Mapping kainate activation of inner neurons in the rat retina

Lisa Nivison-Smith¹, Daniel Sun²,³, Erica L. Fletcher⁴, Robert E. Marc⁵, and Michael Kalloniatis¹,³,⁴,⁶

¹School of Optometry and Vision Science, University of New South Wales, Sydney, New South Wales, 2052, Australia; ²Department of Ophthalmology, Massachusetts Eye & Ear Infirmary, Boston, 02114, Massachusetts, United States; ³Department of Optometry and Vision Science, University of Auckland, Auckland, 1142, New Zealand; ⁴Department of Anatomy and Neuroscience, University of Melbourne, Parkville, 3010, Australia; ⁵University of Utah School of Medicine, Department of Ophthalmology, University of Utah, Salt Lake City, 84113, Utah, United States; ⁶Centre for Eye Health, Sydney, New South Wales, 2052, Australia.

Correspondence:
Professor Michael Kalloniatis, Centre for Eye Health, University of New South Wales, Sydney, 2052, NSW, Australia.
Phone: Int +61 2 81150710
Fax: Int +61 2 81150799
E-mail: m.kalloniatis@unsw.edu.au

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Abstract

Kainate receptors mediate fast, excitatory synaptic transmission for a range of inner neurons in the mammalian retina. However, allocation of functional kainate receptors to known cell types and their sensitivity remains unresolved. Using the cation channel probe 1-amino-4-guanidobutane agmatine (AGB), we investigated kainate sensitivity of neurochemically identified cell populations within the structurally intact rat retina. Most inner retinal neuron populations responded to kainate in a concentration dependent manner. OFF cone bipolar cells demonstrated the highest sensitivity of all inner neurons to kainate. Immunocytochemical localization of AGB and macromolecular markers confirmed Type 2 BCs as part of this kainate sensitive population. The majority of amacrine (ACs) and ganglion cells (GCs) showed kainate responses with different sensitivities between major neurochemical classes (GABA/Glycine ACs > Glycine ACs > GABA ACs; Glu/weakly GABA GCs > Glu GCs). Conventional and displaced cholinergic ACs were highly responsive to kainate whilst dopaminergic ACs do not appear to express functional kainate receptors. These findings further contribute to our understanding of neuronal networks in complex multi-cellular tissues.
Introduction

In the retina, the effects of glutamate are mediated by ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors are integral membrane proteins that form ligand gated cation channels and mediate fast, excitatory synaptic transmission. They are classified as α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) receptors based on pharmacological and electrophysiological characteristics (Hollmann and Heinemann, 1994; Monaghan et al., 1989; Seeburg, 1993). This study focuses on the KA sensitive glutamate receptors - tetrameric proteins formed through combinations of five receptor subunits: GluR5, GluR6, GluR7, KA1, KA2 and two orphan receptor subunits: δ1, δ2 (Dingledine et al., 1999; Hollmann and Heinemann, 1994; Ozawa et al., 1998).

The expression of KA glutamate receptors was first demonstrated in the rodent retina using in situ hybridization at the mRNA level. GluR5 is expressed in the outer half of the inner nuclear layer, suggesting its localization to bipolar cells (BCs) and horizontal cells (Hamassaki-Britto et al., 1993; Hughes et al., 1992; Muller et al., 1992). Some of these cell bodies also labeled for GluR6 and GluR7 (Brandstätter et al., 1994; Hamassaki-Britto et al., 1993). No labeling was seen for KA1 in the rodent retina, but many cell bodies labeled for KA2 (Brandstätter et al., 1994). Using subunit specific immunocytochemical antibodies, processes of both amacrine cells (ACs) and ganglion cells (GCs) post-synaptic to cone BCs in both sublaminae of the inner plexiform layer were shown to express various KA receptor subunits (Brandstätter et al., 1997; Peng et al., 1995; Qin and Pourcho, 2001). At the majority of these cone bipolar cell dyad contacts, immunoreactivity for the subunits was typically confined to only one of the post-synaptic elements, either an AC or a GC (Brandstätter et al., 1997; Qin and Pourcho, 2001). Post-synaptic to rod BCs, KA receptors are expressed on the A1 ACs, but not the AII ACs (Ghosh et al., 2001). Whether these expressed subunits form functional KA receptors remains largely unknown.
Anatomical studies have identified AMPA and KA receptor subunits on OFF cone BCs post-synaptic to photoreceptors but as with other techniques, receptor functionality or identification of OFF BC type has not be determined (Brandstätter et al., 1997; Hack et al., 1999; Haverkamp et al., 2001; Morigiwa and Vardi, 1999; Qin and Pourcho, 1999). Electrophysiological studies demonstrate that morphologically distinct types of OFF BCs in the ground squirrel specifically express either AMPA or KA sensitive receptors (DeVries, 2000).

From this work arises an enduring issue in retinal physiology; to ascribe the locality of glutamate receptor subunits onto known cell types and determine their functionality. One approach for assessing glutamate receptor function is probing cation channel activation based on the entry of the organic cation, 1-amino-4-guanidobutane (AGB). The immunocytochemical localization of accumulated AGB in retinal sections provides a map of neuronal activity with high spatial resolution. The AGB technique also preserves structural integrity of the retina thus allowing for cellular identity. The mechanisms of AGB permeation and its advantages in studying neuronal networks have been expounded in multiple studies (Kalloniatis et al., 2004; Marc, 1999a; b; Marc and Jones, 2002; Marc et al., 2005; Sun and Kalloniatis, 2006; Sun et al., 2003). In the mouse retina, CD15 immunoreactive BCs were AGB permeable secondary to AMPA activation, but not KA activation demonstrating this cation can be use to investigate glutamate receptor functionality in neurochemically identified populations (Sun and Kalloniatis, 2006).

In this study, we describe the KA sensitivity of different neurochemically identified inner retinal neurons within an intact retinal network. We segregated neurons into separate classes by quantitative immunocytochemistry of the amino acids glutamate (Glu), gamma-aminobutyric acid (GABA) and glycine (Gly) and determined the KA sensitivity of these populations by overlapping AGB permeation with amino acid profiles (Kalloniatis et al., 1996; Marc, 1999a; b; Marc and Jones, 2002; Marc et al., 2005; Marc et al., 1990; Marc et al., 1995; Sun et al., 2003). We then used indirect immunofluorescence to

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detect AGB to assigned KA receptor functionality to specific cell classes based on macromolecular markers (Sun et al., 2007a; Sun and Kalloniatis, 2006; Sun et al., 2007c). We show that various retinal neuron populations (i.e. BCs, ACs, GCs) are activated by KA. The combination of macromolecular markers and amino acid immunocytochemistry can be used to assign KA receptor functionality to specific sub-populations e.g. functional activation of the cholinergic AC population, a subgroup of the GABA AC population (Sun et al., 2007a; Sun et al., 2007b; c). We also highlight the need for careful interpretation of AGB labeling when using broad acting glutamate receptor agonists.

Materials and methods

Retinal preparation and incubation
Adult male Sprague-Dawley rats (n = 30) obtained from Animal Resources Centre (Canning Vale, WA, Australia) were deeply anesthetized with an intramuscular injection of ketamine (100 mg/kg; Phoenix Pharm, Auckland, New Zealand) and domitor (1 mg/kg; Novartis Animal Health, Melbourne, Australia) and following the retinal dissection, were killed with an intra-cardial injection of potassium chloride. Isolated retinal samples were mounted on 0.8 µm pore Metricel membrane filters (Gelman Sciences, Ann Arbor, MI) and the retinal pigment epithelium separated from the retina by gently pulling the globe away from the filter paper. Retinal pieces were incubated in vitro in a modified Edwards medium (Edwards et al., 1989) where 25 mM AGB was added and an equimolar reduction in NaCl concentration was made. For the activation studies, various concentrations of KA (1 – 80 µM) were added to the incubation medium. All incubations were performed under normal room lighting (300-400 lux) for 6 min at 37°C with the medium bubbled in 95% O₂ / 5% CO₂. The experimental protocols in this study were approved by The University of Auckland and The University of New South Wales animal ethics committee.
Post-embedding immunocytochemistry
The procedures for post-embedding immunocytochemistry have been described previously (Kalloniatis and Fletcher, 1993; Marc et al., 1990; Marc et al., 1995; Sun et al., 2003). Briefly, retinal pieces were fixed in 2.5% (w/v) glutaraldehyde, 1% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 for 30 min, washed in PB and dehydrated through cold methanol to acetone before impregnating in resin. Resin blocks were sectioned at 250 nm to allow subsequent serial sectioning for post-embedding immunocytochemistry (Sun et al., 2003). Primary antibodies were diluted in 1% goat serum in phosphate buffered saline to the concentrations specified in Table 1.
The primary antibodies were detected with goat anti-rabbit secondary antibodies (British BioCell, UK) coated with a 1 nm gold particle at a dilution of 1:100. The immunogold was visualized by silver intensification (Kalloniatis and Fletcher, 1993; Marc et al., 1990; Sun et al., 2003).

Indirect immunofluorescence
Retinal pieces were fixed in 4% (w/v) paraformaldehyde, 1% (w/v) glutaraldehyde in PB for 30 - 40 min. The samples were cryoprotected in a graded sucrose solution (10 - 30%) overnight then mounted in freezing medium (Reichert, Nussloch, Germany). Vertical sections at 16 μm thickness were collected on positively charged slides (Lomb Scientific, Taren Point, Australia).

The indirect immunofluorescence technique was performed as outlined in Sun and Kalloniatis (2006). Antisera were diluted in PB containing 3% goat serum, 1% bovine serum albumin and 0.5% (v/v) Triton X-100. Descriptions of the antibodies used and their concentrations are presented in Table 1. Sections were initially incubated in PB containing 6% goat serum, 1% bovine serum albumin and 0.5% (v/v) Triton X-100 for 1 h at room temperature followed by incubations with primary antibodies overnight at 4°C. The secondary antibodies were conjugated to Alexa Fluor 405, Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR). The specificity of the secondary antibodies
were confirmed by omitting the primary antibody or using a secondary antibody from a different species.

All the immunofluorescent micrographs were collected using a confocal laser scanning microscope (LEICA Microsystems TCS 4D). The brightness and contrast of the final images were adjusted using Adobe Photoshop (version 6; Adobe Systems, Mountain View, CA, USA). Quantification of AGB labeled cells was performed as previously described (Acosta et al., 2007; Sun and Kalloniatis, 2006). Briefly, cells with distinctly AGB labeled somata from background levels were counted as KA activated cells and were noted to be co-localized if there was overlapping marker immunoreactivity at the same anatomical location. Areas showing obvious anatomical disruptions were excluded. Data was presented as the mean ± standard deviation of five independent retinae and statistical significance was determined using a one-way ANOVA across three KA concentrations (5, 20 and 80 μM).

**Antibody characterization**

*Amino acids (GABA, glutamate and glycine)*: The specificity of the amino acid antibodies has been demonstrated in dot immunoassays. A positive signal for the GABA antibody was obtained for an artificial GABA antigen coupled to bovine serum albumin via glutaraldehyde cross-linking (Marc et al., 1990; Marc et al., 1995). Identical results were seen for glutamate and glycine antibodies with glutaraldehyde cross-linked glutamate and glycine antigens. No cross-reactivity was observed with amino acids other than that of the antibody target (manufacturer’s data sheet, Marc et al., 1990; Marc et al., 1995).

*AGB*: The specificity of the AGB antibody hosted in rabbit has been previously confirmed with dot blot immunoassays which report no cross reactivity with other amino acids (Marc, 1999b). For anti-agmatine IgY hosted in chicken, the immunogen was purified agmatine attached to terminal amino groups on a proprietary carrier peptide via an N-linked pentane spacer. The resulting chicken IgY was purified using serial
polyethylene glycol and octanol precipitations. The specificity was first assayed on previously agmatine-loaded tissue samples and found to be indistinguishable from anti-agmatine IgG labeling published by Marc (1999b). Specificity and cross-reactivity with other potential targets was tested with pattern recognition (Marc et al., 1995), competition assays (Jones et al., 2011) and dot blots (Marc et al., 1990). Figure 1 shows a dot blot of anti-agmatine IgY binding to purified ovalbumin-glutaraldehyde-agmatine and ovalbumin-glutaraldehyde-glutamate conjugates. Glutamate was chosen as it showed the most cross-reactivity of the entire amino acid set against which anti-agmatine IgY was tested. The density measures were converted to linear binding intensity, plotted against first order binding curves. These results are similar to other previously reported anti-amino binding selectivities. The implication is that any cell with an agmatine content of ≈ 3 mM (a very large signal) could have a maximum possible contamination of that signal by endogenous glutamate of ≈ 10% if the cell also contained 3 - 30 mM glutamate. Cells with less glutamate will generate a pure agmatine signal.

**Calretinin:** The specificity of the calretinin antibody was confirmed in Western blots of mouse brain lysate where it presented as a single 31 kDa band (manufacturer’s data sheet).

**Choline acetyl transferase (ChAT):** The specificity of the ChAT antibody was observed in Western blots using purified ChAT from porcine brain, where it yielded an immunoreactive band of 67 kDa (Ostermann-Latif et al., 1992).

**Islet-1:** The specificity of Islet-1 antibody was confirmed in immunostaining which showed Islet-1 immunoreactivity in Chinese hamster ovary (CHO) cells transfected with Islet-1 expression vector but not in normal CHO cells (Thor et al., 1991).
Neurokinin receptor 3 (NK3R): Specificity of NK3R antibody has been previously demonstrated by immunohistochemical staining in rat brain and pre-adsorption studies with NK-3 peptide were the signal was completely abolished by the peptide (manufacturer’s data sheet, personal communication with manufacturer). Labeling patterns observed in this study also matched NK3R immunoreactivity demonstrated in the rat retina using a separate NK3R antibody developed from a synthetic peptide (SSRKKKR) corresponding to amino acids 410-417 of rat NK-3 (Casini et al., 2000; Grady et al., 1996).

Brain nitric oxide synthase (bNOS): bNOS antisera specificity was shown previously in Western blots where the antibody reacted with NOS from rat cerebellum at the expected molecular weight (155 kDa) and not with NOS from macrophages or endothelial cells (manufacturer’s data sheet,(Dinerman et al., 1994).

Parvalbumin (PV): The specificity of antibody for PV has been previously shown in Western blots of rat brain and muscle extracts where the antibody recognises a 12 kDa protein corresponding to PV (Heizmann and Celio, 1987).

Protein kinase C-α (PKCα): The specificity of the monoclonal PKCα antibody raised in mouse was confirmed in a Western Blot of rat glioma extract and NIH 3T3 mouse fibroblast lysate where the antibody reacted with a single 80 kDa band corresponding the expected size for PKCα (Young et al., 1988). The specificity of the polyclonal PKCα antibody raised in rabbit was confirmed in Western blots using rat brain extracts and NIH 3T3 cell lysate as the presence of an 80 kDa band (manufacturer’s data sheet).

Recoverin: Specificity of this antibody was confirmed in Western blots of human adult retina tissue homogenate as the presence of single 26 kDa band (Yan and Wiechmann, 1997).
Tyrosine hydroxylase (TH): The specificity of the antibody for TH was been demonstrated by labeling of a single 62 kDa band in Western blots of brain tissue lysate (Haycock, 1987).

Neuron classification by amino acid labeling patterns

Neurons were classified based on the differential localization of amino acids as described previously (Crooks and Kolb, 1992; Davanger et al., 1991; Kalloniatis et al., 1996; Marc, 1999a; b; Marc and Jones, 2002; Marc et al., 2005; Marc et al., 1990; Marc et al., 1995; Pourcho, 1980; Pow, 2001; Sun et al., 2003). Serial semi-thin sections from identical retina locations were labeled for Glu, GABA and Gly and the cells segregated into the following categories: BCs (OFF cone BCs, i.e. Gly negative), ACs (GABA only, Gly only, and GABA/Gly), GCs (Glu, Glu/weakly GABA) and displaced ACs. The small cell bodies, location and intense GABA staining of displaced ACs allowed them to be separated from GCs (Kalloniatis et al., 1996; Marc and Jones, 2002; Yu et al., 1988). This broad categorization does not allow for the multitude of amino acid classes that can arise from pattern recognition analysis (Kalloniatis et al., 1996; Marc and Jones, 2002; Marc et al., 1995), however, it is less time consuming and allows for a larger sample size appropriate for cell counts and dose response functions.

Dose response curves

For each KA concentration, samples were obtained from four independent retinas. Cells were classified as being immunoreactive for AGB, Glu, GABA, Gly or a combination thereof. For each neurochemical population, two dose response functions were generated. The first, (Figs. 3, 10 and 12, black lines) represents the percentage of cells activated as a proportion of the total number of BCs, ACs or GCs. This function answers the question: Of the total number of activated ACs, what percentage were GABA or Gly immunoreactive ACs? The second function, (Figs. 3, 10 and 12, grey lines) represents the percentage of cells activated as a proportion of the total number of cells belonging to that neurochemical class. This function answers the question: Of all the GABA
immunoreactive ACs, what percentage are activated at the various KA concentrations, and what final percentage are activated at saturating levels? At a saturating level of KA (80 μM), it was difficult to identify the displaced ACs and therefore, both response functions for this population were modeled on only five datum points (Fig. 12D). Procedures for the cell counting and the criterion chosen for considering a cell as being labeled have been described previously (Sun et al., 2003). Response curves were fitted using a Naka-Rushton equation:

\[
\frac{R}{R_{\text{max}}} = \frac{C_{\text{KA}}^n}{C_{\text{KA}}^n + K^n}
\]

where \(R\) is the response, \(R_{\text{max}}\) is the maximum labeling response, \(C_{\text{KA}}\) is the KA concentration, \(n\) is the slope parameter and \(K\) is the KA concentration for a half maximal response.

A non-parametric bootstrap algorithm was used to assess the reliability of the data. This method does not rely on any underlying assumptions about the data and is useful when the sample size is small (Bui et al., 2005; Bui et al., 1998; Efron and Tibshirani, 1986; Foster and Bischof, 1987). Each iterative bootstrap sample consisted of \(n\) random selections taken (with replacement) from the available data in each KA concentration group. A pseudo-replication of the dataset is obtained and each dataset is fitted to obtain estimates of the data descriptive parameters. The bootstrap method allows error estimates to be obtained from the parameters of a fitted function. Although the distribution of the true dataset is not known, the estimate obtained by the resampling strategy provides an approximation of the empirical distribution (Darvas et al., 2004). We applied this bootstrap technique on the dataset of the percentage of activated cells as a function of KA concentration and obtained estimates of the \(R_{\text{max}},\ n,\) and \(K\). The mean parameter value and 95% confidence interval (the 2.5\(^{th}\) and 97.5\(^{th}\) percentiles were taken as the bounds) for each of the parameters were obtained after 1000 bootstrap repetitions. We considered two population parameters as significantly
different when the mean parameter of population $a$ does not lie within the confidence interval of population $b$. In this case, we are determining whether the mean of a parameter for the two populations are significantly different from each other, but not necessarily separable since 2.5% of the parameter probability distribution function may overlap.

Results

**KA induced AGB permeation**

AGB labeling secondary to KA activation was visualized using post-embedding immunocytochemistry (Figs. 2A, C, E, G) and indirect immunofluorescence (Figs. 2B, D, F, H). Short term incubations of isolated retina in medium containing AGB led to no significant neuronal labeling (Fig. 2A-B). This is consistent with there being no measurable endogenous AGB signal in the retina (Kalloniatis et al., 2002; Marc, 1999b; Sun and Kalloniatis, 2006). The addition of KA resulted in a dose dependent AGB entry confined to the inner retina (Figs. 2C-H). Application of 5 µM KA preferentially activated subpopulations of OFF cone BCs (Figs. 2C-D). In Figure 2D, the axon terminals of these BCs could be seen terminating in sublamina $a$ (or the OFF sublamina) of the inner plexiform layer. Light labeling of some ACs and GCs was also evident. Increasing concentrations of KA led to a greater number of AGB labeled cells throughout the inner nuclear and ganglion cell layers (20 µM: Figs. 2E-F; 80 µM: Figs. 2G-H). This was reflected in the increasingly dense punctate staining within the inner plexiform layer. One strata of the inner plexiform layer displayed weak background AGB labeling, most obvious at 5 µM KA and still evident at 80 µM KA (Figs. 2D, F, H; white arrows).

**Characterization of KA activated BC populations**

*Response of OFF BCs to KA*

Dose response curves for KA activation of inner retinal neurons were generated from serial semi-thin sections labeled for AGB, glutamate, GABA and glycine. BCs were
segregated into ON cone BCs (identified by Gly immunoreactive), rod BCs (identified by high basal AGB labeling localized to cell somata and dendritic terminals) and OFF cone BCs (Sun et al., 2007a; Sun et al., 2007c). BC activation showed a dose dependent relationship with KA (Fig. 3). At saturating levels (80 µM KA), 30.7% of all BCs (n = 556) were activated. This population presumably consisted mostly of OFF cone BCs as 88.4% of OFF cone BCs exhibiting functional glutamate receptors responsive to KA. The half maximal concentration for BCs (5.98 µM KA) was significantly less than other inner retinal neurons (see Figs. 10 and 12). This was consistent with previous observations where BCs were strongly labeled at lower KA concentrations compared to ACs and GCs (Marc, 1999b; Sun and Kalloniatis, 2006).

Identification of activated OFF BC types with macromolecular markers

KA activated BCs were further studied by co-localizing AGB with macromolecular markers. ON cone and rod BCs, identified by Islet-1 immunoreactivity, rarely co-localized with AGB at all KA concentrations (Fig. 4, white arrowheads). This was confirmed with cell counts where only 1.6 ± 1.2% of Islet-1 positive BCs co-localized with AGB at 80 µM KA (Fig. 8A), corresponding to basal values observed in non-activated mouse retinae (Sun and Kalloniatis, 2006).

Rod BCs, identified by protein kinase C-α labeling (PKCα; Greferath et al., 1990) also showed very low co-localization with AGB positive BCs at all KA concentrations used (Fig. 5, white arrowheads). In addition, a clear contrast could be seen in the location of the axon terminals of KA activated BCs (in sublamina a) compared to PKCα immunoreactive BC axons terminating in sublamina b (Fig. 5C, black and white arrow respectively). Cell counts indicated only 2.7 ± 1.0% of PKCα immunoreactive BCs were activated at saturation levels (Fig. 8A), matching basal co-localization levels (Sun and Kalloniatis, 2006). These results indicate that rod and ON cone BCs do not express functional KA-sensitive glutamate receptors in the rat retina.
OFF cone BCs were further segregated with the signaling molecule neurokinin 3 receptor (NK3R) and the calcium binding protein, recoverin (Figs. 6 and 7). Previous work suggests NK3R labels type 1 and type 2 OFF cone BCs in the rat retina (Casini et al., 2000; Ghosh et al., 2004; Haverkamp et al., 2003; Oyamada et al., 1999). The activation of NK3R immunoreactive BCs by KA is shown in Figure 6. Faint NK3R immunoreactivity was present in sublamina a (Fig. 6B, white arrow) but not sublamina b which is consistent with NK3R immunoreactive cells being part of the OFF cone BC class. At all KA concentrations, AGB and NK3R co-localized in many BCs (Fig. 6, white arrowheads) but not all (Figs. 6G-I, black arrows) indicating that only some NK3R BCs displayed functional glutamate receptors responsive to KA. This was supported by quantitative analysis which found 56 ± 6.4% of NK3R immunoreactive cells were activated at 80 µM KA (Fig. 8A). Co-localization values below saturation point (5 µM: 59 ± 11%, 20 µM: 57 ± 11%), were not significantly different to saturating KA levels (one-way ANOVA, p = 0.90) suggesting that maximum activation of KA sensitive NK3R BCs occurs at low KA concentrations.

Recoverin labels type 2 OFF cone BCs as well as type 8 ON cone BCs and photoreceptors in the rat retina (Euler and Wässle, 1995; Milam et al., 1993). Consistent with this, we observed recoverin immunoreactive axons in sublamina a and sublamina b indicating the labeling of OFF and ON BC populations respectively (Fig. 7B, white arrows). At low activation conditions, some recoverin BCs were activated (Figs. 7A-C, white arrowheads) but many were not activated (Figs. 7A-C, black arrowheads). This activation increased at 20 µM KA (Figs. 7D-F) and 80 µM KA (Figs. 7G-I). Quantification of activation found 42 ± 7.0%, 48 ± 8.5% and 56 ± 12% of recoverin immunoreactive BCs were activated at 5 µM, 20 µM and 80 µM KA respectively (Fig. 8A). A significant increase in co-localization occurred with increasing KA suggesting dose dependent activation of recoverin immunoreactive cells by KA (one-way ANOVA, p = 0.044).
Recoverin immunoreactive BCs were separated into type 2 and type 8 BCs with the ON cone and rod BC marker Islet-1 (Fig. 9). Type 2 BCs were identified as recoverin positive, Islet-1 negative (Fig. 9C-E, white arrowheads) and type 8 BCs were identified as recoverin and Islet-1 positive (Fig. 9C-E, black arrowheads). Co-localization with AGB confirmed that activated recoverin labeled BCs were exclusively type 2 (Fig. 9F, white arrowheads) and not type 8 (Fig. 9F, black arrowheads). Quantification at saturating KA levels found 45 ± 4.3% of type 2 BCs were activated but only 2.7 ± 0.7% type 8 BCs were activated (Fig. 9G). There was no significant difference between the number of activated recoverin cells in Figure 8A and the number of activated type 2 cells in Figure 9G (student’s t-test, p = 0.11).

The remaining OFF cone BC types (type 3 and 4) were analyzed as activated BCs which did not co-localize with recoverin or NK3R (Fig. 8B). Between 5 µM to 80 µM KA, the number of activated recoverin-negative (types 1, 3 and 4 BCs) and NK3R-negative (type 3 and 4 BCs) cells increased, consistent with dose activation curves (Fig. 3). This increase however did not reach significance (one-way ANOVA, Rec negative: p = 0.12, NK3R negative: p = 0.85). There was also no significant difference between the total number of activated recoverin-negative cells (28 ± 5.0 %) and activated NK3R-negative BCs (40 ± 13%) at saturation point (student t-test, p = 0.11).

**Characterization of KA activated AC populations**

*Response of major neurochemical classes of ACs to KA*

ACs were segregated according to their major neurochemical groupings: GABA immunoreactive ACs, glycine immunoreactive ACs and GABA/glycine immunoreactive ACs. A summary of activation characteristics of each cell class is shown in Table 2. For all populations, activation proceeded in a dose dependent manner. At 80 µM KA, 86.3% of all ACs were activated suggesting that most ACs express functional glutamate receptors responsive to KA (Fig. 10A). The half maximal concentration for ACs was 30.0 µM KA.
GABA immunoreactive ACs represented a significantly larger population (Fig. 10B; 46.2 %) compared to the glycine immunoreactive ACs (Fig. 10C; 36.7%). At saturation, 86.3% of GABA immunoreactive ACs responded to KA, not significantly different from the glycine immunoreactive AC population (85.6%). Comparison of the half maximal KA concentration found that GABA immunoreactive ACs were activated at a significantly higher concentration of KA then glycine immunoreactive ACs (GABA: 38.0 µM, Gly: 30.0 µM). GABA immunoreactive ACs were also activated at a significantly higher concentration when compared to the total AC population. Half maximal concentration values for glycine immunoreactive and total AC population showed overlap in the 95% CI.

The GABA/glycine immunoreactive AC population constituted only 5.3% of the total of ACs were responsive to KA (Fig. 10D). The half maximal KA concentration for this population (23.6 µM) was significantly lower than all other AC populations. Saturating concentrations of KA led to activation of only 60.3% of this population.

**Identification of activated ACs with macromolecular markers**

AC populations that were immunoreactive for brain nitric oxide synthase (bNOS), tyrosine hydroxylase (TH), choline acetyl transferase (ChAT), parvalbumin (PV) and calretinin were further studied (Fig. 11). bNOS identifies three AC classes in the rat retina, two in the conventional AC layer and one displaced AC (Perez et al., 1995; Chun et al., 1999; Kim et al., 1999, 2000). Saturating concentrations of KA did not activate any of the bNOS immunoreactive conventional ACs, suggesting that these cells did not express functional KA responsive glutamate receptors (Fig. 11A-C, white arrowheads). Dopaminergic ACs labeled with TH (Haverkamp and Wässle, 2000) were not activated by a low (5 µM KA) or high (80 µM KA) concentration of KA (Figs. 11D-I, white arrowheads). Cholinergic ACs, immunoreactive for ChAT (Voigt, 1986) showed robust AGB labeling with concentrations of KA as low as 5 µM (Fig. 11J-L, white arrowheads). This was true for both the conventional and displaced AC varieties (52 out of 52 cells). All ACs
immunolabeled with PV (Wässle et al., 1993) did not show AGB labeling with 5 μM KA (Fig. 11M-O, black arrowheads). Some PV labeled cells were activated by 20 μM KA (Fig. 11P-R, white arrowheads) and virtually all PV immunoreactive ACs were activated at 80 μM KA (68 out of 68, Fig. 10S-U, white arrowheads). For calretinin immunoreactive ACs, some were activated at low KA concentrations but not others (Fig. 11V-X, white and black arrowheads respectively). These activated cells were possibly cholinergic ACs as they showed similar activation to ChAT labeled cells. Some calretinin reactive cells in the ganglion cell layer also co-localized with KA activated GCs (white arrow) and others did not (black arrow).

**Characterization of KA activation within the GC layer**

As with ACs, percentage activation of GCs and displaced ACs displayed a dose dependent increase with KA. GCs were considered as a separate population to displaced ACs, differentiated according to soma size and amino acid immunoreactivity. The cumulative GC response curve is shown in Figure 12A. At 80 μM KA, 84.6% of all GCs were activated suggesting that an equal proportion of ACs and GCs express functional glutamate receptors responsive to KA. GCs demonstrated a lower half maximal concentration compared to ACs of 12.7 μM KA.

KA activated GCs was largely represented by the Glu/weakly GABA immunoreactive GCs (52.8%) and Glu only immunoreactive GCs (35.1%; Figs. 12B-C). Glu/weakly GABA immunoreactive GCs were activated at significantly lower KA concentrations compared to Glu only immunoreactive GCs. At saturation, 86.7% of Glu/weakly GABA GCs and 73.4% of Glu only GCs expressed functional KA sensitive glutamate receptors.

Figure 12D shows 80.3% of displaced ACs were activated at saturating levels of KA. Displaced AC responses were dominated by the GABA/Glu immunoreactive ACs. The GABA/Glu displaced ACs represented 73.7% of all activated displaced ACs. Displaced ACs exhibited a half maximal KA concentration of 15.5 μM, almost half that of the GABA AC.
population in the amacrine cell layer (Fig. 10B). This may be due to the high proportion of ChAT ACs present in the displaced AC population which are highly sensitive to KA (Fig. 11D).

Discussion

KA responsive glutamate receptors have been observed in BCs, ACs and GCs in many mammalian retinae (Brandstätter et al., 1994; Brandstätter et al., 1997; Cohen and Miller, 1994; DeVries and Schwartz, 1999; Haverkamp et al., 2001; Marc, 1999a; Massey and Miller, 1988; Qin and Pourcho, 2001). This study has quantified the proportion of neurochemically classified cells expressing functional KA sensitive glutamate receptors based on the entry of the organic cation, AGB. We first determined overall cell activation patterns using amino acid labeling and cell morphometrics followed by the use of macromolecular markers to focus of subpopulations of neurons.

Figure 13 summarizes the differences in KA sensitivity of neuronal populations investigated in this study. The clear distinction in KA activation levels of BCs, ACs and GCs suggests that although many inner retinal neurons express glutamate receptors responsive to KA, receptor kinetics is not equivalent between cell classes. In vitro expression studies indicate that KA affinity of glutamate receptors is dictated by receptor subunit composition (Herb et al., 1992). This would explain differences in subunit expression in the rat retina with some KA subunits localized throughout the inner nuclear and ganglion cell layers (i.e. KA2, GluR7) and others restricted to certain strata (i.e. GluR5 to the outer half of the inner nuclear layer; (Brandstätter et al., 1994; Brandstätter et al., 1997). A similar model has been proposed for differences observed in NMDA thresholds of ACs and GCs in the rat retina (Sun et al., 2003).

AGB as a probe of glutamate receptor function
The use of AGB as a probe of ion flow was instigated by Yoshikami (1981) using autoradiography and subsequently applied to the retina through immunodetection by Marc (1999a, b). The theoretic framework has been reviewed in the past (Marc, 1999b; c; Marc et al., 2005; Sun and Kalloniatis, 2006). AGB labeling secondary to endogenous (basal) or exogenous (ligand) activation reports on the cation entry history of the cell and not the summed excitation/inhibitory history (Marc et al., 2005). Guanidinium compounds like AGB permeate cation channels and are immunodetectable when only a small fraction of glutamate receptors have been activated (Marc et al., 2005; Picco and Menini, 1993) using short incubation times secondary to ligand activation (Acosta et al., 2007; Chang et al., 2010; Chang and Chiao, 2008; Chen and Chiao, 2012; Edwards et al., 2007; Edwards and Michel, 2003; Marc, 1999a; b; Marc et al., 2005; Michel et al., 1999; Mobley et al., 2008; Sun and Kalloniatis, 2006). Factors that will affect AGB labeling include cell volume, channel size, number of open channels, ligand affinity of receptor channel, channel permeation and channel desensitization (Marc, 1999a; b; Marc et al., 2005; Sun and Kalloniatis, 2006).

The use of AGB labeling as a probe of functional glutamate receptors requires careful interpretation. KA is known to activate AMPA receptors at high concentrations (Paternain et al., 1995; Sommer and Seeburg, 1992) and thus recruitment of non-KA receptors is possible. Soma labeling is also possible secondary to mechanical disruption (Marc, 1999a; b) however this and previous studies indicated that such labeling will be below ~5-8% - the error in labeling ON bipolar cells secondary to ionotropic receptor activation (Sun and Kalloniatis, 2006).

**Response of BCs to KA**

Approximately 30% of rat BCs were activated by KA and this population comprised almost exclusively of OFF cone BCs. This agrees with previous studies which estimate 25% of BCs in the rat retina as OFF cone BCs (Euler and Wässle, 1995; Kamphuis et al., 2003; Sun et al., 2007a). The absence of AGB in immunolabeled rod and ON cone BCs
further confirmed that KA activation was limited to OFF cone BCs. Four OFF cone BC subtypes: type 1, 2, 3 and 4 with relatively even distributions have been proposed in the rat (Euler and Wässle, 1995; Hartveit, 1997; Masland, 2001a; b). The KA responsiveness of each cell type however is unknown. In the rabbit retina, OFF cone BCs show heterogeneous responses to KA suggesting different KA sensitive glutamate receptors between OFF cone BC subtypes (Marc, 1999a). The high level of OFF cone BC activation (88.4%) observed in this study points to the likelihood of all four rat OFF cone BC types being activated by high levels of KA. This does not imply that all OFF cone BC display KA receptors, but rather all are activated with KA. KA is known to activate AMPA receptors at higher concentrations (Paternain et al., 1995; Sommer and Seeburg, 1992), and thus, OFF BCs activated at low KA concentrations likely reflect the populations that have KA only receptors.

Recoverin labeling indicated that type 2 BCs present functional KA responsive glutamate receptors in the rat. Although only ~56% of the recoverin BCs were activated at saturating KA levels, overlap with ON cone BC markers confirmed that the majority of inactivated recoverin cells were type 8 ON BCs. Previous studies estimate 65% and 35% of recoverin BCs are type 2 and 8 respectively (Euler and Wässle, 1995; Kamphuis et al., 2003). Thus, activation of 56% of recoverin cells suggests that almost all type 2 BCs are KA responsive. The significant increase in activated cells with KA concentration suggests variability in KA sensitivity within this population. DeVries (2000) showed that two OFF BC types in the ground squirrel (b3 and b7) which were both sensitive to KA, varied in desensitization response and recovery times. Despite this, 42 ± 7.0 % of recoverin BCs were activated at 5 µM KA, indicating that a large proportion of type 2 BCs are highly KA sensitive.

For NK3R reactive BCs (type 1 and 2), ~56% of this population was activated at saturating KA concentrations. This was not significantly different from the recoverin BCs population (p = 0.96). As recoverin immunoreactivity suggests that most type 2 BCs are
KA sensitive, it is possible that the inactivated NK3R population consists of type 1 BCs. Similarly to recoverin OFF cone BCs, NK3R cells were highly sensitive to low KA concentrations further suggesting this population to be type 2 BCs. Unfortunately, overlap of antibody hosts meant we were unable to combine NK3R and recoverin markers to differentiate between type 1 and 2 BCs in this study. Studies in the mouse however indicate that type 1 and 2 BCs do differ in activation characteristics. Type 1 mouse BCs appear to avoid S-cones and may be exclusively involved in the green OFF pathway whilst type 2 BCs form non-selective cone contacts (Breuninger et al., 2011; Puller and Haverkamp, 2011). Type 1 mouse BCs and recoverin negative (i.e. type 1, 3 or 4 but not type 2) BCs in the rat retina also exclusively express the AMPA receptor subunit GluR1 (Breuninger et al., 2011; Kamphuis et al., 2003).

Electrophysiological studies suggest that neurons unresponsive to KA may use AMPA receptors. DeVries (2000) showed that OFF BCs in the ground squirrel retina which were not activated by KA, were highly responsive to AMPA, suggesting that OFF BCs may use only one receptor type as a way to filter visual signals (DeVries, 2000). In this study, we could not assign AMPA receptors to BC populations which were unresponsive to KA as only a single glutamate receptor agonist was used. KA also induces non-desensitizing responses from AMPA receptors and thus at saturating concentrations, AGB labeled cells may arise from non-specific activation of AMPA receptors (Paternain et al., 1995; Sommer and Seeburg, 1992). We are currently performing studies with AMPA, GYKI53655 (selective AMPA receptor antagonist) and SYM2081 (selective KA receptor agonist) to resolve the ambiguity of glutamate receptor functionality.

KA can also desensitize KA receptors (Jones et al., 1997) however this is less likely to affect this study as the AGB technique measures the accumulation of the cation in activated cells and only a fraction of channels need to be open for detectable levels of AGB (Marc et al., 2005). We also used a binary classification system when counting cells.
which did not rely on quantitative difference in AGB entry but whether glutamate channels were functional or not (Acosta et al., 2007; Sun and Kalloniatis, 2006).

Analysis of type 3 and 4 BCs was limited in this study by the availability of reliable immunocytochemical rat BC markers. Markers for these cells have been identified in the mouse (Ghosh et al., 2004; Haeseleer et al., 2000; Haverkamp et al., 2003; Haverkamp et al., 2008; Mataruga et al., 2007; Puthussery et al., 2010) but their reactivity in the rat is either absent or yet to be shown. Other markers, such as hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) which identifies type 3 rat OFF cone BCs (Fyk-Kolodziej and Pourcho, 2007) display immunoreactivity mostly localized to cell axon and dendrites making it difficult to reliably match with the predominately soma based AGB labeling. We assessed type 3 and 4 BCs through the absence of NK3R or recoverin labeling. At saturating KA concentrations, 40 ± 5.0% of activated OFF cone BCs were NK3R immunonegative indicating that at least one of these cell types is responsive to KA. Activation of recoverin negative OFF BCs (type 1, 3 and 4) was not significantly different to the NK3R immunonegative population further suggesting type 1 OFF BCs are not responsive to KA.

Response of ACs and GCs to KA

Cells of the inner nuclear and ganglion cell layer responded to KA dose dependently. Virtually all ACs (86%) showed AGB entry, implying functional KA sensitive glutamate receptors on most rat ACs. The half maximal KA concentration for ACs was 30 μM KA, matching values observed for ACs in the rabbit (Marc, 1999b).

The majority of KA responsive ACs could be divided equally between the GABA and glycine immunoreactive populations. The wide range of responses in the GABAergic population is likely to reflect the heterogeneity within this group (Marc, 1999a; Pourcho and Goebel, 1983). Cholinergic ACs were the most KA sensitive GABAergic cell population matching data from the rabbit retina, where cholinergic ACs were strongly
activated with only 6 μM KA (Marc, 1999a; b). Toxicity studies also indicate high KA sensitivity in cholinergic ACs (Osborne et al., 1995).

TH and bNOS ACs immunoreactive contributed to the ~14% GABAergic ACs that were unresponsive to KA. TH immunoreactive ACs which are dopaminergic (Oyster et al., 1985) were also resistant to KA toxicity in the rabbit and the chicken retina (Ingham and Morgan, 1983; Marc, 1999a; Morgan, 1983). Excitotoxicity studies in the rat retina however, demonstrate conflicting results including KA induced cell death of TH cells (Cheon et al., 2006; Park et al., 2005). Similarly, bNOS immunoreactive ACs were unresponsive to KA in our study but showed sensitivity to KA toxicity in the rabbit retina (Sagar, 1990). In the toxicity studies, cell death occurred 7 - 10 days after KA administration and therefore this effect may not be directly due to KA sensitive receptors on TH and bNOS immunoreactive cells. bNOS immunoreactive cells also show KA induced GABA release in the primate retina (Andrade da Costa et al., 2001) but this discrepancy may be related to species specific differences between primate and rat bNOS reactive cells. Species differences may also explain loss of light responses in mouse TH immunoreactive cells after administration of a KA antagonist (Pang et al., 2010). Additionally, Pang et al. (2010) abolished light responses in using DNQX, an antagonist to non-NMDA receptors (Honore et al., 1988) and therefore these effects cannot be specifically attributed to KA sensitive receptors on TH immunoreactive cells. The theoretical calculations by Marc et al. (2005) would suggest that our experimental paradigm should have resulted in detectable AGB levels if these cells had KA/AMPA receptors on their membranes.

For the glycinergic AC population, the characteristics of the dose response curve (~43% activation at 30 μM KA, ~86% activation at 80 μM KA) closely matched the immunolabeling of All ACs by PV (partial co-localization at 20 μM KA, complete co-localization at 80 μM KA). This suggests the All AC population make a considerable contribution to the glycinergic AC response. Indeed, All ACs are the predominant
glycinergic AC type (MacNeil and Masland, 1998; Macneil et al., 2009). In the rabbit retina, all ACs also contributed most to the glycinergic AC response (Marc, 1999a). Dumitrescu et al. (2006) showed that 77% of mice all ACs possessed AMPA and KA responsive glutamate receptors but all ACs did not express KA responsive glutamate receptors exclusively. Electrophysical recordings from the rat and rabbit retina also indicate that all ACs only possess functional AMPA receptors (Morkve et al., 2002; Veruki et al., 2003; Zhou and Dacheux, 2004). Immunocytochemical staining of all ACs in the macaque monkey and rabbit retina found AMPA receptor subunits GluR 2/3 and 4 post-synaptic to rod BCs (Ghosh et al., 2001). Thus, activation of these cells by KA in this study may be due to non-specific activation of AMPA receptors on these cells rather than glutamate receptors responsive to KA (Paternain et al., 1995; Sommer and Seeburg, 1992). Further work is needed to determine ionotropic glutamate receptor functionality for these cells.

In the ganglion cell layer, most GCs (~85%) were KA responsive, similar to the rabbit (Marc, 1999a; Massey and Miller, 1988), cat (Cohen et al., 1994) and primate (Cohen and Miller, 1994; Zhou et al., 1994). Sun et al. (2003) found 87% of GCs were activated with NMDA suggesting equal use of non-NMDA and NMDA receptors by these cells (Sun et al., 2003). Lack of available selective GC markers limited segregation to amino acids profiles. Glu GCs were more sensitive to Glu/weakly GABA to KA, similar to previous work in the rabbit (Marc, 1999a).

**Concluding remarks**

Numerous reports suggest differences in neurotransmitter receptor sensitivity is a mechanism for filtering and delineating signal pathways (Breuninger et al., 2011; DeVries, 2000; Li and DeVries, 2006). This study sought to further understand these pathways by ascribing functional KA sensitive glutamate receptors to specific cell subpopulations of the inner retina. Beyond understanding signaling pathways, knowledge of receptor sensitivity also provides a roadmap to cell susceptibility to
glutamate neurotoxicity. The pathology of retinal conditions such as ischemia is associated with excessive stimulation of glutamate receptors (Lipton and Rosenberg, 1994; Robin and Kalloniatis, 1992). Whilst NMDA receptors are predominately implicated in glutamate toxicity, KA sensitive receptors may also promote cell death (Sucher et al., 1997). OFF BCs are highly sensitive to KA and therefore are probably at the greatest risk of KA toxicity followed by GCs (Fig. 13). Thus monitoring for dysfunction in these cells may be early indicator of glutamate toxicity.

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Conflict of interest statement
All the authors certify that there is no conflict of interest with any regarding the material presented in this manuscript. R. E. Marc is Chief Executive Officer of Signature Immunologics.

Role of authors
All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: L. Nivison-Smith, D. Sun, E. L. Fletcher, M. Kalloniatis
Acquisition of data: L. Nivison-Smith, D. Sun, R. E. Marc
Analysis and interpretation of data: L. Nivison-Smith, D. Sun, M. Kalloniatis
Drafting of the manuscript: L. Nivison-Smith, D. Sun
Critical revision of the manuscript for important intellectual content: E. L. Fletcher, R.E. Marc, M. Kalloniatis
Statistical analysis: L. Nivison-Smith, D. Sun
Obtained funding: E. L. Fletcher, M. Kalloniatis, R.E. Marc
Administrative, technical, and material support: R. E. Marc
Study supervision: M. Kalloniatis
Literature cited


Michel WC, Steullet P, Cate HS, Burns CJ, Zhainazarov AB, Derby CD. 1999. High-resolution functional labeling of vertebrate and invertebrate olfactory receptor


Table 1: Details of the antibodies used in this study.

**Abbreviations** – Rb, rabbit; Ch, chicken; Ms, mouse, IF, immunofluorescence, ICC, immunocytochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Manufacturer, Cat No</th>
<th>Host</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agmatine (AGB)</td>
<td>Agmatine conjugated to bovine serum albumin</td>
<td>Chemicon (Millipore); AB1568*</td>
<td>Rb;</td>
<td>1:100 (IF);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:800 (ICC)</td>
</tr>
<tr>
<td>Agmatine (AGB)</td>
<td>Agmatine cross-linked to purified fraction V bovine serum albumin with glutaraldehyde</td>
<td>Abcam; ab62667*</td>
<td>Ch;</td>
<td>1:500</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Recombinant rat calretinin</td>
<td>Millipore; MAB1568</td>
<td>Ms;</td>
<td>1:1000</td>
</tr>
<tr>
<td>Choline acetyl transferase (ChAT)</td>
<td>Highly purified ChAT obtained from porcine brain</td>
<td>Chemicon (Millipore); MAB5270</td>
<td>Ms;</td>
<td>1:500</td>
</tr>
<tr>
<td>Gamma-aminobutyric acid (GABA)</td>
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<td>Chemicon (Millipore); AB5016*</td>
<td>Rb;</td>
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<tr>
<td>Glutamate</td>
<td>Glutamate conjugated to bovine serum albumin with glutaraldehyde</td>
<td>Chemicon (Millipore); AB5018*</td>
<td>Rb;</td>
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<td>Glycine</td>
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<td>Rb;</td>
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<td>Islet-1</td>
<td>Truncated rat islet protein corresponding to amino acids 178-349</td>
<td>Developmental Studies Hybridoma Bank; 39.4D5</td>
<td>Ms;</td>
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<tr>
<td>Neurokinin receptor 3 (NK3R)</td>
<td>Synthetic peptide (SSFISPPYTSSVDEYS) corresponding to amino acids 451-465 of rat NK-3 conjugated to bovine thyroglobulin</td>
<td>Novus Biologicals; NB300-102</td>
<td>Rb;</td>
<td>1:4000</td>
</tr>
<tr>
<td>Nitric oxide synthase-brain (bNOS)</td>
<td>Recombinant neuronal NOS fragment (amino acids 1-181) from rat brain</td>
<td>Sigma-Aldrich; N2280</td>
<td>Ms;</td>
<td>1:3000</td>
</tr>
<tr>
<td>Parvalbumin (PV)</td>
<td>Frog muscle parvalbumin</td>
<td>Sigma-Aldrich; P3088</td>
<td>Ms;</td>
<td>1:500</td>
</tr>
<tr>
<td>Protein kinase C-α (PKCα)</td>
<td>Purified bovine brain PKC</td>
<td>Sigma-Aldrich; P5704</td>
<td>Ms; monoclonal</td>
<td>1:400</td>
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<td>---------------------------</td>
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<tr>
<td>Protein kinase C-α (PKCα)</td>
<td>Synthetic peptide (KVNPQVFVHPIQSAV) corresponding to amino acids 659-672 from the C-terminal variable region of rat PKCα</td>
<td>Sigma-Aldrich; P4334</td>
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<td>Recoverin</td>
<td>Recombinant human recoverin</td>
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<td>Rb; polyclonal</td>
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<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>Denatured tyrosine hydroxylase from rat pheochromocytoma (denatured by sodium dodecyl sulfate)</td>
<td>Chemicon (Millipore); AB152</td>
<td>Rb; polyclonal</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

*Antibodies were kindly donated by Dr. R.E. Marc and are licensed to Signature Immunologics and are also commercially available through Abcam and/or Merck Millipore.*
Table 2

Summary of KA activation curve characteristics for cell populations of the rat inner retina. $R_{\text{max}}$ represents the total percentage of cells in the class activated at saturating KA levels (80 μM), $n$ represents the slope parameter and $K$ represents the concentration of KA which activates half of the cell class. Values are presented as mean and bounds of four rat retinae using a 95% confidence interval.

<table>
<thead>
<tr>
<th>Cell class</th>
<th>$R_{\text{max}}$</th>
<th>$n$</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amacrine cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>86.34 (82.01, 86.78)</td>
<td>0.034 (0.031, 0.038)</td>
<td>30.00 (27.67, 30.78)</td>
</tr>
<tr>
<td>GABA</td>
<td>46.21 (41.00, 52.07)</td>
<td>0.023 (0.019, 0.032)</td>
<td>38.00 (30.72, 45.18)</td>
</tr>
<tr>
<td>Glycine</td>
<td>36.70 (32.69, 39.46)</td>
<td>0.036 (0.028, 0.044)</td>
<td>30.00 (24.24, 35.68)</td>
</tr>
<tr>
<td>GABA/Glycine</td>
<td>5.27 (4.10, 6.02)</td>
<td>0.050 (0.027, 0.065)</td>
<td>23.57 (19.19, 28.09)</td>
</tr>
<tr>
<td><strong>Ganglion cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>84.56 (83.31, 85.75)</td>
<td>0.071 (0.064, 0.080)</td>
<td>12.70 (11.83, 13.33)</td>
</tr>
<tr>
<td>Glu</td>
<td>35.13 (30.26, 35.98)</td>
<td>0.038 (0.031, 0.072)</td>
<td>23.00 (13.92, 26.65)</td>
</tr>
<tr>
<td>Glu/weakly GABA</td>
<td>52.75 (51.08, 54.54)</td>
<td>0.078 (0.066, 0.089)</td>
<td>11.42 (10.16, 12.71)</td>
</tr>
<tr>
<td><strong>Displaced amacrine cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu/GABA</td>
<td>73.70 (71.92, 75.39)</td>
<td>0.061 (0.053, 0.069)</td>
<td>15.50 (14.05, 17.23)</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: Characterisation of the chicken anti-AGB antibody. A: Dot blots show anti-agmatine binding to purified ovalbumin-glutaraldehyde-agmatine and ovalbumin-glutaraldehyde-glutamate conjugates. B: Density measures were converted to linear binding intensity, plotted against first order binding curves (white circles-AGB, black circles – glutamate).

Figure 2: AGB immunoreactivity in the adult rat retina at A-B: basal levels, C-D: 5 μM KA activation, E-F: 20 μM KA activation and G-H: 80 μM KA activation. Sections were imaged with brightfield light-microscopy using post-embedding immunocytochemistry (A, C, E, G) or confocal microscopy using indirect immunofluorescence (B, D, F, H). Retinal layers including the inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL) are indicated by white lines and annotations on the left-hand side of the image. The white arrow indicates weakly AGB immunoreactive strata in the IPL. Scale bar is 20 μm.

Figure 3: KA dose response curves for OFF cone and total BC populations. The black line indicates the number KA activated BCs in the cell population as a percentage of the total number of BCs present (n = 556). The grey line indicates the percentage of OFF BCs activated by KA. KA concentration is presented as a log 10 scale on the x-axis and percentage activation on the y-axis is presented as a normalized response where 1.0 indicates activation of 100% of the population. Annotations at the activation curve plateau indicate the mean total percentage of cells activated at saturating KA concentrations (80 μM KA). Vertical black arrows and their annotations delineate the half maximal KA concentration. Bracketed values indicate the bounds of the 95% confidence interval. The data points in the dose response curve have had the proportion of basal AGB labeled cells subtracted so each datum point reflects true KA activation.
**Figure 4:** Confocal micrographs of AGB (green) and Islet-1 (Ist-1; magenta) immunoreactivity after activation with A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA. Activated BCs (white arrowheads) did not co-localize with the Islet-1 positive somata at any KA concentration used. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.

**Figure 5:** Immunoreactivity of AGB (green) and the rod BC marker, protein kinase C-α (PKCα; magenta) secondary to A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA activation. PKCα did not co-localize with activated BCs (white arrowheads) at all KA concentrations shown. PKCα immunoreactive axon terminals localized to sublamina b are indicated by the white arrow and the KA activated terminals are indicated by the black arrow. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.

**Figure 6:** AGB (green) and neurokinin 3 receptor (NK3R; magenta) immunoreactivity after activation with A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA. At all activation concentrations, co-localization of some NK3R and KA activated BCs was observed (white arrowheads). Some activated BCs were not immunoreactive for NK3R (black arrowheads). NK3R immunoreactive BCs which were not activated by KA are marked with a black arrow. NK3R immunoreactive axon terminals were exclusively localized to sublamina a as indicated by the white arrow. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.

**Figure 7:** AGB (green) and recoverin (Rec; magenta) immunoreactivity after activation with A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA. White arrowheads indicate recoverin immunoreactive BCs activated by KA and black arrowheads indicated recoverin BCs which were not activated. White arrows indicate the recoverin positive axon terminals in sublamina a and b. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.
Figure 8: Quantification of KA activated BC populations. A: Co-localization of KA activated cells with BC markers. Each column represents the number of activated cells as a percentage the total number of marker labeled cells. Numbers above each column indicate the total number of cells counted for each marker at that KA concentration and the asterisk indicates a significant difference (one-way ANOVA, p < 0.05). B: KA activated cells void of marker immunoreactivity. Columns represent the number of KA activated but marker negative cells as a percentage of all cells activated. Numbers above each column indicate the total number of activated cells (i.e. AGB positive) at that KA concentration. For both figures, data is represented as the mean ± SD from five independent rat retinae.

Figure 9: Immunoreactivity of A: AGB (green), B: recoverin (magenta) and C: islet-1 (blue) after activation with 80 µM KA. D: Overlap of AGB and recoverin channels shows many recoverin BCs were activated by KA (white arrowheads) but not all (black arrowheads). E: Overlap of recoverin and Islet-1 channels. Type 8 BCs are identified by co-localization of recoverin and Islet-1 (black arrowheads) and type 2 are identified by the absence of Islet-1 staining (white arrowheads). F: Overlap of all three markers. Type 8 BCs are not activated by KA (black arrowheads) whilst type 2 BCs are activated by KA. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm. G: Quantification of recoverin BC populations with functional KA receptors. The two cell types are presented as a percentage of the total recoverin BC population (5 µM: n = 735; 20 µM: n = 599; 80 µM: n = 455). Each column represents the mean ± SD from five independent rat retinae. Abbreviations: Rec: recoverin; Ist-1: islet-1; BC: bipolar cell.

Figure 10: KA dose response curves for neurochemically distinct AC populations in the inner nuclear layer. Activation curves are presented for A: all ACs within the AC layer, B: GABA immunoreactive ACs, C: Gly immunoreactive ACs and D: GABA/Gly immunoreactive AC populations. The black lines indicate the number KA activated cells in the cell population as a percentage of the total number of activated cells present. The
grey lines indicate the percentage of cells within a neurochemical class which are activated by KA. Activation curve annotations and axes labeling are as described in Fig. 3.

**Figure 11:** KA activation of immunocytochemically identified AC populations. A-C: brain nitric oxide synthase (bNOS) immunoreactive ACs were not activated by KA even at saturating levels (white arrowheads). D-F: Tyrosine hydroxylase (TH) immunoreactive ACs also did not co-localize with KA activated ACs at low or saturating KA concentrations (white arrowheads). G-I: Co-localization was evident between AGB and choline acetyltransferase (ChAT) reactive ACs (white arrowheads). M-U: Parvalbumin (PV) positive ACs were activated with KA concentrations exceeding 5 μM. M-O: No co-localization was observed for incubations with 5 μM KA (black arrowheads). P-R: At 20 μM, most PV ACs were co-localized (white arrowheads) but some remained AGB negative (black arrowheads). S-U: All PV immunoreactive cells were co-localized with KA activated cells at 80 μM (white arrowheads). V-X: Co-localization with KA activated cells was observed for some calretinin positive ACs (white arrowheads) but not others (black arrowheads). Similarly, some calretinin reactive cells in the ganglion cell layer co-localized with KA activated GCs (white arrow) and others did not (black arrow). Retinal layer annotations are the same as Fig. 3. Scale bar is 20 μm.

**Figure 12:** KA dose response curves for neurochemically distinct cell populations in the ganglion cell layer. Activation curves are presented for A: all GCs within the ganglion cell layer, B: Glu immunoreactive GCs, C: Glu/weakly GABA immunoreactive GCs and D: displaced ACs. The black lines indicate the number KA activated cells in the cell population as a percentage of the total number of activated cells present. The grey lines indicate the percentage of cells within a neurochemical class are activated by KA. Activation curve annotations and axes labeling are as described in Fig. 3.
Figure 13: Diagrammatic representation of KA sensitivity of various neurochemically identified cell populations in the rat inner retina. Annotations below cell images indicate the neurochemical cell class and the half maximal activation concentration for that class. Data is based on dose activation curves in Figs. 3, 10 and 12. For Chat ACs and Type 2, 3, 4 BCs, estimates of half maximum concentration have been made from immunolabeling. Abbreviations: INL: inner nuclear layer, GCL: ganglion cell layer, BC: bipolar cell, AC: amacrine cell, GC: ganglion cell, GABA: gamma-aminobutyric acid, Gly: glycine, Glu: glutamate.
Figure 1: Characterisation of the chicken anti-AGB antibody. A: Dot blots show anti-agmatine binding to purified ovalbumin-glutaraldehyde-agmatine and ovalbumin-glutaraldehyde-glutamate conjugates. B: Density measures were converted to linear binding intensity, plotted against first order binding curves (white circles-AGB, black circles – glutamate).

82x102mm (300 x 300 DPI)
Figure 2: AGB immunoreactivity in the adult rat retina at A-B: basal levels, C-D: 5 µM KA activation, E-F: 20 µM KA activation and G-H: 80 µM KA activation. Sections were imaged with brightfield light-microscopy using post-embedding immunocytochemistry (A, C, E, G) or confocal microscopy using indirect immunofluorescence (B, D, F, H). Retinal layers including the inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL) are indicated by white lines and annotations on the left-hand side of the image. The white arrow indicates weakly AGB immunoreactive strata in the IPL. Scale bar is 20 µm. 90x96mm (300 x 300 DPI)
Figure 3: KA dose response curves for OFF cone and total BC populations. The black line indicates the number KA activated BCs in the cell population as a percentage of the total number of BCs present (n = 556). The grey line indicates the percentage of OFF BCs activated by KA. KA concentration is presented as a log 10 scale on the x-axis and percentage activation on the y-axis is presented as a normalized response where 1.0 indicates activation of 100% of the population. Annotations at the activation curve plateau indicate the mean total percentage of cells activated at saturating KA concentrations (80 µM KA). Vertical black arrows and their annotations delineate the half maximal KA concentration. Bracketed values indicate the bounds of the 95% confidence interval. The data points in the dose response curve have had the proportion of basal AGB labeled cells subtracted so each datum point reflects true KA activation.
Figure 4: Confocal micrographs of AGB (green) and Islet-1 (Ist-1; magenta) immunoreactivity after activation with A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA. Activated BCs (white arrowheads) did not co-localize with the Islet-1 positive somas at any KA concentration used. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.
Figure 5: Immunoreactivity of AGB (green) and the rod BC marker, protein kinase C-α (PKCα; magenta) secondary to A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA activation. PKCα did not co-localize with activated BCs (white arrowheads) at all KA concentrations shown. PKCα immunoreactive axon terminals localized to sublamina b are indicated by the white arrow and the KA activated terminals are indicated by the black arrow. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.
Figure 6: AGB (green) and neurokinin 3 receptor (NK3R; magenta) immunoreactivity after activation with A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA. At all activation concentrations, co-localization of some NK3R and KA activated BCs was observed (white arrowheads). Some activated BCs were not immunoreactive for NK3R (black arrowheads). NK3R immunoreactive BCs which were not activated by KA are marked with a black arrow. NK3R immunoreactive axon terminals were exclusively localized to sublamina a as indicated by the white arrow. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.
Figure 7: AGB (green) and recoverin (Rec; magenta) immunoreactivity after activation with A-C: 5 µM, D-F: 20 µM and G-I: 80 µM KA. White arrowheads indicate recoverin immunoreactive BCs activated by KA and black arrowheads indicated recoverin BCs which were not activated. White arrows indicate the recoverin positive axon terminals in sublamina a and b. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm.

182x138mm (300 x 300 DPI)
Figure 8: Quantification of KA activated BC populations. A: Co-localization of KA activated cells with BC markers. Each column represents the number of activated cells as a percentage the total number of marker labelled cells. Numbers above each column indicate the total number of cells counted for each marker at that KA concentration and the asterisk indicates a significant difference (one-way ANOVA, p < 0.05). B: KA activated cells void of marker immunoreactivity. Columns represent the number of KA activated but marker negative cells as a percentage of all cells activated. Numbers above each column indicate the total number of activated cells (i.e. AGB positive) at that KA concentration. For both figures, data is represented as the mean ± SD from five independent rat retinae.
Figure 9: Immunoreactivity of A: AGB (green), B: recoverin (magenta) and C: islet-1 (blue) after activation with 80 µM KA. D: Overlap of AGB and recoverin channels shows many recoverin BCs were activated by KA (white arrowheads) but not all (black arrowheads). E: Overlap of recoverin and Islet-1 channels. Recoverin BCs which are type 8 BCs (i.e. ON cone BCs) are identified by co-localization of recoverin and Islet-1 (black arrowheads). Recoverin BCs which are type 2 (i.e. OFF cone BCs) are identified by the absence of Islet-1 staining (white arrowheads). F: Overlap of all three markers. BCs which are immunoreactive for Islet-1 and recoverin (i.e. type 8 BCs) are not activated by KA (black arrowheads) whilst those which were immunoreactive for recoverin but not Islet-1 (i.e. type 2 BCs) are activated by KA. Retinal layer annotations are the same as Fig. 2. Scale bar is 20 µm. G: Quantification of recoverin BC populations with functional KA receptors. The two cell types (as determined by the marker immunoreactivity) are presented as a percentage of the total recoverin BC population (5 µM: n = 735; 20 µM: n = 599; 80 µM: n = 455). Each column represents the mean ± SD from five independent rat retinas. Abbreviations: Rec: recoverin; Ist-1: islet-1; BC: bipolar cell.
Figur 10: KA dose response curves for neurochemically distinct AC populations in the inner nuclear layer.

Activation curves are presented for A: all ACs within the AC layer, B: GABA immunoreactive ACs, C: Gly immunoreactive ACs and D: GABA/Gly immunoreactive AC populations. The black lines indicate the number KA activated cells in the cell population as a percentage of the total number of activated cells present. The grey lines indicate the percentage of cells within a neurochemical class which are activated by KA. Activation curve annotations and axes labelling are as described in Fig. 3.

203x176mm (300 x 300 DPI)
Figure 11: KA activation of immunocytochemically identified AC populations. A-C: brain nitric oxide synthase (bNOS) immunoreactive ACs were not activated by KA even at saturating levels (white arrowheads). D-F: Tyrosine hydroxylase (TH) immunoreactive ACs also did not co-localize with KA activated ACs at low or saturating KA concentrations (white arrowheads). G-I: Co-localization was evident between AGB and choline acetyltransferase (ChAT) reactive ACs (white arrowheads). M-U: Parvalbumin (PV) positive ACs were activated with KA concentrations exceeding 5 µM. M-O: No co-localization was observed for incubations with 5 µM KA (black arrowheads). P-R: At 20 µM, most PV ACs were co-localized (white arrowheads) but some remained AGB negative (black arrowheads). S-U: All PV immunoreactive cells were co-localized with KA activated cells at 80 µM (white arrowheads). V-X: Co-localization with KA activated cells was observed for some calretinin positive ACs (white arrowheads) but not others (black arrowheads). Similarly, some calretinin reactive cells in the ganglion cell layer co-localized with KA activated GCs (white arrow) and others did not (black arrow). Retinal layer annotations are the same as Fig. 3. Scale bar is 20 µm.
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Figure 12: KA dose response curves for neurochemically distinct cell populations in the ganglion cell layer. Activation curves are presented for A: all GCs within the ganglion cell layer, B: Glu immunoreactive GCs, C: Glu/weakly GABA immunoreactive GCs and D: displaced ACs. The black lines indicate the number KA activated cells in the cell population as a percentage of the total number of activated cells present. The grey lines indicate the percentage of cells within a neurochemical class are activated by KA. Activation curve annotations and axes labelling are as described in Fig. 3.
Figure 13: Diagrammatic representation of KA sensitivity of various neurochemically identified cell populations in the rat inner retina. Annotations below cell images indicate the neurochemical cell class and the half maximal activation concentration for that class. Data is based on dose activation curves in Figs. 3, 10 and 12. For Chat ACs and Type 2, 3, 4 BCs, estimates of half maximum concentration have been made from immunolabeling. Abbreviations: INL: inner nuclear layer, GCL: ganglion cell layer, BC: bipolar cell, AC: amacrine cell, GC: ganglion cell, GABA: gamma-aminobutyric acid, Gly: glycine, Glu: glutamate.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Manufacturer, Cat No</th>
<th>Host</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>Agmatine (AGB)</td>
<td>Agmatine conjugated to bovine serum albumin</td>
<td>Chemicon (Millipore); AB1568*</td>
<td>Rb;</td>
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<tr>
<td>Agmatine (AGB)</td>
<td>Agmatine cross-linked to purified fraction V bovine serum albumin with glutaraldehyde</td>
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<td>Calretinin</td>
<td>Recombinant rat calretinin</td>
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<td>Choline acetyl transferase (ChAT)</td>
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<td>Gamma-aminobutyric acid (GABA)</td>
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<td>Glutamate</td>
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<td>Glycine</td>
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<td>Islet-1</td>
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<td>Neurokinin receptor 3 (NK3R)</td>
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<td>Rb;</td>
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<td>$K$</td>
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<tr>
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<td>------------------------</td>
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<td>------------------</td>
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<tr>
<td>Amacrine cells</td>
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<tr>
<td>Total</td>
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<td>30.00 (27.67, 30.78)</td>
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<td>GABA</td>
<td>46.21 (41.00, 52.07)</td>
<td>0.023 (0.019, 0.032)</td>
<td>38.00 (30.72, 45.18)</td>
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<td>Glycine</td>
<td>36.70 (32.69, 39.46)</td>
<td>0.036 (0.028, 0.044)</td>
<td>30.00 (24.24, 35.68)</td>
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<td>GABA/Glycine</td>
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<td>0.050 (0.027, 0.065)</td>
<td>23.57 (19.19, 28.09)</td>
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<td>Ganglion cells</td>
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<tr>
<td>Total</td>
<td>84.56 (83.31, 85.75)</td>
<td>0.071 (0.064, 0.080)</td>
<td>12.70 (11.83, 13.33)</td>
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<td>Glu</td>
<td>35.13 (30.26, 35.98)</td>
<td>0.038 (0.031, 0.072)</td>
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<td>Glu/weakly GABA</td>
<td>52.75 (51.08, 54.54)</td>
<td>0.078 (0.066, 0.089)</td>
<td>11.42 (10.16, 12.71)</td>
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<td>Displaced amacrine cells</td>
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<tr>
<td>Glu/GABA</td>
<td>73.70 (71.92, 75.39)</td>
<td>0.061 (0.053, 0.069)</td>
<td>15.50 (14.05, 17.23)</td>
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</table>
Using the probe agmatine, we found kainate sensitivity is highest in OFF bipolar cells (particularly type 2) followed by ganglion cells. GABA/glycine amacrine cells (ACs) are the most sensitive ACs, followed by glycine and GABA ACs. Cholinergic ACs are highly responsive but dopaminergic ACs do not express functional kainate receptors.
Author/s:
Nivison-Smith, L; Sun, D; Fletcher, EL; Marc, RE; Kalloniatis, M

Title:
Mapping kainate activation of inner neurons in the rat retina

Date:
2013-08-01

Citation:
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