Zebrafish (*Danio rerio*) as a Model for the Study of Glial Calcium Signaling in Awake, Behaving Animals

By

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Abstract

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Glial biology has been studied for over a century, yet the existence of dynamic calcium signaling in astrocytes was only discovered in 1990. Early studies used cultured astrocytes to investigate this phenomenon; however, studies in cultured cells are highly vulnerable to artifactual results that do not accurately represent normal physiological conditions. More recent studies *in vivo* have revealed a broad heterogeneity in astrocyte signaling—the mechanisms that trigger a calcium rise, the sources of that calcium rise, and the downstream effects of astrocyte signaling all vary with different brain regions and types of stimulation. In zebrafish (*Danio rerio*), the primary glial cell type is radial glia that persist into adulthood, which have previously been shown to perform many of the same roles as mammalian astrocytes, including regulating synaptic glutamate uptake and maintaining CNS water balance. This study establishes that zebrafish are an appropriate model for studying glial calcium signaling, which can be triggered by eliciting an acoustic startle response in awake, behaving fish. The glial calcium signal, measured by the genetically encoded calcium indicator GCaMP5, reliably peaks following an escape response and is mediated by the group I metabotropic glutamate receptor mGluR1.

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1. Introduction

For over a century, glia were viewed as simple support cells for neurons, supplying energy sources and regulating metabolite and neurotransmitter levels. Although these roles are indispensible to nervous system function, recent work has shown that glia respond to neurotransmitter release and in turn can modulate neurovascular coupling, release gliotransmitters, and influence neural processing and behavior. The discovery that glia have the ability to actively signal upended the long-held notion that glia were merely passive observers of the nervous system and opened up an entirely new field of study. The morphology of astrocytic networks coupled with their responsiveness to excitatory neural signaling provides a possible mechanism for integration and modulation of neural signaling over large areas of the central nervous system.

Glial cells are present throughout the central nervous system (CNS) and have important roles in normal physiology as well as disease. They fall into three morphologically and genetically diverse categories—astroglia, oligodendroglia, and microglia (**Figure 1**). Microglia are the resident macrophages of the central nervous system that are responsible for monitoring the brain for damage and infection as well as removing dead cells and debris. Oligodendroglia, as well as Schwann cells in the peripheral nervous system, are responsible for the myelination of axons, which enables fast electrical communication between neurons. Myelin is a fatty layer that, when wrapped around axons, increases their resistance, thus slowing current leakage and enabling fast transmission of neuronal signals over long distances[1]. They also provide trophic and structural support to axons, and the loss of myelination causes neuropathy and is a central component of diseases such as multiple sclerosis[2]. Astroglia are a heterogeneous group of cells that participate widely in processes necessary for brain development and function including neurogenesis, neuron outgrowth, axon guidance, and synapse formation[3].

The primary form of glia in the developing nervous system is radial glia, which are named for the radial processes that extend from their somata to the apical and basal surfaces of the developing brain. This morphology allows them to act as tracks that guide developing neurons to migrate from the proliferative ventricular zone to their final positions in the brain[4]. In addition to this developmental support function, radial glia themselves act as precursor cells that generate both neurons and astroglia[5,6]. Upon completion of developmental neurogenesis, radial glia transform intro astrocytes in mammals, but persist into adulthood in amphibians, reptiles, and fish[7,8]. Even after radial glia have fulfilled their developmental functions as precursors and guides, glia are critical for normal synaptogenesis and maintenance of brain health—*in vitro*, astrocytes are required for formation of functional, active synapses[9]. At the same time, astrocytes participate in the elimination of unnecessary synapses through phagocytosis, which is a key step in synaptic remodeling and strengthening[10].

Mature astrocytes further subdivide morphologically into star-shaped fibrous astrocytes of the white matter and protoplasmic astrocytes of the gray matter, and radial Muller glia of the retina and Bergmann glia of the cerebellum. However, studies of physiology and signaling *in vitro* have revealed a high degree of heterogeneity within astrocyte types as well as between and

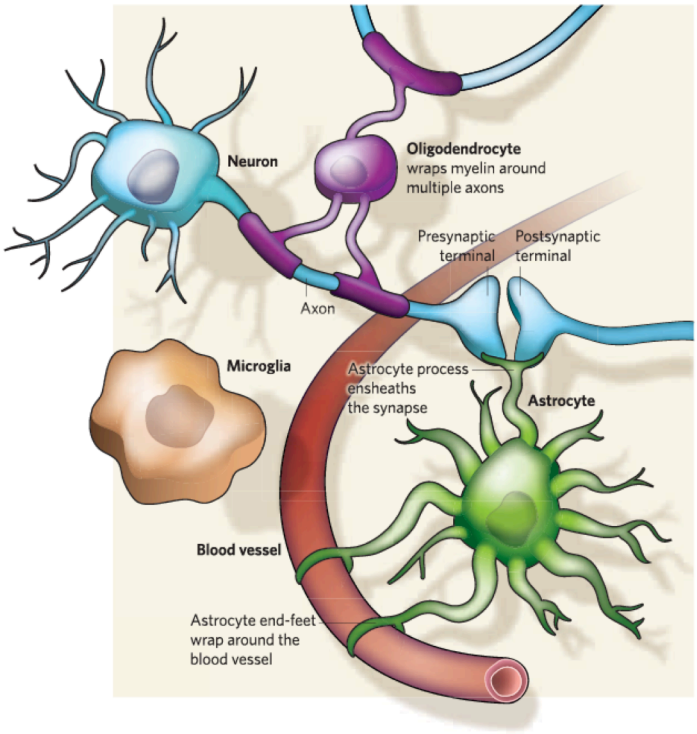

Figure 1. Diversity of glial cell types in the central nervous system.
Glia are broadly classified into three cell types. Microglia are the immune cells of the brain that sense damage or infection. Oligodendrocytes generate myelin to insulate axons and speed up neuronal transmission. Astrocytes contact both blood vessels and synapses and are thought to mediate neurovascular coupling as well as neural signaling and synaptic strength. Illustration from Allen, N. J. & Barres, B. A. (2009)[120].

within astrocytes from different brain regions. This heterogeneity can be seen during development, when cultured astrocytes from different brain regions have different effects on neurite outgrowth and neuronal migration, as well as in physiological processes such as calcium signaling and modulation of neural activity[3]. Recent transcriptome analysis of neurons, astrocytes, and oligodendrocytes confirmed this diversity at the level of gene expression[11]. Here I will focus on astroglia, which are critical for healthy brain function and have been shown to respond to and modulate neural activity in multiple regions of the CNS.

1.1 The "glue" of the nervous system

Early morphological studies of the brain by Camillo Golgi and Santiago Ramón y Cajal revealed a large number of star-shaped cells, named for the Greek word "astro," meaning star, and "glia," meaning glue[3]. The second part of the name reveals an assumption about the function of these cells—that they were the glue of the nervous system, merely there to hold it all together while the neurons performed the important work. It was observed that astrocytes associate closely with synapses as well as cerebral blood vessels, giving morphological clues to their functions, but very little was understood about their physiological roles until the advent of stable astrocyte cultures in the 1980s[12]. Early electrophysiological studies revealed that astrocytes lack excitable membranes, and it was thought that their primary purpose was the control of K^+ concentration in the extracellular space[13]. Although this function is part of the canonical view of astrocyte biology, the mechanism of astrocytic K^+ buffering remains controversial and likely proceeds through more than one pathway[14].

In addition to $[K^+]$ regulation, early observers posited that astrocytes provide a route for movement of necessary metabolic substrates from the circulation to energy-hungry neurons[15]. Glucose uptake into astrocytes, followed by release of the glucose metabolite lactate, was shown to be directly triggered by glutamate applied to cultured astrocytes[16]. This pathway has more recently been described in detail, deepening our understanding of the complex interplay between the vasculature, astrocytes, and neurons. Rouach et al.[17] showed that circulating glucose is taken up by astrocytes through gap junctions, where it is metabolized to lactate and released into the extracellular space to be taken up by neurons. They showed that both glucose and lactate can travel through gap-junction coupled astrocytic networks, efficiently providing energy to neurons located far from the vasculature.

In order to maintain this supply of lactate to active neurons, astrocytes communicate with the blood vessels and modulate cerebral blood flow (CBF) and therefore availability of glucose as well as oxygen[18–20]. Modern techniques have confirmed that astrocyte endfeet contact 99% of the vascular surface in the brain[21,22]. Gordon et al.[23] were the first to demonstrate the interplay of oxygen availability, extracellular lactate concentration, and the astrocytic signaling cascade that leads to the release of the vasodilator prostaglandin E2 (PGE_2) or the vasoconstrictor arachidonic acid. They found that under conditions of high energy use by neurons, low oxygen availability triggers lactate release from astrocytes and the resulting high extracellular lactate concentration reduces the uptake of PGE_2 through prostaglandin transporters in astrocytes and neurons, thereby increasing the concentration of extracellular PGE_2 that triggers vasodilation. This cascade could be stimulated by a metabotropic glutamate (mGluR) agonist, which triggered an astrocytic calcium rise and subsequent increase in glycolysis and lactate release. Conversely, under

conditions of low lactate concentration, PGE_2 transporters effectively removed PGE_2 from the extracellular space, allowing astrocyte-derived arachidonic acid to act as a vasoconstrictor. Elucidating the mechanism of neurovascular coupling is particularly important for our understanding of the blood-oxygen level dependent (BOLD) signal that is measured in functional magnetic resonance imaging (fMRI)[24]. Although fMRI uses cerebral blood flow as a proxy for neural activity, it is not in fact measuring neural activity directly, and understanding the pathways that lead to changes in cerebral blood flow will allow for a more accurate interpretation of the BOLD signal.

One more way in which astrocytes contribute to overall brain health is through a pathway known as the glymphatic system, which is critical for maintaining brain health by removing toxic waste products from the interstitial space. Astrocytes express the water channel aquaporin-4 (AQP4) in endfeet[25], which is necessary for the flux of cerebrospinal fluid (CSF) that drives solute clearance from the interstitial space. Iliff et al.[26] used fluorescent tracers to show that this movement of CSF clears interstitial solutes from the brain, including soluble amyloid β, which is implicated in the pathophysiology of Alzheimer's disease. They found a 70% reduction in solute clearance in AQP4 null mutant mice, demonstrating the critical role of astrocytes in this process. The same group more recently showed that the rate of CSF influx increases dramatically during sleep, suggesting that the primary purpose of sleep may be to clear the brain of harmful metabolites via the glymphatic system[27].

The roles of astrocytes in neurovascular coupling and the glymphatic system are unsurprising given their unique morphology and clear connection to the cerebral vasculature. In addition, morphology provides another clue that these cells do more than interact with blood vessels, which can be seen in the close association of astrocytic processes with synapses. This arrangement is referred to as the tripartite synapse, and it underscores the integral role of astrocytes in supporting neural communication[28]. One of the most critical functions of astrocytic processes at the tripartite synapse is glutamate reuptake, which precisely controls the concentration of glutamate in the synaptic cleft and prevents the glutamate toxicity that occurs at high concentrations. This occurs through the glutamate transporters GLT-1 and GLAST, which are the first step in the glutamate-glutamine cycle that allows the brain to recycle glutamate rather than synthesize it anew, which would require a significant increase in glucose consumption[29]. Mice lacking GLT-1, which is expressed in all astrocytes in the brain and spinal cord[30], experience lethal spontaneous seizures caused by an excess of synaptic glutamate[31].

1.2 Astrocytes respond to neural activity with dynamic calcium signaling

Although glia have long been appreciated as indispensible regulators of metabolism in the central nervous system, it has traditionally been difficult to discern any active signaling happening in glial cells. Unlike neurons, glia do not have excitable membranes and therefore do not spike in response to signals such as neurotransmitters. For decades this was interpreted to mean that glia were merely passive observers of neural function and did not respond to neural activity with any intrinsic signaling mechanisms[32]. Cornell-Bell et al.[33] were the first to observe glutamate-triggered calcium elevations in cultured astrocytes, a discovery that opened up an entirely new field of research on glial signaling mechanisms and function. They observed calcium transients that propagated as a wave through confluent cells, although much more slowly

than the propagation of neural signaling, suggesting that glial calcium waves could provide a mechanism for a distinct form of long range signaling in the CNS.

However, this property is not unique to astrocytes and has been observed in several other cell types in culture, raising the question of whether astrocytic calcium waves exist *in vivo* and what might be their physiological relevance[34]. Porter and McCarthy[35] were the first to demonstrate evoked astrocytic calcium rises *in situ*, confirming that astrocytic calcium signaling is not merely an artifact that occurs in cultured cells. They stimulated the Schaffer collaterals in hippocampal slices while measuring astrocytes loaded with a calcium dye, and the astrocytic responses were abolished by blocking neurotransmitter release or metabotropic glutamate receptors, but not ionotropic glutamate receptors. Nett et al.[36] extended the observation of *in situ* calcium signals to include spontaneous calcium oscillations that occurred in the absence of neural activity, which they found to be initiated by inositol trisphosphate (IP_3) mediated release of calcium from internal stores. These studies confirmed the existence of spontaneous astrocyte calcium signaling *in situ*, and the advent of today's *in vivo* techniques combined with highly sophisticated genetically-encoded calcium indicators has allowed for a more extensive and nuanced observation of glial signaling *in vivo*[37,38]. In particular, the increased level of spatial and temporal resolution has enabled more detailed observations of astrocyte calcium dynamics under physiological conditions. Using these tools, astrocytes *in vivo* have been shown to exhibit a wide variety of calcium signals, ranging from sub-cellular microdomain transients to large waves that spread through multiple cells.

Wang et al. were the first to show *in vivo* astrocyte calcium transients in response to a physiological stimulus, in this case whisker stimulation[39]. Using the calcium dye Fluo-4 AM in conjunction with 2-photon imaging in the mouse barrel cortex while also recording neural local field potential (LFP), they observed slow glial calcium elevations in both processes and cell bodies following neural activity triggered by whisker stimulation. They found that blockers of group I metabotropic glutamate receptors (mGluRs), a class of g-protein couple receptors (GPCRs) that trigger a rise in intracellular calcium upon glutamate binding, reduced but did not abolish the observed glial calcium transient while not affecting the neural LFPs. When antagonists of mGluR1 and mGluR5 were applied together they achieved a 74% reduction in the glial signal, indicating that group I mGluRs are the primary contributors to astrocyte calcium signaling in the cortex *in vivo*.

Bergman glia as a model for neuron-glia signaling

Bergmann glia (BG), the sole type of astroglia found in the cerebellum, are often studied *in vivo* as a model for neuron-glia interactions[40]. The cerebellar cortex contains a relatively simple neural circuit—parallel fibers and climbing fibers synapse onto Purkinje cells, which are the only output neurons, and Purkinje cell dendrites and synapses are ensheathed by Bergmann glial processes, which account for up to 90% of the membrane surface area of the cell[41]. Shibuki et al.[42] used immunostaining and electron microscopy to examine the morphology of Bergmann glia in glial fibrillary acidic protein (GFAP) mutant mice and found no observable differences; however, the mice had impairments in normal long term depression (LTD) at parallel fiber-Purkinje synapses and were deficient in a test of associative learning. Although this study did not provide a mechanism to explain how loss of GFAP, an intermediate filament protein that

provides structural support to glia, affected synaptic plasticity, it did spark considerable interest in the influence of Bergmann glia on cerebellar synaptic plasticity.

Bergmann glia express several neurotransmitter receptors, including mGluR1 and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors[43]. BG AMPA receptors have been shown to be necessary for normal motor coordination in adult mice, a finding that underscores the physiological importance of BG signaling[44]. Beierlein and Regehr[45] observed calcium transients in BG cells following stimulation of parallel fibers in cerebellar slices, which were abolished by an mGluR1 antagonist. In addition, they showed that bath application of ATP led to calcium release from internal stores, which was blocked by an IP_3-receptor antagonist. However, Piet et al.[46] later demonstrated that mGluR1 was not directly responsible for rises in glial calcium, but rather was activating molecular layer interneurons that release ATP, which in turn activate purinergic receptors on glia. They identified two phases of the calcium transient in BG cells—a fast, AMPAR mediated influx of extracellular calcium, followed by a slow, ATP mediated release of calcium from internal stores. These studies indicated that BG cells are capable of responding to neural activity through multiple pathways, but it remained to be seen how BG cells behave under normal physiological conditions.

Nimmerjahn et al. were the first to report calcium transients in Bergmann glia in awake, behaving mice[47]. Using the calcium dye Oregon Green BAPTA-AM, they observed three distinct patterns types of calcium activity, which they termed "flares," "sparkles," and "bursts." Sparkles and bursts both occurred spontaneously in resting animals, with sparkles confined to glial fibers and bursts spreading across a range of 10-40 cells. Flares occurred during locomotion and consisted of a large spreading calcium transient that included all the glial fibers in a field of view. Flares were abolished by blocking either neural activity or glutamatergic signaling, indicating that the Bergmann glia are responding to neural glutamate release occurring during locomotion. Flares and sparkles were also reduced or eliminated when the animals were anesthetized with isofluorane, a finding that underscores the importance of using awake animals for the study of glial signaling *in vivo*.

1.3 Does glial calcium signaling trigger release of gliotransmitters?

In addition to contacting cerebral blood vessels and ensheathing synapses, another intriguing aspect of glial morphology is the fact that a single astrocyte can contact tens of thousands of synapses, and astrocytes are evenly spaced in tiled, non-overlapping regions[48]. This underscores their potential to form a long-range signaling network that could use gap-junction coupling to signal independently of neuronal input, as well as the possibility of affecting neuronal activity over a specified domain of thousands of synapses. Conversely, the existence of glial microdomains that respond to neural signaling suggest that astrocytes could also be exerting a highly localized influence on individual synapses[49]. Both of these possibilities have led researchers to ask whether glia are capable of releasing neurotransmitters or neuromodulators, a phenomenon termed "gliotransmission," and whether that release can modulate signaling at nearby synapses (**Figure 2**). This area of study has been far more controversial than those discussed above for several reasons, including artifacts present in cultured cells and the difficulty of teasing out the astrocytic contributions to neural signaling *in vivo*.

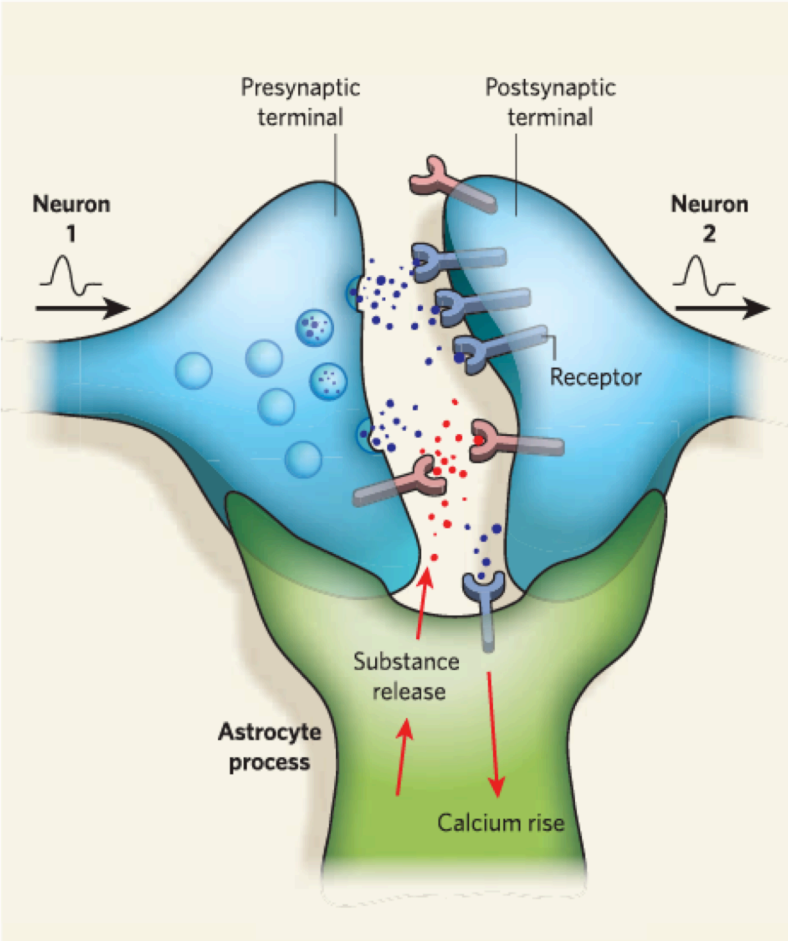

Figure 2. Bi-directional signaling between neurons and astrocytes.
Astrocytes can respond to neurotransmitters (blue) with an intracellular calcium rise, which triggers release of gliotransmitters (red) that can act on pre- and postsynaptic neurons. Several molecules have been shown to active receptors on astrocytes, leading to an intracellular calcium rise, including glutamate, ATP, norepinephrine, and acetylcholine. Possible gliotransmitters include glutamate, D-serine, and ATP. Illustration from Allen, N. J. & Barres, B. A. (2009)[120].

In vitro and *in situ* studies present conflicting results

Early pharmacological studies of gliotransmission *in situ* provided evidence that astrocytes are capable of modulating neural activity[50,51]; however, these results are difficult to interpret due to the broad effects of some of the pharmacological agents and the uncertainty regarding which cells are being directly affected. Modern tools, including caged compounds and the use of non-native GPCRs, have enabled more specific manipulations of glial calcium signaling and gliotransmission. Fiacco and McCarthy[52] uncaged IP_3 in astrocytes in hippocampal slices and were able to reliably trigger an astrocytic calcium wave that coincided with an increase in the frequency of spontaneous AMPAR-mediated excitatory postsynaptic currents (EPSCs) in surrounding neurons. Because uncaging did not alter the kinetics of EPSCs, only the frequency, they suspected that astrocytes were not directly activating AMPARs but could be acting through presynaptic mGluRs, which had been shown to modulate the frequency of EPSCs[53]. When they added antagonists of group I mGluRs, which had no significant effect on spontaneous neuronal currents by itself, they did not see a change in EPSCs in response to IP_3 uncaging in astrocytes, indicating that astrocytes are releasing glutamate that acts on presynaptic mGluRs to increase the probability of spontaneous glutamate release. D'Ascenzo et al.[54] performed a similar study using slices from the nucleus accumbens, where astrocyte signaling, which could be elicited by calcium uncaging in individual astrocytes, triggered slow inward currents in neighboring neurons mediated by N-methyl-D-aspartate receptors (NMDARs) rather than AMPARs. The differences between these two studies highlight the differences in glial signaling in different parts of the brain.

One of the most basic questions that remains controversial is how astrocytes are releasing glutamate or other transmitters. Vesicular glutamate release from astrocytes is an appealing idea due to the calcium-dependence of glutamate release, the known roles of astrocytes in controlling synaptic glutamate concentration, and the ubiquity of glutamate as a modulator of neuronal function. The evidence for vesicular glutamate release from astrocytes is widely variable, and to date no study has conclusively demonstrated its occurrence under physiological conditions. Exocytotic glutamate release from astrocytes was first demonstrated in culture[55], but the existence of glutamatergic vesicles and necessary exocytotic machinery in astrocytes under normal physiological conditions is unclear. The minimum components required for calcium-triggered glutamate exocytosis are soluble *N*-ethylmaleimide sensitive attachment protein receptor (SNARE) proteins to form the fusion complex, synaptotagmins to sense calcium, and vesicles with vesicular glutamate transporters (VGLUTs), and many of these components have been found in cultured astrocytes[56,57]. However, Wilhelm et al.[58] showed that expression of exocytotic proteins differs between astrocytes *in vitro* and *in situ*. Bezzi et al.[59] used a combination of immunogold labeling and single cell transcription profiling in hippocampal astrocytes to look for these; although they did find both mRNA and immunolabeling for VLGUTs 1 and 2, only about a quarter of the cells (identified by expression of the astrocytic marker S100β) expressed at least one VGLUT, and immunolabeling was sevenfold less dense than in synaptic terminals. The VGLUT immunolabeling was present on small vesicles and co-labeled with the SNARE protein cellubrevin. Zhang et al.[60] identified synaptotagmin IV as the calcium sensor present in astrocytes in situ. However, a more recent transcriptome analysis of purified mouse astrocytes failed to detect expression of VGLUT 1 or 2 or synaptotagmin[11].

Despite the inconclusive evidence regarding exocytotic machinery in astrocytes, several studies have succeeded in blocking gliotransmission *in situ* using tetanus neurotoxin light chain (TeNT), which cleaves the SNARE protein synaptobrevin[61,62]. In order to investigate the effect of astrocyte signaling on synaptic plasticity, Perea and Araque used simultaneous stimulation of Schaffer collaterals and single astrocytes in a hippocampal slice[63]. When uncaging calcium in a single astrocyte while providing mild stimulation of Schaffer collaterals, 50% of the time they saw a transient increase in probability of release in the paired pre-synaptic cell, but no change in the kinetics of EPSCs. They were able to block this effect by individually treating astrocytes with TeNT, which prevents glutamate exocytosis in neurons by cleaving synaptobrevin. They then asked if astrocyte signaling can affect long-term synaptic plasticity, and they showed that pairing astrocyte activation by calcium uncaging with postsynaptic depolarization led to a sustained increase in release probability, an effect that was blocked by group I mGluR antagonists. This study supported the idea that astrocytes are able to modulate synaptic plasticity through glutamate exocytosis.

Astrocytes have also been shown to respond to and release purines including ATP[64]. One possible pathway for ATP-triggered gliotransmission is through the $P2X_7$ ion channel, which is activated by ATP binding and can dilate to become permeable to glutamate and ATP[65,66]. Fellin et al.[67] found that a $P2X_7$ agonist triggered a sustained glutamate efflux from astrocytes in hippocampal slices that in turn caused an NMDAR mediated current in neurons. However, expression of $P2X_7$ receptors is undetectable in the hippocampus under normal physiological conditions and is upregulated under pathological conditions such as inflammation, ischemia, and seizure[66,68]. Bowser and Khakh[69] argue that the ATP-binding P2Y GPCRs are contributing to astrocyte calcium elevations in hippocampal slices. Using antagonists of P2Y receptors and group I mGluRs together they were able to block astrocyte calcium transients triggered by neural stimulation, whereas either antagonist along only produced a partial decrease in the response. Although it is unlikely that $P2X_7$ receptors play a role in glial signaling under normal physiological conditions, P2Y receptors provide an alternate route for astrocytic stimulation by ATP that can work in combination with glutamate.

Other studies suggest adenosine as the gliotransmitter responsible for modulation of synaptic transmission in hippocampal slices. Astrocytes can release ATP through SNARE-dependent exocytosis, which is then hydrolyzed to adenosine extracellularly and can suppress nearby synaptic activity *in situ*[70,71]. Panatier et al.[72] point to presynaptic A_{2A} purinergic receptors, which are known to mediate the neuromodulatory effect of adenosine, as the target of ATP released from astrocytes[73]. As in other studies, they documented an increase in synaptic efficiency triggered by calcium signaling in neighboring astrocytes. They were able to block this effect with either an mGluR5 inhibitor, which was acting only on astrocytes, or a blocker of presynaptic A_{2A} purinergic receptors. Because the effects of the two drugs mimicked each other in terms of kinetics, they concluded that mGluR5 and A_{2A} receptors are acting on the same circuit, with astrocytes responding to presynaptic glutamate through mGluR5 signaling and triggering vesicular release of ATP. The time it takes for ATP to hydrolyze to adenosine introduces a ~200 ms delay into this mechanism of signaling that allows diffusion of the signal to more distant synapses, differentiating it from neuronal glutamatergic or GABAergic signaling, which are released and cleared from the synapse in a fast, controlled manner.

Astrocytes have also been show to release D-serine, a co-agonist of NMDA receptors that is required for long-term potentiation (LTP) and is localized in astrocytes in areas of the brain expressing NMDA[74,75]. Cultured astrocytes show a calcium-dependent vesicular release of D-serine following glutamate stimulation[76]. Henneberger et al.[77] were able to suppress LTP in hippocampal synapses *in situ* by clamping calcium concentration in nearby astrocytes, an effect that was rescued by the addition of D-serine. They were also able to block LTP by preventing D-serine synthesis in astrocytes, confirming that those cells are the source of the D-serine. Kang et al.[78] confirmed the findings of that study in the same system, and they went on to show that D-serine release is blocked by light-chain tetanus toxin, indicating that astrocytic D-serine is released through vesicular exocytosis. This finding illustrates another type of glial signaling that can activate neurons via a distinct mechanism that is independent of and also complementary to glutamatergic signaling, whether the glutamate is or neuronal or astrocytic origin.

Although several of the above studies identify group I mGluRs as the triggers of astrcoytic calcium rises, Sun et al. performed cell-sorting and qPCR on adult mouse astrocytes and found very low expression levels of mGluRs 1 and 5 in mice older than 3 weeks, which calls into question the applicability of studies in juvenile animals to adult glial physiology[79]. Additionally, several studies have refuted the claim the astrocyte calcium signaling has any effect on nearby synaptic function. One group has developed genetically encoded tools to selectively activate or abolish glial calcium signaling, and they have repeatedly demonstrated that they can induce or block that signaling with no effect on hippocampal synaptic plasticity *in situ*. For targeted stimulation of astrocytes, they developed mice with GFAP driven expression of the Gq coupled mGluR MrgA1, which is normally expressed in dorsal root ganglia and is activated by Phe-Met-Arg- Phe-NH2 amide (FMRF), a ligand not present in the brain[80]. Although FMRF stimulation triggered astrocytic calcium transients that mimicked those seen with mGluR5 stimulation, FMRF stimulation had no effect on basal signaling or synaptic potentiation[81,82]. For blocking glial calcium, they selectively knocked out the IP_3 receptor 2 isoform (IP_3R2), which abolished spontaneous and agonist-evoked calcium transients in astrocytes but did not affect IP_3R dependent calcium elevations in neurons, which rely on IP_3R isoforms 1 and 3[83]. Using eight different electrophysiological measurements, they did not observe any differences in basal synaptic transmission or potentiation in IP_3R2 knockout mice[82]. These studies highlight the difficulty of using pharmacological tools to study astrocytic and neuronal signaling together, given the high degree of overlap between signaling molecules and receptors that can act on both cell types. By eliminating the uncertainty inherent in pharmacological manipulations, these experiments provide convincing evidence that astrocytes do not modulate neural activity in an IP_3R-dependent manner *in situ*.

In vivo studies highlight heterogeneity in astrocyte signaling mechanisms

A recent paper from Srinivasan et al.[84] directly refutes the claim that IP_3R2 knockout mice lack astrocyte calcium signaling. Using the genetically encoded calcium indicator GCaMP6f, which allows for fast, high-resolution recording of calcium transients in subcellular compartments[85], they recorded astrocytes in brain slices and in awake, behaving mice using two-photon imaging. In hippocampal slices of mice lacking IP_3R2, they were able to observe somatic calcium waves that were smaller, shorter, and less frequent than in WT mice, and in addition they observed previously uncharacterized microdomain calcium fluctuations in astrocytic processes that occurred in both knockout and WT mice, although with different kinetics. Having

established the existence of IP_3R2-independent calcium signals in astrocytes, they used pharmacology *in situ* to demonstrate that the observed microdomain calcium transients in processes are caused by a combination of intracellular calcium release and calcium flux across the membrane.

They then transitioned to looking at cortical astrocytes in un-anesthetized mice *in vivo*, where they also saw reduced spontaneous somatic calcium transients in IP_3R2 knockouts and a decrease in the frequency but no change in the kinetics of spontaneous microdomain transients. Using a sensory stimulus to evoke a startle response, which is known to trigger a broad calcium transient across multiple astrocytes[86,87], they observed a large somatic transient that was abolished and small microdomain transients that were changed but still present in IP_3R2 knockouts. In WT mice, the microdomain transients were multiphasic, consisting of a fast component with a similar time course to somatic transients and a slow component that peaked almost a minute after the startle response. In IP_3R2 knockouts, the fast component was eliminated by the slow component was unchanged, indicating a second, IP_3R2-independent mechanism of astrocyte calcium signaling *in vivo*.

Another study that highlights the importance of studying *in vivo* circuitry found that barrel cortex astrocytes in mice are activated by acetylcholine (ACh) released from the nucleus basalis of Meynert (NBM), and that astrocyte calcium signaling is required for NMDA-dependent plasticity in the barrel cortex. Takata et al.[88] first observed an NMDAR- and muscarinic acetylcholine receptor (mAChR)-dependent potentiation of cortical local field potential (LFP) when they stimulated the NBM concurrently with whisker stimulation. Using the calcium dye Fluo-4 AM, they saw that astrocytes were significantly more likely to experience a calcium transient during co-stimulation than during whisker stimulation alone. This effect was not blocked by an antagonist of mGluR5, suggesting that the cholinergic NBM afferents were acting directly on the astrocytes. They then performed the same co-stimulation on IP_3R2 knockout mice, which lacked somatic calcium transients in response to stimulation, and they did not see potentiation of the LFP as they had in control mice. Given the NMDAR-dependence of that potentiation, they measured extracellular levels of the NMDAR co-agonist D-serine and found a significantly higher concentration in WT mice compared to IP_3R2 knockouts following NMB stimulation. Additionally, they were able to rescue potentiation of the LFP in IP_3R2 knockouts by surface application of D-serine, indicating that IP_3R2-dependent glial calcium activity and D-serine release, which is potentiated by cholinergic stimulation from the NBM, contributes to cortical plasticity.

Cerebellar Bergmann glia were the subject of the first *in vivo* calcium imaging of glia performed in awake, behaving animals[47]. Paukert et al.[86] followed up by more closely investigating the mechanisms behind the BG calcium transients seen during locomotion. In mice expressing the genetically encoded calcium sensor GCaMP3 under the control of the promoter for GLAST, the glial-specific glutamate transporter, they observed that locomotion-triggered calcium transients were not correlated with locomotion speed and did not happen during every bout of locomotion, suggesting that the glutamatergic neural circuit directing locomotion behavior are not the only influence on BG calcium levels. Using pharmacology against a variety of neurotransmitters, they found that an antagonist of adrenergic receptors blocked locomotion-trigger calcium, leading them to suspect norepinephrine, which is known to be released during

physiological arousal[89]. They then extended their study to astrocytes in the visual cortex, where norepinephrine is released during arousal even in the absence of visual input. When a light stimulus was presented during locomotion, the astrocyte calcium response spread to a higher percentage of cells and increased in amplitude, suggesting that norepinephrine primes astrocytes to respond to local neural circuit activity. This work, together with the results in the barrel cortex indicating that astrocytes act as a mediator for ACh signaling in cortical plasticity, suggest that astrocyte calcium signaling can interact with neural activity through a variety of mechanisms that differ depending on the brain region and cells involved.

Although it has been almost 30 years since the discovery of astrocyte calcium transients, many of their reported functions remain controversial and there are still wide gaps in our knowledge of the physiological role of glial signaling. Many of these discrepancies are due to different methods of experimentation, including cultured, slice, and *in vivo* preparations, and much of the difficulty in the field has been in elucidating what phenomena are applicable to normal physiological conditions. The startle response is a defensive reflex that is present in all animals and has been shown to trigger astrocyte calcium increases *in vivo* in both cortical astrocytes and Bergmann glia (**Figure 3**). This study investigates the mechanism of glial calcium signaling during startle behavior in the awake, behaving zebrafish (*Danio rerio*) and provides an experimental protocol for the use of zebrafish as a model for studying glial biology.

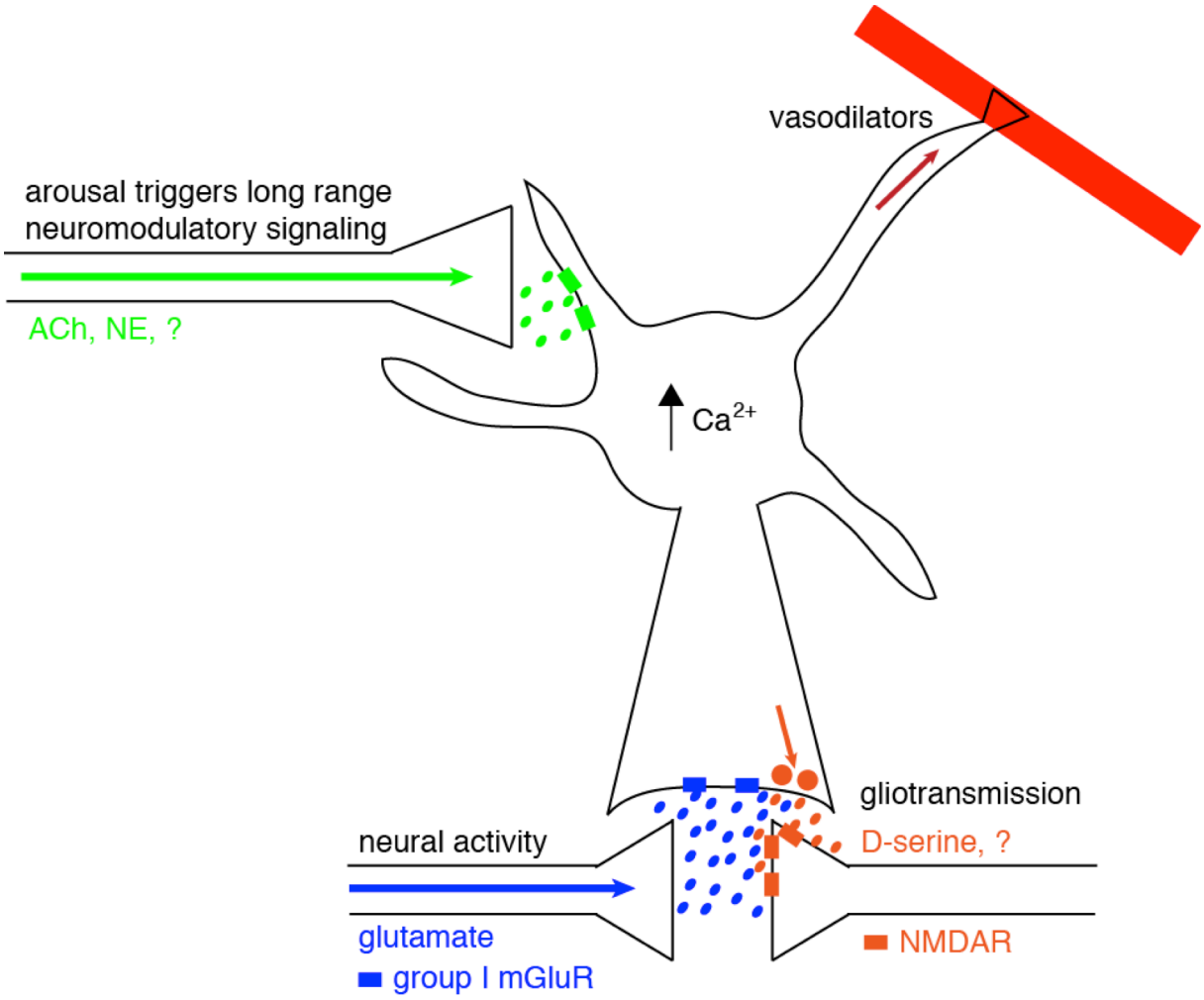

Figure 3. Model for astrocytic signaling during startle reflex.
A physiological state of arousal triggers long-range neuromodulatory signaling through cholinergic or adrenergic afferents. ACh or NE is sensed by astrocytes, which are then primed to respond to local synaptic glutamate with an increase in intracellular calcium. Intracellular calcium increase triggers vasodilation and possible release of gliotransmitters such as D-serine, which can act on postsynaptic NMDARs to modulate synaptic plasticity. mGluR, metabotropic glutamate receptor; NMDAR, N-methyl-D-aspartate receptor; ACh, acetylcholine; NE, norephinephrine.

2. A new system for studying *in vivo* glial calcium signaling in zebrafish

2.1 Introduction

Despite recent advances in our understanding of glial signaling, it remains challenging to elucidate how glia respond to or influence neural network activity *in vivo*. *In vitro* studies have often presented conflicting results regarding the mechanisms that trigger glial calcium signaling as well as the existence and possible function of subsequent glial modulation of neuronal activity. Larval zebrafish are an ideal model system for studying *in vivo* glial function because of their optical clarity, tractable genetics, and the ability to combine *in vivo* live-cell imaging with simultaneous behavioral recording. Our lab has developed and built custom experimental apparatuses that enable cellular-resolution live imaging coupled to simultaneous high-speed behavioral analysis of individual fish as well as high-throughput behavioral analysis (**Figures 4 and 5**).

Zebrafish (*Danio rerio*) are a species of small teleost fish that are popular as a model organism for neuroscience, cell biology, and drug discovery, among other fields of study. Targeted gene expression in the zebrafish was first enabled by the Gal4-UAS system, derived from yeast and initially popularized in *Drosophila*. This system consists of the yeast Gal4 transcriptional activator that binds to the Upstream Activator Sequence (UAS). Gal4 expression is typically driven by the promoter of interest, while the gene of interest is placed downstream of the UAS and is only transcribed in the presence of the Gal4, thus limiting expression to cells in which the Gal4 is expressed[90]. One advantage of this system is the ability to use separate lines of transgenic organisms to mix and match promoters of interest (driving Gal4) with different genes to be expressed under control of the UAS. In zebrafish, transgenesis is achieved using the Tol2 transposase, isolated from the medaka fish, which yields high rates of stable transgenesis and is readily available in plasmids designed specifically this purpose[91,92]. Tol2-Gal4 has been used for gene and enhancer trapping to randomly generate libraries of stable transgenic zebrafish lines with distinct expression patterns, including *Gal4*^{s1107t}, which labels radial glia[93].

Though mammalian systems have been more commonly used in the study of glial biology, the zebrafish presents unique advantages that many other model organisms lack. Astrocytes have been shown to express several neurotransmitter receptors and exhibit measurable calcium signals in response to different neurotransmitters; however, a high throughput analysis of those responses *in vitro* was unable to distinguish patterns of calcium signaling in response to specific agonists, so the physiological relevance of that signaling remains unclear[94]. In mice, astrocytes *in vivo* have been shown to respond to sensory stimulation and motor activity[39,86], but these types of studies are rare due to the difficulty of obtaining data from awake, behaving mice. In the zebrafish larva, glial calcium transients can be visualized clearly with no surgical intervention or complicated head-fixing apparatus. The entire fish, or a portion of it, can be immobilized in agar, and a single fish can yield data from cells in multiple regions of the central nervous system. Additionally, zebrafish are particularly amenable to high-throughput behavioral analysis because data can be collected from dozens of fish simultaneously using a specialized experimental apparatus (**Figure 5**).

Unlike mammals, zebrafish do not have stellate-shaped astrocytes in addition to radial glia. Nonetheless, there is evidence that these radial cells serve many of the same function as mammalian astrocytes in addition to acting as neural progenitor cells[95,96]. Zebrafish radial glia express the water channel aquaporin-4, also found in mammalian astrocytes, along with astrocytes markers glial fibrillary acidic protein (GFAP), glutamine synthetase (GS)[97], and the glutamate transporter EAAT2[98]. These expression patterns indicate that zebrafish radial glia perform many of the same critical functions as mammalian astrocytes including glutamate reuptake and controlling water movement through the CNS. Despite the many available morphological observations, it has not been shown whether these cells exhibit dynamic calcium signaling similar to that observed in mammalian astrocytes both *in vitro* and *in vivo*.

Signaling through metabotropic glutamate receptors (mGluRs) are hypothesized to be one of the primary mechanisms that initiate glial calcium transients[34,99]. The close association of astrocytic processes with the synaptic cleft and their known roles in glutamate reuptake make glutamate a likely candidate for a neurotransmitter that can also trigger glial responses. In culture and slice preparations, astrocytes have shown responses through multiple mGluR subtypes including group I mGluRs 1 and 5[94,100]. In mammals, Bergmann glia of the cerebellum are known as "radial astrocytes," and they have been well characterized due to their relative accessibility and morphological simplicity. Bergman glia have been shown to posses mGluR1 mediated calcium transients in response to neuronal activity[45,46] and are required for normal cerebellar function[42,44]. These cells differ morphologically from stellate astrocytes, which are the most widely studied cell type in investigations of glial calcium signaling, but they posses many of the same properties as astrocytes and are capable of responding to and regulating neural transmission. Although zebrafish lack stellate astrocytes, studies of Bergmann glia demonstrate that a radial morphology is not indicative of a less active glial cell type.

The acoustic startle response (ASR) in zebrafish is a simple behavior that can be reliably elicited by a short sound stimulus[101]. The ASR consists of a short latency defensive behavior, which in zebrafish is a fast, unilateral movement of the tail known as a C-bend [102]. This response is initiated by the Mauthner cells of the hindbrain, which receive a combination of excitatory glutamatergic inputs that respond to sensory stimuli and feed-forward inhibitory glycinergic inputs[103]. The reliable nature of the behavioral output combined with the relative simplicity of the neural circuit involved make the ASR an ideal assay with which to study glial calcium signaling *in vivo*. This study establishes a reliable experimental method for eliciting and recording calcium transients in radial glia of the zebrafish larva.

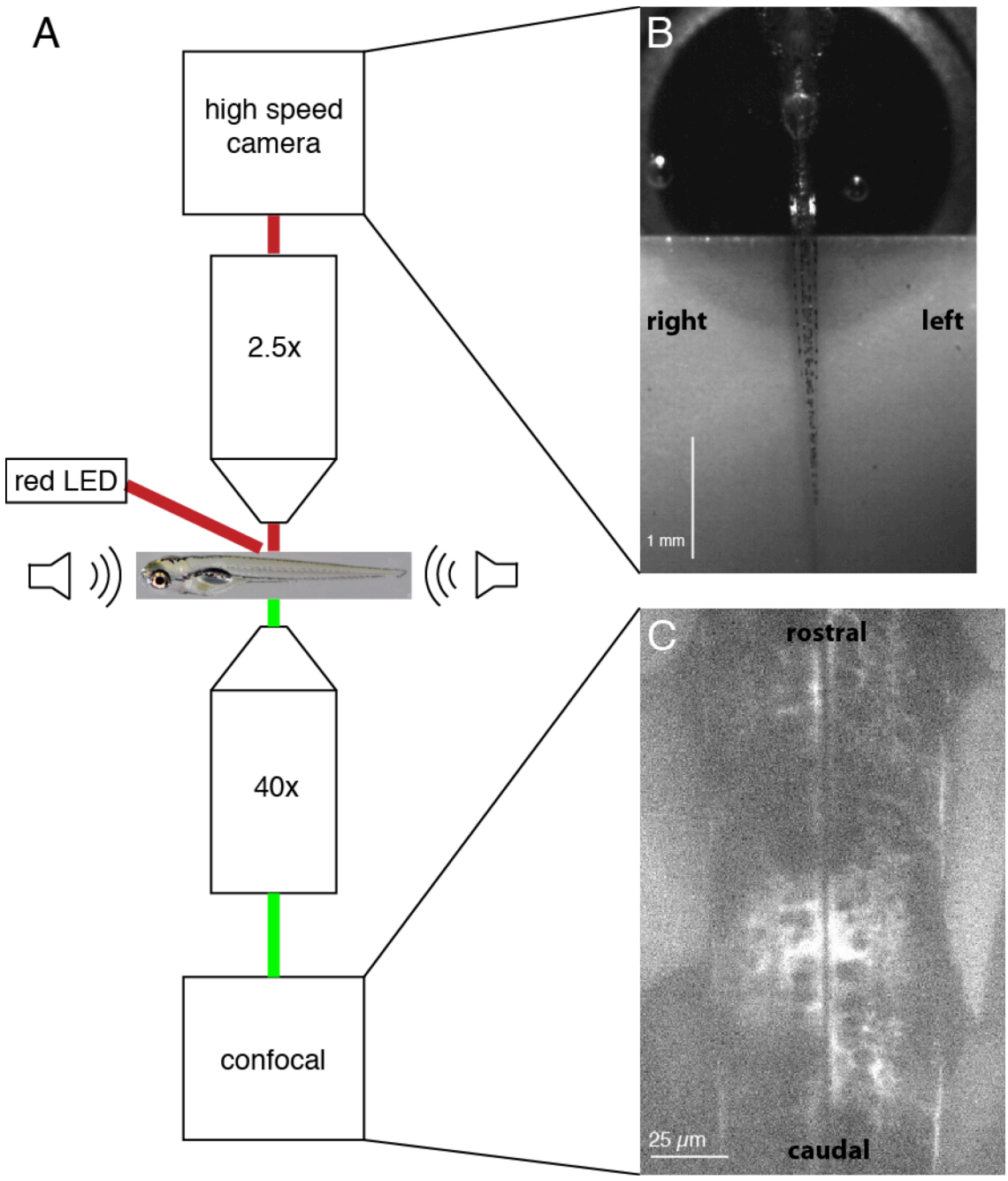

Figure 4. Experimental setup for simultaneous imaging with acoustic stimulus
A. Schematic of two-headed microscope with speakers for acoustic stimulus.
B. Representative view from the high speed behavioral imaging camera through 5x objective, looking down on the ventral aspect of the fish. Fish is mounted on its dorsal side, so fish's right side is to the left in this image. **C.** Representative view from the inverted confocal camera through 40x objective. View is from the dorsal aspect of the fish looking into the spinal cord. GCaMP5 expression can be seen in the muscle on either side of the spinal cord.

2.2 Materials and methods

Zebrafish transgenesis

Stable transgenic zebrafish lines (*Danio rerio*) that label glia were selected from a Gal4 enhancer trap screen (Baier Lab)[104]. *Gal4^{s1107t}/UAS:Kaede* fish were selected based on the clear radial morphology of the labeled cells as well as the lack of cells with a neuronal morphology (Figure 2). They were crossed to *UAS:GCaMP5* fish and offspring were screened for GCaMP5 expression and lack of Kaede. Larvae were then raised to adulthood and in-crossed to generate the larvae used in experiments. *gSAIzGFFD478A /UAS:GFP* fish were obtained from the Kawakami lab, then crossed to wild type fish to generate "dark" *478A* lacking GFP expression, and those fish were raised to adulthood and crossed to *UAS:GCaMP5* fish to generate *478A/UAS:GCaMP5*.

The stable line of *UAS:GCaMP5* fish was created by injecting *Tol2-UAS:GCaMP5* into *Cmlc2:Kal4/cry:red* AB line (in which a modified version of the transcription factor Gal4 is expressed in the larval zebrafish heart) embryos at the one cell stage. The *Tol2-UAS:GCamP5* construct was built by using the following primers to PCR amplify the open reading frame (ORF) of *GCaMP5.003*: 5'- ATATATGGATCCTCACGTTACTAGTATGGGTTCTC -3' and 5'- ATATATGCGGCCGCTCACAAAGATCCTCTAGACTTCG -3'. The *GCaMP5.003* ORF was ligated into the PME-MCS vector, the middle vector in the Tol2 zebrafish transgenesis Gateway® kit. The Multisite Gateway® technology was used to combine the PME-MCS-GC5 vector with the p5E-UAS, p3E-polyA and pDestTol2pA2 vectors to generate the *Tol2-UAS:GCaMP5* fish transgenesis vector. The injections consisted of plasmid DNA (30 ng/ml), RNA for the Tol2 transposase (25 ng/µl) and 0.1% Phenol Red (wt/vol). Injected fish were screened for transgenesis at 5 days post-fertilization (dpf) by looking for GCaMP5 expression in the heart using a fluorescent stereoscope, and positive larvae were raised to adulthood and crossed to wild type fish to generate stably expressing F1 fish.

HuC:GCaMP5;GFAP:RGECO fish were generated by injecting Tol2-GFAP:RGECO into HuC:GCaMP5 fish (Schier lab) at the one-cell stage to produce transient expression of RGECO in glia. To generate the Tol2-GFAP:RGECO construct, the ORF of *RGECO* was PCR amplified with the following primers and ligated into Tol2-GFAP:GFP (Raymond lab[105]):
5'- GATGGCGCGCCACC ATGGTCGACTCTTCACGTC- 3' and
5'- GATGGCGCGCCCTACTTCGCTGTCATCATTTGTAC- 3'.

Calcium imaging

For imaging spontaneous glial calcium transients, *Gal4^{s1107t}/UAS:GCaMP5* larvae at 5 dpf were mounted in a glass well petri dish and immobilized right side down in a solution of 2% agar in embryo water (E3). For simultaneous red and green imaging of glia and neurons, respectively, *HuC:GCaMP5*;*GFAP:RGECO* larvae at 5 to 6 dpf were mounted in the same way. Fish were imaged using an inverted spinning disk confocal through a 40x/1.1 Zeiss objective at 10 fps. Fluorescence excitation was performed with 448 nm (GCaMP alone) or alternating 488 and 560 nm (GCaMP and RGECO) laser lights at 10 fps.

Simultaneous behavioral and calcium imaging

Gal4^{s1107t}*/UAS:GCaMP5* larvae at 5 to 6 dpf were placed in a glass well petri dish and immobilized dorsal side down in a solution of 2% agar in embryo water (E3). The agar was then cut away from a region caudal to the fish's anus in order to allow the tail to move freely. Fish were placed on the microscope and allowed to acclimate for 15 minutes under red light before any stimulus was delivered. Imaging was performed on a 3i Marianas system (Intelligent Imaging Innovations (3i), Inc.) with an inverted spinning disk confocal (Yokogawa) as well as an upright CMOS camera (Mikrotron Eosens 1362) mounted on a Zeiss microscope for simultaneous image capture of cellular resolution fluorescence and whole fish swimming behavior.

For behavior imaging, fish were illuminated from the side with far red light provided by a custom-built red LED light source and were imaged from above with a 2.5x/0.06 air objective (Carl Zeiss, Inc.) at 1000 fps and 800 x 600 pixel resolution. The inverted confocal was used for calcium imaging of spinal glia through a 40x/1.1 Zeiss objective at 8 fps. GCaMP5 fluorescence excitation and detection were performed with continuous 488 nm laser light at low intensity and detection through BP 488-568 nm and BP 520-535 nm to block red light used for behavior imaging. Fluorescence imaging was performed with a 100 ms exposure time in order to maintain the lowest possible laser power due to the sensitivity of glial cells to laser light[39].

Piper software (Stanford Photonics) was used to control the behavior camera as well as to synchronize behavioral and confocal imaging by triggering Slidebook software (Intelligent Imaging Innovations (3i), Inc.). Once triggered, a 1 or 4 s baseline was recorded before delivery of the sound stimulus. The sound stimulus was generated by two speakers (Visaton SC 5.9) resting on the imaging stage with the cone side down. The stimulus was triggered by a sinusoidal wave (1000 Hz, 1-10 ms, 0.4 V) generated by a function waveform generator (Agilent, 33220A) connected to the speakers. Behavioral imaging at 1000 fps continued for 200 ms following the stimulus, while calcium imaging at 8 fps continued for 20 s.

Pharmacology

2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) and 7-(Hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) were obtained from Tocris Biosciences. MPEP was diluted to 4uM in E3, and CPCCOEt was diluted to 10 uM in E3. Fish were placed in a petri dish containing the drug and left for 45 minutes before mounting. Once a fish was mounted in agar, the drug was added to the petri dish and the fish was placed on the microscope for 15 minutes of acclimation before the experiment began, bringing the total time in the drug to one hour before the experiment was performed. For high-throughput behavioral analysis, fish were left in the drug for 45 minutes before being transferred to the 48-well plate in the same solution and placed in the recording apparatus, where they were also given a 15 minute acclimation period.

High throughput behavioral assay

Gal4^{s1107t}*/UAS:GCaMP5* fish at 5 days post-fertilization were subjected to either drug or control treatment for 45 minutes before being sorted into 48 well plates. Each fish was transferred to a single well in 300 μL of E3 with or without drug. Each plate consisted of both control and drug-treated fish in alternating rows in order to minimize any spatial differences in the acoustic stimulus. Plates were then transferred to a custom-built high-throughput behavioral apparatus that delivers acoustic stimuli through two speakers (Visaton SC 5.9) and illuminates the plate for high-speed behavioral recording with a CCD camera (fire-i 780b, Unibrain, 30 fps). The 48-well plate was inserted into a holding device in the apparatus in order to position it for behavioral recording and delivery of the acoustic stimulus by speakers mounted to the platform holding the plate (**Figure 5**). The speakers delivered 900Hz square waves of ~ 3 ms duration, powered by a 15W amplifier. The experiment was run by custom-written MATLAB scripts that trigger stimulus output through a Native Instruments PCI-6229 DAQ.

Fish were allowed to acclimate for 15 minutes under white light once placed in the apparatus, followed by 10 min of spontaneous activity recording, and then a protocol designed to test habituation to the acoustic stimulus. The habituation protocol begins with 10 low-voltage (0.025 V, ~ 75 dB) stimuli with a 90 s inter stimulus interval (ISI), 10 high-voltage (0.4 V, ~ 95 dB) stimuli with a 90 s ISI, and then 100 high-voltage (0.4 V, ~ 95 dB) stimuli with a 5 s ISI. These data were used to quantify the habituation index (HI), defined as the ratio of the probability of response after a series of stimuli at a short ISI (5 s) (P_{after}) to the initial probability of response to a series of stimuli at a long, non-habituating ISI (90 sec) ($P_{initial}$), ($HI = 1 - P_{after} / P_{initial}$).

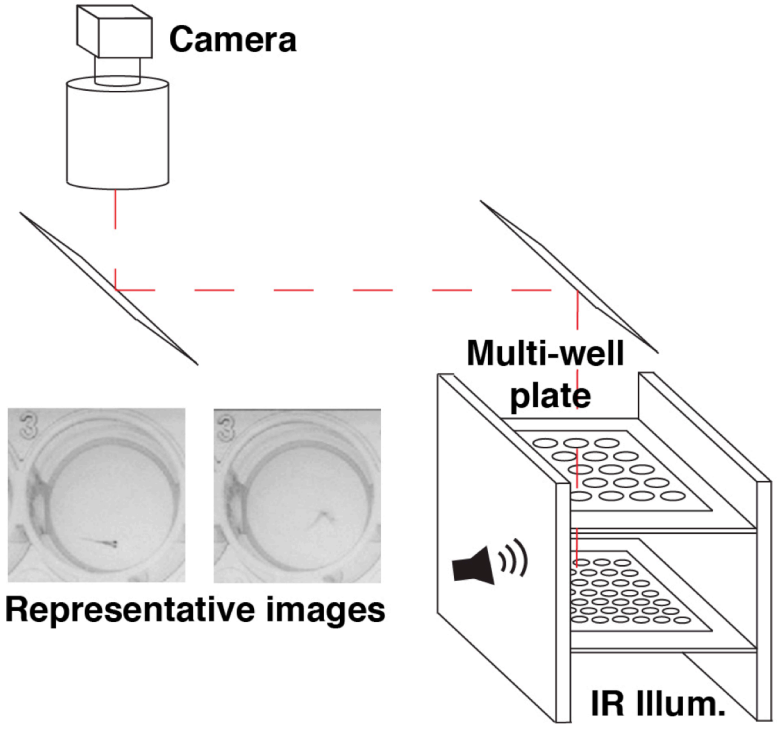

Figure 5. Experimental setup for high throughput behavioral analysis.
Fish are separated into individual wells of 48-well plate, which is placed in the behavioral apparatus and illuminated with infrared (IR) light from below. High speed behavioral imaging is captured at 1000 fps, allowing for detection of escape behavior as seen in representative images. Illustration credit C. Pantoja.

2.3 Results

Zebrafish spinal glia exhibit spontaneous and evoked calcium waves

Larval *Gal4*^{s1107t}*/UAS:GCaMP5* fish were imaged on their side in order to optimally visualize both dorsal and ventral spinal glia (**Figure 6**). When un-anaesthetized fish were imaged undisturbed for 8 minutes, full-body muscle contractions were always followed by a broad glial transients that included all cells in the field of view (total of 17 broad transients in 6 fish). Out of 6 fish, all moved during at least once during the imaging window (avg = 3 ±1 movements per 8 minutes), and the subsequent transients lasted an average of 9s ±1s. Spontaneous sub-cellular transients were also witnessed in between movements, lasting an average of 14.6s ±2.8s (**Figure 7**). Broad transients were only seen following movement. *HuC:GCaMP5*;*GFAP:RGECO* larvae were then used to determine the relative timecourse of neural and glial activity. Fish were given a sound stimulus to trigger neural firing, and movement in response to the sound stimulus was reliably followed by a neural transient and glial transients. Glial transients peaked an average of 1.4s ±.14s following the neural peak (n=4) (**Figure 8**). In this setup, fish only responded to the acoustic stimulus with a movement 15% of the time, and fish were unable to perform a normal escape behavior because they were constrained in agar on their sides. Although the glia are best visualized in this configuration, the low rate of response to the stimulus as well as concerns about effects on the vestibular system made this a difficult and unreliable setup for imaging evoked transients. Additionally, when fish are imaged on their sides, their contractile motion creates artifacts that are difficult to remove during analysis (motion artifacts can be seen in the averaged traces in figure 8).

Zebrafish spinal glia display reliable calcium transients following sound-evoked escape

In order to repeatedly record glial calcium activity, further experiments were performed in an established setup that reliably evokes neural activity and an escape behavior (C. Pantoja, manuscript in preparation). Simultaneous high-speed behavioral imaging and cellular-resolution calcium imaging were used to characterize calcium transients in radial glia of the spinal cord following an acoustic startle response (ASR), which was elicited in 70% of fish by the first acoustic stimulus presented after a 15 minute acclimation (**Figure 4**). *Gal4*^{s1107t}*/UAS:GCaMP5* fish were imaged with a 40x objective and positioned so that 4-6 ventral spinal glial cells were in focus. Radial glia in the spinal cord extend from the central canal to the pial surface in all directions, and the ventral cells were chosen due to their more compact size, which makes them easier to capture in a single focal plane. Out of 26 fish that had an ASR in response to the first sound stimulus, 13 (54%) displayed glial calcium transients (total of 43 cells) with a maximum ΔF/F of 1.31 a.u. (± 0.04 a.u), peaking at 2.3s (±0.08 s) following sound evoked escape, lasting approximately 7 s. Cells were divided into ipsilateral and contralateral groups relative to the direction of the escape response tail flick, based on the observation that one side is often stronger than the other in individual fish. Ipsilateral cells had a maximum ΔF/F of 1.34 a.u. (± 0.05 a.u), and contralateral cells had a maximum ΔF/F of 1.29 a.u. (± 0.06 a.u), but they did not differ significantly ($p > 0.05$). In response to the second sound stimulus, presented after a non-habituating interval of 2 min.[106], 23 fish exhibited a second ASR escape behavior (all 23 had also responded in the first trial) but only 7 (30%) of those had glial calcium transients in a total of 15 cells. Ipsilateral cells had a maximum ΔF/F of 1.31 a.u. (± 0.05 a.u), and contralateral cells had a maximum ΔF/F of 1.27 a.u. (± 0.09 a.u), which did not differ significantly ($p > 0.05$) from each

other or from peaks in the first trial (**Figure 9A**). On the third trial, 22 fish exhibited an ASR escape behavior and 7 (30%) had glial calcium transients. Although the percentage of fish with a glial calcium response was consistent from trial 2 to trial 3, by trial 3 only one cell per fish responded with a calcium transient. From this point on all experiments include only the first trial due to maximum responsiveness to the acoustic stimulus and maximum number of cells per fish responding with a calcium transient.

Due to strong GCaMP5 expression in the muscle of the *Gal4*^{s110t7} fish, all traces include a sharp peak of fluorescence due to bleedthrough of the GCaMP signal from the calcium transient in the muscle. This muscle signal lasted an average of 1.1s. Although this signal obscures the beginning of the glial calcium transient, the *Gal4*^{s110t7} line was chosen for its strong, broad, and consistent expression of GCaMP5 in glial cells and was superior to any other available fish lines with glial expression. Only fish that responded immediately (latency ≤ 14 ms) to the acoustic stimulus with a single escape behavior were used in averaged traces, and the muscle artifact was removed. Example images and trace shown in **Figure 10**.

Glial calcium transients are mediated by mGluR1

The specific mGluR5 antagonist MPEP had no effect on transients **(Figure 9B)**. In fish treated with 4 μM MPEP (IC_{50} 36 nM), 5 of 12 fish that displayed an ASR on the first trial also had a glial calcium transient (42%, n=12) with a maximum ΔF/F of 1.32 a.u. (± 0.05 a.u), peaking at 2.1s (±0.09 s) following sound evoked escape, lasting approximately 7 s. Peak ΔF/F and latency were not significantly different from controls ($p > 0.05$). In contrast, the specific mGluR1 antagonist CPCCOEt abolished glial calcium transients following ASR. In fish treated with 10 μM CPCCOEt (IC_{50} 6.5 μM), none displayed measurable glial calcium transients following sound evoked escape (n=42 cells from 10 fish with ASR) (**Figure 9C**).

CPCCOEt treatment was repeated in *Tph2:Gal4ff/UAS:GCaMP5* fish to control for a possible effect of CPCCOEt on neuronal activity or GCaMP fluorescence. Tph2 expressing cells in the dorsal Raphe nucleus respond reliably to an acoustic stimulus, and that response produces a sharp increase in fluorescence in the dorsal raphe nucleus of *Tph2:Gal4ff/UAS:GCaMP5* fish (C Pantoja, unpublished data). CPCCOEt treated fish (n=4) exhibited a strong and distinct calcium signal, confirming that the effect of CPCCOEt on glial calcium transients is not due to a global effect on neural signaling or a direct effect on GCaMP fluorescence (**Figure 11**).

Inhibition of mGluR1 does not alter swimming or escape behavior

A high throughput behavioral experiment was performed to determine if mGluR1 blockade has any effect on global behavioral patterns in zebrafish. Locomotion in fish is used as a measure of general health, and habituation to ASR is a non-associative form of learning that relies on changes in synaptic strength[106]. If mGluR1 were involved in the neural circuits governing locomotion or startle, we would expect a difference in behavioral measurements between control and drug-treated fish. Individual fish were placed in single wells of a 48 well microplate, with control and drug treated (10 μM CPCCOEt) in alternate rows, and the plate was placed on the behavioral apparatus to record spontaneous and sound evoked activity (**Figure 5**). Spontaneous swimming behavior was recorded for 10 minutes, and there was no significant difference between the two groups (n=96 control, 96 CPCCOEt) (**Figure 12A**). Fish were then

subjected to 20 acoustic pulses at a 90 s ISI followed by 100 pulses at 5 s ISI to determine habituation index (HI) (see Materials and Methods for description). There was no significant difference in HI between the two groups (n=96 control, 96 CPCCOEt) (**Figure 12B**). These results indicate the CPCCOEt is not altering neural activity or synaptic potentiation upstream of the observed glial calcium signaling that occurs in response to acoustic startle.

Vascular calcium transients follow a similar time course to glial transients

Given the role of astrocytes in neurovascular coupling[47], a promising application of this technique would be the use of zebrafish as a model organism for studying neurovascular interaction. In a transgenic line that drives expression in the vasculature (*478A/UAS:GCaMP5*), GCaMP5 fluorescence in vessels lining the spinal cord peaks following ASR. In 17 fish with an ASR on the first stimulus, 11 (65%) displayed vascular calcium transients peaking 2.5 s (±0.24s) following sound evoked escape. A total of 27 cells had transients (**Figure 13**). Although the mechanism of the observed calcium transient is unknown, smooth muscle cells that line the walls of the vasculature are known to exhibit dynamic changes in calcium concentration[107], and arachidonic acid and adenosine, two metabolites release by astrocytes, act as vasodilators by depolarizing smooth muscle cells[108]. Although observed in separate experiments, the fact that the glial calcium signal peaks at 2.3s and the vascular signal peaks at 2.5s suggests that further study of the two phenomena in the same experiment would be valuable in investigating the contributions of glial signaling to neurovascular coupling.

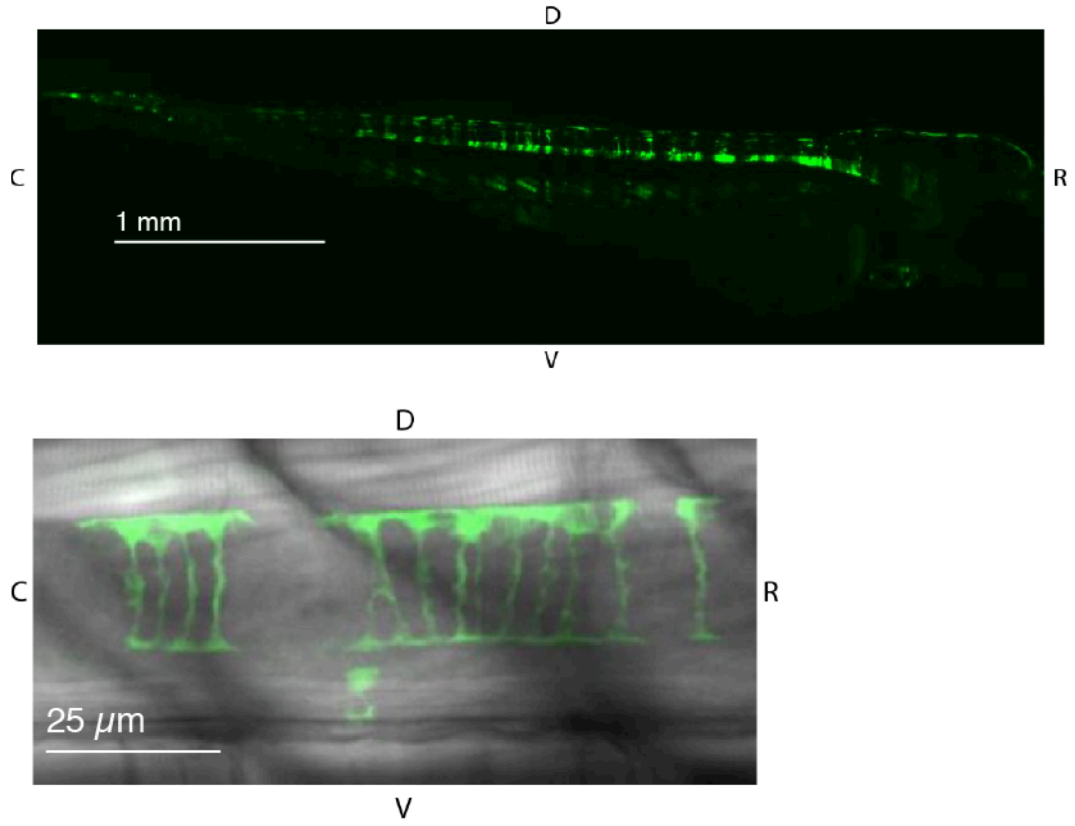

Figure 6. *Gal4*^{s1107t} labels zebrafish spinal radial glia.
Top panel shows an entire *Gal4*^{s1107t}*/UAS:GCaMP5* fish at 5dpf imaged at 5x magnification. Radial glia extend from the central canal to the pial surface of the spinal cord in all directions. Ectopic muscle expression of GCaMP5 can be seen ventral to the spinal cord. Bottom panel shows a *Gal4*^{s1107t}*/UAS:Kaede* fish at 5dpf imaged through a 40x objective, overlaid with a brightfield image showing the boundaries of the spinal cord. Kaede expresses sparsely, giving a clearer image of individual cells.

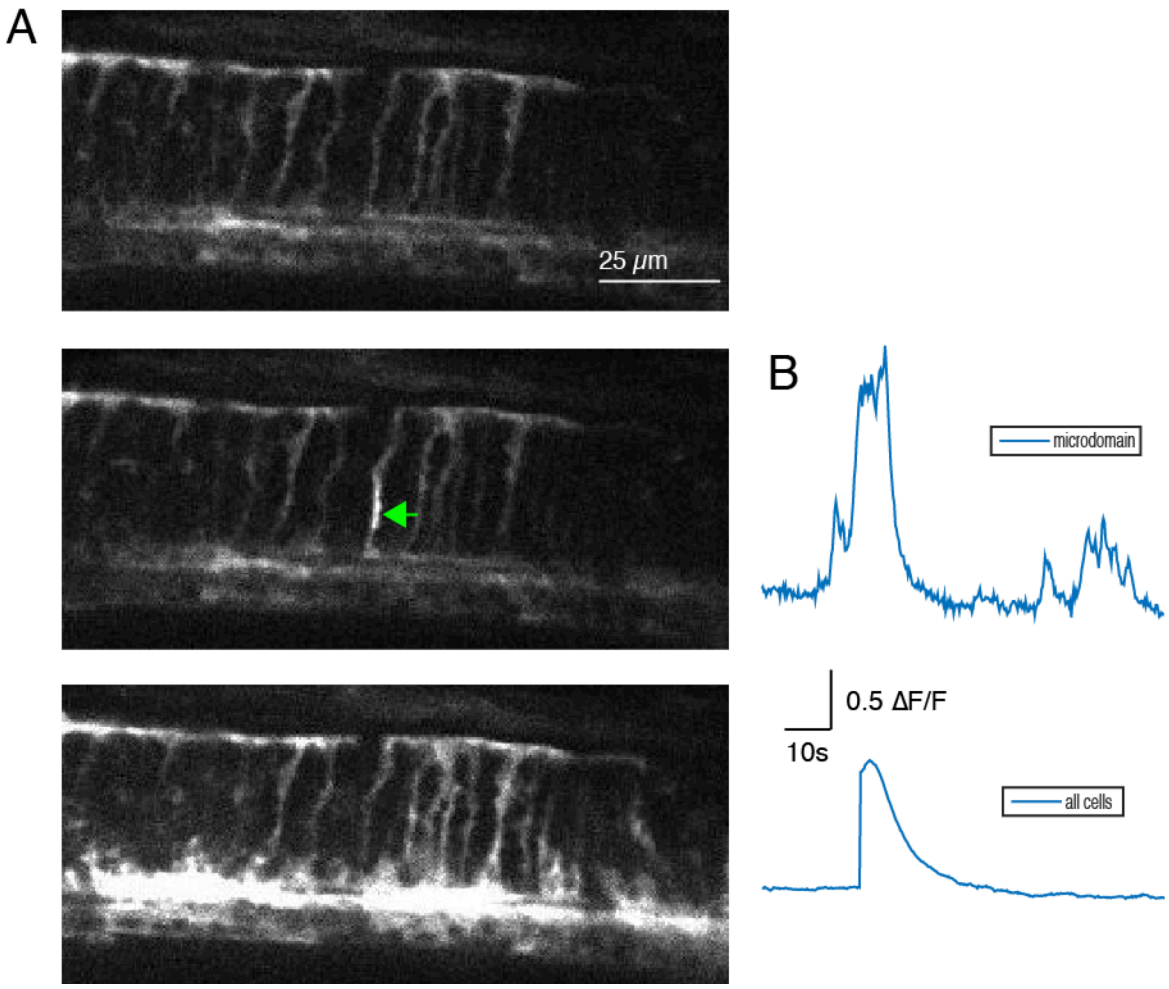

Figure 7. Zebrafish spinal glia exhibit spontaneous calcium transients.
A. Still images from an 8 minute movie of spontaneous activity in a 5dpf *Gal4*^{s1107t}*/UAS:GCaMP5* larva. Top panel shows baseline fluorescence, middle panel shows a spontaneous subcellular transient, and bottom panel shows a broad calcium transient that occurred following spontaneous movement. **B.** Traces of the subcellular domain (top) and whole field (bottom) representing the times shown in the images. Broad transients lasted an average of 9s ±1s, and subcellular transients lasted an average of 14.6s ±2.8s.

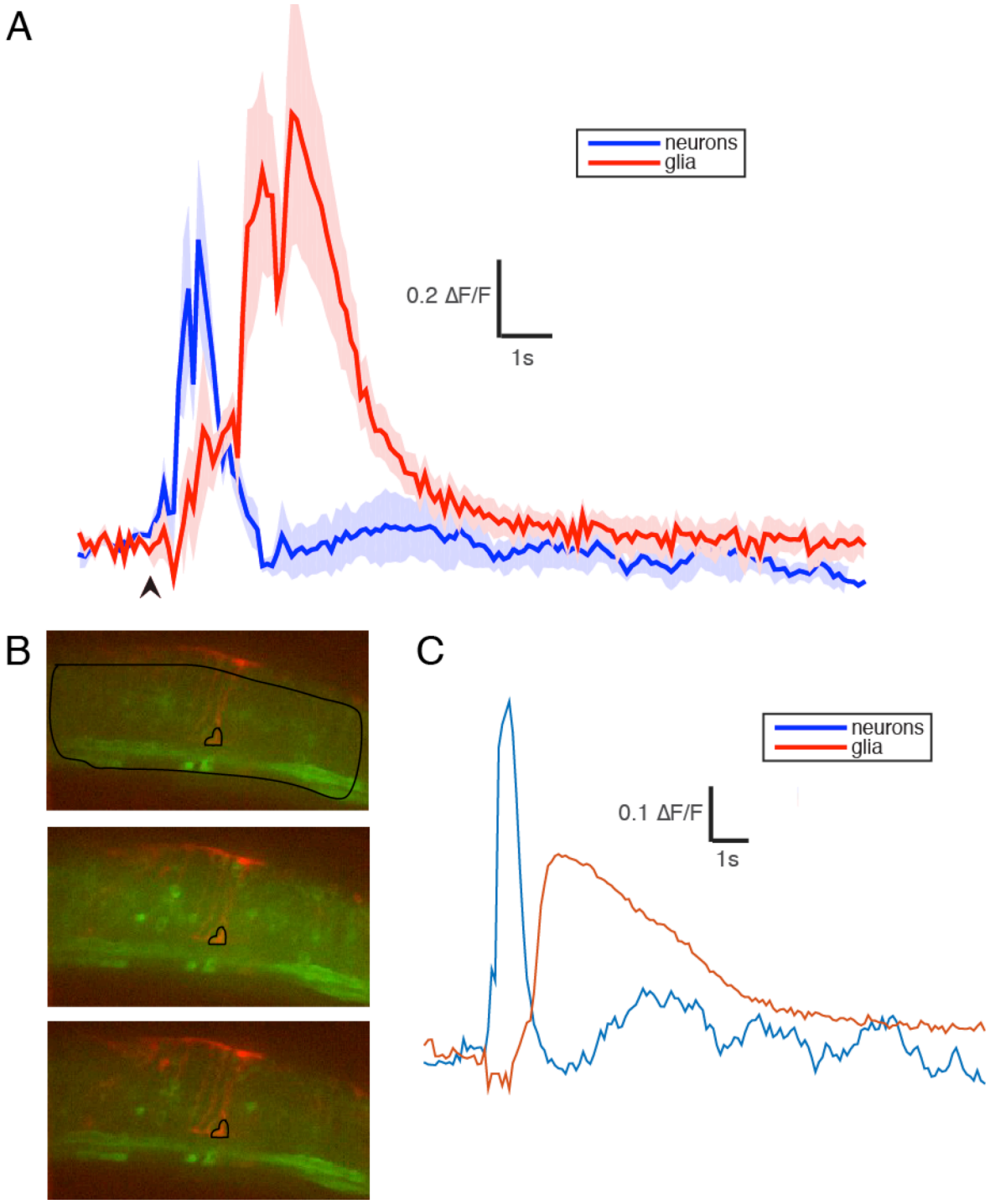

Figure 8. Glial transients follow neural activity.
A. Averaged traces for neural and glial activity in *HuC:GCaMP5;GFAP:RGECO* fish following sound stimulus. Shaded area represents ± s.e.m., arrowhead indicates stimulus (n=8 glial cells from 4 fish. Neural ROI was drawn around whole area of green fluorescence for each fish). Glial transients peaked an average of 1.4s ±.14s following the neural peak. **B.** Representative images of sound-evoked neural and glial transients. ROIs shown in black. Large ROI in the top panel is for neural activity. **C.** Traces of highlighted ROIs.

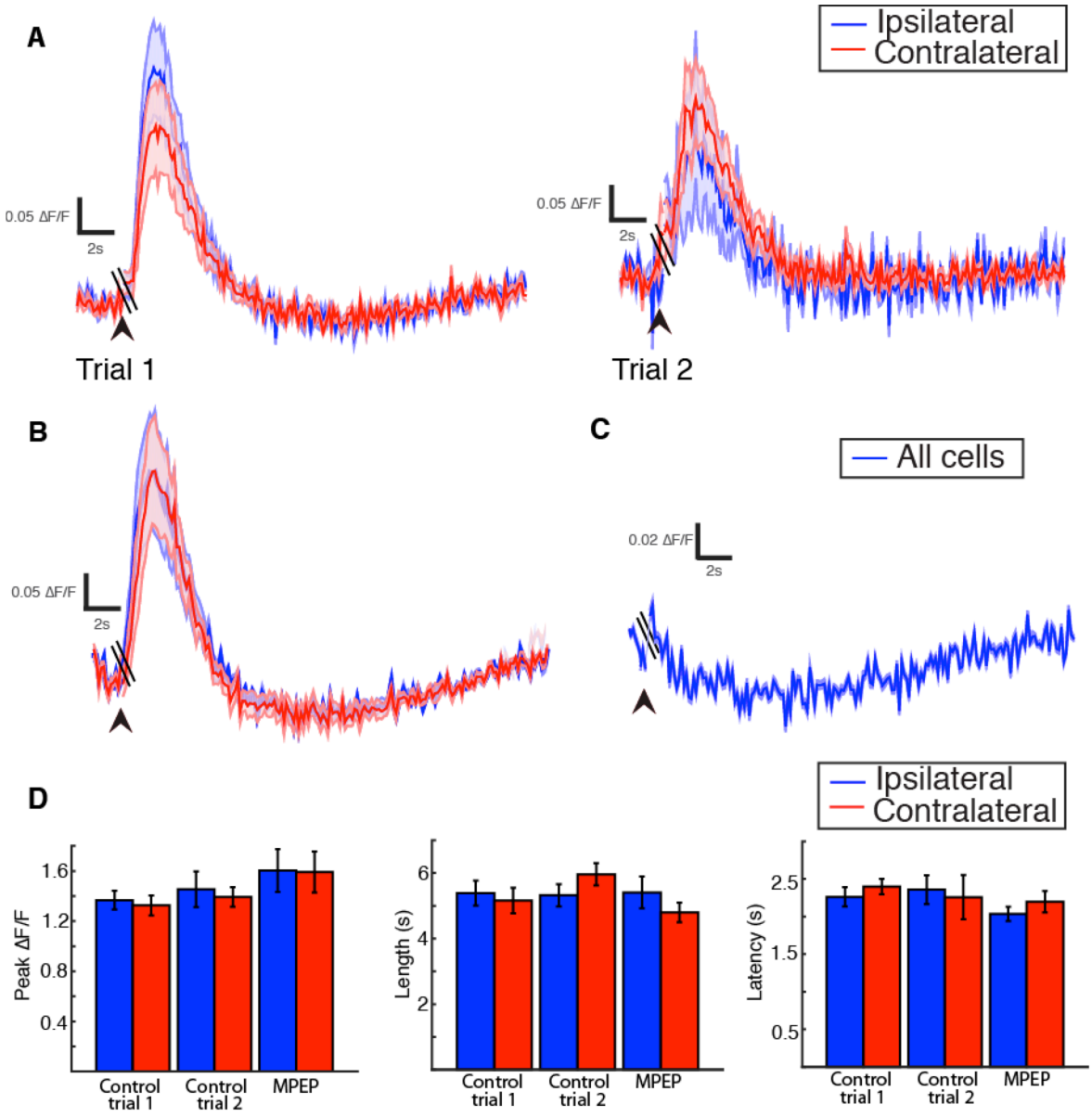
A
Ipsilateral
Contralateral
0.05 ΔF/F
2s
Trial 1
Trial 2
B
C
All cells
0.02 ΔF/F
D
Peak ΔF/F
Length (s)
Latency (s)
Control trial 1
Control trial 2
MPEP

Figure 9. **Glial calcium transient is abolished following CPCCOEt treatment.**
mGluR1 antagonist abolishes glial calcium transients following escape response. **A**. Averaged fluorescence traces from cells contralateral and ipsilateral to the direction of escape movement in control *Gal4*^{s1107t}*/UAS:GCaMP5* fish following the 1st sound stimulus (Trial 1) (contralateral n=14 cells from 8 fish, ipsilateral n=12 cells from 8 fish) and following the 2nd sound stimulus (Trial 2) (contralateral n=11 cells from 6 fish, ipsilateral n=4 cells from 2 fish). Sound stimulus followed a 2s baseline recording and the interval between the two trials was 2 min. **B**. Averaged traces from *Gal4*^{s1107t}*/UAS:GCaMP5* fish treated for 1 hour with 4 µM MPEP (contralateral n=10 cells from 6 fish, ipsilateral n=10 cells from 5 fish). Sound stimulus followed a 1s baseline recording. **C**. Averaged traces from *Gal4*^{s1107t}*/UAS:GCaMP5* fish treated for 1 hour with 10 µM CPCCOEt (n=42 cells from 10 fish). Sound stimulus followed a 1s baseline recording. For all traces, movement artifact was removed totaling 7 time points (0.8s) starting in the frame immediately following the stimulus. Stimulus is indicated by the black arrowhead and omitted time points are indicated by the black bars. Shaded areas represent ± s.e.m. **D**. Peak ΔF/F, length of calcium signal, and latency from peak muscle signal to peak calcium signal for control trials 1 and 2 and MPEP treated fish. No significant differences exist ($p>0.01$).

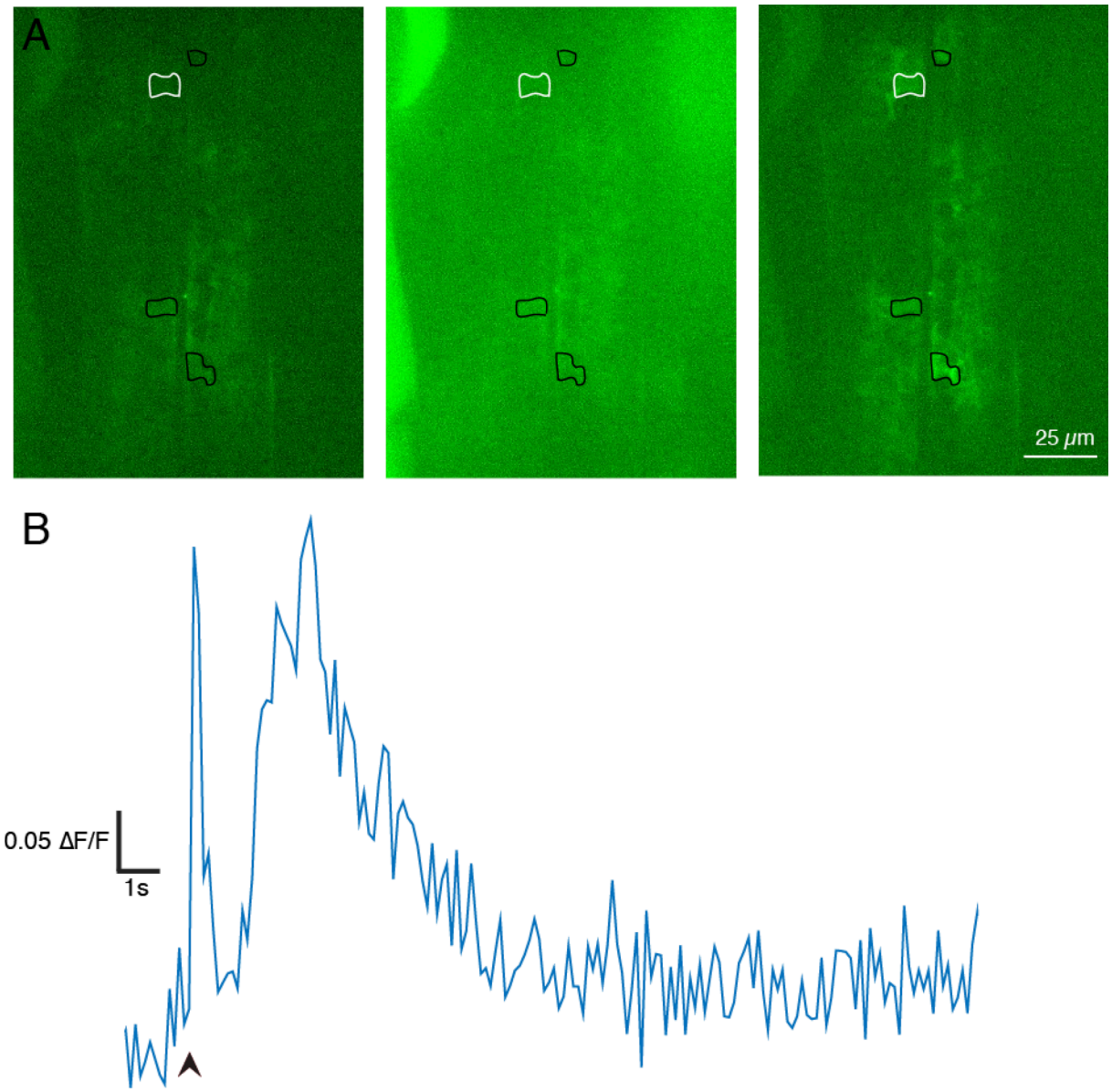

Figure 10. Representative data from control *Gal4^{s1107t}/UAS:GCaMP5* fish.
A. Example fish during baseline (left), during movement (middle), and following movement (right) with ROIs outlined. Ectopic GCaMP expression in the muscle causes a broad fluorescence increase during movement, which creates an artifact in the traces of glial cell calcium transients. **B.** Trace from ROI outlined in white. Motion artifact can be seen in the trace immediately following the stimulus (represented by the black arrowhead).

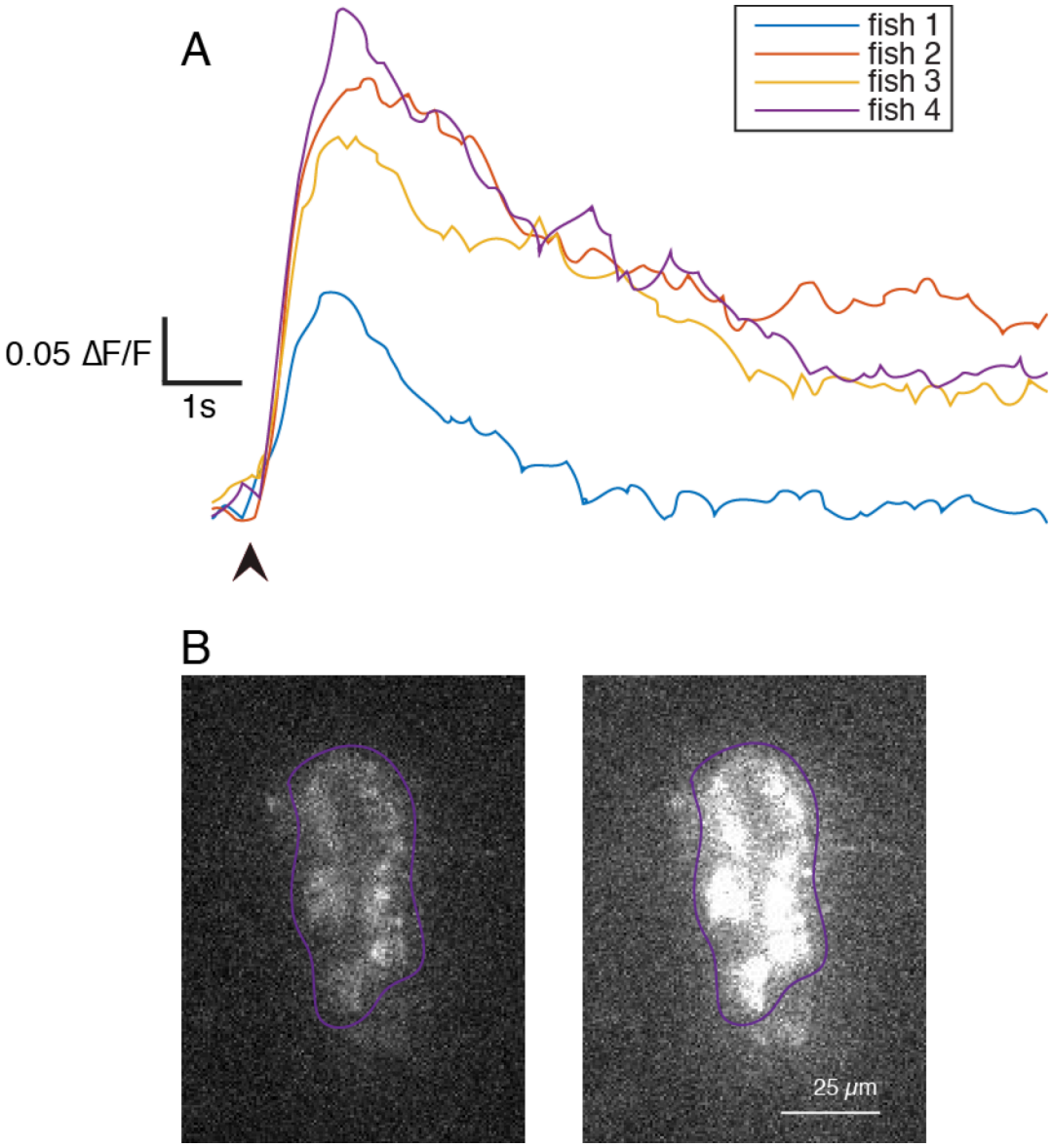

Figure 11. **CPCCOEt treatment does not abolish neural GCaMP signals during escape response. A.** Individual fluorescence traces from *Tph2:Gal4ff/UAS:GCaMP5* fish following acoustic stimulus. The ROI in each fish was drawn around the entire dorsal raphe nucleus. Stimulus is represented by the black arrowhead. **B.** Representative images from fish 4, with ROI outlined, baseline (left) and following acoustic stimulus (right).

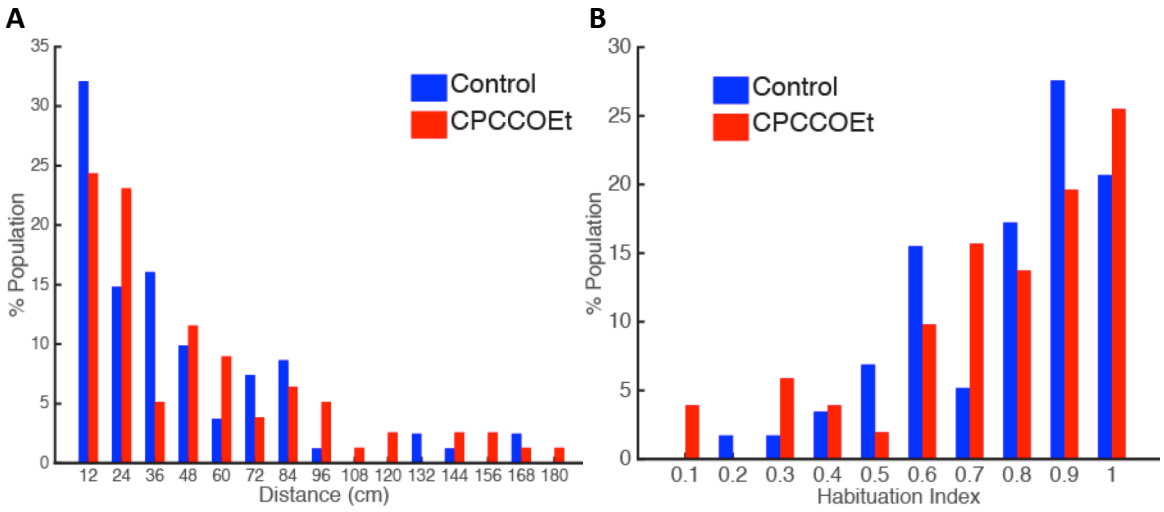

Figure 12. mGluR1 antagonist does not alter swimming behavior.
Control and CPCCOEt treated fish exhibited no significant differences in measures of total swimming distance (**A**) or habituation index (90 s ISI) (**B**) (n=96 in each group). All $p > 0.05$, 2-sided Mann-Whitney U test. ($HI = 1 - P_{after} / P_{initial}$).

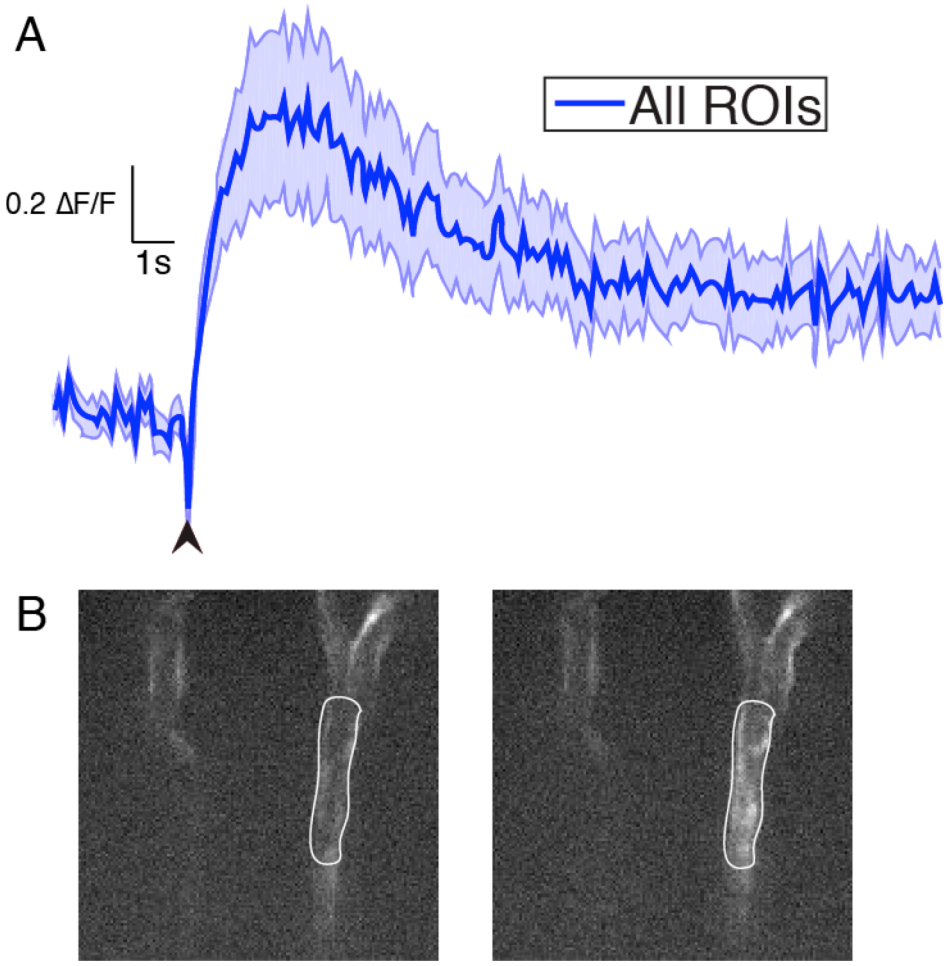

Figure 13. **Spinal blood vessels show calcium transients following escape response.**
A. Averaged fluorescence trace from *478A/UAS:GCaMP5* fish following acoustic stimulus (n=17 ROIs from 8 fish). Black arrow represents acoustic stimulus. Shaded area represents ± s.e.m. Transients peaked an average of 2.5 s (±0.24s) following sound stimulus.
B. Representative images with ROI from *478A/UAS:GCaMP5* fish during baseline (left) and following acoustic stimulus (right).

2.4 Discussion

The data presented here provide an initial characterization of glial calcium signaling in the zebrafish. Zebrafish are chosen as a model organism for their tractable genetics, ease of maintenance and breeding, and most of all for the optical clarity of the larvae. Combined with optogenetic tools, zebrafish can be used for powerful *in vivo* studies with minimally invasive techniques; often, restraint in an agarose matrix is the most disruptive part of the experimental setup. This is ideal for studying glial calcium signaling—glia have been shown to respond to mechanical pressure, stretch, osmotic changes, and even laser light[39,109,110]. This study aimed to better understand the properties of glial calcium signaling in zebrafish and to establish a foundation for further study in this model.

Although zebrafish lack the stellate astrocytes found in mammals[111], their radial glia perform several of the same functions and are similar morphologically to mammalian Bergmann and Muller glia. In mammals, these "radial astrocytes" are well studied due to their relatively simple morphology and accessibility within the central nervous system. This study establishes that zebrafish radial glia produce reliable calcium transients in response to acoustic startle and that the calcium signal is driven by mGluR1 receptors. This result concurs with previous work in astrocytes and Bergmann glia, which both exhibit calcium transients following a startle response[84,86,87]. Additionally, the observation of calcium signals in blood vessels and their similar timing to glial calcium signals suggests that zebrafish are a promising model for studying glia-mediated neurovascular coupling. The time resolution used in these experiments is not sufficient to conclusively determine the order of the glial and vascular signals, but this result supports the idea that glial calcium signals could be linked to changes in vascular tone.

The question that naturally follows this study is whether or not there is a physiological consequence of abolishing glial calcium signaling by blocking mGluR1. In the short term, based on the high throughput behavioral assay, there was no difference in either total swimming activity, which is controlled by spinal central pattern generators under the influence of multiple neuromodulators[112,113], or habituation, a non-associative form of learning which represents a complex interaction of neural circuitry from several different parts of the central nervous system[106]. Although abolishing calcium transients in spinal glia did not have an effect on habituation, it remains an appealing area of investigation due to its reliance on changes in synaptic plasticity, which has been shown to be mediated by glial activity. Given the known heterogeneity among astrocytes in mammals, it is likely that zebrafish glial cells behave differently in different parts of the central nervous system, and it remains to be seen whether glia in the zebrafish brain could have an effect on habituation or other types of synaptic plasticity. Understanding the multi-modal nature of both of these behaviors, there are many factors that need to be teased apart in order to generate a more complete and detailed picture of the possible effects of abolishing glial signaling.

The existence of glial calcium signals following a startle response in zebrafish demonstrates that these cells are actively responding to neural signaling in a manner similar to mammalian astrocytes. A model of glial calcium signaling during startle suggests that physiological arousal triggers the release of neuromodulatory signals that act on glia to prime them to respond to neural signaling (**Figure 3**). In zebrafish, possible neuromodulators include

serotonin and dopamine, which are both known to be involved in motor behaviors[112,114]. Although the observations presented here agree with the published data regarding the behavior of mammalian astrocytes during a startle response, the consequences of glial signaling in zebrafish remain unclear. Future studies will be needed to determine whether mGluR1 mediated glial signaling leads to any downstream effects in terms of neural signaling or synaptic plasticity.

3. Conclusions

This characterization of glial calcium signaling in zebrafish, along with the experimental methodology, can form the basis for many avenues of research in the future. The experiment that could be improved upon most immediately is the question of whether glial calcium signaling mediates neurovascular coupling in the zebrafish. Although well-established in mammalian astrocytes[19], it is unclear whether the radial cells found in organisms lacking astrocytes perform the same function. The optical clarity and available transgenic lines in zebrafish make this a very appealing system for investigating this question. Ideally, expression of two different colored calcium indicators could be combined in one fish; for example, by breeding *478A/UAS:GCaMP5* to a line stably expressing a red calcium sensor under the control of the zebrafish GFAP promoter[105]. This would allow for simultaneous imaging of the glial and vascular signals, which would make it easier to tease apart the relationship between the two. Although this study shows a similar time course of the two signals, a higher temporal resolution is needed to truly determine the order of calcium activity in the two locations. Additionally, the physiological relevance of the vascular calcium signal is unknown, but could be easily measured by observing blood vessel constrictions and flow rate under white light. If the glial calcium increase is found to precede that in the vasculature, pharmacological studies could be undertaken to determine the signaling factors involved, starting with blocking mGluR1 to see if abolishing calcium transients in spinal glia leads to changes in the vascular response.

Identification of mGluR1 as the primary neurotransmitter that drives glial calcium response to startle behavior opens up a wide range of possibilities for future experiments. However, it needs to be confirmed whether CPCCOEt abolishes glial calcium signaling in all regions of the zebrafish CNS or just the spinal cord, and whether or not there are other neurotransmitters that act in other parts of the CNS, alone or in conjunction with glutamate. This will require a different transgenic line that expresses in the brain, either from the library of enhancer trap screens or by generating a *GFAP:GCaMP* stable line. Lower resolution imaging of the whole brain would be sufficient to determine the existence and extent of glial calcium signals in the brain of control fish and fish treated with CPCCOEt, and other pharmacological studies could be used to reveal the possible contributions of neuromodulators such as serotonin or dopamine. In the spinal cord, one line of inquiry would be to vary CPCCOEt concentration, treatment time, and treatment window (relative to both age of the fish and to the timing of behavioral analysis) and use high-throughput behavioral analysis to determine if there is any behavioral effect that can be attributed to the loss of glial calcium signaling. Pharmacology could also be used to stimulate group I mGluR signaling in order to study the effects of non-physiological glial calcium transients. Additionally, our lab has been working on a variety of light-gated receptors including mGluRs[115]. The novel CRISPR/Cas9 genome editing system has been used successfully for gene knock-in in zebrafish and could be used to replace the native mGluR1 with a light-gated version[116]. Although there are two paralogs of mGluR1 in the zebrafish genome, they have different expression patterns that would allow for a comparative study of the importance of mGluR1 signaling in different CNS regions[117]. Obtaining spatial and temporal control over mGluR1 signaling would yield valuable information about the physiological function of mGluR1 in glia. In particular, stimulation of glial calcium transients using a light gated mGluR could be combined with calcium imaging or electrophysiology in neurons to ask whether glia are releasing a transmitter that is affecting neuronal signaling[118].

Additionally, novel optical sensors such as GluSNFR could be employed to visualize release of glutamate[118] and possibly other types of neurotransmitters in the near future.

In order to successfully and meaningfully investigate glial calcium signaling using genetically encoded optical tools, it would first be necessary to determine the expression pattern and timing of relevant proteins such as mGluR1. I propose using fluorescence-activated cell sorting (FACS) to isolate glial cells from the fish CNS, followed by mRNA sequencing to determine expression levels. This would have to be repeated at multiple timepoints to confirm the age at which different mGluRs are expressed in the zebrafish; although expression of mGluR1 has been confirmed in 3 and 5 dpf zebrafish[117], it would be necessary to investigate whether this expression continues past 5 dpf or is lost, as in adult mice[79]. A comprehensive mRNA profile would also provide a list of interesting genes that are enriched in those cells, such as neurotransmitter receptors, exocytotic machinery or channels capable of releasing glutamate or ATP, and proteins involved in intracellular release of calcium, such as IP_3Rs. A molecular characterization of glial cells in zebrafish would provide valuable clues to possible mechanisms of glial signaling, and, combined with the experimental setup described here, would be a powerful tool for generating insights into the physiological function of glial calcium signaling.

A new technique that could be applied to this study is the genetically encoded calcium indicator CaMPARI, which converts from green to red fluorescence in the presence of calcium only when stimulated with violet light[119]. This tool combines the precision and cellular resolution of calcium imaging with high-throughput behavioral analysis by allowing calcium activity to be recorded during a defined window, for instance during a particular behavior. Fish could be placed in the high-throughput behavioral apparatus and exposed to an acoustic stimulus in the presence of violet light, using CaMPARI to record any calcium rises that occurred during the escape behavior. Then, rather than the time consuming method of individually acclimating and imaging each fish on the simultaneous behavioral and calcium imaging setup, calcium signaling could be imaged after the fact by taking a confocal z stack or lightsheet image. This would make for significantly faster data collection, and it could be used as a screening tool to choose interesting developmental timepoints or CNS regions to study. In particular, lightsheet microscopy can provide fast, whole animal 3D images that could be used to compare glial calcium rises in different parts of the CNS during a single behavior. Because it would be faster to collect this data on a large number of fish, this method could also be used to compare fish across different ages to determine when in development glial calcium signaling begins and whether it has a defined window during which it occurs. Using whole-fish imaging, it could also help to define the brain regions where glial signaling is taking place during different behaviors.

The discovery of mGluR1-mediated glial calcium signaling in the radial glia of the zebrafish spinal cord during startle behavior indicates that the larval zebrafish is well-suited to the study of glial signaling. Further investigations stemming from this could help determine the interplay between glial calcium signaling and neurovascular coupling, the patterns of glial excitation during different behaviors, and the possible downstream effects of manipulating neural-glial communication by modulating or abolishing mGluR signaling. Glia have been studied for over a century, but their integral roles in the nervous system were woefully underestimated for most of that time. Zebrafish can be incorporated as an essential tool in the

modern study of glial biology that is driving us towards a deeper understanding of the fundamental workings of the brain.

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