

# A Role for Ligand-Gated Ion Channels in Rod Photoreceptor Development

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## Summary

Neurotransmitter receptors are central to communication at synapses. Many components of the machinery for neurotransmission are present prior to synapse formation, suggesting a developmental role. Here, evidence is presented that signaling through glycine receptor  $\alpha 2$  (GlyR $\alpha 2$ ) and GABA(A) receptors plays a role in photoreceptor development in the vertebrate retina. The signaling is likely mediated by taurine, which is present at high levels throughout the developing central nervous system (CNS). Taurine potentiates the production of rod photoreceptors, and this induction is inhibited by strychnine, an antagonist of glycine receptors, and bicuculline, an antagonist of GABA receptors. Gain-of-function experiments showed that signaling through GlyR $\alpha 2$  induced exit from mitosis and an increase in rod photoreceptors. Furthermore, targeted knockdown of GlyR $\alpha 2$  decreased the number of photoreceptors while increasing the number of other retinal cell types. These data support a previously undescribed role for these ligand-gated ion channels during the early stages of CNS development.

## Introduction

The vertebrate CNS comprises a network of myriad cell types that develop from pools of multipotent progenitor cells. These progenitor cells must produce the correct cell types at the right time, in the right location, and in the correct ratios to generate a functional system. The retina is a particularly well-studied area of the CNS that has been used to address these aspects of neural development. The retina consists of six neuronal cell types and one glial cell type that are generated in a stereotyped order during development (reviewed in Altshuler et al., 1991). One intriguing question concerns the nature of the signals that drive this stereotyped order of genesis, and another concerns the mechanisms of cell fate determination. Both of these questions have been addressed by heterochronic cell mixing experiments. In these experiments, cells at different developmental time points were shown to be able to influence each others' cell fate decisions, thereby demonstrating a role for extrinsic cues in cell fate determination. However, there was a limit to the cell fate changes that could be produced, as it was shown that progenitor cells were limited to production of only those cell fates appropriate to their time in development (Belliveau and Cepko, 1999;

Belliveau et al., 2000). This observation demonstrated the contribution of intrinsic properties to the cell fate determination process.

One extrinsic factor that has been shown to effect cell fate determination in the retina is taurine. Altshuler et al. (1993) showed that rat retinal cultures initiated from postnatal day zero (P0) generate taurine in the medium and that application of additional taurine promotes rod photoreceptor production. Taurine is a cysteine derivative that is present at high levels in many areas of the developing CNS, including the retina (Hilton et al., 1981; Lombardini, 1991; Macaione et al., 1974). When pregnant cats were deprived of dietary taurine, most of the embryos aborted, and those that survived had severely malformed brains and retinae (Imaki et al., 1986; Sturman et al., 1986, 1987). These malformations appeared to stem from problems with the differentiation and migration of developing neurons (Palackal et al., 1986; Sturman et al., 1985).

Although little is known about the mechanism(s) of taurine signaling, it has been shown that taurine can enter cells through a taurine transporter. Blocking taurine transport, however, did not eliminate the rod-promoting effect of exogenously added taurine, indicating that taurine is acting extracellularly, most likely through a receptor (Altshuler et al., 1993). In order to determine which receptor mediates taurine activity, we took a candidate molecule approach and used pharmacological agents to antagonize potential receptors.

It is well established that taurine binds to and activates glycine receptors (Lewis et al., 1991; Rajendra et al., 1995a; Schaeffer and Anderson, 1981; Schmieden et al., 1992). In addition, there is evidence that taurine can activate  $\gamma$ -aminobutyric acid (GABA) receptors (Horikoshi et al., 1988; O'Byrne and Tipton, 2000; Wu and Xu, 2003). Both glycine and GABA(A) receptors are ligand-gated chloride channels whose role in the adult CNS is to mediate inhibitory neurotransmission. Glycine receptors, in particular the glycine receptor  $\alpha 2$  (GlyR $\alpha 2$ ) subunit, are also expressed in the developing CNS, including within regions, such as the developing cortex, that do not have functional glycinergic synapses in the adult (Malosio et al., 1991). GABA(A) receptors are expressed in progenitor cells of the developing CNS as well, including within the progenitor zone of the cerebral cortex (Owens and Kriegstein, 2002). Expression in the cortical progenitor zone at this time is curious, as it occurs prior to the arrival of the majority of the GABAergic interneurons (Anderson et al., 1997; Tamamaki et al., 1997). Moreover, GlyR $\alpha 2$  and GABA(A) receptors are expressed in some regions of the brain before any synapses are formed. Flint et al. (1998) have shown that extrasynaptic glycine receptors are functional in that nonsynaptically released taurine is capable of activating GlyR $\alpha 2$  in the neocortex. In these studies, functional glycine receptors were shown to be expressed on newly postmitotic, migrating neurons in the neocortex. Activation of these receptors is excitatory and results in a rise in intracellular calcium (Flint et al., 1998).

The data discussed above support a role for taurine

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and glycine receptors as well as GABA receptors in development. However, an *in vivo* role for these channels during development has not been clearly demonstrated. Here, we show a role for ligand-gated ion channels in the development of the retina. Specifically, signaling through GlyR $\alpha$ 2 is demonstrated to be necessary for the taurine induction of rods *in vitro* and the genesis of rods *in vivo* at P0 when rod genesis is at its peak. GlyR $\alpha$ 2 also is shown to be limiting for rod production by embryonic progenitor cells, which have GABA(A) receptors but little if any GlyR $\alpha$ 2. These data suggest that GlyR $\alpha$ 2 is one component of the intrinsic properties of progenitor cells that controls their competence to produce particular cell types at specific times.

## Results

### Taurine Acts through Glycine and GABA Receptors to Promote Rod Genesis

Since taurine is able to bind to and activate both glycine and GABA receptors, we hypothesized that taurine may act through one or both of these receptors to promote rod production. Antagonists to glycine and GABA receptors were tested for inhibition of the rod-promoting effect of taurine (Figure 1; Altshuler et al., 1993). When newborn (P0) mouse retinal cells were cultured in dissociated cell cultures at a low density in defined media that lacked taurine, approximately 9% of the cells became rod photoreceptors. When taurine was applied to these cultures at P0, 2.7-fold more rod photoreceptors were produced (Figure 1A). Glycine or GABA alone did not increase the percentage of rod photoreceptors. However, the combination of glycine and GABA induced a 2.5-fold increase. Addition of glycine and GABA to saturating levels of taurine did not induce more rod photoreceptors over taurine alone (data not shown).

Addition of strychnine, a glycine receptor antagonist, with taurine inhibited the rod-promoting effect of taurine when applied at 0.5  $\mu$ M, 1.0  $\mu$ M, and 5.0  $\mu$ M. Reported IC50 values for strychnine inhibition of taurine-induced GlyR $\alpha$ 2 currents range from low nanomolar concentrations to low micromolar concentrations, depending on the experimental protocol and cellular context (Flint et al., 1998; Thio et al. 2003). Bicuculline is an antagonist of the GABA receptor and may also inhibit the glycine receptor in certain contexts (Curtis et al., 1971, 1974; Goldinger and Muller, 1980). Bicuculline blocked the rod-promoting effect of taurine at concentrations of 5.0 and 10.0  $\mu$ M (Figure 1A). We previously reported that strychnine and bicuculline do not inhibit the rod-promoting effect of taurine when applied only on day 0 of the culture period (Altshuler et al., 1993). When strychnine was added only on day 0 in cultures in the current set of experiments, ambiguous results were obtained, i.e., some replicates showed no inhibition of taurine by strychnine, while others showed some inhibition. Repeated addition on days 0, 3, and 6 did not show such irreproducibility, as indicated in Figure 1A. This difference may reflect instability of the antagonists in culture over several days.

In addition to strychnine, picrotoxin (5  $\mu$ M) also inhibited the rod-promoting effect of taurine (Figure 1A). Picrotoxin is a chloride channel blocker that can inhibit

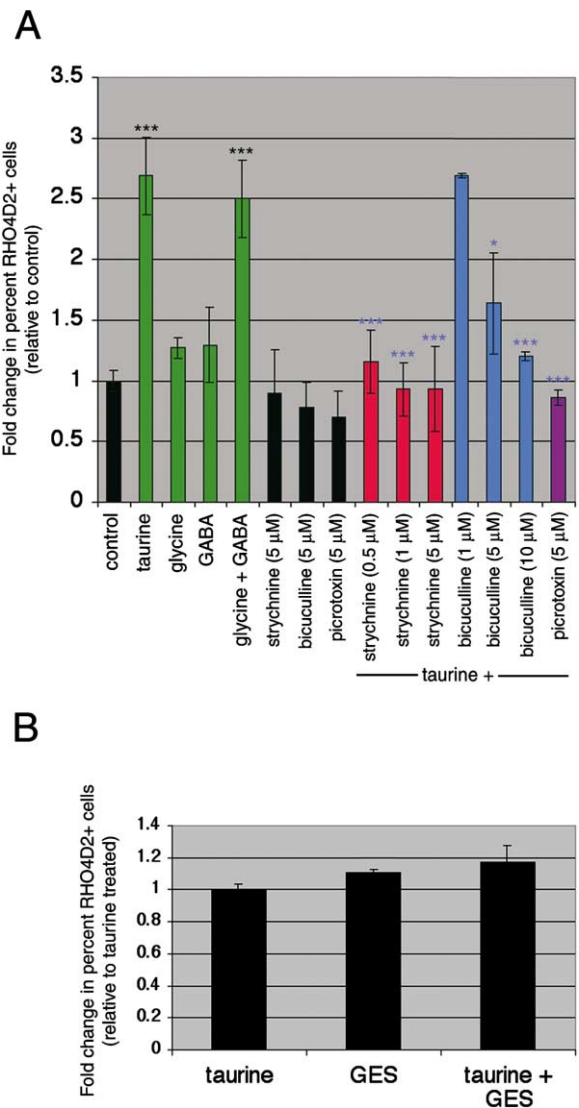


Figure 1. Percentage of Total Retinal Cells Expressing Rhodopsin after Exposure to Pharmacological Agents

P0 mouse retinae were dissociated and cultured in collagen gels with serum-free defined medium. Pharmacological agents were added and cells were cultured for 10 days. Cultures were then dissociated, immunostained with an anti-rhodopsin antibody (RHO4D2) that labels only rod photoreceptors, and quantified. *y* axes represent the fold change in the percent of rod photoreceptors relative to control cultures with no factor added (A) or relative to taurine treated cultures (B). Strychnine, a specific antagonist of glycine receptors, picrotoxin, an antagonist of GABA(A) receptors and homomeric glycine receptors, and bicuculline, an antagonist of GABA receptors, were added to cultures on day 0, day 3, and day 6. Abbreviations: Tau, taurine (100  $\mu$ M); gly, glycine (100  $\mu$ M);  $\gamma$ -amino-n-butyrac acid, GABA (100  $\mu$ M); strych, strychnine (0.5–5  $\mu$ M); picro, picrotoxin (5  $\mu$ M); bic, bicuculline (1–10  $\mu$ M); GES, guanidinoethylsulfinate (100  $\mu$ M). Graph represents data from three to five independent experiments. Significance tests were performed comparing control versus agonists (black asterisks) and taurine treated versus taurine plus antagonists (purple asterisks). \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

both ionotropic GABA receptors and homomeric glycine receptors with reported IC50 in the low micromolar range (Bormann et al., 1993; Etter et al., 1999; Inoue and

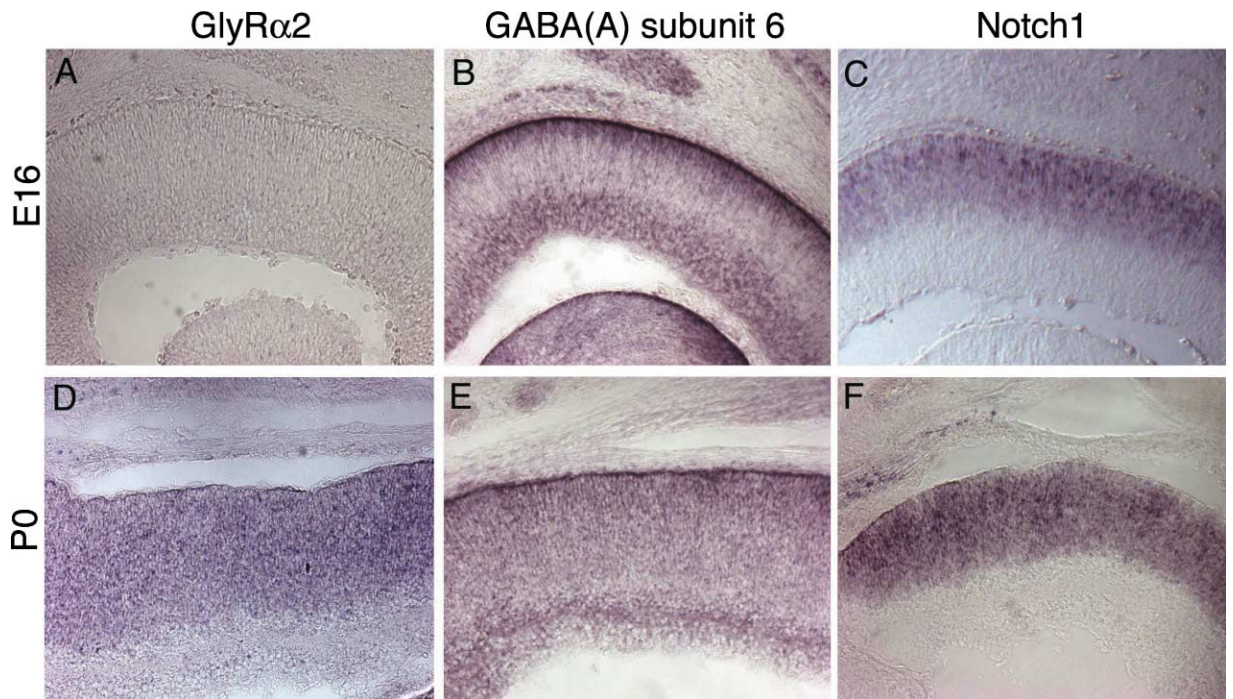


Figure 2. Glycine and GABA Receptor mRNAs Are Expressed in Developing Retinal Cells

In situ hybridization on retinal sections from E16 (A–C) and P0 (D–F). GlyR $\alpha$ 2 (A and D), GABA(A) subunit  $\alpha$  6 (B and E), and Notch1 (C and F). Expression of Notch 1 is included to indicate the location of progenitor cells in the ventricular zone.

Akaike, 1988; Pribilla et al., 1992; Thio et al., 2003). In summary, the inhibition of the rod-promoting effect of taurine by strychnine, bicuculline, and picrotoxin indicated that glycine and GABA receptor activity is required for taurine to promote rod production.

Guanidinoethylsulfinate (GES) blocks cellular taurine uptake by specifically inhibiting sodium-taurine cotransport and has been shown to increase extracellular taurine concentrations (Huxtable et al., 1980; Lombardini and Medina, 1978; Kishi et al., 1988; Lehmann and Hamberger, 1984). GES significantly stimulated rod photoreceptor production over control to levels similar to taurine treatment. Addition of GES to saturating levels of taurine did not further stimulate rod photoreceptor production (Figure 1B).

#### Glycine Receptor $\alpha$ 2 and GABA(A) Subunit $\alpha$ 6 Are Expressed in Retinal Progenitor Cells

Glycine receptor  $\alpha$ 1 (GlyR $\alpha$ 1) is expressed in the adult retina in a subset of amacrine, bipolar, and ganglion cells in rodents (Boos et al., 1993; Greferath et al., 1994; Karschin and Wässle, 1990; Koulen et al., 1996; Sassoe-Pognetto and Wässle, 1997; Sassoe-Pognetto et al., 1994; Suzuki et al., 1990; Tauck et al., 1988). GlyR $\alpha$ 2 is the developmentally expressed glycine receptor subunit in the spinal cord and brain (Becker et al., 1988; Malosio et al., 1991). In situ hybridization analysis showed that GlyR $\alpha$ 2 was expressed in developing retinal cells at P0 (Figure 2D). At P0, GlyR $\alpha$ 2 was expressed in the area of the retina where developing rods as well as mitotic cells reside and was absent from the inner portion of the developing inner nuclear layer, where amacrine and

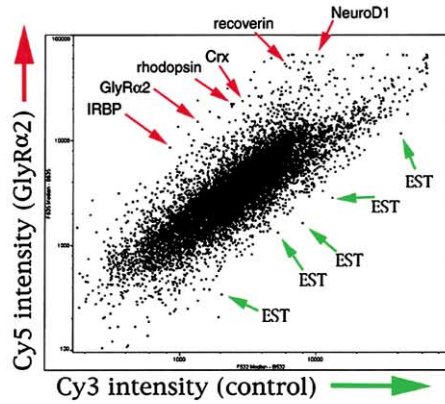
ganglion cells reside. In contrast, GlyR $\alpha$ 2 expression was not detected at embryonic day 16 (E16) in the retina using these in situ hybridization methods (Figure 2A). For comparison, in situ hybridizations using a Notch1 probe, a marker of progenitor cells, are shown (Figures 2C and 2F). The expression of GlyR $\alpha$ 2 at P0 in developing retinal cells has not been previously detected in studies using glycine receptor antibodies. These antibodies often mark puncta of clustered glycine receptors at the synapse. The glycine receptors expressed in immature retinal cells may not be clustered, since synapses have not yet formed at this developmental time point in the retina and may therefore not be detectable with available antibodies. Attempts to detect GlyR $\alpha$ 2 protein expression by Western blots were inconclusive due to the reactivity of the antibody (mAb4) with all subunits of the glycine receptor.

Of the GABA receptor genes tested, GABA(A) subunit  $\alpha$ 6 was found to be expressed in a subset of developing retinal cells in the area where newborn and differentiating photoreceptors reside at both E16 and P0 (Figures 2B and 2E).

#### Misexpression of the Glycine Receptor Embryonically Induces Rod Photoreceptors

In order to test if GlyR $\alpha$ 2 can lead to increased rod photoreceptor production, viral constructs were used to express GlyR $\alpha$ 2 at embryonic day 16 (E16), when GlyR $\alpha$ 2 is not normally expressed. E16 retinal explants were infected with a virus that coexpressed GlyR $\alpha$ 2 and green fluorescent protein (GFP), or GFP alone. Retinae were cultured as explants with media containing fetal

A



B

Genbank ID	Unigene ID	Gene Symbol	Name	exp1	exp2	exp3	exp4	exp5
LC366	Mm.3918	Abca4	ABCR	2.4	1.1	2.2	1.3	ND
LC184	Mm.56987	Opn1sw	blue cone opsin	2.2	1.8	1.7	1.9	2.0
LC368	Mm.56987	Opn1sw	blue cone opsin	2.5	1.9	1.3	2.9	1.5
A1845817	Mm.8008	Crx	cone-rod homeobox containing protein	1.9	1.7	3.8	1.8	2.9
LC55	Mm.8008	Crx	cone-rod homeobox containing protein	5.7	2.1	3.1	2.1	3.7
LC382	Mm.8008	Crx	cone-rod homeobox containing protein	4.6	1.6	2.3	2.1	2.2
LC249	Mm.151852	Rbp3	Retinol binding protein 3, interstitial	2.0	1.5	1.2	1.4	ND
LC242	Mm.4636	NeuroD1	NeuroD1	5.4	1.5	4.0	1.8	3.1
LC211	Mm.4636	NeuroD1	NeuroD1	7.3	1.4	4.2	1.5	2.9
A1835157	Mm.4636	NeuroD1	NeuroD1	6.1	1.4	2.3	1.3	2.6
LC246	Mm.134516	Obx2	Obx2	5.0	1.6	3.4	2.0	3.2
LC166	Mm.157931	Rcovm	recoverin	6.0	1.5	3.0	1.6	2.0
A1839178	Mm.1276	Sag	Retinal S-antigen	1.4	2.1	2.4	1.8	2.9
LC 171	Mm.2965	Rho	rhodopsin	2.2	1.2	3.4	ND	2.9
LC160			Rod arrestin	3.7	1.9	1.5	2.0	1.9
LC169			Rod channel	2.5	1.2	1.8	1.6	1.7
LC355	Mm.679	Rom1	rod outer segment membrane protein 1	7.5	1.5	3.9	2.2	2.6
LC531		GlyRa2		7.9	3.3	2.6	ND	2.1

C

Genbank ID	Unigene ID	Gene Symbol	Name	exp1	exp2	exp3	exp4	exp5
A1839888	Mm.205791	ATP1a1	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 1 polypeptide	0.24	0.81	0.71	0.84	0.72
LC190	Mm.42241	Barx1	Bar1-like homeobox 1	0.66	0.59	0.96	0.68	0.65
A1845051	Mm.7363	Spnb3	Beta-spectrin 3	ND	0.57	0.61	0.74	0.55
A1841978	Mm.6534	Capns1	calpain, small subunit 1	ND	0.62	0.60	0.64	0.54
A1839368	Mm.26940	Cbr1	Carbonyl reductase 1	0.96	0.89	0.47	0.76	0.55
A1836796	Mm.370	C1qa	Complement component 1, q subcomponent	0.51	0.62	0.78	0.76	0.79
LC217	Mm.22569	Ccnb1	cyclin B1	0.67	0.63	0.62	0.85	0.53
A1841974	Mm.52	D11Erd99e	DNA segment, Chr 11, ERATO Doi 99	ND	0.67	0.67	0.70	0.45
A1840556			ESTs	0.61	0.80	0.69	0.72	0.43
A1843248			ESTs	0.39	0.83	0.85	0.75	0.65
A1835330	Mm.260504		ESTs	0.37	0.80	ND	0.95	0.69
A1840241			ESTs	0.68	0.77	0.69	0.68	0.61
A1840354	Mm.30158	0710001C03Rik	ESTs	ND	0.61	0.68	0.63	0.44
A1841981	Mm.33907	1110003H18Rik	ESTs	0.40	0.73	0.68	0.80	0.56
A1847909	Mm.25661	1700030A21Rik	ESTs	ND	0.65	0.65	0.63	0.49
A1845753	Mm.141864	2810422J05Rik	ESTs	0.57	0.84	1.02	0.80	0.64
A1846301	Mm.193073	5730454B08Rik	ESTs	0.80	0.71	0.53	1.06	0.51
A1838289	Mm.250688		ESTs	0.48	0.63	0.67	0.79	0.55
A1835277			ESTs	0.39	0.65	0.33	0.51	0.71
A1841804	Mm.1260	Nme1	expressed in non-metastatic cells 1	ND	0.69	0.59	0.66	0.47
A1836205	Mm.41787	Nme4	expressed in non-metastatic cells 4	ND	0.68	0.56	0.70	0.48
A1840184	Mm.6799	Fhl2	four and a half LIM domains 2	0.37	0.61	0.43	0.88	0.80
A1841957	Mm.4915	Gabra6	Gamma-aminobutyric acid (GABA-A) receptor, subunit 6	ND	0.72	0.52	0.74	0.55
A1845043	Mm.16620	Gad1	Glutamic acid decarboxylase 1	ND	0.57	0.55	0.72	0.51
A1835674	Mm.2338	Glu1	glutamine synthetase	0.29	0.80	0.67	0.77	0.80
A1839176	Mm.2338	Glu1	glutamine synthetase	ND	0.66	0.63	0.63	0.42
A1836495	Mm.24443	Gdi3	guanosine diphosphate (GDP) dissociation inhibitor 3	0.72	0.62	0.66	0.75	0.56
A1841041	Mm.10717	Hap1	Huntingtin-associated protein 1	ND	0.72	0.52	0.74	0.53
A1839182	Mm.70573		hypothetical protein LOC207728	0.25	0.63	0.58	0.79	0.75
A1836400	Mm.3931	Max	Max protein	0.84	0.63	0.72	0.65	0.49
A1849805	Mm.142140	Nef3	neurofilament 3, medium	ND	0.72	0.79	0.73	0.41
A1841479	Mm.29096	Nrbf1	nuclear receptor binding factor 1	ND	0.72	0.58	0.75	0.49
A1849253	Mm.21855	Pglyrp	peptidoglycan recognition protein	0.65	0.65	0.75	0.68	0.57
A1843656	Mm.2314	Prkcd	protein kinase C, delta	1.31	0.49	0.72	0.73	0.44
A1843041	Mm.5163	Ril2	Ras-like without CAAX 2	ND	0.77	0.83	0.74	0.42
A1837976	Mm.22130	Rpn2	ribophorin II	ND	0.79	0.87	0.63	0.80
A1836185	Mm.147693	Rps3	ribosomal protein S3	ND	0.74	0.72	0.89	0.46
A1840050	Mm.24783	Secoc	short coiled-coil pln	1.22	0.64	0.73	0.69	0.44
A1849568	Mm.41552	Tbl3	transducin (beta)-like 3	ND	0.65	0.80	0.65	0.52
A1841564	Mm.1485	Ube2e3	ubiquitin-conjugating enzyme E2E 3	0.44	0.59	0.79	0.85	0.68

Figure 3. Genes that Are Regulated Following Misexpression of GlyR $\alpha$ 2 in the Retina at E16

GlyR $\alpha$ 2 and GFP, or GFP alone (control), were misexpressed by retroviral mediated gene transfer in E16 retinal progenitor cells. Retinal explants were cultured for 12 days in vitro (DIV) and then dissociated and FACs sorted to collect GFP-positive cells. Amplified cDNA was then labeled and hybridized to cDNA microarrays.

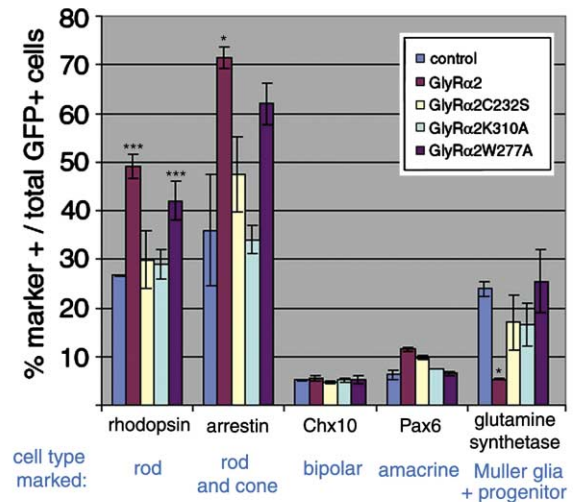
(A) Representative scatter plot of gene expression of GlyR $\alpha$ 2 versus control cells. Each spot represents a single gene. The x axis plots the intensity from control infected cells and the y axis plots the intensity from cells transduced with GlyR $\alpha$ 2. Any gene that lies along the slope of 1 is unchanged upon GlyR $\alpha$ 2 misexpression. Genes that lie above this line are upregulated with misexpressed GlyR $\alpha$ 2. Although the majority of genes are unchanged, many of the known photoreceptor genes on the microarray were upregulated. The majority of downregulated clones are expressed sequence tags (ESTs) representing genes that have not been characterized.

calf serum (FCS), which contains taurine. Retinae were allowed to develop *in vitro* for 12 days and were then dissociated and sorted using fluorescent activated cell sorting (FACS) to collect GFP-positive cells. The production of various retinal cell types was assayed both by cDNA microarrays and immunocytochemistry. Microarray analysis showed that when GlyR $\alpha$ 2 was misexpressed at E16, many photoreceptor genes were upregulated (Figures 3A and 3B). In addition to photoreceptor genes, a number of uncharacterized genes were also upregulated. These genes may be of interest in that they may be previously unidentified photoreceptor genes. Many of the genes downregulated upon GlyR $\alpha$ 2 misexpression were expressed sequence tags (ESTs) or genes whose function has not been previously characterized. Interestingly, one of the known genes found to be downregulated was glutamine synthetase, a marker of progenitor cells as well as Muller glial cells. Although in general the genes found to be downregulated are not known to represent a specific cell type, these data may be of increased value in the future when the gene expression profiles for different retinal cell types are better characterized.

In order to determine if the upregulation of photoreceptor markers reflects an increase in the number of photoreceptors, immunocytochemistry of the virally infected population was carried out with photoreceptor-specific antibodies for rhodopsin (rods) and arrestin (rods and cones) (Figure 4A). Misexpression of GlyR $\alpha$ 2 induced approximately a 2-fold increase in the percentage of rod photoreceptors produced. Interestingly, the microarray data and staining with anti-arrestin together suggest that the percentage of cone photoreceptors may also be increased upon GlyR $\alpha$ 2 misexpression. Misexpression of GlyR $\alpha$ 2 also slightly increased the percentage of amacrine cells produced. In contrast, the percentage of glutamine synthetase-positive cells was reduced (Figure 4A).

Three mutant GlyR $\alpha$ 2 receptors were constructed and misexpressed at E16 using the methods described above (Figure 4B). Residues were chosen for mutation based on studies of human glycine receptor  $\alpha$ 1 (GlyR $\alpha$ 1) (Lynch et al., 1997; Rajendra et al., 1995b). These point mutations in GlyR $\alpha$ 1 affect ligand binding, channel activity, or both. One point mutation is at cysteine 232 (GlyR $\alpha$ 2C232S), a residue that is part of a disulfide bond in the extracellular ligand binding domain that is highly conserved among ligand-gated ion channels and is required for ligand binding (Rajendra et al., 1995b). When this mutant receptor was misexpressed at E16, it did not induce production of rod photoreceptors or inhibit expression of glutamine synthetase (Figure 4A). This result shows that the phenotype seen with the wild-type receptor was not due to nonspecific effects of misexpressing the subunit in these cells. In addition, it suggests that ligand binding is required for the rod-promoting activity of wild-type GlyR $\alpha$ 2.

A



B

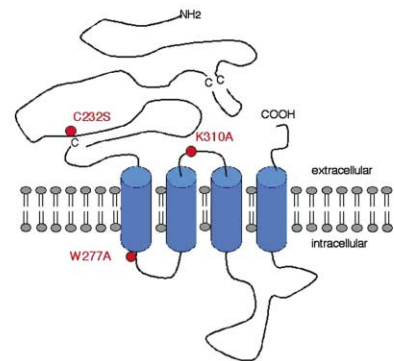


Figure 4. Results of Misexpression of GlyR $\alpha$ 2 in the Retina at E16 (A) E16 retinal explants were infected with retroviruses encoding GFP alone (control), GFP and wild-type GlyR $\alpha$ 2, or GFP and a mutant form of GlyR $\alpha$ 2. After 12 DIV, retinal explants were dissociated and immunostained for markers of different retinal cell types. The percentage of virally infected cells (identified by GFP expression) expressing each marker is plotted on the y axis. Mutant forms of the receptor that putatively destroy ligand binding (GlyR $\alpha$ 2C232S) or disable the ability of taurine to activate the receptor (GlyR $\alpha$ 2K310A) or disable the ability of taurine or glycine to activate the receptor (GlyR $\alpha$ 2W277A) were tested in the same assay in independent infections. Experiments were performed in duplicate or triplicate, with each replicate containing cells pooled from 10–20 retinae. Within each replicate, 100–300 cells were counted for each antibody for each variable. Significance tests were performed comparing control infected cells with GlyR $\alpha$ 2 wild-type or mutant infected cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

(B) Schematic of GlyR $\alpha$ 2 subunit and mutated residues. Glycine receptor  $\alpha$ 2 subunit is proposed to encode four transmembrane domains with extracellular amino and carboxyl termini. Residues chosen for mutational analysis are colored red.

(B and C) Photoreceptor genes upregulated with GlyR $\alpha$ 2 misexpression (B) over five experiments and genes downregulated upon misexpression of GlyR $\alpha$ 2 (C). Values given represent the fold change upon GlyR $\alpha$ 2 misexpression. Experiments 1–3 are independent experiments. Experiments 4 and 5 are replicates of experiments 2 and 3 with the fluorescent labels reversed. Values given represent the ratio of intensity of signal from cells transduced with GlyR $\alpha$ 2 relative to that of control infected cells. Genes that are listed more than once are represented multiple times on the microarray by independent clones. ND, no data; LC, lab clone.

The mutation GlyR $\alpha$ 1K276A eliminates taurine activity and slightly decreases the binding of taurine and glycine (Lynch et al., 1997). Mutation of the homologous amino acid in GlyR $\alpha$ 2, K310A, also eliminated taurine activation of the receptor while only slightly affecting glycine activity when assayed *in vitro* in transfected 293 cells (G. Yellen, personal communication). In the rod induction assay, GlyR $\alpha$ 2K310A neither induced rod photoreceptor production nor decreased the percentage of cells expressing glutamine synthetase (Figure 4A). This result is consistent with the hypothesis that taurine is the primary ligand responsible for activating GlyR $\alpha$ 2 to promote rod photoreceptor production.

An interesting observation was made upon misexpression of a third mutant receptor, which contains a point mutation in the intracellular domain of the glycine receptor. GlyR $\alpha$ 1W243A eliminates the ability of taurine and glycine to properly gate the channel without affecting ligand binding (Lynch et al., 1997). The mechanism by which this point mutation affects receptor activity is unclear. The homologous mutation, GlyR $\alpha$ 2W277A, increased the percentage of rod photoreceptors but did not affect the percentage of glutamine synthetase-positive cells produced (Figure 4A). Although the mechanism by which this mutation changes GlyR $\alpha$ 2 activity is unclear, this result shows that these two aspects of the phenotype of misexpressed GlyR $\alpha$ 2 are separable.

#### Misexpression of the Glycine Receptor Induces Exit from Mitosis

The phenotype induced by misexpression of GlyR $\alpha$ 2 could be due to a cell fate switch, a change in proliferation, an induction of apoptosis, or a combination of these activities. Several assays were conducted to address these possibilities.

In order to determine if misexpression of the glycine receptor affected the number of cells per clone following viral infection, either due to effects on proliferation or cell death, the size of clones marked by infection with a replication incompetent retrovirus was measured. Retinal explants from E16 and P0 were infected with retroviruses coexpressing GlyR $\alpha$ 2 and nuclear LacZ, or nuclear LacZ alone. The retinae developed *in vitro* in the presence of serum, which contains taurine, until the equivalent of P8, and were then fixed, stained for LacZ activity, and sectioned. Retinae were infected with viruses at a concentration that allowed for identification of individual clones, as clones were well separated from one another (Figure 5A). GlyR $\alpha$ 2 misexpression at both E16 (Figure 5B) and P0 (Figure 5C) consistently resulted in an increase in the percentage of smaller clones and a decrease in the percentage of larger clones. In order to investigate if there was differential apoptosis between control and GlyR $\alpha$ 2-infected clones, TUNEL assays were performed 4 days following infection. Very few TUNEL-positive cells were observed in these retinae. In three retinae, 0 TUNEL-positive cells out of >1000 nuclear LacZ-positive cells counted were observed, and these were found only in areas of poor morphology. Taken together, these results suggest that expression of GlyR $\alpha$ 2 reduced proliferation of retinal progenitor cells.

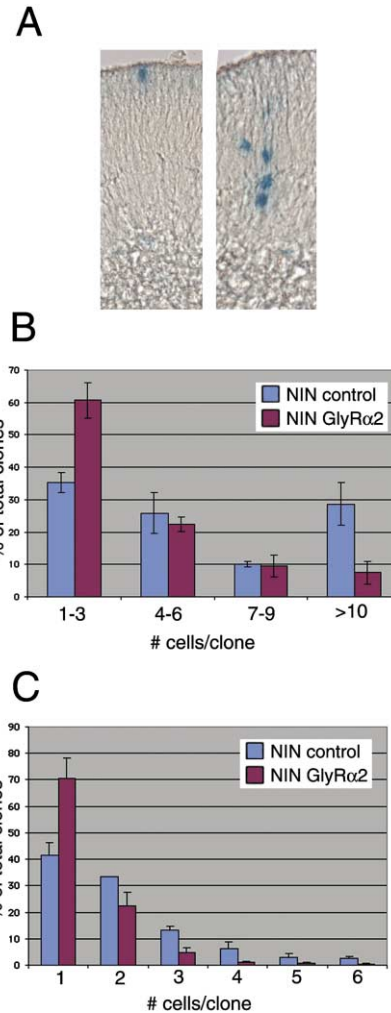


Figure 5. Clone Size Following Misexpression of GlyR $\alpha$ 2 at E16 or P0

Retinal explants at E16 and P0 were infected with replication incompetent retroviruses encoding either nuclear LacZ alone (NIN control) or nuclear LacZ and GlyR $\alpha$ 2 (NIN GlyR $\alpha$ 2). Retinae developed *in vitro* for 12 DIV (E16 infected) or 8 DIV (P0 infected). Explants were stained for  $\beta$ -galactosidase activity with X-gal and sectioned. Clones were identified by spatial separation from one another. The number of cells in each clone was determined by counting the number of blue nuclei in each clone.

(A) Examples of clones identified by nuclear LacZ staining.

(B and C) Quantitation of the percentage of different clone sizes for cells infected with NIN or NIN GlyR $\alpha$ 2 at E16 (B) and P0 (C). Three to five independent retinae were analyzed for each time point and type of virus. For E16 retinae, 286 and 348 clones were counted for NIN control and NIN GlyR $\alpha$ 2, respectively. For P0 retinae, 1017 and 751 clones were counted for NIN and NIN GlyR $\alpha$ 2, respectively.

#### Knockdown of GlyR $\alpha$ 2 Inhibits Rod Photoreceptor Development

A small inhibitory RNA (siRNA) directed against GlyR $\alpha$ 2 was expressed from pBSU6, a vector that promotes expression of a 22 base pair small interfering RNA hairpin (Sui et al., 2002). Such constructs have previously been shown to knock down levels of gene expression in several contexts, including the developing retina (Matsuda and Cepko, 2004; and for general review, see Sui et al., 2002).

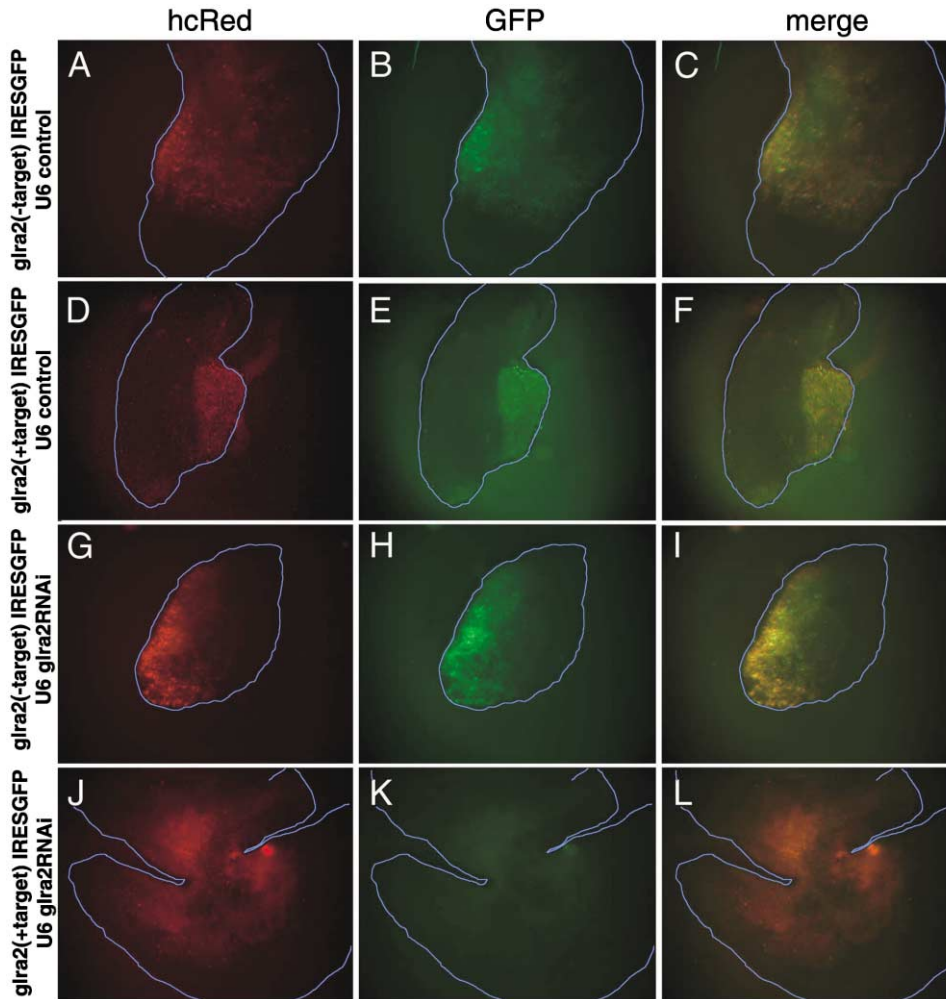


Figure 6. In Vitro Test of GlyRa2-RNAi Activity in P0 Retinae

P0 retinae were co-electroporated in vitro with three plasmids: one encoding CAG hcRED, another with CAG GlyR $\alpha$ 2 IRES GFP with or without the RNAi target sequence, and a third with a U6 promoter without an siRNA (U6 control) or with an RNAi targeting GlyR $\alpha$ 2 (U6 GlyR $\alpha$ 2-RNAi). Retinae were cultured as explants for 4 days and fluorescence was visualized in whole mounted retinae. Retinae shown are representative of 2-3 independent retinae and are positioned to show the region of electroporated tissue. Blue lines were drawn to outline the edges of the retinal tissue.

Constructs that target four different regions of GlyR $\alpha$ 2 were tested for their ability to reduce the level of GlyR $\alpha$ 2 RNA. In preliminary screening by RT-PCR, one siRNA targeting a 22 base pair sequence in the 3'UTR of GlyR $\alpha$ 2 (U6-GlyR $\alpha$ 2-RNAi) appeared to be the most promising candidate. In order to test the ability of this construct to reduce protein levels, we utilized a fluorescent-based assay for siRNA activity previously developed in our lab (Matsuda and Cepko, 2004). This assay employs an RNA with sequences of the gene of interest (GlyR $\alpha$ 2) followed by an IRES GFP. If this RNA contains the target of the siRNA being tested, GFP fluorescence should be reduced when co-expressed with the siRNA construct. In this system, three DNA constructs are co-electroporated into retinae in vitro. One construct encodes hcRed driven by a strong ubiquitous promoter, CAG (Niwa et al., 1991). This construct is a control to visualize the cells that were transfected (Figures 6A, 6D, 6G, and 6J). Another construct directs synthesis of the

RNA driven by the CAG promoter that has GlyR $\alpha$ 2 sequences with or without the siRNA target sequence, followed by IRES GFP. A third construct is either an empty U6 vector or U6-GlyR $\alpha$ 2-RNAi. The electroporated retinae were cultured as explants for several days to allow for expression of these constructs (Figure 6). GFP levels were efficiently reduced only when both the U6-GlyR $\alpha$ 2-RNAi and the GlyR $\alpha$ 2-IRESGFP with the siRNA target sequence were co-electroporated (Figure 6K). Importantly, the GFP expression was not reduced when the GlyR $\alpha$ 2 siRNA target sequence was not included on the RNA (Figure 6H).

The effects of GlyR $\alpha$ 2 knockdown in the developing retina were tested in vivo. U6-GlyR $\alpha$ 2-RNAi was co-electroporated with a ubiquitous promoter (CAG) driving GFP (CAGIG; Matsuda and Cepko, 2004) into P0 rat retinae in vivo. As a control, empty U6 vector was co-electroporated with CAGIG. The animals developed for 2 weeks and the retinae were harvested and sectioned,

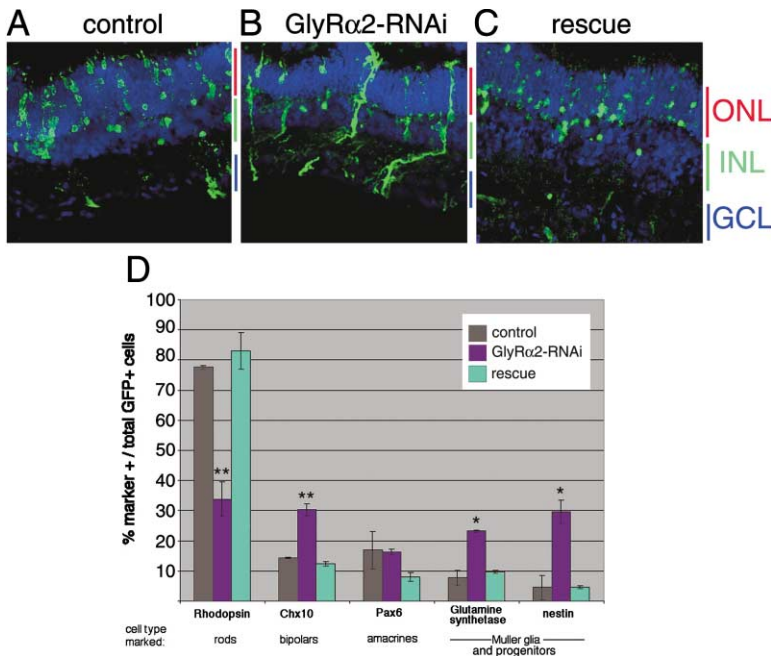


Figure 7. Introduction of RNAi for GlyR $\alpha$ 2 by In Vivo Electroporation into P0 Retinae

P0 retinæ were co-electroporated in vivo with 2 plasmids: one encoding an empty RNAi vector (U6 control) or RNAi targeting GlyR $\alpha$ 2 (U6-GlyR $\alpha$ 2-RNAi) and another encoding the CAG promoter driving either GFP alone or GFP with GlyR $\alpha$ 2 cDNA (without the siRNA target sequence). Animals developed until postnatal day 14 when retinæ were harvested and examined for GFP expression. (A–C) GFP fluorescence marks electroporated retinal cells in retinal sections. Most of the electroporated cells in the control (A) reside in the photoreceptor layer, the outer nuclear layer (ONL). Upon GlyR $\alpha$ 2 RNAi misexpression (B), most of the electroporated cell bodies were observed in the inner nuclear layer (INL), the location of amacrine, bipolar, horizontal, and Muller glial cells. The processes that extend from the ganglion cell layer (GCL) through the ONL to the outer edges of the retina are typical of Muller glia. (C) Retinæ co-electroporated with GlyR $\alpha$ 2-RNAi and the rescue construct encoding GlyR $\alpha$ 2.

(D) Electroporated retinæ were dissociated and stained for markers of different retinal cell types. Percent of electroporated cells (as identified by GFP fluorescence) expressing each marker was determined. Experiments were performed two independent times with each replicate consisting of electroporated cells pooled from two or three retinæ. For each antibody for each replicate, 100–200 cells were counted. Significance tests were performed for control versus GlyR $\alpha$ 2-RNAi electroporated retinæ. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

and GFP-positive cells were visualized (Figures 7A and 7B). In the control retinæ, the majority of GFP-positive cells resided in the photoreceptor layer with cell bodies being distributed throughout the outer nuclear layer (ONL). Fewer GFP-positive cells were present in the inner nuclear layer (INL), which consists of horizontal, amacrine, bipolar, and Muller glial cells (Figure 7A). These controls looked the same as retinæ that were electroporated with RNAi constructs that successfully targeted other genes, but did not have a phenotype (Matsuda and Cepko, 2004). In contrast, when retinæ were electroporated with U6-GlyR $\alpha$ 2-RNAi, very few electroporated cells resided in the photoreceptor layer. The majority of the electroporated cells appeared to be in the inner nuclear layer. In addition, many of the cells appeared to have Muller glial-like processes that spanned the length of the retina (Figure 7B).

In order to confirm that this phenotype was specific to knockdown of the glycine receptor, a rescue experiment was performed by co-electroporating U6-GlyR $\alpha$ 2-RNAi with the CAG promoter driving expression of GlyR $\alpha$ 2 and GFP (CAGIG-GlyR $\alpha$ 2). This construct has the full coding region of GlyR $\alpha$ 2, but does not have the 3' UTR of GlyR $\alpha$ 2, which is the targeted region in the endogenous gene. When these constructs were co-electroporated, the majority of the GFP-positive cells were present in the ONL, similar to control electroporations (Figure 7C). In addition, sections of retinæ electroporated with CAGIG-GlyR $\alpha$ 2 without the RNAi construct also looked like control electroporations, with the majority of the GFP-positive cells residing in the photoreceptor layer (data not shown).

In order to quantify these results, the same experiment was performed, but the retinæ were dissociated and stained for markers of different retinal cell fates. Electro-

poration of U6-GlyR $\alpha$ 2-RNAi decreased the percentage of cells expressing rhodopsin and increased the number of cells expressing Chx10, nestin, and glutamine synthetase (Figure 7D). At this stage in development in vivo, Chx10 antibody labels bipolar cells (Burmeister et al., 1996), while nestin and glutamine synthetase antibodies label Muller glial cells (Walcott and Provis, 2003). These results suggest that knockdown of GlyR $\alpha$ 2 inhibited the production of rod photoreceptors and increased the percentages of bipolar and Muller glial cells produced. This effect was specific to knockdown of GlyR $\alpha$ 2, as CAGIG-GlyR $\alpha$ 2 rescued the phenotypes observed with U6-GlyR $\alpha$ 2-RNAi expression.

## Discussion

### A Role for Ligand-Gated Ion Channels in Retinal Development

It is estimated that taurine is the second most abundant free amino acid in the CNS, after glutamate (reviewed in Sturman, 1988). Previous studies of the effects of taurine-free diets on CNS development demonstrated the importance of taurine, as deprived fetuses had a paucity of differentiated neurons in the areas examined (reviewed in Sturman, 1993). Nonetheless, the specific roles of taurine have been unclear, as well as its mechanism of action. The data presented here suggest that taurine acts through the glycine and GABA(A) receptors to stimulate rod photoreceptor production. In this study, we have focused our attention on the glycine receptor and have demonstrated a role for the  $\alpha$ 2 subunit during development in vivo.

Within E16 progenitor cells, GABA(A) receptors, but not glycine receptors, are present and rod production is relatively low. Misexpression of GlyR $\alpha$ 2 was sufficient

at this time to induce formation of rod photoreceptors. Reduction of GlyR $\alpha$ 2 levels at early postnatal stages *in vivo*, when both GABA(A) receptors and GlyR $\alpha$ 2 are normally expressed and rod production is at its peak, decreased the number of rod photoreceptors produced. These data demonstrate the sufficiency and necessity of GlyR $\alpha$ 2 for rod photoreceptor development. This raises the question of when in photoreceptor development GlyR $\alpha$ 2 acts. Activation of the receptor may instruct mitotic retinal progenitor cells to produce postmitotic daughters, which then differentiate. The primary evidence to support this point of action is that misexpression of GlyR $\alpha$ 2 using a replication incompetent retrovirus induced the production of smaller clones at both embryonic (E16) and early postnatal (P0) stages. In addition, misexpression at E16 induced the production of rod photoreceptors and amacrine cells at the expense of glutamine synthetase-positive cells. At this stage of development, glutamine synthetase marks both late progenitor cells and Muller glial cells, the latest produced cell type. A reduction in each of these populations is expected if GlyR $\alpha$ 2 misexpression instructs cells to exit mitosis and differentiate. Consistent with such an effect on mitosis was the finding that reductions of GlyR $\alpha$ 2 expression at P0 reduced the number of rod photoreceptors produced, which is the primary cell type made at this developmental stage. If loss of GlyR $\alpha$ 2 expression resulted in a prolonged progenitor state, one might expect to see an increase in the number of later produced cell types, such as bipolar and Muller glial cells. In fact, this was the phenotype observed.

Glycine receptors have not previously been shown to have a function in proliferation. However, previous studies have suggested that ionotropic GABA receptors are involved in proliferation as well as differentiation and migration of developing neurons (reviewed in Owens and Kriegstein, 2002). In the proliferative zone of the neocortex, activation of GABA(A) receptors decreased the amount of tritiated thymidine incorporation and the number of bromodeoxyuridine (BrdU)-positive cells, while antagonizing GABA(A) receptor activity with bicuculline increased DNA synthesis (Haydar et al., 2000; LoTurco et al., 1995). Exposure of cortical explants to elevated potassium levels also inhibited DNA synthesis, suggesting that depolarization may be sufficient to inhibit proliferation (LoTurco et al., 1995). During the time in retinal development under study here, glycine receptors, as well as GABA receptors, are excitatory (Vu et al., 2000). An intriguing hypothesis is that depolarization may be a general signal for neural progenitors to exit the cell cycle and differentiate.

In addition to acting on mitotic cells, taurine and GlyR $\alpha$ 2 may also act within postmitotic cells to induce differentiation into rod photoreceptors by modulating a cell fate decision. Several lines of evidence suggest this point of action. The effects of misexpression of wild-type and mutant forms of GlyR $\alpha$ 2 on proliferation and rod photoreceptor cell fate suggest that these two effects are separable. Addition of GlyR $\alpha$ 2W277A induced rods without reducing the number of glutamine synthetase-positive cells. In addition, in our previous work on dissociated retinal cells, addition of taurine near the end of the culture period, when the vast majority of the cells are no longer cycling, still increased the percentage of

cells expressing a rod photoreceptor marker (Altshuler et al., 1993). A role for taurine in neuronal differentiation also has been suggested by taurine deprivation studies in cats. In newborn cats from taurine-deprived mothers, postmitotic neuronal precursors in the visual cortex failed to differentiate and many failed to migrate (Pallack et al., 1986). In addition, GlyR $\alpha$ 2 is expressed in newly postmitotic, migrating neocortical cells rather than in mitotic cells (Flint et al., 1998). The expression data presented here for the retina cannot discriminate between GlyR $\alpha$ 2 expression in mitotic retinal progenitor cells or newly postmitotic, undifferentiated cells. However, the reduction of clone size argues for a role in mitotic cells, while the effects on postmitotic cells in earlier studies argue for an additional role in postmitotic cells.

Our data show that the  $\alpha$ 2 subunit is the primary glycine receptor subunit involved in promoting rod photoreceptor production in the retina. This is in keeping with previous observations of expression of the  $\alpha$ 2 subunit throughout the developing CNS (Malosio et al., 1991). GlyR $\alpha$ 2 RNA is expressed in developing retinal cells between P0 and P6, which is the primary time of rod photoreceptor development. In addition, the target of the GlyR $\alpha$ 2 siRNA is within the 3' UTR of the GlyR $\alpha$ 2 transcript and cannot recognize other glycine receptor subunit messages.

The mature form of the glycine receptor in the adult has been shown to be a heteropentamer of 3 $\alpha$ :2 $\beta$  subunits (Langosch et al., 1988). However, GlyR $\alpha$ 2 homopentamers have previously been shown to be active and responsive to taurine *in vitro* and *in vivo* (De Saint Jan et al., 2001; Flint et al., 1998; Grenningloh et al., 1990; Hoch et al., 1989; Takahashi et al., 1992; Tapia and Aguayo, 1998). In the retina, RT-PCR studies have shown that the  $\beta$  subunit also is expressed during development (data not shown). However, picrotoxin is able to block the rod-promoting effect of taurine in the rod induction assay. Since picrotoxin antagonism of glycine receptors is inhibited by GlyR $\beta$  (Bormann et al., 1993; Pribilla et al., 1992), the rod-promoting effect observed in this assay is likely due to the activity of GlyR $\alpha$ 2 homopentamers.

#### What Are the Signals Downstream of the Glycine and GABA Receptors?

The signaling that occurs following taurine activation of the glycine and GABA receptors may involve depolarization of the cell membrane, changes in intracellular ion concentrations resulting from channel opening, and/or some other as of yet unidentified signaling component of the glycine or GABA receptors. The data from misexpression of mutant forms of the glycine receptor suggest that ligand binding is required for glycine receptor activity in promoting the production of rod photoreceptors. However, it is unclear whether channel opening or membrane depolarization is required. Previous studies have shown that picrotoxin blocks the channel of glycine receptors (Pribilla et al., 1992). Based on these studies, the results showing that picrotoxin blocks taurine induction of rods suggests that channel opening is required for the activity of the glycine receptor. However, it should be noted that other studies have shown that picrotoxin

may also block ligand binding of glycine receptors (Lynch et al., 1995) and that differences in picrotoxin effects may be context dependent. The concentration of taurine used in these studies (100  $\mu$ M) is well below the EC<sub>50</sub> for taurine activation of glycine receptor  $\alpha$ 2 (Flint et al., 1998; Thio et al., 2003). This may suggest that membrane depolarization is not required for the effects of taurine on rod production. Rather, single channel opening may be sufficient to activate a downstream pathway that leads to rod production.

### **Taurine Is an Endogenous Ligand for GlyR $\alpha$ 2 in Development**

While taurine is able to induce rod photoreceptor production in dissociated cell cultures and in explants at P0, it is curious that neither glycine nor GABA alone can do so (Figure 1; Altshuler et al., 1993). In addition, a mutant form of GlyR $\alpha$ 2 that presumably abolished the ability of taurine to activate the receptor, while only mildly affecting the activity of glycine, is unable to induce rod production. These data strongly support taurine as the endogenous ligand for GlyR $\alpha$ 2 in the developing retina.

The concentrations of taurine within the developing retina reach millimolar levels (Hilton et al., 1981; Macai-one et al., 1974), which would be sufficient to activate glycine and GABA receptors. These high levels of retinal taurine can result from either biosynthesis within the retina or via uptake from the circulation through the taurine transporter (Huxtable, 1989; Pasantes-Morales et al., 1998; Sturman, 1993). In the developing retina, both may occur, as the rate-limiting enzyme for taurine synthesis (cysteine sulphinate decarboxylase), as well as taurine transporters, are expressed (Lombardini, 1991; Pasantes-Morales et al., 1976). In contrast, it appears that the developing retina is actively trying to decrease the level of glycine. Recent SAGE data from our laboratory have shown that there is a sharp upregulation of glycine decarboxylase, a catabolic enzyme for glycine, at P0 in the mouse retina, when rod photoreceptor genesis is at its peak (S. Blackshaw et al., submitted).

In the developing retina, immunohistochemistry with antitaurine antisera has shown an intriguing pattern of taurine localization in that cells appear to accumulate taurine as they exit mitosis and initiate their differentiation (Lake, 1994). Once accumulated within the cell, the controlled local release of taurine could be one mechanism by which taurine regulates development *in vivo*. An interesting question is whether taurine release is regulated in the developing retina, and if so, what are the signals that stimulate release? It is known from other regions of the CNS that taurine can be released upon depolarization of cells or application of hyperosmolar solutions (Bockelmann et al., 1998; Deleuze et al., 1998). Release may occur through reversal of the taurine transporter and/or through osmosensitive anion channels, as has been observed in other systems (Bres et al., 2000; Saransaari and Oja, 1999; Strange and Jackson, 1995).

In the developing cortex, endogenous, nonsynaptically released taurine can activate precursor cells (Flint et al., 1998). This was shown using a blocker of the taurine transporter, guanidinoethylsulfinate (GES), that results in the accumulation of extracellular taurine (Hux-

table et al., 1980; Kishi et al., 1988; Lehmann and Hamberger, 1984; Lombardini and Medina, 1978). Similarly, addition of GES in our assay also stimulated rod photoreceptor production, suggesting that endogenous, nonsynaptically released taurine is able to have an effect similar to exogenously added taurine.

Although glycine alone and GABA alone are not able to induce rod photoreceptor production in dissociated retinal cells, glycine and GABA together are capable of rod induction. One possible explanation is that GlyR $\alpha$ 2 expressed in the context of developing retinal cells is more sensitive to taurine than glycine. In this scenario, GABA may potentiate the effect of glycine either through direct binding of glycine receptor (De Saint Jan et al., 2001; Taleb and Betz, 1994) or indirectly through activation of GABA receptors. Another hypothesis is that both glycine and GABA receptors are activated by taurine to potentiate the rod-promoting effect. In this case, activation of both receptors is required for the rod effect, perhaps in a sequential manner, or the effects of activation of these receptors may be additive.

### **GlyR $\alpha$ 2 as a Regulator of the Timing of Rod Photoreceptor Production**

Heterochronic mixing experiments demonstrated that E16 retinal progenitor cells did not make additional rods in response to a rod-inducing environment provided by P0 cells (Belliveau and Cepko, 1999). In contrast, P0 progenitor cells respond to, and in fact require, the soluble components made by P0 cells to make their normal complement of rod photoreceptor cells (Altshuler and Cepko, 1992). This intrinsic difference in response to rod-inducing cues appears to be due, at least in part, to the presence or absence of GlyR $\alpha$ 2. Its expression was observed in P0 progenitor cells but not E16 progenitor cells. Importantly, addition of GlyR $\alpha$ 2 to E16 cells led to production of more rods by these cells in the presence of taurine. Similarly, the fact that reduction of GlyR $\alpha$ 2 in P0 progenitor cells led to reduced rod production provides evidence for the necessity of GlyR $\alpha$ 2 in rod production. The fact that a difference between P0 and E16 cells is expression of a receptor for an extrinsic cue provides a basis for their differential response to the P0 environment. These data provide a molecular mechanism for at least one aspect of the competence model of retinal development (Cepko et al., 1996).

### **Experimental Procedures**

#### **Animals**

Timed pregnant Swiss Webster mice and Sprague Dawley rats were purchased from Taconic farms.

#### **Collagen Gel and Explant Cultures**

For collagen gel cultures, retinae were dissected from Swiss Webster mice at postnatal day 0 (P0) in Hanks Balanced Salt Solution (HBSS) without calcium or magnesium. Retinae were dissociated in papain for 10 min at 37°C. The cells were pelleted (800 g, 3 min) and resuspended in culture media (1:1 DMEM/F-12 Ham's [GIBCO], B27 Supplement [GIBCO], 5  $\mu$ g/ml insulin [Sigma], 100 U/ml penicillin/streptomycin [GIBCO], 5 mM HEPES [pH 7.0] [GIBCO]). Collagen gel cultures were constructed as previously described (Altshuler and Cepko, 1992), with minor modifications. Cell concentration was determined with a hemocytometer, and  $5 \times 10^5$  cells/100  $\mu$ l collagen

gel were plated per well in a 24-well tissue culture plate with a final volume of 500  $\mu$ l. All pharmacological agents were added on day 0 of the cultures and used at the concentrations indicated. Antagonists (strychnine, bicuculline, picrotoxin) were added at day 3 and day 6 of the culture period in addition to day 0. All compounds were purchased from Sigma and Tocris, with the exception of guanidinoethylsulfinate (GES) (see below). Collagen gel experiments were performed in triplicate with two or three wells pooled for each replicate to minimize well-to-well variation. For each replicate, 150–500 cells were counted. Retinal explants were prepared from Swiss Webster mice at embryonic day 16 (E16) or postnatal day 0 (P0) in Hanks Balanced Salt Solution with divalent cations. Retinal explants that were virally infected were cultured on filters as described (Altshuler et al., 1993).

For GFP viral infections for the microarray analysis, experiments were performed five independent times. Each replicate included cells infected and pooled from an entire E16 litter. For dissociated cell immunostaining, experiments were performed in duplicate or triplicate, with each replicate containing cells pooled from 10–20 retinæ. Within each replicate, 100–300 cells were counted for each antibody for each variable.

#### GES Synthesis

Synthesis of GES was as described (Fujii and Cook, 1975). Crystals were filtered on a 45  $\mu$ m filter (Whatman) and dried overnight. Crystals were recrystallized three times from water to obtain pure GES. Purity of GES was confirmed by mass spectrometry in the Harvard Medical School Biopolymer facility.

#### In Vitro and In Vivo Electroporation

Dissected retinæ were electroporated in a micro chamber (Nepagene, model CUY532, 3  $\times$  10  $\times$  5 mm) that contained a DNA solution that consisted of DNA constructs each at a concentration of 0.5–1  $\mu$ g/ml in PBS (CAG-hcRed and GFP constructs at 1  $\mu$ g/ml and U6 constructs at 0.5  $\mu$ g/ml). In vitro electroporations were performed using a Tokiwa CUY-21 square wave electroporator with 25 mV for five pulses of 50 milliseconds each and 950 milliseconds chase. Approximately 5%–30% of total cells were electroporated using this method.

In vivo electroporations of P0 retinæ from Sprague Dawley rats were performed as described (Matsuda and Cepko, 2004). DNA was injected into the subretinal space at a concentration of 1 mg/ml per DNA construct. Approximately 65% of attempted electroporations contained visualizable GFP patches that covered approximately 25%–75% of the surface area of the retina. RNAi experiments were performed two independent times with each replicate consisting of electroporated cells from two or three retinæ. Between 1% and 20% of the total cells in an electroporated retina expressed GFP. For each antibody for each replicate, 100–200 cells were counted.

#### Plasmid Constructions

All viral glycine receptor misexpression studies were performed using full-length human GlyR $\alpha$ 2 cDNA (provided by H. Zoghbi). For point mutations, site-directed mutagenesis was performed using Clontech's Transformer site directed mutagenesis kit.

GlyR $\alpha$ 2-RNAi is directed against the 3'UTR of GlyR $\alpha$ 2 with the sequence GGGTGGGGTTTCTGGCACCTAA, which was cloned into pBSU6 (Sui et al., 2002). Rescue experiments were performed with a full-length mouse GlyR $\alpha$ 2 cDNA from cloned from the start ATG to 12 bp past the stop codon.

#### Replication Incompetent Retroviral Production

Full-length human GlyR $\alpha$ 2 or point mutants were cloned directly into retroviral expression constructs pGFP (gift of Gary Nolan) or pNIN (M. Samson and C.L.C., unpublished). Virus was produced and concentrated as described (Cepko et al., 1998).

#### Microarray Hybridization and Analysis

Microarrays were constructed within the lab and consisted of cDNA inserts from approximately 11,136 clones from the Brain Molecular Anatomy Project clone set (kind gift of Dr. Bento Soares, University of Iowa) and over 600 clones of interest collected in our laboratory (Livesey et al., 2004).

Approximately 5–10  $\mu$ g of cDNA were labeled by incorporation of Cy3- or Cy5-dCTP (Amersham-Pharmacia) as described (Livesey et al., 2000). CDNAs from FACS-sorted cells were amplified for 16–20 cycles using Clontech's SMART amplification protocol. Previous studies have shown that expression data generated by this approach are comparable to results obtained by other methods (Livesey et al., 2000). Pairs of labeled probes were hybridized at 42°C overnight and washed in 0.2 $\times$ SSC/0.1%SDS at room temperature, followed by two room temperature washes in 0.2 $\times$ SSC. Slides were scanned in an Axon GenePix 4000B Scanner (Axon Instruments) and data extracted from the resulting images using the GenePix software package (Axon Instruments).

#### In Situ Hybridization and Immunofluorescence

Section in situ hybridizations were performed on retinal cryosections (20  $\mu$ m) as described (Chen and Cepko, 2002). Probes were as follows: full-length mouse GlyR $\alpha$ 2 cDNA, GABA(A) subunit  $\alpha$  6, GenBank accession number AI841957; Notch 1, GenBank accession number AW047868. Images were taken on a Nikon Eclipse E1000 microscope using a Leica DC200 digital camera.

For immunofluorescent staining, retinal cryosections (20  $\mu$ m) or dissociated retinal cells were blocked with 2% goat serum/2% donkey serum/0.1% Triton X-100 for 1 hr at room temperature. Slides were incubated in primary antibody overnight at 4°C. Primary antibodies used: anti-rhodopsin, Rho4D2 (mouse monoclonal, 1:250 [Molday and MacKenzie, 1983]), anti-Chx10 (rabbit polyclonal, 1:500 [A. Chen and C.L.C., unpublished]), anti-Pax6 (mouse monoclonal, 1:500, University of Iowa Developmental Studies Hybridoma Bank, deposited by Atsushi Kawakami), anti-arrestin (rabbit polyclonal, 1:100, Zymed), anti-nestin (mouse monoclonal, 1:200, PharMingen), anti-glutamine synthetase (mouse monoclonal, 1:200, PharMingen), and anti-GFP (mouse monoclonal, rabbit polyclonal, both 1:500, Molecular Probes). After several washes (PBS, 0.1% Triton X-100), slides were incubated in goat anti-mouse or goat anti-rabbit Cy3 or Cy5 (Jackson ImmunoResearch Laboratory, 1:200) for 1–2 hr at room temperature. Cells were counterstained with DAPI and washed several times. Dissociated samples were visualized and quantified on a Nikon Eclipse E1000 microscope. Immunofluorescent stainings of retinal cryosections were photographed on an Axioplan 2 microscope with a LSM 510 Meta module for confocal imaging.

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