors to excite the glycinergic cell. In turn, this cell (possibly, DAPI-3) would normally inhibit the starburst amacrine cell or reduce its excitation by cone bipolar cells. This inhibition would control acetylcholine release from starburst amacrine cells and thus would reduce their action onto ganglion cells.

Hence, the role of GABA_B receptors in the model would be to dis-inhibit (facilitate) acetylcholine release from starburst cells. This model thus suggests a role for GABA_B receptors in the facilitation of responses to motion (Barlow and Levick, 1965; Grzywacz and Amthor, 1993; Amthor et al., 1996). Here is how we think of the GABA_B-receptor involvement in this motion function. If one suddenly delivers a no-motion stimulus to the starburst cell, then the GABA_B action on the muscarinic synapse may not have time to react. This is because a slow G-protein second-messenger system may mediate this action (Introduction). If instead of sudden stimuli, a motion sweeps through the starburst cell, then a different set of events occurs. Consider a motion sweeping from right to left in a model like that in Figure 6. The depolarization in the right dendrites (not shown) would start the GABA_B loops even before the motion would reach the left bipolar cells. These loops would use the glycinergic cell to bypass the locality of dendritic computation (Euler et al., 2002). (This locality is due, among other things, to expression of Kv3 channels at the proximal processes of starburst cells – Ozaita et al., 2004.) In this case, when the motion finally reaches the left bipolar cells, the muscarinic release to the glycinergic cell is truncated, eliminating the glycinergic feedback onto the starburst cell. Hence, the model predicts that the starburst cell's responses and release of ACh may be larger for motion than for other stimuli. Interestingly, this mechanism for motion facilitation may also aid in models for directional selectivity. Some models involve multiple mechanisms, including preferred-direction facilitation (Grzywacz et al. 1997, 1998a, 1998b; Taylor and Vaney, 2002) and thus, may depend directly on the GABA_B mechanisms postulated here. In contrast, other models depend only on null-direction inhibition (Barlow and Levick, 1965; He and Masland, 1997; Kittila and Massey, 1997; Chiao and Masland, 2002; Fried et al., 2002). In particular, a recent paper presents evidence that null-direction inhibition is mediated by GABAergic release from starburst cells (Fried et al., 2002). The mechanism of delayed GABA_B-mediated dis-inhibition that we propose may also work for GABA release, potentiating null-direction inhibition.

This motion-facilitation mechanism may work in tandem with other such mechanisms suggested by Amthor and colleagues (Grzywacz and Amthor, 1993; Tjepkes and Amthor, 2000). If GABA_B receptors contributed to motion facilitation, then cholinergic action on ganglion cells may have to be mainly from non-GABA_B containing varicosities. If, on the contrary, this action were from GABA_B varicosities, then baclofen would reduce it rather than augment it. Mechanistically, it is possible that cholinergic action on ganglion cells is mainly from non-GABA_B varicosities, since they are more numerous. Hence, these varicosities may dominate overall cholinergic release. Computer simulations show that the model in Figure 6 can produce motion facilitation of acetylcholine release if we assume this segregation of varicosities (Grzywacz and Zucker, 2005). Furthermore, these simulations show that the model is consistent with the Neal-and-Cunningham data.

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Figure 1.

Localization of GABA_B receptors on starburst amacrine cell and ganglion cell bodies and within the inner plexiform layer (IPL) of the rabbit retina. Confocal pairs (A, B - 1.2 μm optical section; and C, D - 3 μm optical section) of rabbit retinal cross sections double labeled for ChAT (green) and for GABA_B receptors (red). In each pair, several ChAT labeled OFF- and ON- starburst amacrine cell bodies (arrows) are seen to also be immunoreactive for GABA_B receptors. In contrast to the starburst amacrine cells, which exhibit significant GABA_B receptor staining along the perimeter of their cell bodies, ganglion cell bodies (stars) exhibit an extensive granular pattern of GABA_B receptor immunoreactivity restricted mostly to the cytoplasmic portion of the cell. Extensive GABA_B receptor staining throughout the inner plexiform layer largely obscures that directly associated with the processes belonging to starburst amacrine cells. Scale bars = 25 μm.

Figure 2.

Demonstration of GABA_B receptors on a subset of starburst amacrine cell varicosities. **A, D:** Confocal images of Alexa-488-filled starburst-cell dendrites (green) double labeled for GABA_B receptors (red). **B, E:** Changing pixels containing both GABA_B receptor and Alexa-488 signal into white illuminates the pattern of receptor staining on starburst amacrine cell processes. Colocalization is determined independently for each optical section (0.7μm) and changed to a third color prior to generating a z-projection of the image stack **C, F:** All identified varicosities are indicated by a white tick mark. Those double-labeled with GABA_B receptors are indicated by the addition of a red tick mark. Scale bars = 20 μm.

Figure 3.

A: Quantitative analysis of GABA_B-receptor labeling on starburst amacrine cell varicosities. GABA_B-receptor immunoreactivity was limited to a distinct subset of starburst-cell varicosities, representing about 26% of the total likely synaptic-release sites. $n = 2,682$; SEM = 1.6%. **B:** Degree of GABA_B-receptor co-localization on labeled varicosities and inter-varicose segments of filled starburst amacrine cells. On average, 25% of the pixels comprising each labeled varicosity were co-localized with GABA_B-receptor immunoreactivity. In contrast, inter-varicose segments and unlabeled varicosities showed essentially no GABA_B-receptor staining. $n = 368$ /SEM = 0.5%; $n = 83$ /SEM = 0.1%.

Figure 4.

DAPI-3 and other glycinergic amacrine cells do not express detectable levels of GABA_B receptors. **A, B and C, D:** DAPI-3 cells labeled for GABA_A-receptor α_1 subunits (green - arrows) are devoid of GABA_B receptors (red). Examples of GABA_B-receptor-positive ganglion cells are also indicated (stars). Scale bar = 25 μ m. **E, F:** Double-labeling for glycine transporter-1 (green) and GABA_B receptors (red) is shown. Several glycine transporter-1 immunoreactive amacrine cell bodies are shown (arrows); all of which are GABA_B-receptor negative. Several GABA_B-receptor labeled amacrine cells are also seen that are glycine transporter-1 immunonegative (stars). The pattern of glycine transporter-1

staining within the inner plexiform layer is qualitatively different from that seen for the GABA_B receptors. Scale bars = 25 μ m.

Figure 5.

A, B: Double-labeling of starburst amacrine cells labeled for choline acetyltransferase (green) and m2-type muscarinic receptors (red). Although amacrine (a) and ganglion cells (star) are clearly labeled for the muscarinic receptors, the starburst amacrine cells do not show any detectable receptor labeling (open arrows). **C, D:** Double-labeling of DAPI-3 cells labeled for GABA_A-receptor subunits (green) and m2-type muscarinic cholinergic receptors (red) shows extensive colocalization of these receptors on the cell body of a DAPI-3 cell (solid arrows). Extensive m2-type muscarinic receptor staining in the inner plexiform layer largely obscures that which would be associated with the processes belonging to starburst amacrine cells. Scale bars = 25 μ m.