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Visualizing the brain's astrocytes with diverse chemical scaffolds

Alyssa N. Preston¹, Joshua D. Farr¹, Brianna K. O'Neill¹, Kaitlyn K. Thompson², Stella E. Tsirka², and Scott T. Laughlin^{1,*}

¹Department of Chemistry, Stony Brook University, Stony Brook, New York 11794, United States

²Department of Pharmacological Sciences, Stony Brook University, Stony Brook, New York, 11794, United States

Abstract

Astrocytes are the most abundant cells in the brain. They support neurons, adjust synaptic strength, and modulate neuronal signaling, yet the full extent of their functions is obscured by the dearth of methods for their visualization and analysis. Here we report a chemical reporter that targets small molecules specifically to astrocytes both *in vitro* and *in vivo*. Fluorescent versions of this tag are imported through an organic cation transporter to label glia across species. The structural modularity of this approach will enable wide-ranging applications for understanding astrocyte biology.

Once considered mere supporters of the brain's neural circuitry, astrocytes have recently emerged as active players in neural circuit function. For example, astrocytes mediate synaptogenesis and adjust the synaptic strength of connected neurons¹. Like neurons, astrocytes experience cytosolic Ca²⁺ spikes upon binding neurotransmitters, a process now recognized as an astrocyte contribution to information processing². Beyond sensing neurotransmitters, astrocytes modulate synaptic activity through the release of gliotransmitters³, thus participating directly in neuronal signaling on a breadth of timescales inaccessible via neuron-centric brain circuitry.

Despite their importance, strategies to mark and study astrocyte function are limited. Indeed, the anionic fluorophore sulforhodamine 101 (SR101)^{4,5} and a coumarin dipeptide (β-Ala-Lys-Nepsilon-Coumarin) that targets the astrocyte-resident peptide transporter PepT2⁶, are the only reported chemical astrocyte markers, and genetic approaches for targeting astrocytes using astrocyte-specific promoters (*e.g.*, GFAP or AldH1L⁷) do not provide the same capabilities or flexibility as small molecule probes. SR101 labels astrocytes via its uptake by an astrocyte-resident organic anion transporting protein⁸. It has seen wide use as a marker for astrocytes, for example, in rat hippocampal slice cultures or the mouse neocortex

*Correspondence: scott.laughlin@stonybrook.edu.

Author Contributions

ANP performed experiments, analyzed data and wrote the manuscript. JDF, BKO, KKT performed experiments and critically read the manuscript. SET critically read the manuscript. STL conceived the project, analyzed data, and wrote the manuscript.

Competing financial interests

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in vivo^{4,9}. However, there are concerns over its specificity for astrocytes due to its redistribution to other glia through gap junction hemichannels¹⁰. Additionally, since its publishing in 2004, no structural analogs that label astrocytes have been described, suggesting that functionalized SR101 analogs do not retain their astrocyte labeling properties. Similarly, the β -Ala-Lys-Nepsilon-Coumarin marker has not been modified with other fluorophores, and its peptide nature may complicate this molecule's use in living animals. While searching for molecular scaffolds that promote astrocyte labeling and permit elaboration with useful functionality, we noted that astrocytes from diverse species also express Organic Cation Transporters (OCTs), which are responsible for transporting drugs and endogenous molecules like neurotransmitters in the brain¹¹. These channels are promiscuous transporters of organic cations in sizes similar to small molecule fluorophores, making them ripe targets for the development of selective astrocyte markers (Fig. 1a).

For our initial evaluation of cationic probes as selective astrocyte markers, we selected 4-di-2-asp. 4-di-2-asp is a small, methylpyridinium-containing fluorophore that is commercially available and has served as a marker of nerve terminals at the neuromuscular junction¹². Additionally, its isomer, 4-di-1-asp, is a substrate for OCTs^{13,14}. To assess its potential as an astrocyte marker, we exposed astrocytes or neurons isolated from rat hippocampus to 4-di-2-asp and characterized the resulting labeling by confocal microscopy. These experiments revealed a 6-fold increase in fluorescence intensity compared to unlabeled astrocytes, suggesting 4-di-2-asp showed some promise (Supplementary Fig. 1). However, its labeling was dim, prone to photobleaching, and, more importantly, not selective for astrocytes over neurons (Supplementary Fig. 1).

Intrigued by the modest uptake of 4-di-2-asp by astrocytes, we pursued as potential astrocyte markers fluorophores with more desirable photo-physical properties and an attached methylpyridinium moiety, a key feature of 4-di-2-asp. As a starting point, we employed rhodamine B, a sulfonate-free relative of the anionic astrocyte marker SR101. We synthesized a methylpyridinium-tagged rhodamine B (compound **1**, Fig. 1b and Supplementary Scheme 1–2)¹⁵ and assayed the compound's aptitude for labeling astrocytes, but not neurons, microglia, or oligodendrocytes, isolated from rat hippocampus or mouse cortex.

First, we confirmed the primary cell cultures contained the appropriate cell type via immunohistochemistry, probing for Glial Fibrillary Acidic Protein (GFAP) or NG2, proteins whose expression is frequently used as a marker for astrocytes (GFAP) or oligodendrocyte precursor cells (NG2)¹⁶ (Supplementary Fig. 2 and 3) or through expression of GFP in microglia isolated from the MacGreen transgenic mouse¹⁷. To determine the optimal concentration for astrocyte labeling, we bathed astrocytes isolated from the mouse cortex in concentrations of compound **1** from 0.1–10 μ M. We observed only a modest increase in signal to noise for concentrations higher than 1 μ M, so we selected the 1 μ M concentration as the working concentrations for future experiments (Supplementary Fig. 4). Next, we bathed cells from each population and the non-neuronal cell line HEK293 in media containing 1 μ M compound **1**. After washing out residual fluorophore, confocal microscopy analysis of the images revealed bright labeling throughout the cytoplasm of astrocytes isolated from rat hippocampus (Fig. 1c, d) and mouse cortex (Fig 1h and Supplementary Fig.

5). Importantly, analysis by confocal microscopy revealed essentially no labeling of rat hippocampal neurons, HEK293 cells (Fig. 1e-f and Supplementary Fig. 1), or mouse cortical oligodendrocyte precursor cells, and only a small amount of internalization from mouse microglia, which was expected based on their tendency to actively endocytose media components since they function as the brain's macrophage¹⁸ (Fig. 1h-j and Supplementary Fig. 5). Indeed, quantification of the fluorescence intensity of astrocytes, neurons, and HEK cells exposed to compound **1** revealed a significant ~11-fold fluorescence increase in labeling over background for rat hippocampal astrocytes compared to neurons (Fig. 1g), and a more modest ~10-fold increase for mouse cortical astrocytes over oligodendrocyte precursors and ~2.5-fold over microglia (due to the molecule's internalization by a subset of cells) (Fig. 1k).

We next sought to determine the mechanism of astrocyte labeling by compound **1**. We hypothesized that OCTs were responsible for its transport into astrocytes in much the same way as an anion transporter imports SR101. To analyze the uptake mechanism, we expressed OCT1 linked to an EGFP expression marker via a self-cleaving P2A peptide¹⁹, or an EGFP-only control, in HEK293 cells, and then treated the cells with compound **1**. Upon analysis by confocal microscopy, we observed distinct punctate labeling with compound **1** in cells expressing OCT1 (Fig. 2a) that was absent from the EGFP-only controls (Fig. 2b,d), suggesting that the exogenously-expressed OCT1 imports compound **1**. To further validate this conclusion, we treated cells expressing Oct1-p2a-EGFP or EGFP-only control with 10 μ M prazosin, an OCT inhibitor²⁰, together with compound **1**. Confocal microscopy analysis revealed that prazosin treatment inhibited the labeling of cells expressing OCT1 (Fig. 2c and Supplementary Fig. 6), and quantification of the fluorescence of cells in each population revealed a significant decrease in the labeling of OCT1 transfected cells exposed to prazosin (Fig. 2d).

Next, we confirmed that OCT was responsible for compound **1**'s uptake by rat hippocampal astrocytes. Importantly, we did not expect pharmacological disruption of compound **1** using prazosin to eliminate labeling due to the varying affinities of prazosin for different OCT isoforms. For example, rat astrocytes express all three OCTs (OCT1–3), which are homologous to the human OCTs (hOCT1–3)^{20–22}, and prazosin is a known inhibitor for rat OCT3 ($IC_{50} = 2 \mu$ M)²³ and hOCT1 and 3, but not hOCT2 ($IC_{50} = 1.84 \mu$ M; $>100 \mu$ M; and 12.6μ M for hOCT1–3, respectively)²⁰. Thus, we expect exposure of astrocytes to prazosin and compound **1** will result in a partial decrease of the observed labeling due to meager inhibition of OCT2 and more robust inhibition of rat OCT1 and 3. To test this hypothesis, we incubated the astrocytes with prazosin and compound **1** and analyzed labeling by confocal microscopy (Fig. 2e–f). Incubation with prazosin resulted in a significant 1.7-fold decrease in the intensity of astrocyte labeling with compound **1** (Fig. 2g and Supplementary Fig. 6–7), suggesting that endogenous OCTs facilitate compound **1**'s uptake by astrocytes.

One of the most exciting prospects provided by this new astrocyte label is the opportunity to generate structural variants with interesting properties. Examples include optimized fluorophores with green- or red-shifted emission, utility in super-resolution imaging, or the ability to report on intra-astrocyte conditions, such as changing Ca^{2+} or metal ion concentrations. Thus, we sought to determine the generality of the pyridinium tag as an

astrocyte labeling strategy. To accomplish this, we synthesized a panel of conjugates, each containing a popular fluorophore and a methyl- or ethyl-pyridinium tag (Supplementary Fig. 8). These fluorophores included diverse chemical scaffolds, including fluoresceins, other rhodamines, cyanines, and bodipys.

We evaluated each pyridinium-modified fluorophore by exposing astrocytes to each compound, washing out residual dye, analyzing the resulting labeling by confocal microscopy, and quantifying the results (Fig. 2h–i and Supplementary Fig. 9). Among the molecules tested, we found several with astrocyte labeling comparable to compound **1**. For example, a methylpyridinium-modified Cy3 with a +2 overall charge at physiological pH (Compound **2**) displayed astrocyte labeling intensities of 50% relative to compound **1**. The substantial difference in structure between the rhodamine and Cy3 scaffolds suggests that it is the appended pyridinium and its cationic charge that promote uptake, not a specific feature of the rhodamine scaffold, indicating that there is room for further modification to these probe's chemical structures. Additionally, the green fluorescent rhodamine 110 variant with a +2 overall charge (Compound **3**) exhibits labeling at ~20% of compound **1** and expands the utility of these molecules into systems requiring a green fluorophore, which was not previously available (Fig. 2h–i and Supplementary Fig. 9).

The compounds with a +1 charge displayed more modest labeling. For example, the rhodamine B analog with a different linker to the methylpyridinium moiety (Compound **4**) provided ~5-fold astrocyte labeling over background, whereas a pyridinium-modified bodipy with a +1 charge (Compound **5**), and a neutral sulfo-cy3 (Compound **6**), yielded no significant labeling of astrocytes (Fig. 2i). Likewise, a negatively-charged fluorescein (Compound **7**) did not label astrocytes, consistent with the notion that the organic anion channels responsible for SR101 uptake are more discerning in their substrate choice (Fig. 2i and Supplementary Fig. 9). Finally, versions of the successful rhodamine B and Cy3 (compounds **1** and **2**) without an attached pyridinium group were moderately (Rhodamine) or strongly (Cy3) cell permeable, precluding their utility as specific astrocyte markers (Supplementary Fig. 10).

Lastly, we were interested in determining the generality of this approach with respect to species and to evaluate it in the context of a whole organism. To accomplish this, we explored glia labeling with compound **1** in living zebrafish larvae. Although, as a vertebrate, many aspects of zebrafish neurobiology mirror other vertebrates, zebrafish are not known to have the classic, stellate astrocytes observed in mammals²⁴. Rather, they have cells resembling radial glia, with a more elongated, as opposed to star-like, morphology²⁵. Nevertheless, these zebrafish glia express the mammalian astrocyte marker GFAP and they possess many of the functions of mammalian astrocytes, including the expression of glutamate receptors at points of contact with the neural synapse and the ability to differentiate into neurons²⁴.

To evaluate the ability of the described cationic fluorophores to label GFAP+ glia in zebrafish larvae, we microinjected 2.3 nl of a 100 μ M solution of compound **1** into the brain ventricle of 4-days-post-fertilization (dpf) zebrafish expressing GFP in astrocytes via the GFAP promoter. After 3 hours, we imaged the fish and observed labeling throughout the

zebrafish brain (Fig. 3a). At first glance, the labeling appeared to be different than that seen in the mammalian astrocytes, with intense regions of fluorescence reminiscent of cell surface labeling as opposed to the more diffuse cytoplasmic labeling of mammalian astrocytes. We considered the possibility that compound **1** in zebrafish adhered to cell surface OCTs instead of being internalized by them. However, the OCT1 used to characterize the labeling mechanism in HEK293 cells (Fig. 2a–c) was derived from zebrafish, indicating that this zebrafish OCT is competent to internalize compound **1**. Scrutiny of the images resulting from compound **1** labeling revealed that its global appearance resulted from prominent labeling of the GFAP+ glia's fine projections and the high density of these cells in the fish, especially in the olfactory bulb, anterior telencephalon, and hindbrain. The projections were too small (~1 μm in diameter) to distinguish membrane vs. internal labeling in these brain regions. However, compound **1** labeling in the spinal cord, which is sparsely populated with well-ordered medial cell bodies projecting outward²⁶, revealed cytosolic labeling, suggesting that the compound can be internalized by this cell population (Supplementary Fig. 11).

To determine the compound's specificity for GFAP+ glia over zebrafish neurons, microglia, and the oligodendrocyte cell lineage, we microinjected compound **1** into the brain ventricle of 4 dpf transgenic fish with GFP expressed in neurons under the control of the HuC promoter, which marks neurons²⁷, the Mpeg1 promoter, which marks microglia²⁸, and the Olig2 promoter, which marks oligodendrocyte precursor cells as well as motor neurons and putative cerebellar Purkinje neurons²⁹. Analysis by confocal microscopy revealed colocalization of compound **1** and GFAP+ glia throughout the larval zebrafish brain. For example, in the anterior telencephalon, we observed cellular-level colocalization with GFAP+ glia, although the GFAP marker and compound **1** exist in separate subcellular locations (Fig. 3b,c). Conversely to the GFAP+ glia colocalization, we observed no colocalization between the neuron marker and compound **1** in this brain region (Fig. 3d). We observed similar results in the hindbrain and anterior telencephalon, where cell-level colocalization between GFAP+ cells and compound **1** was apparent (Fig. 3e), and no colocalization was observed with neurons, microglia, or the oligodendrocyte precursor cells (Fig. 3f–h, Supplementary Figs. 12–13). Indeed, quantification of the degree of colocalization between compound **1** and GFAP+ glia in the anterior telencephalon and hindbrain revealed a strong degree of colocalization, whereas colocalization of compound **1** with neurons, microglia, and oligodendrocytes was minimal (Supplementary Fig. 14–15), confirming the specificity of compound **1** for GFAP+ glia over neurons and other neural cell types in the larval zebrafish brain.

We next explored whether these cationic dyes mark cells or tissues outside the brain. Zebrafish OCTs are also expressed in the kidney, liver, heart, and brain³⁰. We observed no labeling with compound **1** in the heart, liver, pancreas, and gut (Supplementary Fig. 16). We did observe labeling in the kidney (Fig. 3i) and the rostral choroid plexus, which contains ependymal cells (Fig. 3a, j, and Supplementary Fig. 17). Labeling in these regions was not surprising because OCT1 is expressed in the choroid plexus and brain microvessel epithelia, where astrocytic end feet modulate the function of the blood-brain barrier^{31,32}. This finding, together with previous work showing OCT3 expression in rat ependymal cells³³, suggests

that ependymal cells in the rodent brain are also likely to be labeled. Importantly, if labeled, the kidney, choroid plexus, and juxta-ventricular ependymal cells are distinct enough to be distinguished from astrocytes based on anatomical position alone.

In summary, we have developed methylpyridinium-tagged fluorophores with diverse scaffolds as markers of rat and mouse astrocytes and zebrafish GFAP⁺ glia. The mechanism of cellular uptake relies on astrocyte-resident OCTs. The modularity of this approach suggests that it can be applied to other useful small molecules, such as photoactivatable fluorophores, smart fluorophores that report on the state of the cell (*e.g.*, Ca²⁺ sensors), or small-molecule transcription activators like doxycycline or tamoxifen, thus expanding the toolkit for interrogation of the brain's astrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A methylpyridinium tagged-fluorophore specifically labels astrocytes.

(a) Strategy for employing cationic fluorophores that target astrocyte-resident organic cation transporters to label astrocytes but not other cell types in the brain. (b) Structure of the first-generation fluorescent methylpyridinium-tagged astrocyte marker. (c–f) Labeling of rat hippocampal astrocytes (c), single planes of a small collection of cells shown in (d), neurons (e), or HEK293 cells (f) with compound **1**. (g) Measurement of mean compound **1** fluorescence in rat astrocytes, rat neurons, and HEK293 cells. *** $P < 1 \times 10^{-17}$, $n = 20$. (h–j) Labeling of mouse cortical astrocytes (h), microglia (i), or oligodendrocyte precursors (j) with compound **1**. ** $P < 1 \times 10^{-6}$, $n = 20$. Error bars in (g) and (k) represent SEM. Scale bars = 10 μm (d), 50 μm (c, e–f, h–j).

Figure 2. Astrocyte Labeling mechanism and the strategy's applicability to diverse fluorophore scaffolds.

(a–b) Compound **1** labels HEK293 cells that express OCT1-p2a-EGFP (a) but not cells that express a EGFP-only control (b). (c) Concurrent exposure of OCT1-p2a-EGFP to both compound **1** and the OCT1 inhibitor prazosin inhibits cellular uptake of compound **1**. (d) Measurement of integrated density (ID) fluorescence in HEK293 cells +/- OCT1 and +/- prazosin shows a significant dependence of compound **1** cellular uptake on OCT1 expression and a significant decrease in uptake upon treatment with prazosin. *** $P < 0.001$, $n = 50$. (e–f) Labeling of rat hippocampal astrocytes by compound **1** alone (e) or with the OCT inhibitor prazosin (f) shows that OCT inhibition decreased compound **1** uptake by astrocytes. (g) Measurement of integrated density (ID) fluorescence in astrocytes bathed in compound **1** with or without prazosin. (h) Methylpyridinium-tagged Cy3 and rhodamine 110 analogs **2** and **3** label rat hippocampal astrocytes. (i) Measurement of integrated density fluorescence of methylpyridinium-tagged fluorophores show a trend of increased astrocyte labeling with increased positive charge, *** $P < 0.001$, $n = 10$. Error bars in (d) and (g) represent SEM. Scale bars = 10 μm (a–c); 50 μm (e–f, h).

Figure 3. Compound 1 marks GFAP+ glia and not neurons in larval zebrafish.

(a) Compound 1 labels throughout the entire brain of 4 dpf larval zebrafish after injection into the ventricle. (b) Colocalization is observed between GFAP+ cells and compound 1-marked cells in the anterior telencephalon. (c) Cellular colocalization is seen in GFAP+ glial processes of the telencephalon. (d) Colocalization is not observed between HuC+ neurons and compound 1-marked cells in the same brain region as b. (e) Compound 1 marks GFAP+ glia in the hindbrain. (f) Lack of colocalization between compound 1 and HuC+ neurons in the hindbrain. (g) Lack of colocalization between compound 1 and Mpeg1 (+) microglia in the anterior telencephalon. (h) Lack of colocalization between compound 1 and Olig2 (+) oligodendrocytes in the anterior telencephalon. (i) Compound 1 has minor uptake in the larval zebrafish kidney, where OCTs are known to be expressed. (j) Compound 1 labeling is observed in the choroid plexus which contains OCT-expressing epithelial cells. Dorsal view (a, e, f, j); Frontal view (b–d, g, h). Scale bars = 50 μ m (a, i); 25 μ m (b–h, j).