Published in final edited form as:

Methods Enzymol. 2019 ; 622: 129–151. doi:10.1016/bs.mie.2019.02.006.

Visualizing the brain's astrocytes

Alyssa N. Preston¹, Danielle A. Cervasio², Scott T. Laughlin^{1,3,*}

¹Department of Chemistry, Stony Brook University, Stony Brook, NY, United States, 11790

²Department of Pharmacological Sciences, Stony Brook University, Stony Brook, NY, United States, 11790

³Institute of Chemical Biology and Drug Discovery, Stony Brook University, Stony Brook, NY, United States, 11790

Abstract

Astrocytes are the most abundant cell type in the brain and are a crucial part of solving its mysteries. Originally assumed to be passive supporting cells, astrocyte's functions are now recognized to include active modulation and information processing at the neural synapse. The full extent of the astrocyte contribution to neural processing remains unknown. This is, in part, due to the lack of methods available for astrocyte identification and analysis. Existing strategies employ genetic tools like the astrocyte specific promoters glial fibrillary acidic protein (GFAP) or Aldh1L1 to create transgenic animals with fluorescently labeled astrocytes. Recently, small molecule targeting moieties have enabled the delivery of bright fluorescent dyes to astrocytes. Here, we review methods for targeting astrocytes, with a focus on a recently-developed methylpyridinium targeting moiety's development, chemical synthesis, and elaboration to provide new features like light-based spatiotemporal control of cell labeling.

Keywords

Brain imaging; neuroimaging; glia imaging; astrocytes; small molecules; cationic fluorophore; photoactivatable

1. INTRODUCTION

Traditionally, neurons have been the focus of neuroscience research, as they are the principal units of neural computation, sending signals through neural circuits. However, findings over the last two decades have identified active roles for astrocytes in modulating synaptic activity. Astrocytic processes have been shown to be in contact with a synapse's pre- and post-synaptic neurons forming a structure called the tripartite synapse in several regions of the brain (Araque, Parpura, Sanzgiri, & Haydon, 1999) (Figure 1A). This close, structural relationship between an astrocyte and neurons has motivated inquiry into the role of astrocytes in synaptic transmission.

*Corresponding author: scott.laughlin@stonybrook.edu.

Although astrocytes cannot be electrically stimulated like neurons, they do experience cytosolic Ca^{2+} fluctuations upon binding the neurotransmitters glutamate or GABA (Guerra-Gomes, Sousa, Pinto, & Oliveira, 2017; Khakh & McCarthy, 2015). This represents an astrocytic version of synaptic transmission called gliotransmission that can have a direct influence on neuronal function (Savtchouk & Volterra, 2018) (Figure 1B). Indeed, gliotransmission has been shown to have a wide range of roles, such as modulating synaptic transmission, plasticity, and NMDA receptor activity (Halassa, Fellin, & Haydon, 2007; Harada, Kamiya, & Tsuboi, 2015). The cytosolic Ca^{2+} rise spreads through the activated astrocyte and into nearby astrocytes connected through gap junctions causing a cascade of intracellular events. For example, an increased Ca^{2+} concentration stimulates the release of glutamate, ATP, D-serine, and GABA that act as gliotransmitters, inducing a variety of effects on nearby neurons. The release of astrocytic glutamate activates NMDA receptors and synchronizes the excitability of spatially relevant neurons, regulating synaptic signaling (Fellin et al., 2004) (Figure 1C). ATP released from astrocytes into the synapse is converted to adenosine, which binds the pre-synaptic adenosine A1 receptor, suppressing neural signaling (Zhang et al., 2003) (Figure 1D). The above processes are all examples of astrocyte's ability to process information and control synaptic function, all supporting the conclusion that astrocytes are active participants in neural processing.

As neuroscience moves past the classical model of solely neuron-based computation to a balance of both neurons and astrocytes through the tripartite synapse, it is crucial to develop new methods to study the astrocyte's roles in these processes. Current strategies to target, label and study astrocytes fall into two categories, genetic and small molecule. Genetic strategies utilize promoters of proteins upregulated in astrocytes to create transgenic animals that express fluorescent reporter proteins in astrocytes, or to target fluorescent antibodies to astrocytes for labeling in fixed samples. Small molecule strategies utilize astrocyte-resident transporters to travel into and specifically label astrocytes *in vitro* and *in vivo*. The following sections provide an overview of both genetic- and small-molecule-approaches to label astrocytes as well as an introduction to a new methylpyridinium targeting moiety that can be used to direct a variety of functional small molecules to astrocytes.

2. GENETIC STRATEGIES TO LABEL ASTROCYTES

In the central nervous system, there are several proteins that are specifically and highly upregulated in astrocytes. Labeling these endogenous proteins immunohistochemically, or exploiting their promoter to control transgene expression, provides general strategies for revealing the presence of astrocytes in the brain. The most popular astrocyte-specific proteins are Glial Fibrillary Acidic Protein (GFAP), S100 β , and Aldh1L1. Such genetic methods for probing astrocytes are useful for studying populations that express the classic marker proteins, but they can be problematic due to the variability in protein expression among astrocytes. Possibly the most limiting caveats for these genetic strategies are the requirement of fixation in immunohistochemical analysis and timely genetic engineering for promoter-driven gene expression. Below, we describe the strengths and weaknesses of the popular genetic methods that have been used to target astrocytes.

2.1 Glial fibrillary acidic protein (GFAP)

The most popular astrocyte targeting strategy employs glial fibrillary acidic protein (GFAP). In mature astrocytes, GFAP contributes to glial scar formation in response to injury and is the most abundant intermediate filament protein (Fawcett & Asher, 1999; Pekny et al., 1999). Indeed, GFAP upregulation and subsequent assembly of the intermediate filament network in mature astrocytes is considered a hallmark of reactive astrocytes (Zhu et al., 2004). This upregulation is evident in both the astrocytic processes and soma, making GFAP expression a reliable marker for reactive astrocytes. Accordingly, GFAP serves as an excellent protein to target for immunohistochemical detection of astrocytes and its promoter enables the creation of transgenic cells and organisms that express the desired reporter or detectors like GCaMP in astrocytes (Shigetomi et al., 2013).

However, exploiting GFAP's promoter is not a perfect solution for targeting astrocytes because it does not mark the cell population in its entirety. GFAP is likely regulated at the level of translation, evident in the disconnect between promoter-linked expression and the endogenously expressed protein. In other words, many cells produce GFAP mRNA without transcribing it (Nolte et al., 2001). For example, when used immunohistochemically, it only labels the intermediate filament cytoskeleton of mature astrocytes, limiting its use to cells in brain regions where the intermediate filament protein is present (Nolte et al., 2001). Additionally, GFAP detection via immunohistochemistry is more prominent in white vs. gray matter astrocytes and does label all astrocytic processes (Bushong, Martone, & Ellisman, 2004). Conversely, GFAP promoter-driven expression of fluorescent reporter proteins can reveal gray matter astrocytes in the cortex, hippocampus, and cerebellum as well as their fine astrocytic processes (Hirrlinger et al., 2005; Nolte et al., 2001). Finally, astrocytes display a wide range of GFAP expression due to their inherent heterogeneity, thus producing variable transgene expression or endogenous protein labeling (Emsley & Macklis, 2006; Grass et al., 2004; Kimelberg, 2004; Matthias et al., 2003; Meng et al., 2015; Nolte et al., 2001) (Figure 2A, B).

2.2 S100 β

S100 β is a calcium, copper, and zinc-binding protein, which mediates the interactions between glial cells and neurons, enabling visualization of astrocytes using an engineered transgene or immunohistochemistry-based strategy. Murine S100 β expression levels increase in the telencephalon as organisms age, defining a period in which GFAP positive glia lose their neural stem cell potential and acquire a more mature phenotype and therefore, a useful marker for developing glia (Raponi et al., 2007). Like GFAP, S100 β is not a universal marker for all astrocytes. S100 β labels the cell nucleus and cytoplasm (Figure 2C) of both gray matter and white matter astrocytes in many areas of the brain, but fails to label astrocytes in germinal zones of the brain (Cahoy et al., 2008; Deloulme et al., 2004; Schitine, Nogaroli, Costa, & Hedin-Pereira, 2015). Finally, perhaps the most significant caveat to using S100 β as a target for astrocytes is that it is also present in ependymal cells (Raponi et al., 2007; Steiner et al., 2007), Schwann cells (Stefansson, Wollmann, & Moore, 1982), subsets of neurons (Steiner et al., 2007; Vives, Alonso, Solal, Joubert, & Legraverend, 2003), oligodendrocytes, oligodendrocyte precursor cells (Deloulme et al.,

2004; Steiner et al., 2007), and neural precursor cells in embryonic brains (Deloulme et al., 2004).

2.3 Aldh1L1

Recently, the astrocyte-specific gene, aldehyde dehydrogenase 1 family, member L1 (Aldh1L1), has been utilized as a genetic astrocyte marker. Aldh1L1 is a 10-formyltetrahydrofolate dehydrogenase, a participant in astrocyte folate metabolism. This protein has been identified in both immature and mature astrocytes in gray and white matter within the central nervous system, exhibiting a broader expression pattern in comparison to GFAP (Cahoy et al., 2008). Intracellularly, it is expressed in the astrocyte's cell body and morphologically significant fine processes compared to GFAP which is only able to reveal the cell's main processes (Srinivasan et al., 2016) (Figure 2C, D). Importantly, Aldh1L1 promoter-driven expression overlaps consistently with the immunohistochemical pattern of the protein. This allows researchers to definitively identify astrocytes through visualization of not only an astrocyte's projections but within its cell body as well. Conversely, Aldh1L1 expression decreases in an age-dependent manner within the spinal cord (Yang et al., 2011) and is also expressed in radial glia during embryogenesis (Anthony & Heintz, 2007; Yang et al., 2011) and is also expressed in radial glia during embryogenesis (Anthony & Heintz, 2007).

2.4 Other promoters and proteins used for genetic targeting of astrocytes

There are other proteins whose expression can be used to identify astrocyte populations, but they are not as widely used as GFAP, S100 β , and Aldh1L1. For example, glutamate transporter 1 (GLT1) and glutamate-aspartate transporter (GLAST), are two glutamate transporter proteins present within astrocytes. However, they are not as specific as the aforementioned markers and show expression in organs outside of the CNS (Berger & Hediger, 2006; Chaudhry et al., 1995; Slezak et al., 2007). GLT1 positive cells exhibit substantial overlap with GFAP and Aldh1L1 (Yang et al., 2011) labeling, yet GLT1 is able to identify a subpopulation of GFAP-negative astrocytes (Lovatt et al., 2007). Although there are transgenic GLT1 lines and this marker labels a more diverse population of astrocytes, its promoter is also active in a subset of hippocampal neurons (Berger & Hediger, 2006). GLAST is another glutamate uptake protein that is highly expressed in immature astrocytes, but also present in radial glia, populations of oligodendrocytes, and progenitor cells destined to become neurons. GLAST demonstrates especially high activity in radial glia of the sub-granule layer of the dentate gyrus and oligodendrocytes of white matter tracts, making it non-specific for mature astrocytes. After differentiation of neural progenitors into astrocytes, GLAST is primarily restricted to the cerebellum, white matter, and various forebrain niches (Regan et al., 2007), making it a good marker for progenitor cells, but not mature astrocytes.

Aquaporin 4 (AQP4) is a water channel concentrated in the perivascular end-feet of astrocytes surrounding blood vessels where it helps to regulate water and potassium homeostasis. Although AQP4 is highly astrocyte-specific, it is preferentially expressed in astrocyte processes that are in direct contact with blood vessels. Immunohistochemically, this results in a labeling pattern more consistent with the morphology of the blood vessel than the astrocyte. Due to its importance in central nervous system water transport, it is not

surprising that the AQP4 protein is also present in subpopulations of ependymal cells that line the ventricles (Nielsen et al., 1997).

Gap junction proteins, connexin 30 (Cx30) and 43 (Cx43), are also preferentially expressed in astrocytes. Specific labeling has been achieved in mice by replacement of Cx30 and Cx43 exons with lacZ or a Cre-dependent lacZ cassette (Gosejacob et al., 2011). This approach proved successful in labeling astrocytes, however, Cx30 is only present heterogeneously in gray matter astrocytes and Cx43 is additionally seen in endothelial and ependymal cells (Nagy, Patel, Ochalski, & Stelmack, 1999).

3. SMALL MOLECULE STRATEGIES TO LABEL ASTROCYTES

Fluorescent small molecules are a simple, user-friendly option for labeling and studying astrocytes in living systems. Where antibodies require a fixed sample, non-toxic small molecules can be used to bath cells or brain slices, or they can be injected *in vivo*. Unfortunately, there are very few astrocyte-specific chemical markers available for targeting and labeling astrocytes. For example, cell permeable fluo-4 preferentially labels rat hippocampal astrocytes over neurons in slice culture and has been used to measure Ca^{2+} fluctuations in response to low K^{+} (Dallwig & Deitmer, 2002). However, care must be taken in its application, as fluo-4 is also a widely used small molecule Ca^{2+} indicator in a large variety of other cell types, including isolated neurons and mammalian cultured cells like HEK293.

Over the past two decades, two markers have distinguished themselves with the ability to target astrocytes, Sulforhodamine 101 and β -Ala-Lys- N_ϵ -AMCA. Since then, no derivatives of these markers, or new markers have been published, until our methylpyridinium-tagged rhodamine B astrocyte-specific marker. In the following sections, we describe the benefits and drawbacks of Sulforhodamine 101 and β -Ala-Lys- N_ϵ -AMCA as astrocyte markers before detailing our recently-designed cationic markers.

3.1 Sulforhodamine 101 (SR101)

Since its first use in 2004 to specifically label astrocytes, sulforhodamine 101 has been the standard small molecule marker for targeting astrocytes. The Texas red rhodamine fluorophore accompanied by two sulfate groups can target astrocytes *in vitro* and *in vivo* in rodents (Figure 3A). Early in its use, SR101 was applied through a topical application of a dilute solution to an exposed mouse brain to label cortical astrocytes (Nimmerjahn, Kirchhoff, Kerr, & Helmchen, 2004). After this successful experiment in the mouse cortex, SR101 was applied to other species and brain areas such as the hippocampus, ventral lateral medulla, and brain stem. As more brain regions were assayed with SR101, it became clear that SR101 labeling can differ between different brain regions, where labeled astrocytes are only visualized in the hippocampus and cortex and SR101 labeling was non-specific or non-existent in other areas. For example, in the rat spinal cord, SR101 can label both astrocytes and neurons (Cina & Hochman, 2000) and in the ventral lateral medulla, SR101 labeling is insufficient due to an extremely low signal to background (Schnell, Hagos, & Hülsmann, 2012). Additionally, SR101 had been previously shown to label oligodendrocytes in the

rabbit retina (Ehinger, Zucker, Bruun, & Adolph, 1994) as well as oligodendrocytes in the mouse cortex after *in vivo* application (Nimmerjahn et al., 2004).

Originally, gap junctions were believed to be the mechanism of uptake for SR101 in astrocytes and its labeling of oligodendrocytes. In fact, when mouse hippocampal brain slices were co-stained with both SR101 and the gap junction inhibitor carbenoxolone, the result was decreased, but not abolished, SR101 labeling (Schnell et al., 2012). As further evidence for a non-gap junction import mechanism, double knockout mice for the gap junction proteins connexins 30 and 43 (Cx30^{-/-}Cx43^{-/-}) did not have completely reduced SR101 labeling (Pannasch et al., 2011). Only recently were Schnell et al. able to verify that the uptake mechanism of SR101 is active transport through an organic transporting polypeptide, OATP1C1, which is a bidirectional transporter for the endogenous thyroid hormone, thyroxine (Schnell et al., 2015). In addition to their expression in astrocytes, these transporters are also expressed in the vascular endothelium, allowing SR101 to travel through the blood-brain barrier and enter astrocytes if first injected into the vasculature (Schnell et al., 2015). Upon the discovery of SR101's uptake mechanism, Hagos et al. were able to determine that upon entering astrocytes, SR101 travels through hemichannel gap junctions to label oligodendrocytes (Hagos & Hülsmann, 2016) (Figure 3B). Although the uptake mechanism of SR101 through OATP1C1 has been determined, its ability to also travel through gap junctions between cells decreases its utility as an unequivocal astrocyte marker.

Due to the variety of strategies required by researchers to label astrocytes with SR101, there have also been questions about the excitatory side effects of SR101 in neurons. The concentration of SR101 required to efficiently label astrocytes ranges from 1 μ M to upwards of 250 μ M. It has been shown that, upon bathing at the lower concentration of 1 μ M SR101, neurons can undergo long-term potentiation of intrinsic excitability that continues past the bathing time of the cells (Kang et al., 2010). Additionally, the bolus loading of SR101 at 100 and 250 μ M in the mouse cortex can induce seizure-like activity in the short period of 10 minutes post-loading (Rasmussen, Nedergaard, & Petersen, 2016). These side effects should be taken into consideration by researchers using SR101, especially when bathing or injecting at concentrations at or above 100 μ M.

Ultimately, SR101 has several caveats; making it difficult to use as a specific astrocyte marker. Its ability to travel into not only astrocytes, but oligodendrocytes and neurons does not allow researchers to solely target one cell-type. The range of labeling ability depending on brain region restricts experiments to areas of the brain with the most robust labeling. Therefore, experiments are limited to regions such as the cortex and hippocampus where SR101 labeling is most prominent. Finally, SR101 has been seen to induce activity in neurons and, at high concentrations, induce seizures in animals. All of these issues are important considerations researchers should note when using SR101 as an astrocyte-specific small molecule marker.

3.2 β -Ala-Lys-N_e-AMCA

A second known astrocyte marker is β -Ala-Lys-N_e-AMCA; a fluorescent dipeptide. Bauer and coworkers creatively designed this marker based on the known uptake of carnosine, a β -

Ala-His dipeptide, by astrocytes, which express the peptide transporter PepT2 (Schulz, Hamprecht, Kleinkauf, & Bauer, 1987). To create their fluorescent astrocyte marker, Beaur and coworkers used a β -Ala-Lys dipeptide where the amino-terminal β -Ala makes the compound resistant to most peptidases and the lysine residue provides a simple attachment point to which a variety of fluorophores could be attached and tested for astrocyte labeling (Figure 3C). Unfortunately, the PepT2 transporter was more selective of the fluorophore than anticipated and only the coumarin derivative successfully labeled astrocytes.

Using their coumarin dipeptide derivative, Bauer and coworkers were able to label primary rat astrocytes and 0–2 progenitor cells, an oligodendrocyte precursor cell, and show a lack of labeling in neurons. Interestingly, they saw a relationship between the intensity of AMCA labeling and the expression of GFAP in labeled cells; the higher levels of GFAP expressed by the cells, the more intense AMCA labeling was observed. This finding was confirmed years later by Hatton and coworkers who visualized a decrease in β -Ala-Lys-N_e-AMCA labeling in astrocytes in the supraoptic nucleus with decreased GFAP expression (Wang & Hatton, 2009).

Since the design of β -Ala-Lys-N_e-AMCA in 1999, there are limited references to its use for specifically labeling astrocytes. Over the past two decades, it has been used to study the Ca²⁺ signaling of astrocytes in the rat supraoptic nucleus (Espallergues et al., 2007) (Figure 3D) and the human PepT2 transporter in neoplastic glial cells (Zimmermann & Stan, 2010). In addition to its expression in astrocytes, PepT2 is most abundantly expressed in the small intestine and kidney of rodents (Lu & Klaassen, 2006). Researchers have taken advantage of β -Ala-Lys-N_e-AMCA as a fluorescent substrate to study PepT2 transport in these areas. For example, it was intravenously injected into mice to study its uptake in the kidney (Rubio-Aliaga et al., 2003). More recently, Moens and coworkers measured the activity of brush border membrane vesicles in the cortex and medulla of the rat kidney (Alghamdi, King, Jones, & Moens, 2018).

Although β -Ala-Lys-N_e-AMCA has seen wide-use in studying the PepT2 transporter, its lack of use in the brain to label astrocytes could be contributed to some of its drawbacks. One of these drawbacks is its dependence on the coumarin fluorophore for astrocyte labeling, making it difficult to modify for specific experiments required by researchers. Additionally, it lacks astrocyte specificity as it can readily label oligodendrocyte precursor cells, which are another type of glial cell. Lastly, di-peptides can be readily metabolized and therefore can be difficult to implement in *in vitro* and *in vivo* systems.

3.3 Cationic pyridinium astrocyte markers

Recently, we developed a method for targeting small molecules like fluorophores to astrocytes by attaching a methylpyridinium moiety (Preston et al., 2018). Expanding on the knowledge of SR101 and β -Ala-Lys-N_e-AMCA utilizing astrocyte-resident ion transporters to travel specifically into astrocytes, we chose to target the organic cation transporter, another astrocyte-specific transporter protein (Furihata & Anzai, 2017). To create a cationic, astrocyte-specific small molecule, we started our search with commercially available 4-di-2-asp, a methylpyridinium containing fluorescent small molecule known to stain live nerve terminals at the neuromuscular junction (Magrassi, Purves, & Lichtman, 1987). While 4-

di-2-asp was able to label primary astrocytes from the rat hippocampus, it also labeled primary neurons and would not provide the specificity for astrocytes we hoped to achieve. Therefore, we chose to utilize the methylpyridinium from 4-di-2-asp as a targeting moiety, providing a permanent positive charge, to allow a variety of fluorophores to travel through the astrocyte-resident organic cation transporter. We synthesized a library of compounds containing common, commercially available fluorophores such as rhodamine, bodipy, and cyanine tagged with a methylpyridinium moiety. The most successful marker, a methylpyridinium-tagged rhodamine B was shown to specifically label primary cultures of mouse and rat astrocytes over neurons and other glial cell types such as microglia or oligodendrocyte precursors (Figure 4A, C). Additionally, it has been introduced through bolus ventricle injection into the larval zebrafish brain and visualized solely in astroglia, the resident GFAP-positive cell type in zebrafish.

The original marker was based on a methylpyridinium-tagged rhodamine B, but it is possible that other permanently cationic species, or functional groups expected to be protonated at physiological pH, could also direct chemical cargo to astrocytes. Accordingly, one area of future interest is in the evaluation of a rhodamine B fluorophore modified with functional groups with varying charge, size and structure. Given the mechanism of astrocyte entry by organic cation transporters, we expect most cations that are small enough to transit the transporter will work in this system, although there may be significant differences in their performances.

In addition to evaluating different targeting moieties, another area of interest is the evaluation of the methylpyridinium targeting moiety to deliver other cargo, such as functionalized fluorophores, into astrocytes. One particularly interesting functionalized fluorophore is a photoactivatable one, which would provide spatiotemporal control of the signal to further enhance the experiments possible with the cationic small molecule astrocyte markers. In addition to an overall methods introduction to the methylpyridinium astrocyte labels, we describe in this article the synthesis and evaluation of a photoactivatable astrocyte label: NVOC₂-Q-Rhodamine with an attached the methylpyridinium (Figure 4B).

4. METHODS

4.1 Synthesis of Rhodamine B methylpyridinium astrocyte marker

1.1 Rhodamine B 4-(3-Carboxypropionyl)piperazine amide (0.010 g, 0.016 mmol, 1 equiv) and N-hydroxysuccinimide (0.002 g, 0.016 mmol, 1 equiv) were dissolved in dichloromethane (500 μ l) and cooled to 0 °C in an ice water bath.

1.2 N,N'-dicyclohexylcarbodiimide (0.004 g, 0.018 mmol, 1.1 equiv) was dissolved in dichloromethane (500 μ l), cooled to 0 °C, and added dropwise to the reaction.

1.3 The reaction was stirred at 0 °C for 5 h and then at RT overnight.

1.4 The reaction was filtered and the solid was washed with dichloromethane. The filtrate was collected, evaporated under vacuum and used without further purification.

1.5 Crude Rhodamine B succinimidyl ester (0.012 g, 0.0169 mmol, 1 equiv) was measured into a conical vial and evaporated under vacuum.

1.6 2-(2-aminoethyl) methylpyridinium (0.003 g, 0.0186 mmol, 1.1 equiv) was dissolved in dimethylformamide (500 μ l) and added to the conical vial while stirring.

1.7 Triethylamine (0.004 g, 0.0356 mmol, 2.1 equiv, 0.005 ml) was added and the reaction was stirred at RT for 3 h.

1.8 The reaction was concentrated *in vacuo* and purified using HPLC (R_t = 19.8 min, 10–100% MeOH/H₂O + 0.1% TFA over 25 min, flow rate = 1 mL/min) to give the product as a red/pink solid (0.002 g, 12.5 % over 2 steps).

1.9 ¹H NMR (500 MHz, MeOD) δ 8.84 (d, J = 6.5 Hz, 1H), 8.42 (t, J = 7.9 Hz, 1H), 7.98 (d, J = 7.8 Hz, 1H), 7.88 (t, J = 7.4 Hz, 1H), 7.80 – 7.76 (m, 2H), 7.72 – 7.69 (m, 1H), 7.54 – 7.50 (m, 1H), 7.28 (d, J = 9.5 Hz, 2H), 7.08 (dd, J = 9.6, 2.4 Hz, 2H), 6.97 (d, J = 2.4 Hz, 2H), 4.38 (s, 3H), 3.69 (q, J = 7.1 Hz, 8H), 3.66 – 3.60 (t, 2H), 3.39 (s, 8H), 3.33 (t, J = 6.6 Hz, 2H), 2.56 (t, J = 6.3 Hz, 2H), 2.41 – 2.37 (t, 2H), 1.31 (t, J = 7.1 Hz, 12H).

1.10 HRMS (ESI): Calculated for C₄₄H₅₄N₆O₄²⁺ [M]²⁺ 365.2098, found: 365.2096.

4.2 Synthesis of NVOC₂-Q-Rhodamine methylpyridinium photo-activatable astrocyte marker

2.1 NVOC₂-5-carboxy-Q-Rhodamine (0.0025 g, 0.0027 mmol, 1 equiv), HATU (0.001 g, 0.0032 mmol, 1.2 equiv), and diisopropylethylamine (0.0007 g, 0.0057 mmol, 2.1 equiv, 1 μ l) were dissolved in dimethylformamide (500 μ l) and stirred at RT for 30 min.

2.2 2-(2-aminoethyl) methylpyridinium (0.0004 g, 0.0032 mmol, 1.2 equiv) was added in DMF (100 μ L) and the reaction was stirred at RT overnight.

2.3 The reaction was concentrated *in vacuo* and purified using HPLC (R_t = 10.7 min, 50–90% Acetonitrile/H₂O + 0.1% TFA over 25 min, flow rate = 20 mL/min) to give the product as a white solid (0.0014 g, 45%).

2.4 ¹H NMR (500 MHz, Acetonitrile-*d*₃) δ 8.60 (d, J = 6.0 Hz, 1H), 8.35 (t, J = 7.5 Hz, 1H), 8.31 (s, 1H), 8.08 (dd, J = 8.1, 1.4 Hz, 1H), 7.93 (d, J = 8.2 Hz, 1H), 7.85 – 7.81 (m, 1H), 7.80 (s, 2H), 7.71 (s, 2H), 7.28 (d, J = 8.0 Hz, 1H), 7.15 (s, 2H), 6.59 (s, 2H), 5.58 (s, 3H), 4.32 (s, 2H), 3.88 (d, J = 10.6 Hz, 12H), 3.85 – 3.82 (m, 2H), 3.81 – 3.76 (m, 4H), 3.40 (t, J = 6.8 Hz, 4H), 2.67 – 2.59 (m, 4H), 2.56 – 2.48 (m, 4H).

2.5 HRMS (ESI): Calculated for C₅₅H₅₁N₆O₁₆⁺ [M]¹⁺ 1051.3356, found: 1051.3363.

4.3 Labeling primary astrocytes with methylpyridinium-tagged astrocyte markers

3.1 Passage astrocytes into a poly-d-lysine coated Millipore 8-chamber culture slide and seed at 16,000 cells/well in 400 μ L NbASTRO® media.

3.2 After 24 h, remove 250 μ L of conditioned media from each well to prepare bathing solution of 1 μ M marker and 1 μ g/mL Hoechst 33342. Spin the solution at 17,000 $\times$ g for 10 min to pellet any precipitate before use.

3.3 Remove remaining conditioned media from each well, save, and add bathing solution to well. Incubated at 37 °C in air with 5% CO₂ for 20 min.

3.4 Remove bathing solution and add 400 µL of fresh NbASTRO® to each well. Remove media and replace with 1:2 conditioned to fresh media and incubate at 37 °C in air with 5% CO₂ for 20 min.

3.5 Remove media and add L-15 media for imaging using confocal microscopy.

3.6 If using the caged marker, image cells pre- and post-uncaging to determine the extent of uncaging. To uncage marker, illuminate a z-stack of 26 slices encompassing the top and bottom of the astrocyte for 2000 ms per slice using the DAPI excitation filter.

4.4 Labeling astroglia of larval zebrafish with methylpyridinium-tagged photoactivatable marker

4.1 Anesthetize 4 days post fertilization (dpf) larval zebrafish using 0.064 M tricaine.

4.2 Mount zebrafish dorsal up in 50 µl of 1.5% low melting point agarose in E3 solution on a microscope slide.

4.3 Using a Nanoject II auto-nanoliter injector positioned on a micromanipulator, inject 2.3 nL of 100 µM marker into the ventricle near the optic tectum of the fish. After injection, add E3 media to the top of the fish.

4.4 Remove the agarose from around the fish using a scalpel until free to swim.

4.5 Transfer fish into a fresh PTU-containing E3 solution to recover for 3 hours before imaging.

4.6 To image using confocal microscopy, fish were mounted dorsal in 50 µl of a 2% low melting point agarose in E3 solution on a microscope slide.

4.7 If using the caged marker, image cells pre- and post-uncaging to determine the extent of uncaging. To uncage marker, illuminate a z-stack of 70 slices encompassing the top and bottom of the larval zebrafish brain for 2000 ms per slice using the DAPI excitation filter.

4.5 General Materials and Methods

5.1 Chemical synthesis—All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise specified. HPLC was performed using a Shimadzu HPLC (FCV-200AL) equipped with an Agilent reversed phase Zorbax Sb-Aq C18 column (4.6 × 250 mm or 21.2 × 250 mm) fitted with an Agilent stand-alone prep guard column. NMR spectra (¹H and ¹³C) were obtained using a 500 MHz Bruker spectrometer and analyzed using Mestrenova 9.0. ¹H chemical shifts (δ) were referenced to residual solvent peaks. High-resolution (ESI) mass spectra were obtained at the Stony Brook University Institute for Chemical Biology and Drug Discovery (ICB&DD) Mass Spectrometry Facility with an Agilent LC/MSD and Agilent LC-UV-TOF spectrometer respectively. Rhodamine B 4-(3-Carboxypropionyl)piperazine amide (Nguyen

& Francis, 2003) and 2-(2-aminoethyl) methylpyridinium (Preston et al., 2018) were synthesized as described previously.

5.2 Cell culture maintenance—Astrocytes were maintained in Neurobasal medium supplemented with 10% horse serum and 2 mM GlutaMAX™ (NbASTRO®) at 37 °C in air with 5% CO₂. Cell media was changed every 3–4 days and passaged every 7–8 days when the cells reached approximately 90% confluency.

5.3 Zebrafish maintenance and husbandry—Adult zebrafish were housed at 28.5 °C on a 14-h light 10-h dark light cycle. Embryos were produced from natural crosses between one male and one female zebrafish (zebrafish line: GFAP:GFP). Fertilized embryos were sorted and seeded at 50 embryos per 10 cm petri dish in 1X embryo media (E3) and placed in an incubator (28.5 °C, 14-h light, 10-h dark light cycle) to mature. At 22-hours-post-fertilization (hpf), embryos were transferred to a solution containing 197 µM phenylthiourea (PTU) in 1X E3. Larval zebrafish were observed for manipulation and microinjection using a Leica MZ 10 F fluorescence dissecting microscope equipped with a 1× PlanApo objective. Confocal microscopy was performed using a Zeiss Axio Examiner.D1 modified with an Andor Differential Scanning Disk confocal unit and a 40× NA 1.0 or 20× NA 0.5 water immersion objective. Confocal images were analyzed using ImageJ (NIH) and prepared for presentation using Adobe Illustrator.

5. RESULTS AND DISCUSSION

Upon completing the synthesis of the photocaged rhodamine putative astrocyte marker, we sought to determine its abilities to label astrocytes as compared to our original uncaged, Rhodamine B methylpyridinium. Primary rat hippocampal astrocytes were bathed in 1 µM of the photocaged compound and then uncaged using 365 nm light and imaged using confocal microscopy to observe uncaged rhodamine fluorescence inside the cells. Astrocytes were indeed labeled with the caged fluorophore, leading to punctate fluorescence of the Q-rhodamine fluorophore after uncaging. However, this signal was not as successful as our original uncaged molecule that gave bright, cytosolic labeling throughout the entire astrocyte (Figure 4D). Additionally, we found the solubility of our compound to be very poor in cell culture media. A minimum of 0.5% DMSO was required to maintain solubility of our compound, a concentration that has the potential to negatively impact the health of the cells. There are several potential reasons for the limited labeling of astrocytes by the new caged rhodamine methylpyridinium-tagged marker. Due to the addition of the NVOC photolabile groups on the nitrogens of the xanthene core, the Q-rhodamine fluorophore has a net neutral charge as compared to the rhodamine B fluorophore with a +1 charge. Because of this, the NVOC₂-Q-rhodamine methylpyridinium has an overall charge of +1, lower than the +2- overall charge of the rhodamine B methylpyridinium. A lower overall charge could decrease the affinity of the marker for the organic cation transporter, prohibiting its entry into astrocytes. Another reason for decreased astrocyte labeling could be insufficient uncaging of the rhodamine fluorophore and therefore a lower fluorescence signal to detect. Insufficient uncaging could be occurring due to a problem in our uncaging protocol, however, we believe we have optimized the uncaging protocol for our confocal microscopy setup and this is not the reason for our lack of Q-rhodamine signal.

Undeterred by our minimal astrocyte labeling by the caged rhodamine marker in primary astrocytes in culture, we sought to determine its ability to label astrocytes *in vivo* through bolus injection in the larval zebrafish brain. This strategy was previously used with Rhodamine B methylpyridinium, which gave bright labeling of astroglia throughout the zebrafish brain (Figure 4E). Using a Nanoject II system, 100 μ M NVOC₂-Q-Rhodamine methylpyridinium was injected into the ventricle of a 4-days-post-fertilization larval zebrafish. After 3 hours, to allow the compound to distribute throughout the brain, the fish were imaged using confocal microscopy. Larval zebrafish were mounted dorsal and illuminated with the DAPI filter for 2000 ms per slice to uncage the compound. While uncaging of our compound was visible in the zebrafish, only a small subset of astrocytes were labeled by the compound (Figure 4F). The variability in labeling is likely due to the compound's +1 overall charge, limiting its transport through the organic cation transporter. Although this molecule did not work as expected, its modest success provides a path to the creation of a second version in the future, potentially using a bipyridinium targeting moiety to create a photo-labile marker with an overall +2 charge instead of +1.

6. CONCLUSIONS

Astrocytes have become an important aspect of studying brain function, making it necessary for scientists to create new tools to target and study them. Genetic methods using astrocyte specific proteins such as GFAP and S100 β allow researchers to utilize transgenic animals to study astrocyte biology, while small molecules such as SR101 and β -Ala-Lys-N_e-AMCA are a simple method to quickly and efficiently label astrocytes. Our modular method using a cationic methylpyridinium targeting moiety allows for the transport of various small molecule fluorophores in astrocytes, permitting us to create a fluorescent and pro-fluorescent astrocyte marker. While the first iteration of our photo-labile marker was only minimally successful, it provides insight on best practices for the creation of future cationic astrocyte markers.

References

- Alghamdi OA, King N, Jones GL, & Moens PDJ (2018). A new use of β -Ala-Lys (AMCA) as a transport reporter for PEPT1 and PEPT2 in renal brush border membrane vesicles from the outer cortex and outer medulla. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1860(5), 960–964. 10.1016/j.bbamem.2017.12.021 [PubMed: 29291378]
- Anthony TE, & Heintz N (2007). The folate metabolic enzyme ALDH1L1 is restricted to the midline of the early CNS, suggesting a role in human neural tube defects. *The Journal of Comparative Neurology*, 500(2), 368–383. 10.1002/cne.21179 [PubMed: 17111379]
- Araque A, Parpura V, Sanzgiri RP, & Haydon PG (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends in Neurosciences*, 22(5), 208–15. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10322493> [PubMed: 10322493]
- Berger UV, & Hediger MA (2006). Distribution of the glutamate transporters GLT-1 (SLC1A2) and GLAST (SLC1A3) in peripheral organs. *Anatomy and Embryology*, 211(6), 595–606. 10.1007/s00429-006-0109-x [PubMed: 16868771]
- Bushong EA, Martone ME, & Ellisman MH (2004). Maturation of astrocyte morphology and the establishment of astrocyte domains during postnatal hippocampal development. *International Journal of Developmental Neuroscience*, 22(2), 73–86. 10.1016/j.ijdevneu.2003.12.008 [PubMed: 15036382]

- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, ... Barres BA (2008). A Transcriptome Database for Astrocytes, Neurons, and Oligodendrocytes: A New Resource for Understanding Brain Development and Function. 10.1523/JNEUROSCI.4178-07.2008
- Chaudhry FA, Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, & Storm-Mathisen J (1995). Glutamate transporters in glial plasma membranes: Highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron*, 15(3), 711–720. 10.1016/0896-6273(95)90158-2 [PubMed: 7546749]
- Cina C, & Hochman S (2000). Diffuse distribution of sulforhodamine-labeled neurons during serotonin-evoked locomotion in the neonatal rat thoracolumbar spinal cord. *The Journal of Comparative Neurology*, 423(4), 590–602. 10.1002/1096-9861(20000807)423:4<590::AID-CNE5>3.0.CO;2-L [PubMed: 10880990]
- Dallwig R, & Deitmer JW (2002). Cell-type specific calcium responses in acute rat hippocampal slices. *Journal of Neuroscience Methods*, 116(1), 77–87. 10.1016/S0165-0270(02)00030-4 [PubMed: 12007985]
- Deloulme JC, Raponi E, Gentil BJ, Bertacchi N, Marks A, Labourdette G, & Baudier J (2004). Nuclear expression of S100B in oligodendrocyte progenitor cells correlates with differentiation toward the oligodendroglial lineage and modulates oligodendrocytes maturation. *Molecular and Cellular Neuroscience*, 27(4), 453–465. 10.1016/J.MCN.2004.07.008 [PubMed: 15555923]
- Ehinger B, Zucker CL, Bruun A, & Adolph A (1994). In vivo staining of oligodendroglia in the rabbit retina. *Glia*, 10(1), 40–48. 10.1002/glia.440100106 [PubMed: 7507886]
- Emsley JG, & Macklis JD (2006). Astroglial heterogeneity closely reflects the neuronal-defined anatomy of the adult murine CNS. *Neuron Glia Biology*, 2(3), 175–86. 10.1017/S1740925X06000202 [PubMed: 17356684]
- Espallargues J, Solovieva O, Técher V, Bauer K, Alonso G, Vincent A, & Hussy N (2007). Synergistic activation of astrocytes by ATP and norepinephrine in the rat supraoptic nucleus. *Neuroscience*, 148(3), 712–723. 10.1016/J.NEUROSCIENCE.2007.03.043 [PubMed: 17693027]
- Fawcett JW, & Asher RA (1999). The glial scar and central nervous system repair. *Brain Research Bulletin*, 49(6), 377–91. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10483914> [PubMed: 10483914]
- Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, & Carmignoto G (2004). Neuronal Synchrony Mediated by Astrocytic Glutamate through Activation of Extrasynaptic NMDA Receptors. *Neuron*, 43(5), 729–743. 10.1016/J.NEURON.2004.08.011 [PubMed: 15339653]
- Furihata T, & Anzai N (2017). Functional Expression of Organic Ion Transporters in Astrocytes and Their Potential as a Drug Target in the Treatment of Central Nervous System Diseases. *Biological & Pharmaceutical Bulletin*, 40(8), 1153–1160. 10.1248/bpb.b17-00076 [PubMed: 28768996]
- Gosejacob D, Dublin P, Bedner P, Hüttmann K, Zhang J, Tress O, ... Theis M (2011). Role of astroglial connexin30 in hippocampal gap junction coupling. *Glia*, 59(3), 511–519. 10.1002/glia.21120 [PubMed: 21264956]
- Grass D, Pawlowski PG, Hirrlinger J, Papadopoulos N, Richter DW, Kirchhoff F, & Hülsmann S (2004). Diversity of functional astroglial properties in the respiratory network. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 24(6), 1358–65. 10.1523/JNEUROSCI.4022-03.2004 [PubMed: 14960607]
- Guerra-Gomes S, Sousa N, Pinto L, & Oliveira JF (2017). Functional Roles of Astrocyte Calcium Elevations: From Synapses to Behavior. *Frontiers in Cellular Neuroscience*, 11, 427. 10.3389/fncel.2017.00427 [PubMed: 29386997]
- Hagos L, & Hülsmann S (2016). Unspecific labelling of oligodendrocytes by sulforhodamine 101 depends on astrocytic uptake via the thyroid hormone transporter OATP1C1 (SLCO1C1). *Neuroscience Letters*, 631, 13–18. 10.1016/j.neulet.2016.08.010 [PubMed: 27519929]
- Halassa MM, Fellin T, & Haydon PG (2007). The tripartite synapse: roles for gliotransmission in health and disease. *Trends in Molecular Medicine*, 13(2), 54–63. 10.1016/j.molmed.2006.12.005 [PubMed: 17207662]
- Harada K, Kamiya T, & Tsuboi T (2015). Gliotransmitter Release from Astrocytes: Functional, Developmental, and Pathological Implications in the Brain. *Frontiers in Neuroscience*, 9, 499. 10.3389/fnins.2015.00499 [PubMed: 26793048]

- Hirrlinger PG, Scheller A, Braun C, Quintela-Schneider M, Fuss B, Hirrlinger J, & Kirchhoff F (2005). Expression of reef coral fluorescent proteins in the central nervous system of transgenic mice. *Molecular and Cellular Neuroscience*, 30, 291–303. 10.1016/j.mcn.2005.08.011 [PubMed: 16169246]
- Kang J, Kang N, Yu Y, Zhang J, Petersen N, Tian GF, & Nedergaard M (2010). Sulforhodamine 101 induces long-term potentiation of intrinsic excitability and synaptic efficacy in hippocampal CA1 pyramidal neurons. *Neuroscience*, 169(4), 1601–1609. 10.1016/J.NEUROSCIENCE.2010.06.020 [PubMed: 20600669]
- Khakh BS, & McCarthy KD (2015). Astrocyte calcium signaling: from observations to functions and the challenges therein. *Cold Spring Harbor Perspectives in Biology*, 7(4), a020404 10.1101/cshperspect.a020404 [PubMed: 25605709]
- Kimelberg HK (2004). The problem of astrocyte identity. *Neurochemistry International*, 45(2–3), 191–202. 10.1016/J.NEUINT.2003.08.015 [PubMed: 15145537]
- Lovatt D, Sonnewald U, Waagepetersen HS, Schousboe A, He W, Lin JH-C, ... Nedergaard M (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 27(45), 12255–66. 10.1523/JNEUROSCI.3404-07.2007 [PubMed: 17989291]
- Lu H, & Klaassen C (2006). Tissue distribution and thyroid hormone regulation of Pept1 and Pept2 mRNA in rodents. *Peptides*, 27(4), 850–857. 10.1016/J.PEPTIDES.2005.08.012 [PubMed: 16202478]
- Magrassi L, Purves D, & Lichtman JW (1987). Fluorescent probes that stain living nerve terminals. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 7(4), 1207–14. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/3572476> [PubMed: 3572476]
- Matthias K, Kirchhoff F, Seifert G, Hüttmann K, Matyash M, Kettenmann H, & Steinhäuser C (2003). Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 23(5), 1750–8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12629179> [PubMed: 12629179]
- Meng X, Yang F, Ouyang T, Liu B, Wu C, & Jiang W (2015). Specific gene expression in mouse cortical astrocytes is mediated by a 1740bp-GFAP promoter-driven combined adeno-associated virus2/5/7/8/9. *Neuroscience Letters*, 593, 45–50. 10.1016/J.NEULET.2015.03.022 [PubMed: 25778419]
- Nagy J., Patel, Ochalski PA., & Stelmack G. (1999). Connexin30 in rodent, cat and human brain: selective expression in gray matter astrocytes, co-localization with connexin43 at gap junctions and late developmental appearance. *Neuroscience*, 88(2), 447–468. 10.1016/S0306-4522(98)00191-2 [PubMed: 10197766]
- Nguyen T, & Francis MB (2003). Practical synthetic route to functionalized rhodamine dyes. *Organic Letters*, 5(18), 3245–8. 10.1021/ol035135z [PubMed: 12943398]
- Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, & Ottersen OP (1997). Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 17(1), 171–80. 10.1523/JNEUROSCI.17-01-00171.1997 [PubMed: 8987746]
- Nimmerjahn A, Kirchhoff F, Kerr JND, & Helmchen F (2004). Sulforhodamine 101 as a specific marker of astroglia in the neocortex in vivo. *Nature Methods*, 1(1), 31–7. 10.1038/nmeth706 [PubMed: 15782150]
- Nolte C, Matyash M, Pivneva T, Schipke CG, Ohlemeyer C, Hanisch U-K, ... Kettenmann H (2001). GFAP promoter-controlled EGFP-expressing transgenic mice: A tool to visualize astrocytes and astrogliosis in living brain tissue. *Glia*, 33(1), 72–86. 10.1002/1098-1136(20010101)33:1<72::AID-GLIA1007>3.0.CO;2-A [PubMed: 11169793]
- Pannasch U, Vargová L, Reingruber J, Ezan P, Holcman D, Giaume C, ... Rouach N (2011). Astroglial networks scale synaptic activity and plasticity. *Proceedings of the National Academy of Sciences of the United States of America*, 108(20), 8467–72. 10.1073/pnas.1016650108 [PubMed: 21536893]

- Pekny M, Johansson CB, Eliasson C, Stakeberg J, Wallén A, Perlmann T, ... Frisén J (1999). Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. *The Journal of Cell Biology*, 145(3), 503–14. 10.1083/JCB.145.3.503 [PubMed: 10225952]
- Preston AN, Farr JD, O'Neill BK, Thompson KK, Tsirka SE, & Laughlin ST (2018). Visualizing the Brain's Astrocytes with Diverse Chemical Scaffolds. *ACS Chemical Biology*, 13(6), 1493–1498. 10.1021/acscchembio.8b00391 [PubMed: 29733639]
- Raponi E, Agenes F, Delphin C, Assard N, Baudier J, Legraverend C, & Deloulme J-C (2007). S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. *Glia*, 55(2), 165–77. 10.1002/glia.20445 [PubMed: 17078026]
- Rasmussen R, Nedergaard M, & Petersen NC (2016). Sulforhodamine 101, a widely used astrocyte marker, can induce cortical seizure-like activity at concentrations commonly used. *Scientific Reports*, 6(1), 30433. 10.1038/srep30433 [PubMed: 27457281]
- Regan MR, Huang YH, Kim YS, Dykes-Hoberg MI, Jin L, Watkins AM, ... Rothstein JD (2007). Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 27(25), 6607–19. 10.1523/JNEUROSCI.0790-07.2007 [PubMed: 17581948]
- Rubio-Aliaga I, Frey I, Boll M, Groneberg DA, Eichinger HM, Balling R, & Daniel H (2003). Targeted disruption of the peptide transporter Pept2 gene in mice defines its physiological role in the kidney. *Molecular and Cellular Biology*, 23(9), 3247–52. 10.1128/MCB.23.9.3247-3252.2003 [PubMed: 12697824]
- Savtchouk I, & Volterra A (2018). Gliotransmission: Beyond Black-and-White. *The Journal of Neuroscience*, 38(1), 14–25. 10.1523/JNEUROSCI.0017-17.2017 [PubMed: 29298905]
- Schitine C, Nogaroli L, Costa MR, & Hedin-Pereira C (2015). Astrocyte heterogeneity in the brain: from development to disease. *Frontiers in Cellular Neuroscience*, 9, 76. 10.3389/fncel.2015.00076 [PubMed: 25852472]
- Schnell C, Hagos Y, & Hülsmann S (2012). Active sulforhodamine 101 uptake into hippocampal astrocytes. *PloS One*, 7(11), e49398. 10.1371/journal.pone.0049398 [PubMed: 23189143]
- Schnell C, Shahmoradi A, Wichert SP, Mayerl S, Hagos Y, Heuer H, ... Hülsmann S (2015). The multispecific thyroid hormone transporter OATP1C1 mediates cell-specific sulforhodamine 101-labeling of hippocampal astrocytes. *Brain Structure and Function*, 220(1), 193–203. 10.1007/s00429-013-0645-0 [PubMed: 24129767]
- Schulz M, Hamprecht B, Kleinkauf H, & Bauer K (1987). Peptide uptake by astroglia-rich brain cultures. *Journal of Neurochemistry*, 49(3), 748–55. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/3612123> [PubMed: 3612123]
- Shigetomi E, Bushong EA, Hausteiner MD, Tong X, Jackson-Weaver O, Kracun S, ... Khakh BS (2013). Imaging calcium microdomains within entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses. *The Journal of General Physiology*, 141(5), 633–47. 10.1085/jgp.201210949 [PubMed: 23589582]
- Slezak M, Göritz C, Niemiec A, Frisén J, Chambon P, Metzger D, & Pfrieger FW (2007). Transgenic mice for conditional gene manipulation in astroglial cells. *Glia*, 55(15), 1565–1576. 10.1002/glia.20570 [PubMed: 17823970]
- Srinivasan R, Lu T-Y, Chai H, Xu J, Huang BS, Golshani P, ... Khakh BS (2016). New Transgenic Mouse Lines for Selectively Targeting Astrocytes and Studying Calcium Signals in Astrocyte Processes In Situ and In Vivo. *Neuron*, 92(6), 1181–1195. 10.1016/J.NEURON.2016.11.030 [PubMed: 27939582]
- Stefansson K, Wollmann RL, & Moore BW (1982). Distribution of S-100 protein outside the central nervous system. *Brain Research*, 234(2), 309–317. 10.1016/0006-8993(82)90871-X [PubMed: 7059833]
- Steiner J, Bernstein H-G, Bielau H, Berndt A, Brisch R, Mawrin C, ... Bogerts B (2007). Evidence for a wide extra-astrocytic distribution of S100B in human brain. *BMC Neuroscience*, 8(1), 2. 10.1186/1471-2202-8-2 [PubMed: 17199889]

- Vives V, Alonso G, Solal AC, Joubert D, & Legraverend C (2003). Visualization of S100B-positive neurons and glia in the central nervous system of EGFP transgenic mice. *The Journal of Comparative Neurology*, 457(4), 404–419. 10.1002/cne.10552 [PubMed: 12561079]
- Wang Y-F, & Hatton GI (2009). Astrocytic plasticity and patterned oxytocin neuronal activity: dynamic interactions. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 29(6), 1743–54. 10.1523/JNEUROSCI.4669-08.2009 [PubMed: 19211881]
- Yang Y, Vidensky S, Jin L, Jie C, Lorenzini I, Frankl M, & Rothstein JD (2011). Molecular comparison of GLT1+ and ALDH1L1+ astrocytes in vivo in astroglial reporter mice. *Glia*, 59(2), 200–7. 10.1002/glia.21089 [PubMed: 21046559]
- Zhang J, Wang H, Ye C, Ge W, Chen Y, Jiang Z, ... Duan S (2003). ATP Released by Astrocytes Mediates Glutamatergic Activity-Dependent Heterosynaptic Suppression. *Neuron*, 40(5), 971–982. 10.1016/S0896-6273(03)00717-7 [PubMed: 14659095]
- Zhu L, Ramboz S, Hewitt D, Boring L, Grass DS, & Purchio AF (2004). Non-invasive imaging of GFAP expression after neuronal damage in mice. *Neuroscience Letters*, 367, 210–212. 10.1016/j.neulet.2004.06.020 [PubMed: 15331155]
- Zimmermann M, & Stan AC (2010). PepT2 transporter protein expression in human neoplastic glial cells and mediation of fluorescently tagged dipeptide derivative β -Ala-Lys-N_e-7-amino-4-methyl-coumarin-3-acetic acid accumulation. *Journal of Neurosurgery*, 112(5), 1005–1014. 10.3171/2009.6.JNS08346 [PubMed: 19612975]

Figure 1. Role of astrocytes in the brain.

(A) Depiction of the tripartite synapse, which is magnified for B–D. (B) Release of the neurotransmitter glutamate from a presynaptic neuron can bind to the astrocytic glutamate receptor, GLT1, and cause Ca^{2+} excitability, increasing intracellular $[Ca^{2+}]$. (C) Ca^{2+} excitability also causes the release of gliotransmitter glutamate, which activates post-synaptic NMDA receptors, regulating synaptic signaling. (D) Ca^{2+} excitability releases the gliotransmitter ATP, where it is converted to adenosine in the synapse and binds the presynaptic A1 receptor to induce synaptic suppression.

Figure 2. Differences in expression by astrocyte-specific promoters and proteins.

(A) GFP expression driven by a truncated 1740-bp GFAP promoter (green) with immunostaining against GFAP (red) showing incomplete overlap between promoter-driven expression and protein labeling in mouse cortex. (B) Inset of (A) showing GFP⁺ astrocytes in hippocampal stratum. Arrowheads indicate GFP-expressing astrocytes. (C, D) GFP expression driven by the astrocyte-specific Aldh1L1 promoter (green), with S100β (C) or GFAP (D) co-immunostaining (red) in mouse hippocampal astrocytes. Aldh1L1 reveals the very fine astrocytic processes that S100β or GFAP cannot.

Panels (A, B): Reprinted with permission from Meng, X., Yang, F., Ouyang, T., Liu, B., & Jiang, W. (2015). Specific gene expression in mouse cortical astrocytes is mediated by a 1740bp-GFAP promoter-driven combined adeno-associated virus. *Neuroscience Letters*, 593, 45–50.

Panels (C, D): Srinivasan, R., Lu, T.-Y., Chai, H., Xu, J., Huang, B. S., Golshani, P., ... Khakh, B. S. (2016). New Transgenic Mouse Lines for Selectively Targeting Astrocytes and Studying Calcium Signals in Astrocyte Processes In Situ and In Vivo. *Neuron*, 92(6), 1181–1195. <https://doi.org/10.1016/J.NEURON.2016.11.030>.

Figure 3. Fluorescent small molecules to label astrocytes.

(A) Structure of the small molecule SR101. (B) SR101 can label oligodendrocytes in the mouse hippocampus seen through co-localization between SR101 and a marker for oligodendrocytes, proteolipid-protein (PLP-GFP mice). Colocalization denoted with arrows. (C) Structure of the small molecule β -Ala-Lys-N ϵ -AMCA. (D) β -Ala-Lys-N ϵ -AMCA is capable of labeling astrocytes in the supraoptic nucleus of the rat hypothalamus as seen by colocalization with GFAP immunolabeling.

Panel (B): Reprinted with permission from Hagos, L., & Hülsmann, S. (2016). Unspecific labelling of oligodendrocytes by sulforhodamine 101 depends on astrocytic uptake via the thyroid hormone transporter OATP1C1 (SLCO1C1). *Neuroscience Letters*, 631, 13–18. <https://doi.org/10.1016/j.neulet.2016.08.010>.

Panel (D): Reprinted with permission from Espallergues, J., Solovieva, O., Técher, V., Bauer, K., Alonso, G., Vincent, A., & Hussy, N. (2007). Synergistic activation of astrocytes by ATP and norepinephrine in the rat supraoptic nucleus. *Neuroscience*, 148(3), 712–723. <http://doi.org/10.1016/J.NEUROSCIENCE.2007.03.043>.

Figure 4. Cationic methylpyridinium markers label primary rat hippocampal astrocytes and astroglia in the larval zebrafish.

(A) Structure of Rhodamine B Methylpyridinium marker. (B) Structure of NVOC₂-Q-Rhodamine Methylpyridinium photolabile marker. (C–D) Primary rat hippocampal astrocytes are robustly labeled by Rhodamine B Methylpyridinium (C) while after illumination with 365 nm light, NVOC₂-Q-Rhodamine Methylpyridinium shows moderate, punctate labeling (D). (E–F) Rhodamine B Methylpyridinium labels astroglia throughout entire larval zebrafish brain (E) while after illumination with 365 nm light, NVOC₂-Q-Rhodamine Methylpyridinium shows labeling in a small subset of the zebrafish midbrain (F). Scale bar 50 μ m.