Developmental Changes in the Neurotransmitter Regulation of Correlated Spontaneous Retinal Activity

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Synchronized spontaneous rhythmic activity is a feature common to many parts of the developing nervous system. In the early visual system, before vision, developing circuits in the retina generate synchronized patterns of bursting activity that contain information useful for patterning connections between retinal ganglion cells and their central targets. However, how developing retinal circuits generate and regulate these spontaneous activity patterns is still incompletely understood. Here we show that in developing retinal circuits, the nature of excitatory neurotransmission driving correlated bursting activity in ganglion cells is not fixed but undergoes a developmental shift from cholinergic to glutamatergic transmission. In addition, we show that this shift occurs as presynaptic glutamatergic bipolar cells form functional connections onto the ganglion cells, implicating the role of bipolar cells in providing endogenous drive to bursting activity later in development. This transition coincides with the period when subsets of ganglion cells (On and Off cells) develop distinct activity patterns that are thought to underlie the refinement of their connectivity with their central targets. Here, our results suggest that the differences in activity patterns of On and Off ganglion cells may be conferred by differential synaptic drive from On and Off bipolar cells, respectively. Taken together, our results suggest that the regulation of patterned spontaneous activity by neurotransmitters undergoes systematic change as new cellular elements are added to developing circuits and also that these new elements can help specify distinct activity patterns appropriate for shaping connectivity patterns at later ages.

Key words: retinal development; ferret retina; spontaneous activity; retinal waves; activity dependent; APB; glutamate

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Electrical activity in the developing nervous system is characterized by spontaneous periodic bursts of action potentials that are synchronized among neighboring cells (Feller, 1999; O’Donovan, 1999; Wong, 1999). Such activity occurs in the immature nervous systems of different species (Masland, 1977; Galli and Maffei, 1988; Meister et al., 1991; Gummer and Mark, 1994; Sernagor and Grzywacz, 1996; Wong et al., 1998; Zhou, 1998) and has been implicated in the development and refinement of neuronal connectivity (Katz and Shatz, 1996; Wong, 1999). Because of this functional importance, recent work has focused on how coordinated network oscillations are produced and regulated across development.

Spontaneous rhythmic activity in structures from the spinal cord to the hippocampus and retina often requires excitatory neurotransmission (O’Donovan, 1999). Intriguingly, spontaneous rhythmic activity occurs before and throughout the period when synaptic networks are assembled (Wong et al., 1993; Spitzer et al., 1995; Catsicas et al., 1998; Milner and Landmesser, 1999), raising the question of whether unique or even transient mechanisms are required for its production. Additionally, as new synaptic elements are incorporated across development, they may also exert regulatory influences that modify activity patterns in ways appropriate for establishing connectivity at each stage of development.

These questions can be studied readily in the retina because its anatomy, circuitry, function, and development are well understood. Figure 1 schematically represents retinal circuits at two major phases of development in the ferret. In the first 2 postnatal weeks, retinal ganglion cells (RGCs) receive synaptic input from a lateral network of GABAergic and cholinergic amacrine cells. At this stage, RGCs undergo synchronized bursting activity in the form of propagating waves with all cells exhibiting a common bursting pattern. This activity is driven by cholinergic transmission (Feller et al., 1996; Penn et al., 1998) and modulated by GABAergic transmission (Fischer et al., 1998). Later, in the third and fourth week, the vertical pathway comprising glutamatergic bipolar and photoreceptor cells becomes assembled (Greiner and Weidman, 1981). Around this time, RGCs differentiate into On and Off subclasses with their dendritic arbors stratifying into different sublaminae in the inner plexiform layer (IPL) (Wong and Oakley, 1996; McCarthy et al., 1998) and their axonal terminals segregating into On and Off sublaminae in the dorsal lateral geniculate nucleus (Linden et al., 1981; Hahn et al., 1999). In addition, On and Off RGC populations begin to develop distinct bursting patterns. These contain cues that are appropriate for specifying the segregation of On and Off axonal arbors into separate thalamic sublaminae (Lee and Wong, 1996; Miller, 1996; Wong and Oakley, 1996).

In this study, we examined how the development of retinal circuits contributes to the regulation of spontaneous rhythms. We monitored spontaneous bursting in RGCs using calcium imaging and whole-cell recordings and determined pharmacologically at each stage the relative contributions from the lateral cholinergic and the vertical glutamatergic networks. We also examined the functional development of the bipolar circuitry in relation to its potential contribution to shaping differential On and Off activity patterns.
incubating the tissue in 10 
retinae, cells in the ganglion cell layer were loaded with fura-2 by 
mounts maintained in oxygenated Ringer's solution at 35°C. For P7–P8 
of black Millipore (Bedford, MA) filter paper as described previously 
The retinae were floated onto a clean glass slide and held flat by a piece 
and 20 mM HEPES (all reagents from Sigma, St. Louis, MO), pH 7.4. 
parts of this paper have been published previously (Miller et 
Figure 1. Schematic drawing showing the nature of inputs to ferret 
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MATERIALS AND METHODS
Preparation of tissue. Ferret kits were obtained from Marshall Farms and 
with the ages of postnatal day 7 (P7) and P23. The kits were 
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Calcium imaging. Calcium imaging was performed on retinal whole 
Ringer's solution for 30 min at room temperature and 30 min at 30°C. In the older 

The doses of antagonists used in the study were those that have been
RESULTS
Contribution of cholinergic transmission to spontaneous activity across development
Rhythmic bursting activity in the ganglion cells is characterized by periodic increases in $[\text{Ca}^{2+}]$, (Fig. 2). Calcium imaging of whole-mounted retinae reveals that early in development (P7–P8), blockade of nicotinic cholinergic transmission by the bath application of the antagonist DHβE resulted in a significant decrease in the bursting rates in RGCs (Fig. 2A). By contrast, the same drug application in older retinae (P20–P23) did not reduce the rate of bursting activity (Fig. 2B). Quantification of the results shows that although DHβE suppressed the mean bursting rate of ganglion cells in the younger retina to 66.1 ± 2.1% (± SEM) of the baseline rate, it failed to suppress bursting activity in the older retina, even appearing to increase it slightly to 111.1 ± 2.5% of baseline rates (Fig. 2C). This may be an indirect effect arising from a small degree of excitatory cholinergic drive onto inhibitory GABAergic or glycinergic neurons that are known in the later ages to suppress spontaneous bursting in ganglion cells (Fischer et al., 1998). The changing effect of cholinergic blockade was also observed with the other antagonists used. In younger retinae, mecamylamine reduced bursting to a comparable 69.9 ± 2.4% of the baseline rate, whereas curare, a commonly used antagonist, had a larger effect and abolished activity altogether (0 ± 0%) in all cells (Fig. 2C). In marked contrast, even curare failed to decrease bursting frequency in the older retina (97.3 ± 5.2%). These results indicate that although excitatory nicotinic cholinergic transmission is important in driving correlated bursting activity early on, its contribution diminishes later in development.

Contribution of glutamatergic transmission to spontaneous activity across development
To assess the contribution of glutamatergic transmission across the same period of development, we monitored spontaneous bursting activity in the absence and presence of antagonists to ionotropic glutamate receptors at the two age groups using calcium imaging. At P7–P8, applications of APV and NBQX separately decreased bursting rates by a small amount; the combined application of both antagonists decreased the bursting rate to a greater extent but did not eliminate bursting activity entirely (Fig. 3). In older retinae, at P20–P23, separate applications of NBQX and APV in each case reduced the bursting rate more effectively than in the younger retina, but bursting activity was similarly not eliminated (Fig. 4). However, the combined application of both NBQX and APV abolished bursting activity completely.

Previous experiments have demonstrated that the spontaneous intracellular calcium elevations in ganglion cells correspond to bursts of action potentials measured by electrophysiology (Penn et al., 1998; Wong et al., 1998). To confirm that the application of NBQX and APV abolished activity by blocking neurotransmission, we performed whole-cell patch recordings of ganglion cells in the absence and presence of these antagonists. Under voltage clamp at the reversal potential for $\text{Cl}^-$, periodically occurring postsynaptic currents (PSCs) were observed for both age groups (Fig. 5). Although the combined addition of NBQX and APV to younger retinae did not eliminate these currents, the same drug application in older retinae abolished all spontaneous PSCs. Thus, the absence of periodic spontaneous calcium elevations in the presence of NBQX and APV arises from the blockade of spontaneous cationic currents.

Quantification of the results from calcium imaging and voltage-clamp recordings are summarized in Figure 6. In younger P7–P8 retinae, NBQX and APV each reduced mean bursting rates by

Figure 2. Changing dependence of spontaneous bursting activity in RGCs on cholinergic transmission as evaluated with calcium imaging in the ganglion cell layer. A, B. Examples of the effect of DHβE, a nicotinic cholinergic antagonist, on the bursting activity of cells at P8 (A) and P20 (B) (duration of drug application is denoted by horizontal bar). C. Quantitative summary of the effects of cholinergic blockade with various antagonists on the rate of spontaneous bursting at P7–P8 and P20–P22. Burst rates in the various cholinergic antagonists are expressed as a percentage of the rate under control conditions (Ringer’s solution) immediately preceding the bath application of the drug. Effects of drugs at each age were significant, except for curare at P20–P22 (Mann–Whitney test, p < 0.001). The concentrations of drugs used were 100–200 μM (DHβE), 20–50 μM (curare), and 80–160 μM (mecamylamine) (Mec) ($r$ = number of recordings; $n$ = number of cells monitored).
similar amounts (81.4 ± 2.9 and 82.1 ± 1.9% respectively), whereas the combination of the antagonists decreased bursting to 55.9 ± 1.2% of the baseline (Fig. 6A). Addition of DHβE to NBQX and APV further reduced bursting activity to 28.7 ± 1.3% of baseline rates (Fig. 6A). This suggests that for this early age group, both acetylcholine and glutamate contribute to spontaneous bursting activity of the ganglion cells and that the excitatory drives mediated via cholinergic, glutamatergic AMPA/kainate receptors and glutamatergic NMDA receptors are additive in nature. In older P20–P23 retinae, NBQX alone decreased mean bursting rates to 42.6 ± 1.8%, whereas APV alone reduced mean bursting rates to 74.9 ± 2.6%, suggesting that at the older ages, the excitatory drive to spontaneous bursting as mediated via AMPA/kainate receptors is larger than that mediated via NMDA receptors. When NBQX and APV are applied together, the bursting rate in older retinae was reduced to 1.1 ± 0.4% of baseline values. This trend is also corroborated by measurements of charge transfer for the postsynaptic currents in patch-clamp recordings; when NBQX and APV were applied together to younger P7–P8 retinae, the average charge transfer per minute decreased to 63 ± 9% of baseline values. In contrast, the same application to older P20–P23 retinae had a much larger effect, reducing the average charge transfer per minute more markedly to 5 ± 1% of baseline values (Fig. 6B).
Collectively, these results show that, contrary to the developmental trend observed with cholinergic transmission, the contribution of excitatory glutamatergic transmission to spontaneous bursting activity increases across development and becomes essential to bursting activity per se in older neonates. This requirement for glutamatergic transmission is absolute even in the absence of inhibitory drive from GABAergic and glycinergic amacrine cells. The combination of NBQX and APV continues to suppress all bursting activity even in the presence of antagonists to GABAergic (bicuculline, 150 μM) and glycinergic (strychnine, 1–5 μM) transmission, as assessed by calcium imaging in whole-mount retina (data not shown).

**Maturation of functional connections between bipolar interneurons and ganglion cells**

Our results thus far indicate that spontaneous activity in the ganglion cells becomes totally dependent on glutamatergic transmission as the glutamatergic bipolar interneurons mature and make synaptic contact onto ganglion cells. However, the maturation of the bipolar cell circuitry has thus far been inferred only from anatomical observations (Greiner and Weidman, 1981). To implicate bipolar cells further as a source of transmission driving spontaneous activity in ganglion cells in the older neonates, we examined the time at which bipolar cells make functional glutamatergic connections onto ganglion cells. We assayed for the presence of bipolar-to-ganglion cell connections at different ages by stimulating the dendrites of bipolar cells in the outer plexiform layer and measuring evoked responses in cells in the ganglion cell layer (Fig. 7). This experiment was performed in retinal slices in which puffs of potassium chloride were delivered to the outer plexiform layer via a patch pipette during calcium imaging. The presence of a functional drive from bipolar cells was indicated by the ability to evoke repeatedly a rise in [Ca\(^{2+}\)]\(_i\) in the surrounding ganglion cells and amacrine cells. Because our previous anatomical analysis of the development of bipolar cells suggests the presence of morphologically differentiated bipolar cells by P10–P13 (Miller et al., 1999), we stimulated retinal slices from P11 to P21 animals. Intracellular calcium responses were evaluated in multiple cells in each retinal slice recording; the number of retinai recorded at each age were as follows: P11–P12, n = 3; P13–P14, n = 3; P15–P19, n = 3; and P19–P22, n = 6. We found that evoked responses in the ganglion cell layer can be elicited beginning from P13 but not in younger P11 retinae, even though...
ganglion cells at this age were responsive to direct applications of exogenous glutamate (Fig. 8). The glutamatergic nature of the transmission of the bipolar-to-ganglion cell connection was confirmed by the ability of NBOX and APV to block reversibly the evoked responses in the ganglion cell and inner nuclear layers.

These observations suggest that bipolar cells can provide functional glutamatergic drive to retinal ganglion cells and amacrine cells beginning around P13. This result is consistent with the interpretation that the increasing dependence of spontaneous bursting in ganglion cells on glutamatergic transmission during neonatal development occurs as the functional glutamatergic drive from bipolar cells to ganglion cells develops and matures.

Effects of APB on bursting activity of On and Off ganglion cells

To implicate further the role of endogenous glutamatergic signaling from bipolar cells in driving spontaneous bursting activity in ganglion cells, we examined ganglion cell activity using an intervention that suppresses endogenous bipolar activity. The metabotropic glutamate receptor mGluR6, which binds to the agonist APB, is expressed in On bipolar cells and localized to its dendrites but is not expressed at all in Off bipolar cells (Nomura et al., 1994; Ueda et al., 1997). In the absence of light stimulation, On bipolar cells are hyperpolarized by glutamate via activation of APB-sensitive mGluR6 receptors on these cells. By contrast, Off bipolar cells, lacking the APB-sensitive receptors, are depolarized after activation of ionotropic glutamate receptors (Slaughter and Miller, 1981; Nawy and Jahr, 1990; de la Villa et al., 1995). Thus, the application of APB to older P20–P23 retinae will suppress endogenous glutamatergic drive originating from the On bipolar cells but not that from Off bipolar cells. By measuring spontaneous bursting activity in On and Off ganglion cells in the absence of APB, we were able to examine the role of endogenous bipolar signaling in driving bursting activity.

Figure 9 shows the effects of APB on the activity patterns of On and Off RGCs in a P21 retina at the age when glutamatergic transmission is absolutely required for spontaneous bursting activity in the ganglion cells. Putative On and Off ganglion cells were classified according to their relative burst rates and by their dendritic stratification patterns after intracellular dye filling (Wong and Oakley, 1996). To reveal the effects of APB on bipolar cells, we first removed inhibition by GABA and glycine from amacrine cells using the antagonists bicuculline and strychnine. As demonstrated previously (Fischer et al., 1998), On cells showed a marked increase in bursting rates after disinhibition (Fig. 9A). Subsequently, when APB was applied, the activity in On ganglion cells was abolished, whereas that in Off cells persisted. These results are confirmed by the quantification of the data from recordings from several retinas (Fig. 9B). This preferential total suppression of bursting activity in On ganglion cells by the hyperpolarization of On bipolar cells indicates that in the On pathway at least, endogenous synaptic drive from bipolar cells is absolutely required for bursting activity in ganglion cells after the formation of functional bipolar-to-ganglion cell connections.

DISCUSSION

Neurotransmitter control of spontaneous activity during development

In this study, we demonstrate that the regulation of synchronized spontaneous activity in the ferret retina undergoes progressive change as the retina develops. Taken together with previous work, the results in this study enable us to put together a more unified picture of how patterned spontaneous activity is regulated in the developing ferret retina (Burgi and Grzywacz, 1994; Feller et al., 1997; Fischer et al., 1998; Butts et al., 1999). In the early phase of development, during the first 2 postnatal weeks, ganglion cells receive synaptic input primarily from a lateral network of amacrine cells (Fig. 10A). These amacrine cells drive spontaneous correlated bursting in ganglion cells using excitatory GABAergic (Fischer et al., 1998) and cholinergic (Feller et al., 1996) transmission. As glutamatergic bipolar cells are introduced into the retinal circuitry late in the second postnatal week and make initial contact with ganglion cells, excitatory glutamatergic transmission becomes increasingly important in driving spontaneous bursting in ganglion cells, whereas cholinergic transmission diminishes in importance. It is also at this stage that GABAergic transmission from amacrine cells switches from depolarizing to hyperpolarizing and begins to exert an inhibitory modulatory influence on spontaneous bursting (Fischer et al., 1998). These changing ef-
Effects of glutamate, acetylcholine, and GABA on regulating spontaneous bursting activity are represented in Figure 10B. Thus, the overall mechanism seems to be one in which patterned spontaneous bursting is regulated by various forms of neurotransmission acting in an additive and coordinated manner (see Sernagor and Grzywacz, 1999). These regulatory effects change during development to accommodate new elements of retinal circuitry as they are added, to sustain a continuous and relevant output to retinal targets in the brain where retinogeniculate connections continue to be refined.

What are the actual retinal connections responsible for neurotransmitter regulation of spontaneous activity? In the earlier developmental period, at P7–P8, spontaneous bursting is driven additively by glutamate, acetylcholine, and GABA. Although cholinergic and GABAergic amacrine-to-ganglion cell connections have been documented at this time (Greiner and Weidman, 1981), the source of glutamate at this stage is less clear. The absence of bipolar synapses at this time raises the possibility that glutamate may be secreted in a paracrine manner by ventricular cells or precursors to bipolar cells (Pow et al., 1994). Alternatively, potential transient connections between immature glutamatergic photoreceptors, observed to extend synaptophysin-positive processes into the IPL at this stage, may act as a source of glutamatergic transmission (Johnson et al., 1999). The additive nature of glutamatergic and cholinergic drives at this early stage also suggests that glutamate, at least in part, exerts a direct effect on ganglion cells, as opposed to acting solely via intermediate cholinergic cells presynaptic to the ganglion cells. At the later stage, at P17–P23, when endogenous glutamatergic drive from bipolar cells becomes essential for spontaneous activity, the waning of cholinergic nicotinic regulation indicates that glutamatergic excitation must also occur directly onto ganglion cells. However, despite the decrease in cholinergic nicotinic drive at this stage, it is unlikely that a dismantling of connections from cholinergic amacrine cells to ganglion cells occurs because these connections are present at maturity, subserving important functions in adult vision (Vaney, 1990; Peters and Masland, 1996; He and Masland, 1997).

Figure 9. Effect of APB on On and Off ganglion cell activity in the absence of GABAergic and glycinergic inhibition as evaluated with calcium imaging. A, Application of APB (indicated by horizontal bar) abolishes bursting activity in On ganglion cells but not Off ganglion cells in P21 retina. Bursting activity recovers after the washout of APB. B, Quantitative summary of the effect of APB in On and Off ganglion cells in P17–P22 retina is shown. Burst rates in APB are expressed as a percentage of the rate calculated for the period immediately preceding the application of APB under conditions of GABAergic and glycinergic blockade. APB reduced the burst rate in Off ganglion cells but preferentially abolished bursting altogether in On ganglion cells. These effects were statistically significant (Mann–Whitney U test, \( p < 0.001 \); \( r \) = number of recordings; \( n \) = number of cells monitored). Bic, Bicuculline; Stry, strychnine.

Figure 10. Changing effects of neurotransmission on spontaneous rhythmic bursting in RGCs. A, Forms of neurotransmission from presynaptic cells and their effects on spontaneous activity in ganglion cells at P7 and at P20 (+, excitation; —, inhibition; X, nonparticipating via nicotinic receptors). B, Schematic showing developmental changes in the effect of glutamatergic, cholinergic, and GABAergic neurotransmission on the spontaneous bursting activity. GC, RGC; GCL, ganglion cell layer; INL, inner nuclear layer.
Developmental changes in the neurotransmitter regulation of spontaneous activity have also been observed in the retina of the embryonic chick (Sernagor and O'Donovan, 1997; Catiasca et al., 1998; Wong et al., 1998), as well as in other developing systems, such as in the hippocampus (Garaschuk et al., 1998) and spinal cord (Chub and O'Donovan, 1998; Milner and Landmesser, 1999). It is possible that conserved mechanisms exist in which excitatory cholinergic and GABAergic circuits are first assembled and then followed by the subsequent introduction of glutamatergic connections, coinciding with the excitatory-to-inhibitory reversal of GABAergic transmission (Ben Ari et al., 1997; Leinekugel et al., 1997). The changing regulatory control of spontaneous activity described here may be a reflection of the developmental rules by which many neuronal circuits are assembled at each stage. Lastly, other modes of transmission such as those mediated via muscarinic receptors may also impinge on the regulation of spontaneous bursting (Zhou and Zhao, 1999).

Mechanisms generating diversity in spontaneous activity patterns

In the early stage of development, before bipolar synaptogenesis, spontaneous activity propagates across the retina in the form of waves, with RGCs of all classes sharing a uniform activity pattern. The spatial and temporal features of this patterned activity are thought to contain information necessary to refine the connectivity of ganglion cell arbors in the lateral geniculate nucleus, namely, the segregation of RGC arbors into eye-specific laminae and the refinement of retinotopic maps (Wong, 1999). However, later in development, at the time of bipolar synaptogenesis, a diversification in bursting rhythms develops between neighboring cells; ganglion cells belonging to the On and Off subclasses begin to display two sets of distinct bursting rhythms (Wong and Oakley, 1996). The development of this diversity in patterned activity is suitable for directing the activity-dependent segregation of rod bipolars. Therefore, despite the possibility of additional loci for APB effects, we believe that the observed differences in APB response between On and Off bipolar cells are likely to provide separate glutamatergic drives onto On and Off ganglion cells (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1999). It is possible that conserved mechanisms exist in which glutamatergic signaling onto On bipolar cells through the mGluR6 receptor would hyperpolarize these cells. By contrast, Off bipolar cells, which lack the mGluR6 receptor altogether, will be depolarized by glutamate released in the OPL. As a result, On bipolar cells may produce a smaller synaptic output relative to Off bipolar cells, resulting in a relatively lower bursting rate in Off ganglion cells. Although maturation of the glutamatergic pathway appears to contribute to diversification of On and Off rhythms, changes in the intrinsic physiological properties of ganglion cells also need to be considered (Wang et al., 1997).

Our results also show that although Off ganglion cell activity persists in the presence of APB, it is slightly reduced from that in the controls (Fig. 9B), indicating that APB does not affect Off ganglion cell bursting solely. It is possible that APB may exert direct effects on ganglion cells via metabotropic glutamate receptors in addition to mGluR6 (Duvoisin et al., 1995). Other studies however have suggested that these direct effects may be small (Liets and Chalupa, 1996). In addition, rod bipolar cells, which are also hyperpolarized by APB, may exert an indirect effect on Off ganglion cells via all amacrine cells (Kolb and Famiglietti, 1974; McGuire et al., 1984) that form inhibitory glycinergic inputs onto Off ganglion cells. Indeed, the application of APB to mature retina has been found to increase the maintained discharge in Off ganglion cells, while decreasing that in On ganglion cells (Bolz et al., 1984). In the present study, we performed our APB experiments under conditions of glycinergic blockade that eliminate the indirect contribution of rod bipolar cells to spontaneous activity in ganglion cells. As such, the effects of APB on ganglion cell activity described here are unlikely to arise from the hyperpolarization of rod bipolars. Therefore, despite the possibility of additional loci for APB effects, we believe that the observed differences in APB response between On and Off ganglion cells are attributable primarily to a differential response on the part of On and Off cone bipolar cells.

Chronic in vivo applications of APB have been demonstrated to result in structural changes in the dendrites of developing ganglion cells (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995; Bisti et al., 1998). Thus, an intriguing question that remains is how spontaneous activity driven by On and Off bipolar signaling contributes not only to the activity-dependent remodeling of the axonal terminals of the On and Off ganglion cells but also to the shaping of the dendritic arbors of these ganglion cells.

REFERENCES


Wong et al. • Neurotransmitter Regulation of Spontaneous Retinal Activity


