Excitatory neurotransmission activates compartmentalized calcium transients in Müller glia without affecting lateral process motility

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Abstract

Neural activity has been implicated in the motility and outgrowth of glial cell processes throughout the central nervous system. Here we explore this phenomenon in Müller glia, which are specialized radial astroglia that are the predominant glial type of the vertebrate retina. Müller glia extend fine filopodia-like processes into retinal synaptic layers, in similar fashion to brain astrocytes and radial glia which exhibit perisynaptic processes. Using two-photon volumetric imaging, we found that during the second postnatal week, Müller glial processes were highly dynamic, with rapid extensions and retractions that were mediated by cytoskeletal rearrangements. During this same stage of development, retinal waves led to increases in cytosolic calcium within Müller glial lateral processes and stalks. These comprised distinct calcium compartments, distinguished by variable participation in waves, timing, and sensitivity to an M1 muscarinic acetylcholine receptor antagonist. However, we found that motility of lateral processes was unaffected by the presence of pharmacological agents that enhanced or blocked wave-associated calcium transients. Finally, we found that mice lacking normal cholinergic waves in the first postnatal week also exhibited normal Müller glial process morphology. Hence, outgrowth of Müller glial lateral processes into synaptic layers is determined by factors that are independent of neuronal activity.

Introduction

Bidirectional signaling between neurons and glia is essential for circuit formation and function throughout the nervous system. Across developmental steps from neurogenesis to circuit maturation, glia monitor their environment and in turn regulate neuronal production, migration, and differentiation, promote synapse turnover, and regulate synaptic function via neurotransmitter uptake and ion buffering¹. In the vertebrate retina, for example, Müller glia exhibit neurogenic potential²⁻⁴, promote circuit-specific wiring via secretion of synaptogenic molecules⁵, regulate phagocytosis of neuronal debris⁶, and limit neurotransmitter spillover via transporter activity⁷.

There is extensive evidence that neuronal signaling influences glial physiology in the adult brain. Individual astroglia extend fine processes that contact thousands of synapses and express an array of neurotransmitter receptors⁸. This enables glia to rapidly integrate neuronal activity, often involving intracellular calcium mobilization. Neuronal activity-evoked calcium events in astroglia range in size from small membrane-proximal microdomains to global cytosolic events mediated by various transmembrane proteins and calcium sources^{9,10}. Pharmacological studies suggest compartmentalized and global calcium events are mediated by separate mechanisms and evoke different functional responses within astroglia, which lead to differential effects on neurotransmission¹¹⁻¹³.

Here we use the mouse retina as a model to explore mechanisms and a possible function of neuron-glia signaling during development. In the retina, Müller glia are the predominant glial type, tiling the entire retinal space and interacting with every retinal cell type¹⁴. Similar to Bergmann glia of the cerebellum^{15,16}, Müller glia exhibit a radial structure with a stalk extending from the soma, lateral processes extending from the stalk within synaptic layers, and endfeet

contacting neuronal somata, axons, and vasculature. In the adult, this complex morphology enables Müller glia to mediate neurovascular coupling, maintain pan-retinal ion homeostasis, and modulate neuronal signaling in the adult^{17–19}. During both mouse²⁰, and zebrafish²¹ development, Müller glia undergo calcium transients in response to retinal waves, a term used to describe spontaneous bursts of depolarization that propagate across the retina. The functional relevance of wave-associated calcium signaling in Müller glia is not known.

One potential role for wave-associated calcium-signaling in Müller glia is in modulating outgrowth of lateral processes, which initiates and progresses during the same developmental window as retinal waves¹⁴. In other brain regions and model systems, neuronal activity-evoked calcium transients lead to morphological changes in astroglial processes. For example, in the hippocampus and somatosensory cortex, perisynaptic astrocytic processes undergo spatially localized, glutamate-evoked calcium transients which are essential for activity-evoked ensheathment of synapses²². In the cerebellum, Bergmann glial processes are highly motile during synaptogenic periods, and their ensheathment of Purkinje cells is impaired when glial glutamate transporters are knocked out^{16,23}. Radial glia of the *Xenopus* optic tectum exhibit neuronal activity-evoked calcium transients and motility during visual system development²⁴. In many of these systems, when glial motility is blocked, synaptic development, function, and plasticity are impaired²⁵, highlighting the importance of glial dynamics in setting up and maintaining neural circuits.

Here, we combine morphological and calcium imaging, electrophysiology, and pharmacology to characterize Müller glial lateral process outgrowth during retinal waves and to determine the impact of neuronal activity on Müller glial morphology.

Results

Müller glial lateral process growth is non-uniform and dynamic across the IPL

Our goal is to determine whether spontaneous activity driven by retinal waves influences the morphological development of Müller glial cells. After their differentiation from retinal progenitor cells, which occurs during the first postnatal week, Müller glia extend processes laterally from their stalk into the inner plexiform layer (IPL)²⁶. To visualize this process, we used the *Slc1a3*-CreER;mTmG reporter line to sparsely label Müller glia (Figure 1A). Lateral processes exhibited sublayer-specific outgrowth and distribution, and they reached an adult level of complexity soon after eye opening around postnatal day 14 (P14) (Figure 1B,D; see Tables 1 and 2 for statistical comparisons across sublayer and age, respectively). During the first days of Müller glia differentiation, lateral processes preferentially occupied the borders of the IPL (putative S1/S5), with most outgrowth occurring in the prospective ON half of the IPL (S3-S5) until about P10. By eye opening, after P12, processes arborized throughout the IPL and began to resemble their adult distribution, characterized by fewer processes in IPL sublayers S2/S4 than in S1/S3/S5. These observations are consistent with previous findings using the same mouse line and immunohistochemistry in fixed tissue¹⁴.

During outgrowth from the primary stalks, we observed that Müller glial lateral processes across the IPL were highly motile. To characterize process motility, we conducted volumetric two-photon imaging of sparsely labeled processes at a rate of roughly 1 volume every 2 minutes for 10-minute epochs. We characterized motility that occurred during the imaging period by grouping events into distinct categories as follows: new processes that branched from stalks or from existing processes, extension of existing processes, retraction of processes without elimination, elimination of existing processes, or stable for processes that did not change length.

We made several observations based on this analysis (Figure 1E). First, Müller glial cell lateral processes were highly motile across the IPL throughout the entire second postnatal week. Second, there was a slight bias toward new process sprouting rather than process loss. Third, we found there were roughly equal proportions of extending and retracting processes over 10-minute epochs of imaging, highlighting their rapid turnover during this period of development. Finally, we observed a sharp developmental transition toward stability a few days after eye opening (Figure 1C,E,F; Figure 1-figure supplement 1; Table 3).

To assure that we could observe and quantify changes in process motility, we applied two manipulations. First, we slowed process motility with pharmacological agents that disrupt cytoskeletal proteins. Process motility was reduced by bath application of the microtubule-disrupting agent nocodazole (10 μ M) and the actin polymerization inhibitor cytochalasin-D (5 μ M), the combination of which led to processes stabilization (Figure 1G; Table 4). It is unlikely that this loss of motility resulted from damaged cell health, as Müller glial process morphology remained normal in the presence of cytochalasin and nocodazole. Müller glia, like astroglia throughout the brain, exhibit reactive gliosis associated with hypertrophy and expression of filamentous membrane processes when exposed to damage²⁷, which we did not observe here. Second, we enhanced process motility by bath application of epidermal growth factor (EGF; 1 unit (100ng)/mL; Figure 1H; Table 5), which activates EGF receptors (EGFRs). EGFRs are expressed by Müller glia during the second postnatal week²⁸ and increase motility in other cells via activation of Rac- and Rho-GTPase-dependent pathways^{29,30}. Together, these results indicate that lateral process motility is facilitated by turnover of actin filaments and microtubules and may be modulated by growth factor signaling, pathways which potentially interact with neuronal activity^{31,32}.

Retinal waves activate compartmentalized calcium transients in Müller glia

Much of the morphological development of Müller glia occurs when retinal waves are present (Figure 1 and [14]). Furthermore, previous studies have demonstrated that retinal waves induce increases in intracellular calcium in Müller glia^{20,21}. However, these studies did not assess whether there are distinct calcium compartments within Müller glia which could potentially have distinct impacts on lateral process motility. The two compartments in Müller glia we focused on were the central stalks and the lateral processes within the IPL.

We conducted simultaneous two-photon calcium imaging of Müller glial stalks and processes and voltage clamp recordings from retinal ganglion cells (RGCs). Several strategies for calcium imaging from Müller glia were used. First, retinas were isolated from *Slc1a3*-CreER; cyto-GCaMP6f^{flox} mice, which express genetically encoded calcium indicator selectively in Müller glia. Second, a subset of retinas were isolated from WT mice and bath-loaded with the chemical calcium dye Cal520, which selectively labels Müller glia^{20,33}. Using these two approaches, we were able to clearly identify the boundaries of Müller glial stalks, while calcium in lateral processes was assessed using regions of interest (ROIs) within the areas of IPL intervening glial stalks (Figure 2A). Third, retinas were isolated from mice expressing a membrane-bound calcium reporter in Müller glia (Lck-GCaMP6f in *Slc1a3*-CreER mice)³⁴, which enabled resolution of lateral processes but poor detection of cytosolic calcium transients within stalks (Figure 2-figure supplement 1). Using all of these approaches, we verified that Müller glial stalks and lateral processes exhibited calcium transients in response to retinal waves as previously reported²⁰, as well as spontaneous, non-wave-associated calcium transients.

We observed several differences in wave-associated calcium transients between stalks and lateral processes. Wave-associated stalk transients propagated throughout the stalk and involved many

processes, which we refer to as a global transient. Lateral processes participated in some global transients but also exhibited wave-associated calcium transients independent of stalks (Figure 2A,B and Figure 2-figure supplement 1). Hence, lateral processes and stalks differentially participated in waves, with a greater proportion of lateral process ROIs responding to waves than stalk ROIs. In addition, wave-associated calcium transients in lateral processes were consistently observed from P9 to P12, while those in stalks were significantly reduced in terms of $\Delta F/F$ amplitude and proportion of stalks participating by P11/12 (Figures 2C, 3A; Table 6). Finally, the subset of stalks that responded to retinal waves did so with a slight delay relative to lateral processes (Figure 2B,D; Table 7). Hence, Müller glial stalks and lateral processes can undergo spatiotemporally distinct calcium signaling events.

We next sought to test whether wave-associated calcium transients in stalks vs. lateral processes are mediated via activation of distinct signaling pathways. Muscarinic acetylcholine receptors (mAChRs) have been implicated in Müller glial responses to retinal waves,²⁰ and isolated Müller glia undergo M1 mAChR-dependent responses to cholinergic agonists³⁵. Bath application of pirenzepine (5 μ M), a selective antagonist of M1 mAChRs, led to a significant reduction in wave-associated calcium transients in stalks and lateral processes, (Figure 3A). The magnitude of this effect was age- and compartment-dependent, as wave-associated transients in Müller glial stalks had greater sensitivity to M1 mAChR block at P9/10 than at P11/12, and pirenzepine sensitivity was significantly greater in stalks than in lateral processes at early ages (Figure 3B,C; Tables 8-11). Interestingly, we occasionally detected wave-associated stalk calcium transients that were insensitive to pirenzepine. Pirenzepine-insensitive stalk responses exhibited similar amplitude and latency to those of lateral processes (Figure 3C,D; Table 12). These data suggest that wave-associated responses in stalks are controlled by multiple mechanisms in addition to

activation of M1 mAChRs. Note, pirenzepine had minimal impact on the amplitude and frequency of compound excitatory postsynaptic currents (EPSCs) and inter-wave-interval (IWI, Figure 3E; Table 13), indicating pirenzepine had a selective effect on Müller glial M1 mAChRs, rather than on wave-generating circuits.

To confirm that M1 mAChR signaling preferentially impacts wave-associated stalk transients, we enhanced acetylcholine (ACh) release during waves by bath-applying the GABA_A receptor antagonist gabazine (5 μ M)³⁶. As expected, the presence of gabazine increased the amplitude of EPSCs recorded from RGCs during retinal waves (Figure 4E; Table 19). Gabazine also increased the proportion of stalks and lateral process ROIs that underwent wave-associated calcium transients (Figure 4A,B; Table 14), but with differential compartment-specific effects: there was a significantly larger gabazine-induced fold change in proportion of stalks compared with lateral processes responding to waves, and gabazine led to a nearly 2-fold increase in the amplitude of wave-evoked fluorescence change in stalks, while only slightly increasing the response amplitude in lateral processes (Figure 4C; Tables 15-17). Subsequent application of pirenzepine reduced the extent, magnitude, and latency of stalk responses to waves while largely sparing lateral process responses (Figure 4B-D; Tables 14-18) and without altering wave properties (Figure 4E; Table 19). This effect was independent of age from P9 to P12; pirenzepine suppressed wave-associated transients in stalks following gabazine-mediated upregulation of responses throughout this period. Taken together, these data indicate that M1 mAChRs mediate wave-associated global calcium transients in Müller glial stalks and support the conclusion that stalks and lateral processes are functioning as distinct calcium compartments, with lateral processes undergoing non-M1 mAChR-mediated calcium transients during retinal waves.

Müller glial process motility occurs independent of wave-associated calcium transients

Is Müller glial lateral process motility impacted by neuronal activity? To test whether neuronal activity influences Müller glial lateral process motility, we assessed the impact of multiple pharmacological agents on process motility after the first postnatal week (Figure 5). First, we assessed the impact of agents that modulated mAChR signaling resulting from neural activity (Figure 5A). Gabazine, which potentiated calcium transients in Müller glial processes and stalks, in part via activation of M1 mAChRs, did not change the proportion of processes undergoing motility. Similarly, there was no significant change in lateral process motility in the presence of pirenzepine, which blocks M1 mAChRs and preferentially abolished wave-associated calcium transients in Müller glial stalks (Figure 5A,F; see Table 20 for summary statistics). Second, we assessed the impact of glutamatergic signaling, which is also implicated in wave-associated Müller glial calcium transients^{20,21}. TBOA blocks glutamate transporters and enhances wave-associated calcium transients in Müller glia by increasing glutamate spillover, while DNQX and AP5 block glutamatergic retinal waves and reduce calcium transients in Müller glia^{20,37} (Figure 3-figure supplement 1). Similar to our results in gabazine and pirenzepine, the presence of TBOA or DNQX/AP5 had no significant impact on motility (Figure 5B,F). These observations together indicate that lateral process motility does not arise from wave-associated calcium transients in stalks or processes.

To further explore a role for ACh and glutamate in influencing motility, we tested whether local release of neurotransmitter would alter the motility of nearby processes, as is the case for perisynaptic astrocytic processes elsewhere in the brain, where it is postulated that local elevations in neurotransmitter promote process growth and synapse coverage^{22,38}. We locally perfused via continuous iontophoresis the AChR agonist carbachol or glutamate onto Müller glial processes in *Slc1a3*-CreER;mTmG retinas with sparse Cre-mediated recombination. In

parallel experiments using retinas from *Slc1a3*-CreER;GCaMP6f^{flox} mice, we confirmed that this method induced strong calcium transients in stalks and lateral processes. Despite this, we observed no impact of local perfusion of agonists on Müller glial lateral process motility (Figure 5C,D,F).

As a final test for whether intracellular calcium signaling in Müller glia is required for motility, we assessed motility after bath-loading the potent calcium chelator, BAPTA-AM. Despite blocking retinal waves and all calcium transients in Müller glia (Figure 5-figure supplement 1), the presence of BAPTA did not alter the proportion of processes exhibiting motility. Together these data suggest that motility of Müller glial lateral processes persists in absence of intracellular calcium transients or neuronal activity (Fig. 5E,F). We note however that some of these observations were made using relatively small numbers of cells, and we cannot rule out a small effect of these pharmacological perturbations on process motility given our sample size and high cell-to-cell variability in lateral process motility.

Long-term blockade of cholinergic retinal waves does not alter Müller glial morphology

Thus far, we have assessed the role of neuronal activity and calcium signaling on Müller glial lateral process motility using live imaging during the second postnatal week, while there is lateral process outgrowth. However, during the first postnatal week, there are wave-associated calcium transients in Müller glia also mediated by activation of mAChRs²⁰. To test whether retinal wave-associated calcium signaling during this first postnatal week influences later process outgrowth during the second postnatal week, we assessed Müller glial morphology in retinas isolated from mice lacking the $\beta 2$ nicotinic acetylcholine receptor subunit ($\beta 2$ -nAChR-KO). $\beta 2$ -nAChR-KO mice exhibit significantly reduced cholinergic retinal waves^{39–41} and therefore have reduced M1 mAChR-induced signaling in glial cells.

Morphology of individual Müller glial cells was assessed by filling them with Alexa-488 via sharp pipette in P12 and P30 wild type and $\beta 2$ -nAChR-KO mice. Filled cells were visualized via 2-photon volumetric imaging in live tissue, and processes were traced for subsequent morphological analysis (Figure 6A-C). At both P12 and P30, individual dye-filled Müller cells exhibited lateral processes throughout the IPL, with more processes in the ON-half of the IPL than in the OFF-half prior to eye opening, in agreement with Figure 1. Detailed assessment shows that by P12, Müller glial lateral processes exhibited nearly the same level of complexity as observed in the adult. In addition, we found no significant difference between wild type control and $\beta 2$ -nAChR-KO Müller glia in terms of several morphological parameters including complexity, number, area, and length of lateral processes. However, we did observe a significant reduction in number of primary branches projecting from the stalk in $\beta 2$ -nAChR-KO Müller glia before eye opening, while in mature retina all morphological properties of in $\beta 2$ -nAChR-KO Müller glia appeared normal (Figure 6D,E; Table 21). This suggests that although retinal waves may play a role in process sprouting from Müller glial stalks during development, other mechanisms produce lateral processes in normal numbers and appearance as the retina matures.

Discussion

In this study, we showed that as Müller glial lateral processes emerge during the second postnatal week of development, they exhibit both rapid motility and retinal wave-associated calcium transients that are partially compartmentalized. Global calcium transients in stalks were delayed relative to transients in lateral processes, were inhibited by an M1 mAChR antagonist, and became smaller and less frequent after P10. In contrast, wave-associated calcium transients in lateral processes often occurred locally and independent of stalk transients, were less sensitive to M1 mAChR block, and persisted throughout the second postnatal week. However, in contrast to

astrocytes and radial glia in other regions of the central nervous system (CNS)^{12,16,22,24}, Müller glial lateral process motility and outgrowth occurred independent of neuronal signaling and calcium transients. These results indicate that Müller glial process morphology in the IPL is not regulated by neuronal activity.

Mechanisms underlying Müller glial process motility

Glial morphology is critical for normal development of circuits. For example, a plexus of glial processes provides a diffusional barrier for neurotransmitters and other signaling molecules^{7,42}, undergoes contact-mediated signaling with neurites to modulate synapse formation and function^{43,44}, and participates in synaptic pruning via activation of phagocytic pathways in glia⁴⁵. Across brain regions, neuron-glia signaling impacts glial morphology in diverse ways. We found in the retina that Müller glial lateral process motility during development was not impacted by neuron-glia signaling via release of neurotransmitter. This is similar to Bergmann glia, radial glia of the cerebellum, whose process motility is developmentally regulated and not directly affected by perturbations of neuronal activity or calcium influx¹⁶. Similarly in microglia, another highly motile cell type, chelation of calcium with BAPTA-AM slightly slowed but did not block process motility⁴⁶. In striatal astrocytes, FRET-based synaptic proximity assays have shown that although fine branches interact with synapses, these interactions are stable and unaffected by neuronal activity⁴⁷. These findings stand in contrast to perisynaptic processes of hippocampal astrocytes, in which metabotropic glutamate receptor (mGluR) activation leads to localized increase in intracellular calcium which promotes process motility and coverage of dendritic spines²². This divergence between Müller glia and hippocampal astrocytes might be reflective of differences in ultrastructural interactions between astroglial processes and synapses in these two systems: perisynaptic processes of hippocampal astrocytes engage in true tripartite synapses in a

brain region with high plasticity^{48,49}, while there is currently no published evidence revealing similar structures in the retina. Further, astrocytic processes express specific actin-binding proteins such as ezrin which enable coupling of cytoskeletal dynamics with signaling through mGluRs⁵⁰, which were previously shown to have minimal contribution to retinal wave-associated responses in Müller glia²⁰.

Multiple pathways independent of neuronal activity may regulate morphological dynamics in Müller glial processes. One possibility is the release of growth factors from neurons or other nearby cells, which has been implicated in morphological changes among astrocytes in other systems^{51,52}. This hypothesis is supported by our finding that application of exogenous EGF enhanced process motility prior to eye opening. Interestingly, EGFR expression in Müller glia is high prior to eye opening and declines with a developmental time course that matches our observed decline in motility²⁸. Another intriguing possibility is that repulsive homotypic interactions between lateral processes from neighboring Müller glia underly their motility. This idea is supported by single-cell photoablation experiments in zebrafish Müller glia and mouse Schwann cells, in which processes from non-ablated cells filled in the territory vacated by ablated cells^{53,54}. Further study will be required to determine whether these or other alternative pathways mediate Müller glial lateral process motility during development.

Calcium compartments in Müller glia

We observed two distinct calcium compartments in Müller glia activated by retinal waves. Compartmentalization of calcium within Müller glial stalks was achieved via activation of glial M1 mAChRs, while lateral processes exhibited non-M1 mAChR-mediated transients with a smaller latency than those in stalks. We observed an age-dependent reduction in stalk participation in waves, while lateral processes continued to respond through P12. (Note, our

299 observation that lateral processes continue to respond to retinal waves from P9 – P12 contrasts
300 with a previous study that reported an overall reduction in Müller glial responses to waves at
301 these ages²⁰. We attribute this difference to improved sensitivity of GCaMP6f over GCaMP3).
302 The reduced stalk participation observed in older animals may be due to downregulation of M1
303 mAChRs prior to eye opening, or to a reduction in volume release of acetylcholine during the
304 transition to glutamatergic waves⁵⁵. Our observation that gabazine application during
305 glutamatergic waves caused wave-associated stalk transients to return in an M1 mAChR-
306 dependent manner suggests that the latter is true.

307 Gabazine application also enhanced wave-associated calcium transients in lateral processes via a
308 mechanism independent of M1 mAChR activation. We suspect this is due to enhanced release of
309 other excitatory neurotransmitters following relief of GABA_A receptor-mediated inhibition in the
310 presence of gabazine during retinal waves. This is similar to the action of TBOA, which blocks
311 glutamate transporters and subsequently increases retinal wave frequency and wave-associated
312 Müller glial calcium transients due to an increase in extracellular glutamate levels²⁰.

313 Because astroglia and neurons both express neurotransmitter receptors and transporters, it is
314 interesting to consider which of these cell populations is targeted by the antagonists used in this
315 study. Current RNA-sequencing datasets suggest that Müller glia do not express GABA
316 receptors at an appreciable level and therefore it is unlikely that gabazine directly impacts
317 signaling in these cells. That said, Müller glia express GABA transporters which might
318 contribute to signaling during retinal waves^{7,56}. They also express glutamate transporters
319 (EAAT1) which are targeted by TBOA. At 25 μ M, the concentration of TBOA used to modulate
320 retinal waves in this study was likely too low to effectively inhibit glial EAAT1 (IC₅₀: 70 μ M),
321 and more likely affects retinal wave properties via neuronal EAATs⁵⁷. Furthermore, although we

did not test whether pirenzepine acts on neuronal mAChRs, it is possible that blockade of non-Müller glial M1 mAChRs by pirenzepine indirectly affects glial responses to retinal waves, as previous studies have revealed expression of these receptors in retinal interneurons^{56,58,59}. However, our observation that retinal wave-associated EPSC amplitude and inter-wave interval remained normal in the presence of pirenzepine is consistent with interpretation that pirenzepine acts directly on glial M1 mAChRs rather than on wave-generating circuits (Figure 3E, 4E).

Roles of calcium compartmentalization in astroglia

Subcellular calcium compartmentalization plays a variety of roles in neuron-glia signaling and tissue homeostasis⁶⁰. Hippocampal astrocytic processes undergo at least two types of spatiotemporally distinct calcium transients in response to nearby synaptic activity, depending on the type of synaptic activity that occurs. This calcium signaling is thought to regulate synaptic transmission within the astrocytic territory¹¹. Similarly, compartmentalized calcium transients during startle response frequently occur in distal branches, and less frequently in somata of hippocampal astrocytes in awake mice. Distinct signaling pathways, including those involving transmembrane calcium channels or transporters, adrenergic receptors, and IP3 receptors, differentially contribute to calcium transients in branches vs. somata⁶¹. Another study used a computational approach to reveal how calcium influx in fine astrocytic branches can be mediated by glutamate transporter-dependent activity of Na⁺/Ca⁺⁺ exchangers, while somatic calcium can be modulated by mGluR-dependent activation of IP₃ signaling⁶². Compartmentalization between Müller glial stalks and processes may arise by a similar mechanism.

We found that wave-associated compartmentalized calcium activity is not required for Müller glial process motility, so this activity likely plays a role in other functions of Müller glia during retinal development. These functions include calcium-dependent neurovascular coupling⁶³,

345 release of gliotransmitters such as ATP or D-serine^{17,18,64}, or secretion of synaptogenic molecules
346 such as thrombospondin or growth factors^{5,65}. Our identification of M1 mAChR as a driver of
347 wave-associated calcium transients in stalks provides a target for selective perturbation in Müller
348 glia to better define a role for these transients in retinal circuit development.

349 Materials and methods

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (<i>Mus musculus</i> ; male & female)	C57BL/6J (wild type)	The Jackson Laboratory	RRID:IMS R_JAX:00 0664	Background strain used for mouse crosses
Strain, strain background (<i>Mus musculus</i> ; male & female)	<i>Slc1a3</i> -CreER; Tg(<i>Slc1a3</i> -cre/ERT)1Nat/J; also known as GLAST-CreER	The Jackson Laboratory	RRID:IMS R_JAX:01 2586	Expresses tamoxifen-inducible Cre/ERT in Müller glia
Strain, strain background (<i>Mus musculus</i> ; male & female)	mTmG; mT/mG; B6.129(Cg)- <i>Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)^{Luo}/J</i>	The Jackson Laboratory	RRID:IMS R_JAX:00 7676	Used to measure morphology/motility in Müller glia
Strain, strain background (<i>Mus musculus</i> ; male & female)	GCaMP6f ^{flox} ; B6.129S(Cg)- <i>Gt(ROSA)26Sor^{tm95.1}(CAG-GCaMP6f)^{Hze}/J</i>	The Jackson Laboratory	RRID:IMS R_JAX:02 4105	Used to measure calcium dynamics in Müller glial stalks and processes
Strain, strain background (<i>Mus musculus</i> ; male & female)	Lck-GCaMP6f ^{flox} ; C57BL/6N- <i>Gt(ROSA)26Sor^{tm1}(CAG-GCaMP6f)^{Khakh}/J</i>	The Jackson Laboratory	RRID:IMS R_JAX:02 9626	Used to measure calcium dynamics in Müller glial processes
Strain, strain background (<i>Mus musculus</i> ; male & female)	<i>PDGFRa</i> -Cre; C57BL/6-Tg(<i>Pdgfra</i> -cre)1Clc/J	The Jackson Laboratory	RRID:IMS R_JAX:01 3148	Used to verify loss of calcium transients following BAPTA-AM application
Strain, strain background (<i>Mus musculus</i> ; male & female)	β 2-nAChR-KO; C57BL/6J- <i>Chrn2^{tm1Mdb}</i>	Bansal et al., <i>J Neurosci</i> , 2000. doi: https://doi.org/10.1523/J	RRID:MG I:2663180	Targeted deletion generated by Xu et al. (<i>J Neurosci</i> , 1999) ⁶⁶

female)		NEUROSCI.20-20-07672.2000		and maintained in our lab since
Chemical compound, drug	4-hydroxytamoxifen (4-OHT, 50:50 E:Z isomers)	Sigma-Aldrich	T176	Injected at variable doses to induce uniform or sparse Cre-mediated recombination in Müller glia
Chemical compound, drug	Cytochalasin-D (from <i>Zygosporium mansonii</i>)	Avantor/VWR	supplier no.: 250255 catalog number: 80055-214	Disrupts actin filaments and inhibits actin polymerization
Chemical compound, drug	Nocodazole	Sigma-Aldrich	M1404	Disrupts microtubule assembly/disassembly
Chemical compound, drug	Epidermal growth factor (EGF) (Rac/Cdc42 Activator II)	Cytoskeleton, Inc.	CN02	EGF receptor agonist
Chemical compound, drug	Pirenzepine dihydrochloride	Tocris	1071	M1 mAChR antagonist
Chemical compound, drug	Gabazine (SR 95531 hydrobromide)	Tocris	1262	GABA _A receptor antagonist
Chemical compound, drug	DL-TBOA	Tocris	1223	Glutamate transporter (EAAT) antagonist
Chemical compound, drug	DNQX disodium salt	Tocris	2312	AMPA antagonist
Chemical compound, drug	DL-AP5	Tocris	3693	NMDAR antagonist
Chemical compound, drug	BAPTA-AM	Tocris	2787	Intracellular calcium chelator
Chemical compound, drug	Carbachol (carbamoylcholine chloride)	Tocris	2810	Cholinergic agonist
Chemical compound, drug	L-glutamic acid (Glu)	Sigma-Aldrich	56-86-0	Glutamatergic agonist

Chemical compound, drug	Alexa fluor 488/594 hydrazide, sodium salt (Alexa-488/594)	ThermoFisher Scientific	A10436/A10438	Used for filling Müller glia to visualize processes, and as a counterdye for focal agonist application
Software, algorithm	ScanImage	Vidrio Technologies, LLC	RRID:SC R_014307	2-photon image acquisition software
Software, algorithm	FIJI/ImageJ	Created at National Institutes of Health; Schindelin et al., <i>Nature Methods</i> , 2012. doi:10.1038/nmeth.2019	RRID:SC R_002285	Used for image processing and analysis
Software, algorithm	MATLAB	MathWorks	RRID:SC R_001622	Custom scripts used for data processing, analysis
Software, algorithm	NormCorre: Non-Rigid Motion Correction	Flatiron Institute, Simons Foundation; Pnevmatikakis and Giovannucci, <i>J Neurosci Methods</i> , 2017. doi: https://doi.org/10.1016/j.jneumeth.2017.07.031		For 2D registration of calcium imaging movies
Software, algorithm	FIJI plugin: Distance Transform Watershed	Legland, et al., <i>Bioinformatics</i> , 2016. doi: 10.1093/bioinformatics/btw413		For segmentation of Müller glial stalks in calcium imaging movies
Software, algorithm	FIJI plugin: Correct 3D Drift	Parslow et al., <i>J Vis Exp</i> , 2014. doi: 10.3791/51086.		For 3D registration of glial morphology time courses
Software, algorithm	FIJI plugin: Simple Neurite Tracer	Arshadi et al., <i>Nat Methods</i> , 2021. doi:10.1038/s41592-021-01105-7	RRID:SC R_016566	For tracing of dye-filled glial processes and stalks
Software, algorithm	R; RStudio	R Project for Statistical Computing; RStudio	RRID:SC R_001905; RRID:SC R_000432	Used for statistical analyses

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351

Animals

All mice were purchased from The Jackson Laboratory and were maintained on mixed C57BL/6 backgrounds. For motility experiments, P8-P116 *Slc1a3*-CreER;mTmG mice were generated by cross breeding *Slc1a3*-CreER mice (strain 012586; also known as GLAST-CreER) with *Rosa26^{mTmG}* mice (strain 007676)¹⁴. *Slc1a3*-CreER mice express tamoxifen-inducible Cre recombinase under control of a Müller glia-specific promoter. mTmG is a dual-fluorescence reporter line which constitutively expresses membrane-bound tdTomato, and upon Cre-mediated recombination expresses membrane-bound green fluorescent protein (mGFP). For calcium imaging experiments, P9-P12 *Slc1a3*-CreER;GCaMP6f^{flox} mice were generated by crossing *Slc1a3*-CreER mice with GCaMP6f^{flox} (cytosolic) mice (strain 024105) or Lck-GCaMP6f^{flox} (membrane-bound)³⁴ mice (strain 029626) to enable specific and inducible calcium indicator expression in Müller glia. To verify loss of calcium transients in neurons and Müller glia following BAPTA-AM application, we include imaging results from *PDGFRα*;cyto-GCaMP6f^{flox} mice (*PDGFRα*-Cre: strain 013148), which label Müller glia and a subset of neurons with cytosolic GCaMP6f, as well as results from *Slc1a3*-CreER;Lck-GCaMP6f^{flox} retinas (Figure 5 – figure supplement 1). For sharp fills of Müller glial cells with fluorescent dye, we used C57BL/6 mice as controls for comparison with mice lacking the $\beta 2$ subunit of the nicotinic acetylcholine receptor ($\beta 2$ -nAChR-KO).

Cre-mediated recombination was induced via intraperitoneal injection of 4-hydroxytamoxifen (50:50 E and Z isomers, Sigma-Aldrich) dissolved in sunflower seed oil. For uniform expression across the retina in neonates, injections of 0.5 mg tamoxifen were made 2 and 4 days before each experiment. For sparse expression to enable resolution of individual Müller glia during imaging, a single injection of 0.1 mg tamoxifen was made 2 days before each experiment.

375 All animal procedures were approved by the University of California, Berkeley Animal Care and
376 Use Committee and conformed to the NIH Guide for the Care and Use of Laboratory Animals,
377 the Public Health Service Policy, and the SFN Policy on the Use of Animals in Neuroscience
378 Research.

379 *Retinal preparation*

380 For all experiments, male and female mice were anesthetized via isoflurane inhalation and
381 decapitated. Eyes were enucleated and retinas dissected in oxygenated (95% O₂/5% CO₂)
382 artificial cerebrospinal fluid (ACSF) at room temperature under bright field (less than P10) or
383 infrared (P10 and above) illumination. ACSF contained [in mM] 119.0 NaCl, 26.2 NaHCO₃, 11
384 glucose, 2.5 KCl, 1.0 K₂HPO₄, 2.5 CaCl₂, and 1.3 MgCl₂. Isolated retinas were mounted
385 ganglion cell side up on filter paper (Millipore) and transferred into the recording chamber of an
386 upright microscope for imaging and electrophysiological recording. Retinas were continuously
387 superfused with oxygenated ACSF (2-4 ml/min) at 32°-34°C for the duration of experiments and
388 kept in the dark at room temperature in oxygenated ACSF when not imaging or recording. In a
389 subset of experiments, wild type retinas were bath-loaded with the organic calcium dye Cal520
390 (12 µM) for 1.5-2 hours prior to performing calcium imaging.

391 *Two-photon imaging*

392 Two-photon imaging of Müller glia in the IPL was performed using a modified movable
393 objective microscope (MOM; Sutter Instruments) equipped with an Olympus 60X, 1.0 NA,
394 LUMPlanFLN objective (Olympus America). Two-photon excitation was evoked with an
395 ultrafast pulsed laser (Chameleon Ultra II; Coherent) tuned to 920 nm for all fluorophores. The
396 microscope was controlled by ScanImage software (www.scanimage.org). Scan parameters were

[pixels/line x lines/frame (frame rate in Hz)]: 256 x 256 (1.48–2 .98), at 1–2 ms/line. When imaging GCaMP6f fluorescence in Müller glial stalks and processes, the focal plane was set to ~1/3 the distance from the ganglion cell layer to the inner nuclear layer. Volumetric imaging of motility in mGFP-expressing Müller glial processes and of surrounding tdTomato-expressing cells was achieved by acquiring sequential two-channel Z-stacks through the entire IPL, with slices 1 μ m apart and averaging 4 frames per slice. Volumes were taken every 2 minutes for a total of 10 minutes of imaging lateral process dynamics. A similar procedure was used for imaging motility in a subset of cells after filling them with fluorescent dye.

Electrophysiological recordings

Whole-cell voltage clamp recordings were made from whole mount retinas while simultaneously imaging GCaMP6f fluorescence. Under infrared illumination, RGC somas were targeted for voltage clamp recordings using glass microelectrodes with resistance of 3–5 M Ω (PC-10 pipette puller; Narishige) filled with an internal solution containing [in mM] 110 CsMeSO₄, 2.8 NaCl, 20 HEPES, 4 EGTA, 5 TEA-Cl, 4 Mg-ATP, 0.3 Na₃GTP, 10 Na₂Phosphocreatine, and QX-Cl (pH 7.2 and 290 mOsm). The liquid junction potential correction for this solution was -10 mV. Signals were acquired using pCLAMP10 recording software and a MultiClamp 700A amplifier (Molecular Devices), sampled at 20 kHz and low-pass filtered at 2 kHz.

Pharmacology

For pharmacology experiments, after 5–10 minutes of recording data in ACSF, pharmacological agents were added to the perfusion, and experimental recordings were obtained 5 minutes afterward. Drug concentrations were as follows: 5 μ M cytochalasin-D (Avantor), 10 μ M nocodazole (Sigma-Aldrich), 1 unit (100ng)/mL EGF (Cytoskeleton, Inc.), 5 μ M pirenzepine

(Tocris), 5 μ M gabazine (Tocris), 25 μ M DL-TBOA (Tocris), 20 μ M DNQX (Tocris), 50 μ M AP5 (Tocris), 200 μ M BAPTA-AM (Tocris). DL-TBOA and BAPTA-AM were prepared in 0.1% DMSO. For calcium chelation experiments, whole mount retinas were incubated in BAPTA-AM or vehicle (ACSF/0.1% DMSO) for 1.5-2 hours, and then moved to ACSF for 30 minutes prior to imaging⁶⁷. To verify that BAPTA-AM loading abolished retinal waves and residual calcium activity in cytosolic and membrane-proximal compartments, we measured calcium activity in neurons and Müller glia using cyto-GCaMP6f^{flox} (crossed with *PDGFR α -Cre*) or Lck-GCaMP6f^{flox} (crossed with *Slc1a3-CreER*) at P10-P12 before and after BAPTA-AM loading.

Focal agonist iontophoresis

For focal agonist application to mGFP-expressing lateral processes in *Slc1a3-CreER;mTmG* retinas, carbachol (10 mM, Tocris) or glutamic acid (10 mM, Sigma-Aldrich) was loaded into sharp electrodes pulled on a P-97 Micropipette Puller (Sutter) with a resistance of 100-150 M Ω . Electrodes also contained 2 mM Alexa-594 for verification of iontophoresis and to visualize electrodes under 2-photon illumination. While imaging at 920nm and visualizing fluorescence from mGFP and Alexa-594, electrodes were driven into the IPL at a $\sim 15^\circ$ angle using a micromanipulator set to the 'DIAG' function. For each cell, the tip of the electrode was placed 1-5 μ m from the arbor of lateral processes of interest. Iontophoresis of agonist or control was achieved by applying continuous current using pCLAMP10 software. To apply carbachol, which is positively charged at physiological pH, continuous current of +4 nA was used, while -4 nA current was used to apply glutamate, which is negatively charged. Current of opposite polarity to that used for each respective agonist application was applied as a control, and the order of control vs. agonist application was shuffled to negate potential effects resulting from damage

caused by the electrode. In a separate set of experiments, iontophoresis of agonist was performed in *Slc1a3*-CreER;cyto-GCaMP6f^{flox} or *Slc1a3*-CreER;Lck-GCaMP6f^{flox} retinas to verify that this method reliably evoked calcium transients in lateral processes.

Müller glial sharp fills

For a subset of experiments testing the role of retinal wave-evoked calcium transients in Müller glial motility (Figure 5), and to compare Müller glial morphology in wild type and β 2-nAChR-KO retinas (Figure 6), sharp electrodes were pulled as described above, and the tip was bent 15-20° using a microforge. Electrodes were loaded with 2mM Alexa-488 in water, and iontophoresis of dye into single Müller glial cells was achieved by applying a -10 nA pulse for 500 ms in MultiClamp 700A software while the electrode was on the membrane of Müller glial endfeet in the ganglion cell layer. Electrodes were withdrawn as soon as cells started to fill with dye, and cells were imaged 1-5 minutes after filling⁶⁸. 2-photon volumetric images of dye-filled lateral processes of single Müller glial cells in the IPL were acquired using the same image parameters used for imaging motility, as described above.

Image processing and analysis: Calcium imaging

All images were processed using custom scripts in FIJI/ImageJ (National Institutes of Health)⁶⁹ and MATLAB (MathWorks). For calcium imaging movies, following non-rigid motion correction⁷⁰, regions of interest (ROIs) were semi-automatically placed over stalks using a filtering algorithm based on a Laplace operator and segmented by applying a user-defined threshold⁷¹. The Distance Transform Watershed function⁷² in FIJI was used to segregate nearby stalks in binarized images. This method defined most of the ROIs that an experienced user would recognize by eye. Manual adjustments were made to include stalks that were missed and to

remove ROIs that were erroneously placed on structures other than stalks, which were defined as semiregularly spaced, punctate regions of fluorescence 2-3 μm in diameter in average intensity projection images. Lateral process ROIs were subsequently defined by randomly placing 250 2.5 μm x 2.5 μm squares in regions that did not overlap with stalk ROIs. Fluorescence intensity is reported as the average intensity across all pixels within the area of each ROI, and normalized as the relative change in fluorescence ($\Delta F/F$) as follows:

$$\frac{\Delta F}{F} = (F - F_0)/F_0,$$

where F is the instantaneous fluorescence at any time point and F_0 is the baseline fluorescence, defined as the median fluorescence value over the duration of the trace.

$\Delta F/F$ traces were smoothed using a 2-frame median filter and analyzed to detect calcium transients using custom MATLAB code. Traces were Z scored, and a threshold Z score of 3 was used to define calcium transients, which were then further defined as wave-associated if they occurred within 3 seconds of the peak of a wave-associated EPSC. Intercompartment latency was defined as the difference, in seconds, between wave-associated transients in stalks and the median wave-associated transient time for all lateral processes within the FOV, for each retinal wave.

Image processing and analysis: Müller glial morphology

For volumetric images of Müller glial process morphology, images were bandpass filtered in XY space to reduce noise while maintaining structure of fine processes, and registered using the Correct 3D Drift plugin in FIJI⁷³. 10-minute volumetric time series were corrected for photobleaching using the ‘Histogram matching’ setting within FIJI’s Bleach Correction plugin. Time series were collapsed into temporally color-coded images to facilitate identification of

motile and stable processes in 3D. The Cell Counter tool within FIJI was used to count lateral processes and define their morphological dynamics as one of the following: extending, retracting, new process, lost process, extension followed by retraction, retraction followed by extension, and stable. Locations of process tips within the IPL were recorded and binned into one of five equally sized sections, corresponding to putative sublayers S1 through S5, defined by their distance from the INL and GCL borders as identified using membrane-tdTomato fluorescence on somas.

Volumetric images of sharp-filled Müller glia were traced using the Simple Neurite Tracer plugin (FIJI)⁷⁴. We traced each glial stalk and subsequently any visible processes branching from the stalk, creating distinct paths for each process and preserving branch order relationships. Measurements including number of tips, total process length, total branch points, and primary branches from stalk were derived from traced paths. For Sholl and convex hull analyses, paths were converted to binary skeletons and registered to correct for XY displacement of the stalk between the GCL and INL. Sholl analysis was performed using concentric rings spaced 1 μm apart⁷⁵. For the XY plane, the center of each Sholl radius was placed on the registered stalk, and intersections were counted at each radius overlayed on a maximum projection image. For the XZ and YZ planes, the center of each Sholl radius was placed on the end of the stalk closest to the INL in orthogonal projections of traced cells. Sholl radii were normalized to the total IPL thickness. Convex hull area was defined as the area of the smallest convex polygon enclosing the entire skeleton in an XY maximum projection image.

Statistical analysis

Group measurements are expressed as mean $\pm$ standard error of mean (SEM) except when nonparametric test results are reported, in which case median, 1st, and 3rd quartiles are reported.

MATLAB and RStudio were used to carry out all statistical tests. Chi-squared tests of goodness-of-fit were used to test for nonuniformity in lateral process distribution across the IPL within each age. Chi-squared tests for independent proportions were used to test for age-dependent differences in process distribution across the IPL (Tables 1 and 2). When comparing proportion of total stable processes between control and experimental manipulations, we applied Wilcoxon signed rank tests when cells were paired between conditions, and Wilcoxon rank sum tests for unpaired cells. When there were less than 5 cells in a particular condition, we pooled process counts between cells and report chi-square test statistics for these comparisons as well (Tables 3-5, 20). To test for age-, compartment-, and drug-dependent differences in retinal wave-associated calcium transients (Tables 6, 8-11, 14-17), we applied two- or three-way mixed ANOVAs followed by post hoc t-tests with Benjamini-Hochberg correction for multiple comparisons, when appropriate. Paired t-tests were used to compare fold-changes between compartments in response to pirenzepine and gabazine (Tables 9, 11, 15, 17). Intercompartment latency of wave-associated calcium transients was compared between ages and conditions using Wilcoxon rank sum tests, and within each condition tested for significant difference from zero using Wilcoxon signed rank tests (Tables 7, 12, 18). Wave EPSC amplitude and IWI were compared between control and drug conditions using paired t-tests (Tables 13 and 19). Sholl intersection profiles between wild type and $\beta 2$ -nAChR-KO Müller glia were compared using two-way repeated measures mixed ANOVA to test for genotype-associated differences in complexity across Sholl radii (Figure 6). Two-sample t-tests were used for comparison of other morphological measurements between wild type and $\beta 2$ -nAChR-KO Müller glia (Table 21).

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References

1. Allen, N. J. & Lyons, D. A. Glia as architects of central nervous system formation and function. *Science* **362**, 181–185 (2018).
2. Bernardos, R. L., Barthel, L. K., Meyers, J. R. & Raymond, P. A. Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. *J. Neurosci.* **27**, 7028–7040 (2007).
3. Ji, H. P. *et al.* MicroRNA-28 potentially regulates the photoreceptor lineage commitment of Müller glia-derived progenitors. *Sci. Rep.* **7**, 1–9 (2017).
4. Guimarães, R. P. de M. *et al.* Evidence of Müller Glia Conversion Into Retina Ganglion Cells Using Neurogenin2. *Front. Cell. Neurosci.* **12**, 410 (2018).
5. Koh, S. *et al.* Thrombospondin-1 Promotes Circuit-Specific Synapse Formation via β 1-Integrin. *bioRxiv* 866590 (2019). doi:10.1101/866590
6. Bejarano-Escobar, R., Sánchez-Calderón, H., Otero-Arenas, J., Martín-Partido, G. & Francisco-Morcillo, J. Müller glia and phagocytosis of cell debris in retinal tissue. *Journal of Anatomy* **231**, 471–483 (2017).
7. Bringmann, A., Grosche, A., Pannicke, T. & Reichenbach, A. GABA and glutamate uptake and metabolism in retinal glial (Müller) cells. *Front. Endocrinol. (Lausanne)*. **4**, (2013).
8. Allen, N. J. & Eroglu, C. Cell Biology of Astrocyte-Synapse Interactions. *Neuron* **96**, 697–708 (2017).
9. Shigetomi, E., Patel, S. & Khakh, B. S. Probing the Complexities of Astrocyte Calcium Signaling. *Trends Cell Biol.* **26**, 300–312 (2016).
10. Bindocci, E. *et al.* Three-dimensional Ca^{2+} imaging advances understanding of astrocyte biology. *Science (80-.).* **356**, (2017).
11. Di Castro, M. A. *et al.* Local Ca^{2+} detection and modulation of synaptic release by astrocytes. *Nat. Neurosci.* **14**, 1276–1284 (2011).
12. Chen, J., Poskanzer, K. E., Freeman, M. R. & Monk, K. R. Live-imaging of astrocyte morphogenesis and function in zebrafish neural circuits. *Nat. Neurosci.* 1–10 (2020). doi:10.1038/s41593-020-0703-x
13. Srinivasan, R. *et al.* Ca^{2+} signaling in astrocytes from *Ip3r2(-/-)* mice in brain slices and during startle responses in vivo. *Nat. Neurosci.* **18**, 708–17 (2015).
14. Wang, J. *et al.* Anatomy and spatial organization of Müller glia in mouse retina. *J. Comp.*

- 565 *Neurol.* **525**, 1759–1777 (2017).
- 566 15. De Zeeuw, C. I. & Hoogland, T. M. Reappraisal of Bergmann glial cells as modulators of
567 cerebellar circuit function. *Front. Cell. Neurosci.* **9**, 246 (2015).
- 568 16. Lippman, J. J., Lordkipanidze, T., Buell, M. E., Yoon, S. O. & Dunaevsky, A.
569 Morphogenesis and regulation of Bergmann glial processes during Purkinje cell dendritic
570 spine ensheathment and synaptogenesis. *Glia* **56**, 1463–77 (2008).
- 571 17. Newman, E. A. Glial cell inhibition of neurons by release of ATP. *J. Neurosci.* **23**, 1659–
572 66 (2003).
- 573 18. Newman, E. A. Glial cell regulation of neuronal activity and blood flow in the retina by
574 release of gliotransmitters. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **370**, (2015).
- 575 19. Reichenbach, A. & Bringmann, A. New functions of Müller cells. *Glia* **61**, 651–678
576 (2013).
- 577 20. Rosa, J. M. *et al.* Neuron-glia signaling in developing retina mediated by neurotransmitter
578 spillover. *Elife* **4**, 1–20 (2015).
- 579 21. Zhang, R. wei, Du, W. jie, Prober, D. A. & Du, J. lin. Müller Glial Cells Participate in
580 Retinal Waves via Glutamate Transporters and AMPA Receptors. *Cell Rep.* **27**, 2871-
581 2880.e2 (2019).
- 582 22. Bernardinelli, Y. *et al.* Activity-dependent structural plasticity of perisynaptic astrocytic
583 domains promotes excitatory synapse stability. *Curr. Biol.* **24**, 1679–88 (2014).
- 584 23. Miyazaki, T. *et al.* Glutamate transporter GLAST controls synaptic wrapping by
585 Bergmann glia and ensures proper wiring of Purkinje cells. doi:10.1073/pnas.1617330114
- 586 24. Sild, M., Van Horn, M. R., Schohl, A., Jia, D. & Ruthazer, E. S. Neural Activity-
587 Dependent Regulation of Radial Glial Filopodial Motility Is Mediated by Glial cGMP-
588 Dependent Protein Kinase 1 and Contributes to Synapse Maturation in the Developing
589 Visual System. *J. Neurosci.* **36**, 5279–88 (2016).
- 590 25. Van Horn, M. R. & Ruthazer, E. S. Glial regulation of synapse maturation and
591 stabilization in the developing nervous system. *Curr. Opin. Neurobiol.* **54**, 113–119
592 (2019).
- 593 26. MacDonald, R. B., Charlton-Perkins, M. & Harris, W. A. Mechanisms of Müller glial cell
594 morphogenesis. *Curr. Opin. Neurobiol.* **47**, 31–37 (2017).
- 595 27. Graca, A. B., Hippert, C. & Pearson, R. A. Müller Glia Reactivity and Development of
596 Gliosis in Response to Pathological Conditions. *Adv. Exp. Med. Biol.* **1074**, 303–308
597 (2018).
- 598 28. Close, J. L., Liu, J., Gumuscu, B. & Reh, T. A. Epidermal growth factor receptor
599 expression regulates proliferation in the postnatal rat retina. *Glia* **54**, 94–104 (2006).
- 600 29. Pena, J. *et al.* Controlled microenvironments to evaluate chemotactic properties of
601 cultured Müller glia. *Exp. Eye Res.* **173**, 129–137 (2018).

- 602 30. Liu, B. & Neufeld, A. H. Activation of epidermal growth factor receptor causes astrocytes
603 to form cribriform structures. *Glia* **46**, 153–168 (2004).
- 604 31. Georgiou, J., Robitaille, R. & Charlton, M. P. Muscarinic Control of Cytoskeleton in
605 Perisynaptic Glia. *J. Neurosci.* **19**, 3836–3846 (1999).
- 606 32. Lavoie-Cardinal, F. *et al.* Neuronal activity remodels the F-actin based submembrane
607 lattice in dendrites but not axons of hippocampal neurons. *Sci. Reports 2020 101* **10**, 1–17
608 (2020).
- 609 33. Uckermann, O. *et al.* Selective staining by vital dyes of Müller glial cells in retinal
610 wholemounts. *Glia* **45**, 59–66 (2004).
- 611 34. Srinivasan, R. *et al.* New Transgenic Mouse Lines for Selectively Targeting Astrocytes
612 and Studying Calcium Signals in Astrocyte Processes In Situ and In Vivo. *Neuron* **92**,
613 1181–1195 (2016).
- 614 35. Da Silva, N. *et al.* Metabotropic Receptor-Activated Calcium Increases and Store-
615 Operated Calcium Influx in Mouse Müller Cells. *Investig. Ophthalmology Vis. Sci.* **49**,
616 3065 (2008).
- 617 36. Wang, C. T. *et al.* GABAA receptor-mediated signaling alters the structure of spontaneous
618 activity in the developing retina. *J. Neurosci.* **27**, 9130–9140 (2007).
- 619 37. Blankenship, A. G. *et al.* Synaptic and extrasynaptic factors governing glutamatergic
620 retinal waves. *Neuron* **62**, 230–41 (2009).
- 621 38. Arizono, M. *et al.* Structural basis of astrocytic Ca²⁺ signals at tripartite synapses. *Nat.*
622 *Commun. 2020 111* **11**, 1–15 (2020).
- 623 39. Bansal, A. *et al.* Mice Lacking Specific Nicotinic Acetylcholine Receptor Subunits
624 Exhibit Dramatically Altered Spontaneous Activity Patterns and Reveal a Limited Role
625 for Retinal Waves in Forming ON and OFF Circuits in the Inner Retina. *J. Neurosci.* **20**,
626 7672–7681 (2000).
- 627 40. Burbidge, T. J. *et al.* Visual Circuit Development Requires Patterned Activity Mediated
628 by Retinal Acetylcholine Receptors. *Neuron* **84**, 1049 (2014).
- 629 41. McLaughlin, T., Torborg, C. L., Feller, M. B. & O’Leary, D. D. M. Retinotopic Map
630 Refinement Requires Spontaneous Retinal Waves during a Brief Critical Period of
631 Development. *Neuron* **40**, 1147–1160 (2003).
- 632 42. Syková, E. Glial diffusion barriers during aging and pathological states. *Prog. Brain Res.*
633 **132**, 339–363 (2001).
- 634 43. Filosa, A. *et al.* Neuron-glia communication via EphA4/ephrin-A3 modulates LTP
635 through glial glutamate transport. *Nat. Neurosci. 2009 1210* **12**, 1285–1292 (2009).
- 636 44. Murai, K. K., Nguyen, L. N., Irie, F., Yamaguchi, Y. & Pasquale, E. B. Control of
637 hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. *Nat.*
638 *Neurosci. 2002 62* **6**, 153–160 (2002).
- 639 45. Chung, W.-S. *et al.* Astrocytes mediate synapse elimination through MEGF10 and

640 MERTK pathways. *Nat. 2013 5047480* **504**, 394–400 (2013).

641 46. Pozner, A. *et al.* Intracellular calcium dynamics in cortical microglia responding to focal
642 laser injury in the PC::G5-tdT reporter mouse. *Front. Mol. Neurosci.* **8**, 12 (2015).

643 47. Ochteau, J. C. *et al.* An optical neuron-astrocyte proximity assay at synaptic distance
644 scales. *Neuron* **98**, 49 (2018).

645 48. Ventura, R. & Harris, K. M. Three-Dimensional Relationships between Hippocampal
646 Synapses and Astrocytes. *J. Neurosci.* **19**, 6897–6906 (1999).

647 49. Haber, M., Zhou, L. & Murai, K. K. Cooperative Astrocyte and Dendritic Spine Dynamics
648 at Hippocampal Excitatory Synapses. *J. Neurosci.* **26**, 8881–8891 (2006).

649 50. Lavialle, M. *et al.* Structural plasticity of perisynaptic astrocyte processes involves ezrin
650 and metabotropic glutamate receptors. *Proc. Natl. Acad. Sci.* **108**, 12915–12919 (2011).

651 51. Wu, B., Li, J., Chou, Y.-H., Luginbuhl, D. & Luo, L. Fibroblast growth factor signaling
652 instructs ensheathing glia wrapping of Drosophila olfactory glomeruli. *Proc. Natl. Acad.*
653 *Sci.* **114**, 7505–7512 (2017).

654 52. Liu, B. & Neufeld, A. H. Activation of epidermal growth factor receptors in astrocytes:
655 From development to neural injury. *J. Neurosci. Res.* **85**, 3523–3529 (2007).

656 53. Williams, P. R. *et al.* In vivo development of outer retinal synapses in the absence of glial
657 contact. *J. Neurosci.* **30**, 11951–61 (2010).

658 54. MS, B., JW, L., W, T., Y, Z. & T, M. Spatial constraints dictate glial territories at murine
659 neuromuscular junctions. *J. Cell Biol.* **195**, 293–305 (2011).

660 55. Ford, K. J., Félix, A. L. & Feller, M. B. Cellular mechanisms underlying spatiotemporal
661 features of cholinergic retinal waves. *J. Neurosci.* **32**, 850–63 (2012).

662 56. Macosko, E. Z. *et al.* Highly parallel genome-wide expression profiling of individual cells
663 using nanoliter droplets. *Cell* **161**, 1202–1214 (2015).

664 57. Jabaudon, D. *et al.* Inhibition of uptake unmasks rapid extracellular turnover of glutamate
665 of nonvesicular origin. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8733 (1999).

666 58. Strang, C. E., Renna, J. M., Amthor, F. R. & Keyser, K. T. Muscarinic acetylcholine
667 receptor localization and activation effects on ganglion response properties. *Invest.*
668 *Ophthalmol. Vis. Sci.* **51**, 2778–2789 (2010).

669 59. Strang, C. E., Long, Y., Gavrikov, K. E., Amthor, F. R. & Keyser, K. T. Nicotinic and
670 muscarinic acetylcholine receptors shape ganglion cell response properties. *J.*
671 *Neurophysiol.* **113**, 203–217 (2015).

672 60. Wang, Z., Tymianski, M., Jones, O. T. & Nedergaard, M. Impact of Cytoplasmic Calcium
673 Buffering on the Spatial and Temporal Characteristics of Intercellular Calcium Signals in
674 Astrocytes. *J. Neurosci.* **17**, 7359–7371 (1997).

675 61. Denizot, A., Arizono, M., Valentin Nägerl, U., Soula, H. & Berry, H. Simulation of
676 calcium signaling in fine astrocytic processes: Effect of spatial properties on spontaneous

activity. *PLoS Comput. Biol.* **15**, e1006795 (2019).

62. Oschmann, F., Mergenthaler, K., Jungnickel, E. & Obermayer, K. Spatial separation of two different pathways accounting for the generation of calcium signals in astrocytes. *PLOS Comput. Biol.* **13**, e1005377 (2017).
63. Biesecker, K. R. *et al.* Glial Cell Calcium Signaling Mediates Capillary Regulation of Blood Flow in the Retina. *J. Neurosci.* **36**, 9435–9445 (2016).
64. Sullivan, S. J. & Miller, R. F. AMPA receptor mediated d-serine release from retinal glial cells. *J. Neurochem.* **115**, 1681–1689 (2010).
65. RA, de M. R., AL, V., CS, S., MC, de M. & FG, de M. Müller glia as an active compartment modulating nervous activity in the vertebrate retina: neurotransmitters and trophic factors. *Neurochem. Res.* **33**, 1466–1474 (2008).
66. Xu, W. *et al.* Multiorgan autonomic dysfunction in mice lacking the $\beta 2$ and the $\beta 4$ subunits of neuronal nicotinic acetylcholine receptors. *Journal of Neuroscience* **19**, 9298–9305 (1999).
67. Shigetomi, E., Bowser, D. N., Sofroniew, M. V. & Khakh, B. S. Two Forms of Astrocyte Calcium Excitability Have Distinct Effects on NMDA Receptor-Mediated Slow Inward Currents in Pyramidal Neurons. *J. Neurosci.* **28**, 6659 (2008).
68. Ding, H., Smith, R. G., Poleg-Polsky, A., Diamond, J. S. & Briggman, K. L. Species-specific wiring for direction selectivity in the mammalian retina. *Nat. 2016 535* **535**, 105–110 (2016).
69. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods 2012 97* **9**, 676–682 (2012).
70. Pnevmatikakis, E. A. & Giovannucci, A. NoRMCorre: An online algorithm for piecewise rigid motion correction of calcium imaging data. *J. Neurosci. Methods* **291**, 83–94 (2017).
71. Dorostkar, M. M., Dreosti, E., Odermatt, B. & Lagnado, L. Computational processing of optical measurements of neuronal and synaptic activity in networks. *J. Neurosci. Methods* **188**, 141–150 (2010).
72. Legland, D., Arganda-Carreras, I. & Andrey, P. MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. *Bioinformatics* **32**, 3532–3534 (2016).
73. A, P., A, C. & RJ, B.-R. Sample drift correction following 4D confocal time-lapse imaging. *J. Vis. Exp.* (2014). doi:10.3791/51086
74. Arshadi, C., Günther, U., Eddison, M., Harrington, K. I. S. & Ferreira, T. A. SNT: a unifying toolbox for quantification of neuronal anatomy. *Nat. Methods 2021 184* **18**, 374–377 (2021).
75. Ferreira, T. A. *et al.* Neuronal morphometry directly from bitmap images. *Nat. Methods 2014 1110* **11**, 982–984 (2014).

Figure Legends

Figure 1. Müller glial lateral processes are highly motile during development.

(A) Orthogonal projections of two-photon volumetric images of Müller glia expressing membrane-GFP (mGFP, green) in sparsely recombined *Slc1a3*-CreER;mTmG retinas at P7 (left) and P47 (right). Cells without Cre-mediated recombination express membrane-tdTomato (magenta). Scale bar 10 μ m. IPL: inner plexiform layer. (B) Orthogonal projections of mGFP-expressing Müller glia from P8 to P23. Scale bar 10 μ m. (C) Temporally color-coded projections of two-photon Z-stack time series showing motile processes at P12 (top) and stable processes at P23 (bottom). The location and dynamics of lateral processes at each time point can be determined by referencing the color time scale at bottom. Images on right are enlarged insets from images on left to highlight individual processes. Left scale bar 10 μ m; right scale bar 5 μ m. See Figure 1-figure supplement 1 for enlarged images of these cells in sublayers S1, S3, and S5. (D) Distribution of lateral processes across IPL sublayers through development. See Table 1 for summary statistics and goodness-of-fit test statistics, and Table 2 for tests for independent proportions across age groups. (E) Proportion of lateral processes in each sublayer that underwent extension (blue), sprouting (light blue), retraction (red), elimination (light red), a combination (purple), or that were stable (grey). (F) Proportion of total stable processes across development. See Table 3 for statistical comparisons. (G) Proportion of total processes that were motile (left) and stable (right) in the absence and presence of blockers of cytoskeletal turnover at P11/12. cyto-D: cytochalasin-D (5 μ M); noco: nocodazole (10 μ M). See Table 4 for statistical comparisons. (H) Proportion of total processes that were motile (left) and stable (right) in the absence and presence of epidermal growth factor (EGF, 1 unit (100ng)/mL) at P8 and P17. See Table 5 for statistical comparisons. * corresponds to $p < 0.05$ for all comparisons. Source data available in Figure 1-source data 1 and 2.

Figure 1-figure supplement 1. Temporally color-coded projections of the cells shown in Figure 1C, highlighting motile and stable processes in sublayers S5 (top), S3 (middle), and S1 (bottom). The location and dynamics of lateral processes at each time point can be determined by referencing the color time scale at bottom. Scale bar 10 μ m.

Figure 2. Retinal wave-associated calcium transients are compartmentalized within Müller glial stalks and lateral processes.

(A) *Left*, diagram of experimental setup; cytosolic GCaMP6f is expressed in Müller glia (green) via *Slc1a3*-CreER, and a two-photon microscope is used to image calcium transients in glial stalks and lateral processes in the IPL (dashed line indicates focal depth in whole mount retina). Excitatory currents are recorded from a retinal ganglion cell (RGC) to detect retinal waves. *Right*, average projection of a field of view (FOV) showing GCaMP6f-expressing Müller glia in retinal whole mount. Upper left panel is the full FOV; upper right panel is a magnified portion of this FOV overlayed with regions of interest (ROIs) for stalks (blue) and lateral processes (yellow); bottom panels show the extent of Müller glial activation by two retinal waves (active regions overlayed in magenta). (B) Example traces of fractional change in fluorescence of GCaMP6f-expressing Müller glia showing wave-associated calcium transients in stalks (blue) and processes (yellow) and wave-associated EPSCs recorded from an RGC (black). *Right*, Müller glial calcium response during a retinal wave, expanded in time to highlight temporal delay of stalk transients relative to lateral process transients. Black trace is a voltage-clamp recording from an RGC held at -60mV. (C) Proportion of total stalk and lateral process ROIs that respond to retinal waves at P9/10 and P11/12. Grey dashed line indicates the proportion of ROIs undergoing spontaneous calcium transients at randomly selected times. See Table 6 for summary statistics. (D) Intercompartment response latency, calculated as latency of stalk responses from the median lateral process response time for retinal waves at P9/10 and P11/12. See Table 7 for summary statistics. Image scale bars 10µm. Source data available in Figure 2-source data 1.

Figure 2-figure supplement 1. Visualization of calcium compartments using *Slc1a3*-CreER;Lck-GCaMP6f^{fllox}.

(A) *Left*, diagram of *Slc1a3*-CreER;Lck-GCaMP6f^{fllox} retina used for imaging calcium in glial processes. Black dashed line indicates 2-photon imaging focal plane. *Right*, example FOV showing two spontaneous calcium events (left and middle; transients denoted as magenta regions) and one wave-associated calcium event (right) in Müller glia (MG) at P10. Three stalks (blue circles) and three neighboring lateral process ROIs (yellow squares) have been denoted as MG 1-3. (B) Normalized fluorescence traces showing calcium activity in MG 1-3. Arrowheads indicate time points shown in images in (A).

Figure 3. M1 mAChRs mediate retinal wave-associated calcium transients in Müller glial stalks but not in lateral processes.

(A) Example traces of fractional change in fluorescence of GCaMP6f-expressing Müller glia (top) showing wave-associated calcium transients in stalks (blue) and processes (yellow), and wave-associated EPSCs recorded from a retinal ganglion cell (RGG, bottom) in the absence and presence of pirenzepine (5 μ M) at P9 (left) and P11 (right). (B) *Left*, cumulative distribution of proportion of stalks and lateral processes that exhibited wave-associated calcium transients in absence (solid line) and presence of pirenzepine (dotted line). *Middle*, box plots showing proportion of ROIs that exhibited wave-associated calcium transients. Grey dashed line indicates the proportion of ROIs that exhibited spontaneous calcium transients at randomly selected times. See Table 8 for summary statistics. *Right*, fold-change in proportion of ROIs that exhibited wave-associated calcium transients in presence of pirenzepine. See Table 9 for summary statistics. (C) *Left*, cumulative distribution of the $\Delta F/F$ amplitude of wave-associated calcium transients in the absence and presence of pirenzepine. See Table 10 for summary statistics. *Middle*, box plots showing $\Delta F/F$ amplitudes separated by age. *Right*, fold-change in wave-associated $\Delta F/F$ amplitude in pirenzepine. See Table 11 for summary statistics. (D) Intercompartment response latency in each age and condition. See Table 12 for summary statistics. (E) Summary data for magnitude (top) and interval between (bottom) compound excitatory post-synaptic current (EPSC) amplitude associated with retinal waves. See Table 13 for summary statistics. Source data available in Figure 3-source data 1.

Figure 3-figure supplement 1. DNQX reduces glial participation in waves among stalks and lateral processes.

(A) *Left*, cumulative distribution of proportion stalk ROIs (blue) or lateral process ROIs (yellow, LPs) undergoing wave-associated calcium transients for all waves in control (solid lines) and 20 μ M DNQX (dotted lines). *Right*, box plots showing median and 25%-75% interquartile range for data shown in *left*. Colored boxes indicate control, while grey boxes indicate DNQX. Grey dashed line indicates proportion of ROIs undergoing calcium transients at randomly selected times. N (waves): 20, ACSF; 24, DNQX across 2 mice each. * indicates $p < 0.05$, unpaired t-test. (B) *Left*, cumulative distribution of Z scored wave-associated $\Delta F/F$ amplitude in stalks and LPs in control and DNQX. *Right*, box plots showing median and 25%-75% interquartile range for data shown in *left*. N (wave-associated transients): 466 in stalks/ACSF; 658 in LPs/ACSF; 274 in stalks/DNQX; 259 in LPs/DNQX. * indicates $p < 0.05$, unpaired t-test. Source data available in Figure 3-source data 2.

Figure 4. GABA_A receptor block potentiates wave responses in stalks via M1 mAChR activation.

(A) Example traces of fractional change in fluorescence of GCaMP6f-expressing Müller glia showing wave-associated calcium responses (top) and wave-associated EPSCs recorded from an RGC (bottom) in control (left), 5 μ M gabazine (middle), and gabazine plus 5 μ M pirenzepine (right) at P11. (B) *Left*, cumulative distributions and inset box plots of proportion of stalk ROIs (top) and lateral process ROIs (bottom) that exhibited wave-associated calcium transients in control (solid line, A), gabazine (dotted line, G), and gabazine/ pirenzepine (dashed line, G/P) from P9 to P12. Grey dashed line in box plots indicates the proportion of ROIs undergoing spontaneous calcium transients at randomly selected times. *Right*, fold-change (FC) in proportion ROIs responding in gabazine vs. ACSF (top) and in pirenzepine vs. gabazine (bottom). Grey dashed line indicates fold-change of 1. See Tables 14 and 15 for summary statistics. (C) *Left*, cumulative distributions of normalized wave-associated calcium transient amplitudes in ACSF, gabazine, and gabazine/pirenzepine from P9 to P12, with inset box plots generated from the same data. *Right*, fold-change in normalized calcium response amplitude after gabazine (top) and pirenzepine (bottom). See Tables 16 and 17 for summary statistics. (D) Intercompartment latency in wave-associated calcium transients in control, gabazine (gbz), and gabazine/pirenzepine (gbz+pir), from P9 to P12. See Table 18 for summary statistics. (E) Summary data for magnitude of (top) and interval between (bottom) compound excitatory post-synaptic currents (EPSCs) associated with retinal waves in control, gabazine, and gabazine/pirenzepine for paired FOVs from P9 to P12. See Table 19 for summary statistics. Source data available in Figure 4-source data 1.

Figure 5. Müller glial lateral process motility is unaffected by manipulations of retinal waves.

(A) Temporally color-coded projections of two-photon Z stack time series showing motile and stable processes in control (ACSF), gabazine (5 μ M), and gabazine plus pirenzepine (5 μ M) at P11. Scale bar 10 μ m. (B) Same as A in control (ACSF), TBOA (25 μ M), and TBOA plus DNQX (20 μ M) and AP5 (50 μ M) as pharmacological manipulations of retinal waves. (C) and (D) show motility (left, middle) and calcium responses (right, scale bar 20 μ m) to iontophoretically applied carbachol (P10) and glutamate (P9). A sharp electrode (indicated in magenta) was filled with 10 mM agonist and current continuously applied to eject agonist into the IPL near mGFP-expressing processes to image motility, or near GCaMP6f-expressing processes to image calcium responses. (E) *Left*, motility persisted at P12 even when intracellular calcium was chelated via 200 μ M BAPTA-AM bath application, which greatly reduced baseline calcium (F_0) and eliminated neuronal activity (see Figure 5 – figure supplement 1). Scale bars 10 μ m. *Right*, average projections (top) and normalized traces of Lck-GCaMP6f fluorescence (bottom) in DMSO and BAPTA. (F) Summary data showing proportion of total processes exhibiting stability (top) and other categories of motility (bottom) for drug conditions shown in A-E. * gabazine was included in the control condition for a subset of experiments using pirenzepine, as in (A). † TBOA was included in the control condition for a subset of experiments using DNQX/AP5, as in (B). These trials are denoted by dotted lines connecting control and drug conditions. See Figure Table 20 for summary statistics. Source data available in Figure 5-source data 1.

Figure 5-figure supplement 1. Bath loading with BAPTA-AM during development eliminates glial and neuronal calcium activity and retinal waves. (A) *Left*, average projection of GCL in *PDGFR α ;cyto-GCaMP6f^{flox}* retinas at P12 following incubation in vehicle control or 200 μ M BAPTA-AM. Cyto-GCaMP6f fluorescence is localized to Müller glia and a subset of neurons. Scale bar 10 μ m. *Right*, average projection of IPL in *Slc1a3-CreER;Lck-GCaMP6f^{flox}* retinas at P10 following incubation in vehicle or 200 μ M BAPTA-AM. Lck-GCaMP6f fluorescence is localized to Müller glial processes. Baseline fluorescence (F_0) is greatly reduced in the presence of BAPTA. (B) Cytosolic calcium transients are eliminated in neurons and glia (left), and membrane-localized calcium transients are eliminated in glia (right) following incubation in BAPTA-AM. Related to Figure 5E.

Figure 6. Chronic perturbation of retinal waves does not impact distribution, length, or complexity of Müller glial lateral processes.

(A) Orthogonal projections of two-photon Z stacks obtained after sharp-filling single Müller glial cells with Alexa-488 at P12 (top) and at P30 (bottom) in wild type (WT, left) and in $\beta 2$ nicotinic acetylcholine receptor knockout retinas ($\beta 2$ KO, right). Image scale bar is 10 μ m. (B) XZ projections of skeletons from cells shown in (A). (C) XY projections of skeletons from cells shown in (A). (D) Morphological measurements obtained from traced and skeletonized Müller glia. See Table 21 for summary statistics. Source data available in Figure 6-source data 1. (E) Sholl intersection profiles for wild type (black) and $\beta 2$ KO (red) at P12 (top) and P30-39 (bottom). Left plots are Sholl profiles carried out on XY projections, middle plots are from XZ projections, and right plots are from YZ projections. For XZ and YZ projections, the center of the Sholl radius was placed on portion of the stalk closest to the inner nuclear layer (INL). Two-way mixed ANOVA revealed no significant effect of genotype on number of intersections at any Sholl radii for both ages. GCL; ganglion cell layer. Source data for Sholl analyses available in Figure 6-source data 2.

Tables

Table 1. Analysis of differences in lateral process outgrowth between sublayers; separate tests for each age group

		P8-9	P10-P11	P12-P17	P20+
n (processes; cells; animals)		1895; 32; 6	1822; 24; 5	2546; 30; 6	970; 18; 5
Chi-square value (goodness-of-fit)		404.53	137.36	127.4	58.67
p-value from goodness-of-fit test		1.17×10^{-85}	2.07×10^{-28}	1.87×10^{-26}	5.5×10^{-12}
Average $\pm$ SEM proportion processes in each sublayer; residual difference from uniform distribution	S1	0.23 ± 0.04 ; 0.87	0.21 ± 0.04 ; 0.45	0.21 ± 0.04 ; 1.68	0.22 ± 0.04 ; 1.15
	S2	0.07 ± 0.01 ; -12.58	0.12 ± 0.03 ; -7.04	0.14 ± 0.03 ; -6.35	0.11 ± 0.02 ; -4.67
	S3	0.13 ± 0.02 ; -5.29	0.19 ± 0.04 ; -0.7	0.20 ± 0.04 ; -1.29	0.21 ± 0.04 ; 0.43
	S4	0.21 ± 0.04 ; 2.47	0.18 ± 0.04 ; -1.85	0.18 ± 0.03 ; -2.71	0.17 ± 0.03 ; -2.37
	S5	0.36 ± 0.06 ; 14.54	0.30 ± 0.06 ; 9.15	0.28 ± 0.05 ; 8.68	0.28 ± 0.06 ; 5.46

Chi-square goodness-of-fit tests were used to test for nonuniform distribution of processes across the IPL at each age. p-values were corrected using Benjamini-Hochberg adjustment. Related to Figure 1D. Source data available in Figure 1-source data 1.

Table 2. Analysis of age-dependent differences in lateral process outgrowth within each sublayer

Chi-square value (independent proportions)				112.51			
Overall p-value				1.90×10^{-18}			
n same as in Table 1				P8-9	P10-P11	P12-P17	P20+
statistics from post hoc comparisons between ages; separate comparisons for each sublayer	S1	$X^2 = 0.88$	$p = 0.83$	-0.19	-0.58	0.43	0.37
	S2	$X^2 = 59.85$	$p = 3.1 \times 10^{-12}$	-6.07	0.93	3.66	1.29
	S3	$X^2 = 22.79$	$p = 5.6 \times 10^{-5}$	-3.59	1.2	0.93	1.87
	S4	$X^2 = 23.36$	$p = 5.6 \times 10^{-5}$	3.67	-0.8	-1.48	-1.63
	S5	$X^2 = 30.68$	$p = 2.4 \times 10^{-6}$	3.85	-0.39	-2.2	-1.28

Following an overall Chi-square test for independent proportions across ages, separate Chi-square tests for independent proportions were performed for each sublayer to test for sublayer-specific developmental changes in process outgrowth. p-values were corrected using Benjamini-Hochberg adjustment. Residual differences in proportions across ages are reported for each Chi-square test. Related to Fig. 1D. Source data available in Figure 1-source data 1.

900 **Table 3.** Analysis of age-dependent differences in proportion stable processes

Overall statistics: Kruskal-Wallis rank sum test	overall $X^2 = 77.72$; $p = 1.41 \times 10^{-13}$	Median [1 st quartile/3 rd quartile] proportion stable processes for each age								
	n (processes; cells; animals)	P8/9 0.10 [0.075/ 0.15]	P10/11 0.19 [0.13/ 0.22]	P12/13 0.18 [0.14/ 0.20]	P14 0.28 [0.24/ 0.31]	P16 0.42 [0.40/ 0.50]	P17 0.82 [0.74/ 0.87]	P20 0.86 [0.84/ 0.87]	P23/24 0.74 [0.65/ 0.83]	P40+ 0.86 [0.83/ 0.90]
P8/9	240; 32; 6	Wilcoxon rank sum p-values from pairwise comparisons								
P10/11	319; 24; 5	0.0030								
P12/13	207; 12; 3	0.0027	0.8							
P14	148; 6; 1	0.00075	0.0036	0.0016						
P16	182; 6; 1	0.00036	3.0×10^{-5}	3.7×10^{-4}	0.0030					
P17	245; 6; 1	0.00030	3.0×10^{-5}	3.7×10^{-4}	0.0030	0.0030				
P20	233; 6; 1	0.00036	3.0×10^{-5}	3.7×10^{-4}	0.0030	0.0030	0.34			
P23/24	337; 8; 2	9.7×10^{-5}	6.9×10^{-6}	9.7×10^{-5}	0.0015	0.0015	0.26	0.05		
P40+	212; 4; 2	2.6×10^{-3}	3.7×10^{-4}	2.2×10^{-3}	0.012	0.012	0.50	0.91	0.084	

901 Following overall Kruskal Wallis rank sum test for age-dependent changes in proportion of total stable processes,
902 separate pairwise Wilcoxon rank sum tests were performed to determine developmental time course of process
903 stabilization. Reported p-values are false discovery rate-corrected. Related to Fig. 1F. Source data available in
904 Figure 1-source data 1.

Drug		Cytochalasin-D vs. ACSF	Nocodazole vs. ACSF	Cytochalasin-D + nocodazole vs. ACSF
n (processes; cells)		474; 7	208; 3	161; 3
Statistics (Chi-square test for independent proportions)		overall $X^2 = 444.36$; $p = 8.0 \times 10^{-93}$	overall $X^2 = 98.10$; $p = 6.3 \times 10^{-19}$	overall $X^2 = 356.06$; $p = 7.7 \times 10^{-74}$
Chi-square statistics by motility category	Extension	$X^2 = 25.66$; $p = 7.09 \times 10^{-7}$	$X^2 = 2.10$; $p = 0.17$	$X^2 = 29.56$; $p = 1.3 \times 10^{-7}$
	New process	$X^2 = 20.54$; $p = 6.8 \times 10^{-6}$	$X^2 = 5.52$; $p = 0.033$	$X^2 = 10.83$; $p = 0.0014$
	Retraction	$X^2 = 67.21$; $p = 8.5 \times 10^{-16}$	$X^2 = 10.15$; $p = 0.0051$	$X^2 = 45.98$; $p = 4.2 \times 10^{-11}$
	Lost process	$X^2 = 22.89$; $p = 2.4 \times 10^{-6}$	$X^2 = 4.71$; $p = 0.042$	$X^2 = 8.80$; $p = 0.0035$
	Extension -> retraction	$X^2 = 62.41$; $p = 6.5 \times 10^{-15}$	$X^2 = 6.66$; $p = 0.023$	$X^2 = 27.48$; $p = 2.8 \times 10^{-7}$
	Retraction -> extension	$X^2 = 2.9197$; $p = 0.088$	$X^2 = 0.49329$; $p = 0.48$	$X^2 = 2.14$; $p = 0.14$
	Stable	$X^2 = 431.2$; $p = 6.25^{-95}$	$X^2 = 92.86$; $p = 3.9 \times 10^{-21}$	$X^2 = 352.17$; $p = 1.0 \times 10^{-77}$
Statistics for proportion stable processes (median [1 st quartile/3 rd quartile]; Wilcoxon rank sum p-value)		ACSF: 0.22 [0.17/0.23] cyto.: 0.77 [0.75/0.79] $p = 0.00024$	noco.: 0.50 [0.46/0.60] $p = 0.0088$	Cyto+noco: 0.94 [0.93/0.97] $p = 0.0088$

Table 4. Analysis of motility in blockers of cytoskeletal rearrangements

Chi-square tests for independent proportions were used to test for changes in motility in presence of Cytochalasin-D and nocodazole. Controls were in ACSF (n = 967 processes; 12 cells). Post hoc Chi-square tests were used to test for differences in proportions of processes undergoing each type of motility in control vs. drug. Pairwise comparison of proportion stable processes was performed using Wilcoxon rank sum test. p-values were corrected using Benjamini-Hochberg adjustment. Related to Fig. 1G. Source data available in Figure 1-source data 2.

Table 5. Analysis of motility in exogenous EGF

Age		P8/9	P17
n (cells; processes)		ACSF: 7; 553; EGF: 7; 623	ACSF: 6; 306; EGF: 6; 325
Statistics		overall $X^2 = 64.06$; $p = 6.7 \times 10^{-12}$	Fisher's exact $p = 0.76$
Chi-square statistics by motility category	Extension	$X^2 = 3.78$; $p = 0.12$	Fisher's exact $p = 0.90$
	New process	$X^2 = 0.0013$; $p = 0.97$	Fisher's exact $p = 1$
	Retraction	$X^2 = 0.91$; $p = 0.60$	Fisher's exact $p = 0.20$
	Lost process	$X^2 = 0.61$; $p = 0.61$	Fisher's exact $p = 1$
	Extension -> retraction	$X^2 = 7.74$; $p = 0.019$	Fisher's exact $p = 0.73$
	Retraction -> extension	$X^2 = 0.32$; $p = 0.67$	Fisher's exact $p = 1$
	Stable	$X^2 = 57.33$; $p = 2.6 \times 10^{-13}$	Fisher's exact $p = 0.50$
Statistics for proportion stable processes (median [1 st quartile/3 rd quartile]; Wilcoxon signed rank p-value)		ACSF: 0.16 [0.15/0.20] EGF: 0.033 [0.027/0.056] $p = 0.031$	ACSF: 0.82 [0.74/0.87] EGF: 0.78 [0.74/0.83] $p = 0.063$

Chi-square tests for independent proportions were used to test for changes in overall process motility in the presence of exogenous EGF. Post hoc Chi-square tests were used to test for differences in proportions of processes undergoing each type of motility in control vs. EGF. Pairwise comparison of proportion stable processes was

performed using Wilcoxon signed rank test. p-values were corrected using Benjamini-Hochberg adjustment for multiple comparisons. Related to Fig. 1H. Source data available in Figure 1-source data 2.

Table 6. Analysis of calcium compartmentalization: proportion of ROIs participating in waves

Age	P9/10	P11/12		Two-way mixed ANOVA (with in
n (waves; FOVs; animals)	130 waves; 19 FOVs; 8 animals	182 waves; 17 FOVs; 5 animals	Pairwise comparison across ages (unpaired t-test)	
Stalks	0.141 ± 0.012	0.065 ± 0.002	p = 2.8 x 10 ⁻⁸	
LPs	0.175 ± 0.012	0.148 ± 0.007	p = 0.051	
Pairwise comparison across compartments (paired t-test)	p = 7.0 x 10 ⁻⁸	p = 7.7 x 10 ⁻³⁰		

group: compartment) revealed significant main effects of age ($p = 7.2 \times 10^{-6}$) and compartment ($p = 9.6 \times 10^{-33}$), with a significant interaction between these factors ($p = 7.6 \times 10^{-8}$). Unpaired t-tests were used to compare proportion ROIs responding between ages, and paired t-tests were used to compare proportion responding between stalks and LPs within ages. Related to Fig. 2C. Source data available in Figure 2-source data 1.

Table 7. Analysis of intercompartment latency in ACSF

Age	P9/10	P11/12	
n wave-assoc. stalk transients	1424	158	Wilcoxon rank sum p-value
Inter-compartment latency (s)	0.17 [0.0/0.68]	0.0 [0.0/0.34]	p = 6.4 x 10 ⁻⁵
Wilcoxon signed rank p-value (BH corrected)	p = 1.45 x 10 ⁻⁴⁹	p = 0.20	

Inter-compartment latency was calculated by subtracting the median wave-associated transient time among LPs during a retinal wave from individual wave-associated transient times among stalks responding to that wave.

Wilcoxon signed rank tests were used to test for a non-zero latency at each age, and rank sum test was used to test

Age	P9/10 (4 animals)	P11/12 (3 animals)		for an age-dependent change in latency. Intercompartment latency is
n (waves; FOVs)	ACSF: 56 waves; 8 FOVs pirenzepine: 56 waves; 10 FOVs	ACSF: 138 waves; 9 FOVs pirenzepine: 218 waves; 9 FOVs		
ACSF	stalks: 0.214 ± 0.022 LPs: 0.243 ± 0.020	stalks: 0.059 ± 0.003 LPs: 0.117 ± 0.009		
Pirenzepine	stalks: 0.086 ± 0.008 LPs: 0.148 ± 0.016	stalks: 0.050 ± 0.002 LPs: 0.099 ± 0.005		
Pairwise comparison across conditions (two-sample t-test, p-value BH corrected)	Stalks: p = 2.5 x 10 ⁻⁶ LPs: p = 5.8 x 10 ⁻⁴	Stalks: p = 0.013 LPs: p = 0.068		

reported as median [1st quartile/3rd quartile]. Related to Fig. 2D. Source data available in Figure 2-source data 1.

Table 8. Proportion of total ROIs participating in retinal waves: ACSF vs. pirenzepine

Three-way mixed ANOVA (within group: compartment) revealed significant main effects of drug ($p = 2.7 \times 10^{-13}$), age ($p = 2.7 \times 10^{-13}$), and compartment ($p = 2.7 \times 10^{-13}$) on proportion ROIs participating in waves, with a significant interaction between drug condition and age ($p = 5.88 \times 10^{-9}$). Two-sample t-tests were used for post hoc analysis of pirenzepine effect on proportion ROIs participating within stalks and LPs. Related to Fig. 3B, *middle*. Source data available in Figure 3-source data 1.

Table 9. Fold change in proportion ROIs participating in retinal waves: ACSF vs. pirenzepine

Age (n paired FOVs)	P9/10 (6 FOVs)	P11/12 (6 FOVs)	Pairwise comparison across ages (unpaired t test)
stalks	0.475 ± 0.046	0.888 ± 0.078	$p = 0.0036$
LPs	0.677 ± 0.071	0.930 ± 0.070	$p = 0.030$
Pairwise comparison across compartments (paired t-test, p-value BH corrected)	$p = 0.033$	$p = 0.54$	

Two-way mixed ANOVA (within group: compartment) revealed significant main effects of age ($p = 0.003$) and compartment ($p = 0.017$) on fold change in proportion ROIs participating in waves during pirenzepine application. Paired t-tests were used for post hoc comparison of pirenzepine effect between stalks and LPs, and unpaired t-tests were used to compare pirenzepine effect between ages. Related to Fig. 3B, *right*. Source data available in Figure 3-source data 1.

Age	P9/10	P11/12
n (wave-assoc. transients)	ACSF: 4938 stalk; 4181 LP pirenzepine: 2137 stalk; 2708 LP	ACSF: 3128 stalk; 5292 LP pirenzepine: 3205 stalk; 5788 LP
ACSF	stalks: 5.14 ± 0.03 ; LPs: 4.30 ± 0.02	stalks: 3.84 ± 0.02 ; LPs: 3.96 ± 0.01
Pirenzepine	stalks: 4.01 ± 0.03 ; LPs: 4.08 ± 0.02	stalks: 3.76 ± 0.02 ; LPs: 3.81 ± 0.01
Pairwise comparison across conditions (unpaired t test, p-value BH corrected)	stalks $p = 2.9 \times 10^{-141}$ LPs $p = 6.4 \times 10^{-13}$	stalks $p = 0.0043$ LPs $p = 1.9 \times 10^{-15}$

Table 10. Z scored wave-associated $\Delta F/F$ amplitude: ACSF vs. pirenzepine

Three-way repeated measures ANOVA revealed significant main effects of drug ($p = 2.3 \times 10^{-127}$), age ($p = 2.1 \times 10^{-20}$), and compartment ($p = 1.7 \times 10^{-234}$) on wave-associated transient amplitude, with significant interactions between drug:age ($p = 2.2 \times 10^{-37}$), drug:compartment ($p = 6.6 \times 10^{-65}$), and age:compartment ($p = 1.4 \times 10^{-47}$). Two-sample t-tests were used for post hoc analysis of pirenzepine effect on wave-associated transient amplitude within stalks and LPs. Related to Fig. 3C, *middle*. Source data available in Figure 3-source data 1.

Table 11. Fold change in wave-associated $\Delta F/F$ amplitude: ACSF vs. pirenzepine

Age	P9/10 (6 FOVs)	P11/12 (6 FOVs)	Pairwise comparison across ages (unpaired t-test, p-value BH corrected)
stalks	0.819 ± 0.031	0.982 ± 0.009	$p = 0.0046$
LPs	0.945 ± 0.026	0.970 ± 0.017	$p = 0.45$
Pairwise comparison across compartments (paired t-test, p-value BH corrected)	$p = 0.057$	$p = 0.31$	

Two-way mixed ANOVA (within group: compartment) revealed significant main effects of age ($p = 0.003$) and compartment ($p = 0.023$) on fold change in wave-associated transient amplitude during pirenzepine application, with a significant interaction between age and compartment ($p = 0.009$). Paired t-tests were used for post hoc comparison of pirenzepine effect between stalks and LPs, and unpaired t-tests were used to compare pirenzepine effect between ages. Related to Fig. 3C, *right*. Source data available in Figure 3-source data 1.

Table 12. Intercompartment latency in ACSF vs. pirenzepine

Age	P9/10	P11/12	
n wave-assoc. stalk transients	ACSF: 1141 pirenzepine: 133	ACSF: 79 pirenzepine: 46	Pairwise comparison across age (Wilcoxon rank sum)
ACSF latency; signed rank p-value	0.34 [0.0/0.68] $p = 4.4 \times 10^{-44}$	0.0 [0.0/0.34] $p = 0.093$	$p = 0.018$
Pirenzepine latency; signed rank p-value	0.0 [-0.34/0.68] $p = 0.093$	0.0 [0.0/0.34] $p = 0.46$	$p = 0.93$
Pairwise comparison across conditions (Wilcoxon rank sum p-value)	$p = 0.013$	$p = 0.75$	

Wilcoxon signed rank tests were used to test for a non-zero latency within each condition and age, and rank sum tests were used to test for effects of pirenzepine and age on latency. Intercompartment latency is reported as median [1st quartile/3rd quartile]. Related to Fig. 3D. Source data available in Figure 3-source data 1.

Table 13. Wave properties in ACSF vs. pirenzepine

	EPSC magnitude	IWI
ACSF	172.41 +/- 30.25 pA	55.12 +/- 6.77 sec
pirenzepine	163.63 +/- 33.44 pA	71.47 +/- 11.55 sec
paired t-test p-value	$p = 0.36$	$p = 0.056$

Wave properties are averages per RGC voltage clamp recording (n = 13 cells). Wave-associated EPSC and inter-wave interval (IWI) were compared using paired t-tests. Related to Fig. 3E. Source data available in Figure 3-source data 1.

Table 14. Proportion ROIs participating in retinal waves in ACSF, gabazine, and gabazine + pirenzepine

Age	P9/10	P11/12
n (waves; FOVs)	ACSF: 97 waves; 9 FOVs gbz: 98 waves; 9 FOVs gbz+pir: 54 waves; 7 FOVs	ACSF: 76 waves; 6 FOVs gbz: 76 waves; 6 FOVs gbz+pir: 55 waves; 6 FOVs
ACSF	stalks: 0.046 ± 0.003 LPs: 0.076 ± 0.005	stalks: 0.058 ± 0.003 LPs: $0.148 \pm .015$
gabazine	stalks: 0.247 ± 0.015 LPs: 0.202 ± 0.018	stalks: 0.183 ± 0.017 LPs: 0.357 ± 0.026
gabazine + pirenzepine	stalks: 0.073 ± 0.008 LPs: 0.159 ± 0.019	stalks: 0.085 ± 0.006 LPs: 0.304 ± 0.022
Pairwise comparison: ACSF vs. gbz	stalks $p = 3.3 \times 10^{-23}$ LPs $p = 2.9 \times 10^{-9}$	stalks $p = 9.7 \times 10^{-10}$ LPs $p = 8 \times 10^{-10}$
Pairwise comparison: gbz vs. gbz+pir	stalks $p = 1.5 \times 10^{-18}$ LPs $p = 0.12$	stalks $p = 6.8 \times 10^{-7}$ LPs $p = 0.12$

Three-way mixed ANOVA (within group: compartment) revealed significant main effects of drug ($p = 6.55 \times 10^{-32}$), age ($p = 1.4 \times 10^{-6}$), and compartment ($p = 1.8 \times 10^{-50}$) on proportion ROIs participating in waves, with significant interactions between drug:compartment ($p = 9.59 \times 10^{-12}$), and age:compartment ($p = 1.6 \times 10^{-31}$). Two-sample t-tests were used for post hoc analysis of gabazine and pirenzepine effect on proportion ROIs participating within stalks and LPs. Related to Fig. 4B, *left*. Source data available in Figure 4-source data 1.

Table 15. Fold change in proportion ROIs participating in waves in ACSF vs. gabazine, and in gabazine vs. gabazine + pirenzepine

Age	P9/10 (9 FOVs)	P11/12 (6 FOVs)	Pairwise comparison across ages (unpaired t-test)
stalks (ACSF to gbz)	6.97 ± 1.11	5.78 ± 1.74	p = 0.748
LPs (ACSF to gbz)	3.47 ± 0.82	3.87 ± 0.904	p = 0.748
Pairwise comparison across compartments (paired t-test)	p = 0.00118	p = 0.119	
stalks (gbz to gbz+pir)	0.291 ± 0.042	0.456 ± 0.063	p = 0.11
LPs (gbz to gbz+pir)	0.646 ± 0.052	0.762 ± 0.061	p = 0.18
Pairwise comparison across compartments (paired t-test)	p = 0.0013	p = 0.021	

Two-way mixed ANOVA (within group: compartment) revealed significant effects of compartment ($p = 0.00037$ for ACSF to gabazine; $p = 5.12 \times 10^{-5}$ for gabazine to gabazine + pirenzepine), and of age ($p = 0.03$ for gabazine to gabazine + pirenzepine) on fold change in proportion ROIs participating in waves. Paired t-tests were used for post hoc comparison of gabazine and pirenzepine effect between stalks and LPs, and unpaired t-tests were used to compare drug effects between ages. Related to Fig. 4B, *right*. Source data available in Figure 4-source data 1.

Table 16. Z scored wave-associated $\Delta F/F$ amplitude in ACSF, gabazine, and gabazine + pirenzepine

Age	P9/10	P11/12
n (wave-assoc. transients)	ACSF: 1537 stalk; 2339 LP gbz: 6814 stalk; 5266 LP gbz+pir: 1149 stalk; 2299 LP	ACSF: 1460 stalk; 3022 LP gbz: 4734 stalk; 8160 LP gbz+pir: 1585 stalk; 4687 LP
ACSF	stalks: 3.72 ± 0.031 ; LPs: 3.79 ± 0.023	stalks: 3.78 ± 0.034 ; LPs: 3.97 ± 0.018
gabazine	stalks: 6.71 ± 0.04 ; LPs: 4.37 ± 0.023	stalks: 5.66 ± 0.04 ; LPs: 4.23 ± 0.013
gbz + pirenzepine	stalks: 3.80 ± 0.03 ; LPs: 3.92 ± 0.02	stalks: 3.77 ± 0.037 ; LPs: 4.16 ± 0.02
Pairwise comparison: ACSF vs. gbz	stalks $p < 0.00001$; LPs $p = 1.32 \times 10^{-71}$	stalks $p = 1.28 \times 10^{-252}$ LPs $p = 1.41 \times 10^{-31}$
Pairwise comparison: gbz vs. gbz+pir	stalks $p < 0.00001$; LPs $p = 1.4 \times 10^{-5}$	stalks $p = 2.1 \times 10^{-236}$ LPs $p = 2.5 \times 10^{-4}$

Three-way repeated measures ANOVA revealed significant main effects of drug ($p < 0.00001$), age ($p = 2.2 \times 10^{-109}$), and compartment ($p = 7.3 \times 10^{-8}$) on wave-associated transient amplitude, with significant interactions between drug:age ($p < 0.00001$), drug:compartment ($p = 3.0 \times 10^{-67}$), and age:compartment ($p = 2.5 \times 10^{-22}$). Two-sample t-tests were used for post hoc analysis of gabazine and pirenzepine effect on wave-associated transient amplitude within stalks and LPs. Related to Fig. 4C, *left*. Source data available in Figure 4-source data 1.

Table 17. Fold change in wave-associated transient amplitude: ACSF vs. gabazine; gabazine vs. gabazine + pirenzepine

Age	P9/10 (9 FOVs)	P11/12 (6 FOVs)	Pairwise comparison across ages (unpaired t-test)
stalks (ACSF to gbz)	1.95 ± 0.10	1.46 ± 0.09	p = 0.0054
LPs (ACSF to gbz)	1.20 ± 0.09	1.07 ± 0.04	p = 0.22
Pairwise comparison across compartments (paired t-test)	p = 0.000087	p = 0.0099	
stalks (gbz to gbz+pir)	0.543 ± 0.022	0.713 ± 0.065	p = 0.094
LPs (gbz to gbz+pir)	0.899 ± 0.046	0.975 ± 0.016	p = 0.16
Pairwise comparison across compartments (paired t-test)	p = 0.00011	p = 0.0081	

Two-way mixed ANOVA (within group: compartment) revealed significant main effects of age ($p = 0.014$) and compartment ($p = 1.8 \times 10^{-6}$) on fold change in wave-associated transient amplitude during gabazine and pirenzepine application, with a significant interaction between age and compartment ($p = 0.024$). Paired t-tests were used for post hoc comparison of gabazine and pirenzepine effects between stalks and LPs, and unpaired t-tests were used to compare drug effects between ages. Related to Fig. 4C, *right*. Source data available in Figure 4-source data 1.

Table 18. Intercompartment latency in ACSF, gabazine, and gabazine + pirenzepine

	ACSF (79 responses, 5 FOVs)	Gabazine (3200 responses, 18 FOVs)	Gabazine + pirenzepine (75 responses, 12 FOVs)	Kruskal-Wallis test result
Intercompartment latency (sec)	1.01 [0.17/1.69]	1.18 [0.34/1.69]	0.34 [0.0/1.01]	$X^2 = 26.05$ $p = 2.2 \times 10^{-6}$
Pairwise comparison across conditions (Wilcoxon rank sum)	vs gabazine: $p = 0.33$	vs gbz + pir: $p = 1.4 \times 10^{-6}$	vs. ACSF: $p = 0.0075$	

Kruskal-Wallis test was used to test for overall drug effect on intercompartment latency between all conditions, with pairwise post hoc tests performed using Wilcoxon rank sum. Intercompartment latency is reported as median [1st quartile/3rd quartile]. Related to Fig 4D. Source data available in Figure 4-source data 1.

Table 19. Wave properties in ACSF, gabazine, and gabazine + pirenzepine

	EPSC magnitude	IWI
ACSF	200.22 ± 52.47 pA	64.10 ± 11.48 sec
gabazine	432.18 ± 142.66 pA	88.48 ± 21.20 sec
gbz + pirenzepine	439.84 ± 157.96 pA	97.73 ± 17.71 sec
one-way ANOVA results	F = 4.69; $p = 0.11$	F = 2.82; $p = 0.086$

Wave properties are averages per RGC voltage clamp recording ($n = 10$ cells). Wave-associated EPSC and inter-wave interval (IWI) were compared using paired t-tests. Related to Fig. 4E. Source data available in Figure 4-source data 1.

Table 20. Comparisons of proportion stable processes across conditions

	Control; median [1 st quartile/3 rd quartile] (n cells; processes)	Drug; median/1 st quartile/3 rd quartile (n cells; processes)	Chi-square statistics for stable processes	p-value for proportion stable in control vs. drug (Wilcoxon signed rank for paired cells; rank sum for unpaired cells)
Gabazine	0.079 [0.044/0.11] (7, 301)	0.083 [0.077/0.12] (7, 388)	$X^2 = 0.53$ $p = 0.47$	$p = 0.38$
Pirenzepine	0.12 [0.081/0.25] (14, 945)	0.17 [0.11/0.19] (14; 928)	$X^2 = 0.72$ $p = 0.39$	$p = 0.22$
TBOA	0.10 [0.074/0.16] (7, 506)	0.12 [0.075/0.19] (7, 532)	$X^2 = 0.21$ $p = 0.64$	$p = 0.30$
DNQX + AP5	0.11 [0.079/0.25] (13, 953)	0.17 [0.11/0.20] (13; 905)	$X^2 = 3.2$ $p = 0.074$	$p = 0.19$
local carbachol	0.10 [0.082/0.14] (16, 1258)	0.13 [0.068/0.18] (16, 1133)	$X^2 = 3.86$ $p = 0.05$	$p = 0.35$
local glutamate	P9: 0.15 [0.12/0.17] (2; 183) P24: 0.76 [0.71/0.81] (2; 56)	P9: 0.14 [0.12/0.15] (2; 171) P24: 0.70 [0.70/0.70] (2; 76)	P9: $X^2 = 2.7 \times 10^{-30}$ $p = 1$ P24: $X^2 = 0.49$ $p = 0.48$	p (P9) = 1 p (P24) = 1 p (combined) = 0.88
BAPTA	0.20 [0.19/0.22] (5; 335)	0.20 [0.18/0.29] (6; 408)	$X^2 = 0.12$; $p = 0.73$	$p = 0.93$

Chi-square tests were used to test for differences in proportions of stable processes in control vs. drug using pooled count data. Pairwise comparison of proportion stable processes was performed using Wilcoxon signed rank test for paired cells and ranks sum test for unpaired cells. Related to Fig. 5F. Source data available in Figure 5-source data 1.

Table 21. Comparisons of morphological properties between wild type and $\beta 2$ -nAChR-KO Müller glia

Age	P12/13			P30+		
Genotype	WT (n = 26)	KO (n = 24)	p-value	WT (n = 13)	KO (n = 18)	p-value
# branch points	71.27 $\pm$ 3.55	80.75 $\pm$ 4.32	0.095	100.85 $\pm$ 10.05	99.33 $\pm$ 6.29	0.89
stalk length (μ m)	46.44 $\pm$ 1.09	46.38 $\pm$ 1.14	0.97	46.13 $\pm$ 2.02	46.05 $\pm$ 0.97	0.97
process length (μ m)	553.16 $\pm$ 28.47	604.20 $\pm$ 37.86	0.28	717.86 $\pm$ 69.45	709.03 $\pm$ 46.66	0.91
# tips	84.81 $\pm$ 4.36	93.04 $\pm$ 4.81	0.21	119.23 $\pm$ 12.18	113.67 $\pm$ 7.56	0.69
convex hull area (μ m ²)	343.78 $\pm$ 23.71	411.57 $\pm$ 34.99	0.11	333.03 $\pm$ 26.95	344.78 $\pm$ 36.64	0.81
# branches off stalk	37.92 $\pm$ 1.33	32.21 $\pm$ 1.13	0.0021	45.77 $\pm$ 3.01	41.22 $\pm$ 2.14	0.21

Unpaired t-tests were used to compare morphological properties between wild type (WT) and $\beta 2$ -nAChR-KO retinas. Related to Fig. 6D. Source data available in Figure 6-source data 1.

A

B

A

B

C

D

E