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Review Article

OLIG2-ASTROCYTES: A COMPREHENSIVE MINI-REVIEW OF A DISTINCT ASTROCYTIC SUBPOPULATION

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Abstract: ***Astrocytes, the most populous glial cell type within the central nervous system (CNS), are recognized to form diverse cohorts characterized by unique morphologies, gene expressions, and functionalities. Moreover, these cells are allocated to geographically discrete domain regions throughout development. Such regional heterogeneity implies a significant role of astrocytes in modulating the postnatal establishment of neural circuits, thereby orchestrating the ultimate neuronal activity. In our lineage tracing investigation of Olig2-expressing cells utilizing Olig2^{CreER}; EYFP (or tdTomato) transgenic mice, we unearthed Olig2 lineage mature astrocytes within the adult forebrain. Adequate recombination induction was accomplished via prolonged administration of tamoxifen, unveiling a predilection of Olig2 lineage astrocytes to amass in specific adult brain nuclei. Intriguingly, these nuclei predominantly receive GABA inputs. Concurrently, in these regions, the expression of glial fibrillary acidic protein (GFAP), a principal cytological indicator of astrocytes, was remarkably faint, and a unique localization was observed with GFAP-positive astrocytes. In essence, Olig2 lineage mature astrocytes appear to constitute a distinct subpopulation, exhibiting a differential brain distribution and separate functionalities from GFAP-positive astrocytes. This manuscript presents a synthesis of our current understanding regarding the Olig2 lineage mature astrocyte subpopulation that we have discerned.***

Keywords: Olig2-astrocyte, GFAP-astrocyte, inhibitory synapse, GAT-3, slc7a10

INTRODUCTION

The cerebrum encompasses an extensive array of cells, which, despite originating from identical stem cells as neurons, exhibit no electrical activity. These cells, collectively designated as glial cells, significantly outnumber neurons. Among this group, astrocytes represent the most abundant cell population within

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Dedication: This work is dedicated to Dr. P.D. Gupta on his 85th birthday.

the cerebral structure. Astrocytes initially appeared in the early evolutionary stages of vertebrates equipped with a central nervous system, and it is postulated that subsequent stages witnessed further evolution and specialization [1]. In mammals, ranging from rodents to humans, the astrocyte count has exponentially escalated during the evolutionary trajectory, resulting in a highly intricate and specialized morphology. This observation has given rise to the hypothesis that astrocytes substantially contribute to advanced cerebral functions. Over the preceding two decades, astrocyte research has achieved remarkable strides. Besides elucidating their functions and alterations under pathological circumstances [2, 3], significant progress has been made in comprehending their physiological roles, such as their contribution to the maintenance of neural circuit homeostasis [4]. Consequently, it has been unveiled that astrocytes actively modulate neural activity by reabsorbing neurotransmitters from synaptic clefts and releasing transmitters, termed gliotransmitters [5-7]. This discovery has led to the conception of the "tripartite synapse," a neural transmission unit composed of the presynaptic nerve terminal (axon), the postsynaptic dendrites, and the fine processes of astrocytes that contact or envelop them [8,9]. A research area of astrocytes that has garnered substantial attention in recent years is their 'heterogeneity'. It has been established that the environment surrounding astrocytes significantly influences the properties of individual astrocytes, thereby accumulating evidence that astrocytes are not a homogeneous cell population [10,11]. Moreover, single-cell RNAsequencing analysis, which has advanced quickly in recent years, has published site-specific and environment-specific gene expression profiles of astrocytes, revealing the diversity of their gene expression [12]. Presently, research is transitioning from elucidating "molecular-level heterogeneity" to uncovering "functional-level heterogeneity" [13].

Our research has thus far concentrated on Olig2, a basic-helix-loop-helix (bHLH) transcription factor, by tracking the dynamics of Olig2-positive glial progenitor cells and the glial cells derived from them using the inducible CreER / loxP system for genetic marking. This investigation utilized double transgenic mice (hereafter referred to as Olig2^{CreER}:YFP or Olig2^{CreER}:tdTomato), generated by crossing Olig2^{CreER} mice with reporter mice that express reporter proteins (EYFP or tdTomato, respectively). We have previously documented findings such as the differentiation of Olig2-positive glial progenitor cells into reactive astrocytes, which constitute part of the glia scar in damaged brains [14,15], and their active differentiation into oligodendrocytes in demyelination foci in demyelination models [16]. Through the progression of this series of studies, we discovered that under physiological conditions, Olig2 lineage astrocytes (hereafter referred to as Olig2-AS), constitute a subset that exhibits characteristic localization within the mature brain. In this manuscript, we consolidate our recent findings on Olig2-AS, one of the subsets of astrocytes that we have been focusing on.

Adult brains harbor Olig2-positive astrocytes. Olig2 plays a crucial role in the differentiation of oligodendrocytes and motor neurons in the embryonic spinal cord, as well as in the maturation of oligodendrocytes during development [18,19]. In Olig2-deficient mice, astrocyte differentiation is observed in regions and periods where oligodendrocytes should typically differentiate [20], and it has been reported that enforced expression of Olig2 in precursor cells inhibits differentiation into astrocytes in in vitro experiments [21]. From these findings, it has been postulated that the Olig2 signal, which promotes differentiation into oligodendrocytes, suppresses the differentiation signal into astrocytes through interactions between transcription factors.

However, while conducting detailed lineage experiments using Olig2^{CreER}:TG mice, we have consistently observed Olig2-AS in the adult brain [15,17,22,23]. As illustrated in Fig. 1, by administering tamoxifen over an extended period to Olig2^{CreER}:YFP mice to induce sufficient recombination, the distribution of Olig2-AS in specific brain nuclei can be visualized (Fig. 1a). In these mice, cells of the oligodendrocyte lineage also express the reporter protein, but due to their poor processes, they are challenging to identify at low magnification. Therefore, the distribution of YFP fluorescence at this magnification reflects the distribution of Olig2-AS [17]. They exhibit the bushy morphology of protoplasmic astrocytes and express s100b (Fig. 1b) and sox9 (Fig. 1c), markers of mature astrocytes. Furthermore, immunoelectron microscopic observations using a GFP antibody revealed immunoreactive processes attached to capillaries in the form of endfeet (Fig. 1d). At higher magnification,

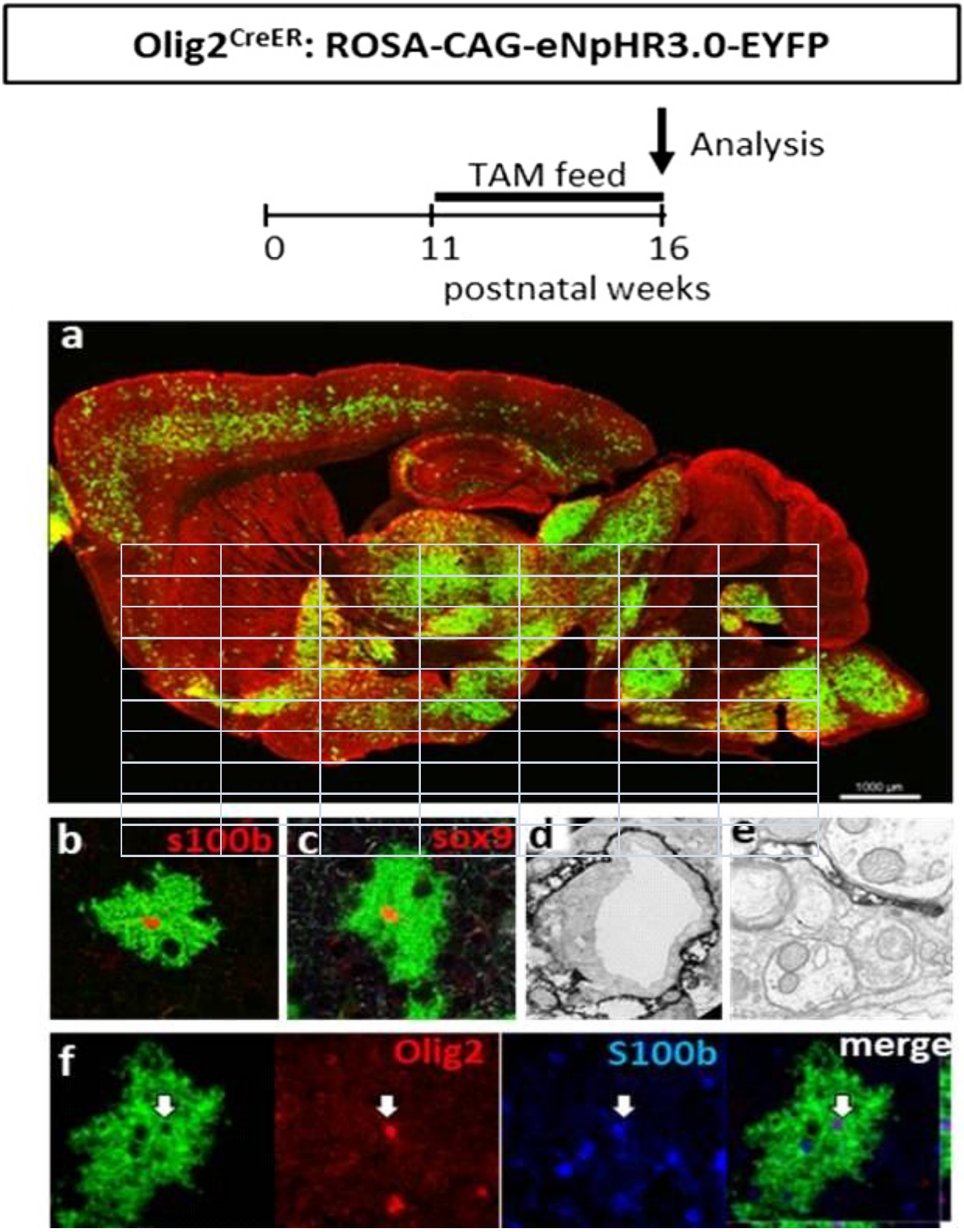


Fig. 1: Adult brains harbor Olig2-positive astrocytes: **a:** A low-magnification view of YFP-positive Olig2-lineage cells (green) with Nissl counterstain (red) is presented in a sagittal section of Olig2^{creER}:YFP mice. **b-c:** Representative images of immunostaining with mature astrocytic markers are shown. Olig2-lineage cells (green) with bushy morphology express s100b (b) and sox9 (c). **d-e:** Immunoelectron microscopy with GFP antibody reveals astrocytic features at the ultrastructural level. An immunoreactive process attached to a capillary is observed in the form of an endfoot (d), and a higher magnification view exhibits the processes extending into the neuropil (e). **f:** Olig2 lineage astrocytes expressing s100b sustain the expression of Olig2 in the nucleus.

the fine processes extend into the neuropil (Fig. 1e), demonstrating the characteristics of mature astrocytes at the ultrastructural level.

Moreover, immunohistochemical analysis using an Olig2 antibody has demonstrated that these Olig2-AS are not temporarily expressing the Olig2 gene and expressing the reporter protein, but are maintaining the expression of Olig2 (Fig. 1f). In other words, while it has been traditionally believed that Olig2-positive precursor cells differentiate into astrocytes accompanied by downregulation of Olig2, there exists a subset that follows the fate of astrocytes regardless of the presence of Olig2. The existence of this Olig2-AS has begun to be reported by other groups following our group, and its existence is currently being recognized [24, 25].

The Olig2-AS preferentially modulates inhibitory neural circuits: Typically, the differentiation of astrocytes in the early postnatal period coincides with the development of neuronal networks, and astrocytes have been demonstrated to be indispensable for proper synapse and circuit formation in the postnatal brain [4,26]. Therefore, astrocytes are subjected to continuous regulation and fine-tuning through various pathways [27]. This is substantiated by the significant alterations in the astrocyte transcriptome within a few weeks after birth [28]. Recently, in addition to this developmental heterogeneity, considerable heterogeneity has been reported between brain regions [29]. These factors are considered to contribute to the substantial diversity in astrocytes, reflecting functional adaptation to local neural networks [30,31].

To elucidate the involvement of Olig2-AS in neural networks, we focused on its distribution map across the entire brain [1]. Furthermore, we created a similar gene marking-based distribution map for glial fibrillary acidic protein (GFAP) positive astrocytes (hereinafter, GFAP-AS), which has been traditionally used as an astrocyte marker [17]. As a result, we found that Olig2-AS and GFAP-AS demonstrate exclusive distributions within the mature brain (Fig. 2a and 2b)[1]. This exclusive arrangement was also observed in the same neural nuclei where both types of astrocytes coexist, such as the lateral globus pallidus (LGP) (Fig. 2c and 2d). This suggests that Olig2-AS form a distinct subpopulation from GFAP-AS and may possess unique functions. Upon examining the distribution of Olig2-AS in the brain to explore their function, we noticed a tendency for them to localize in neural nuclei that receive GABA inputs. When we focused on astrocyte transporters, we found that Olig2-AS express the GABA transporter (GAT-3) (Fig. 3a), while the expression of glutamine transporters (GLT-1, GLAST) was very weak (Fig. 3b). Importantly, detailed immunoelectron microscopic measurements revealed that in the LGP, where inhibitory and excitatory synapses coexist (Fig. 3c and 3d), Olig2-AS preferentially envelop vGAT-positive inhibitory synapses (Fig. 3e) over VGluT1/2-positive excitatory synapses (Fig. 3f and 3e). This strongly suggests that Olig2-AS may be a subtype preferentially involved in the modulation of inhibitory neural circuits.

Olig2-AS are enriched with membrane-related genes: Olig2-AS exhibit significant localization within the basal ganglia circuits that control voluntary movements, predominantly receiving inhibitory inputs, such as the LGP, medial globus pallidum, subthalamic nucleus, and substantia nigra reticulum [1,2]. We therefore explored the relationship between basal ganglia circuit activity induced by motor load and Olig2-AS, focusing on the LGP, which forms the indirect pathway of the basal ganglia circuit. When a running wheel was introduced into the mouse cage to stimulate spontaneous movement, the individual volume of Olig2-AS in the LGP significantly increased, and its morphology became more complex [23]. To elucidate the causal relationship between Olig2-AS morphological changes and spontaneous movement, we halted the wheel rotation after a 3-week period of spontaneous movement, then inhibited movement for another 3 weeks to observe whether the morphology would alter again. Morphological measurements via electron microscopy revealed that the form of astrocytes 6 weeks later was significantly simpler than that at the 3-week mark, demonstrating that Olig2-AS's fine processes exhibit plastic changes correlating with the amount of movement. This suggests that the fine processes of astrocytes may actively participate in neural function regulation through the reuptake of neurotransmitters and release of gliotransmitters. Indeed, the overall expression of GAT-3 in the LGP fluctuated in correlation with the amount of movement, and the number of inhibitory synapses inputting into the LGP also changed correspondingly. This is a fascinating finding suggesting that astrocytes are involved in the

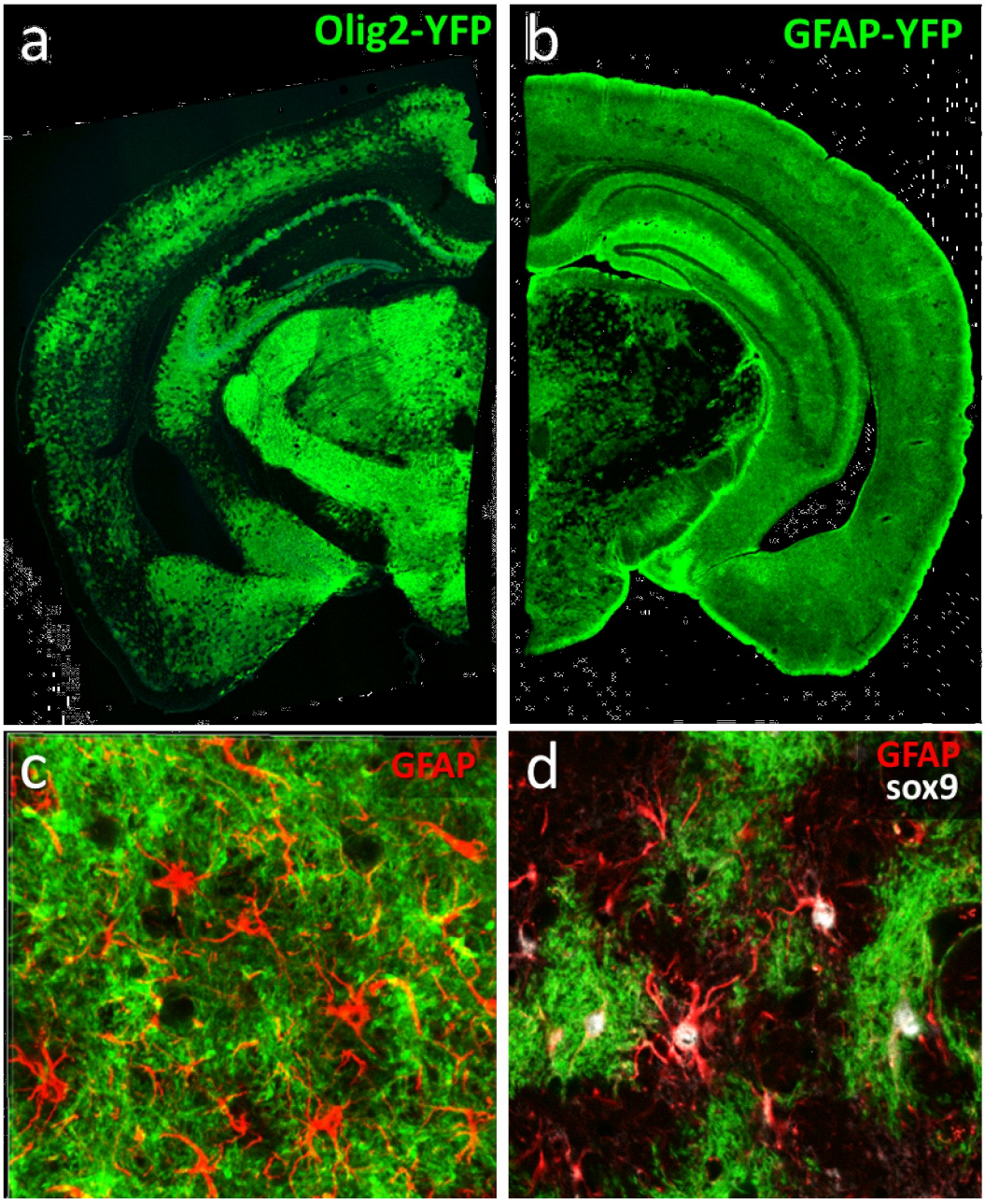


Fig.2: Olig2-AS and GFAP-AS exhibit exclusive distributions within the mature brain: . **a-b:** A representative distribution map of Olig2- and GFAP-AS in coronal sections at the same rostro-caudal position in $Olig2^{CreER}$ (a)- and $GFAP^{Cre}$-YFP (b) mice is presented. **c:** Orthogonal views (x-y, y-z and x-z planes) reveal that Olig2-AS (green) and GFAP-immunoreactive astrocytes (red) occupy adjacent but non-overlapping territories in the LGP. **d:** A view at higher magnification reveals that both Olig2-AS and GFAP-immunoreactive astrocytes, which express Sox9, a marker of mature astrocytes, seemingly occupy discrete territories.

regulation of neuronal activity by altering their morphology [2].

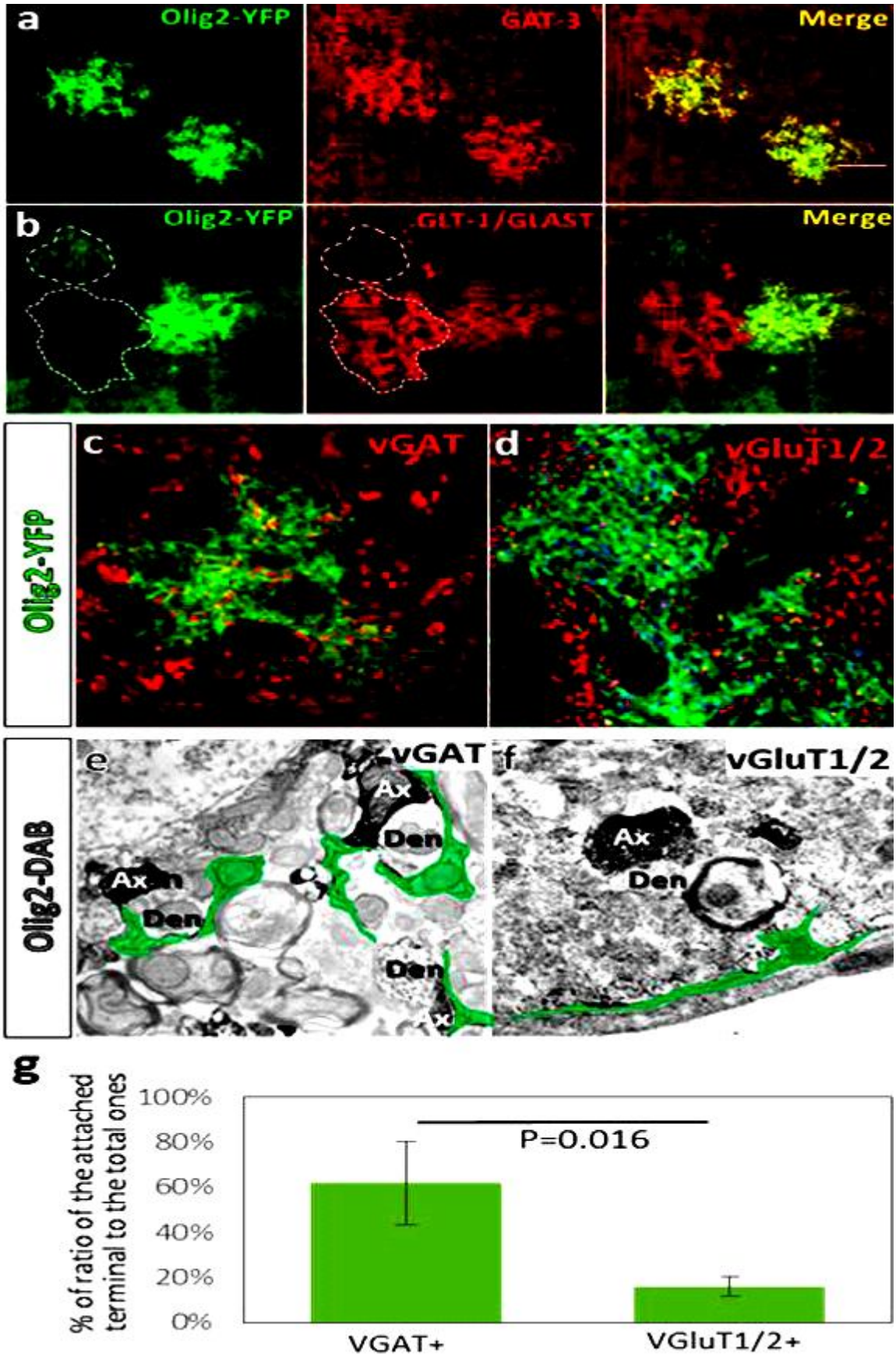
Olig2-AS are enriched with GAT-3 (slc6a13) and ASC-1 (slc7a10) genes: In recent years, the single-cell RNAsequencing method has rapidly advanced, and many transcriptome data have been made public [32,33]. We extracted data from astrocytes in the LGP from a site-specific mouse brain single-cell RNA-seq database [34] with the objective of revealing the specific functions of Olig2-AS. As a result of UMAP analysis, astrocytes in the LGP were classified into twelve clusters (Fig. 4a). Olig2-AS ($Olig2^{high}GFAP^{low}$) (Fig. 4b) and GFAP-AS ($GFAP^{high}Olig2^{low}$) (Fig. 4c) were distributed in different astrocyte clusters, revealing differences in gene expression. Subsequent gene ontology analysis showed that Olig2-AS were more likely to be distributed in clusters rich in membrane-related genes compared to GFAP-AS (Fig. 4d). Among these, focusing on transporters, Olig2-AS showed high expression of the neutral amino acid transporter gene ASC-1 (slc7a10). To complement this *in silico* data analysis, we dissected Olig2-AS and GFAP-AS from the LGP of mouse brain frozen sections using laser micro dissection (Fig. 4e), and compared gene expression using quantitative reverse transcription PCR. After confirming that there was almost no cross-contamination between the two types of cells by laser microdissection, we compared the expression of transporters and found that Olig2-AS expressed significantly higher levels of ASC-1 mRNA than GFAP-AS, which corroborated the *in silico* data (Fig. 4f). Furthermore, we confirmed the expression of SLC7A10 in Olig2-AS by *in situ* hybridization and immunohistochemistry [35]. We also confirmed the high expression of GAT-3 (slc6a13) mRNA, which we have been focusing on (Fig. 4f). Traditionally, SLC7A10 has been known as the sodium-independent alanine-serine-cysteine transporter-1, which has a high affinity for D-serine and glycine and is thought to be primarily expressed in neurons [36]. However, a new perspective has been reported in a study using SLC7A10 knockout mice that SLC7A10 is abundant in a subset of astrocytes and is scarcely expressed in neurons [37]. Interestingly, these SLC7A10-expressing astrocytes are predominantly located in areas with many glycine-acting inhibitory synapses, such as the brainstem and spinal cord, and it has been suggested that they may play a role in maintaining glycine storage in cooperation with GAT-3. Our report of SLC7A10 expression in Olig2-AS is the first report in forebrain astrocytes [35]. Further research is needed to understand how Olig2-AS are involved in inhibitory synapses in the forebrain, but conditional knockout of the SLC7A10 gene in Olig2-AS could be an intriguing project.

CONCLUSIONS

Approximately 160 years ago, astrocytes, a type of glial cell, were considered to merely fill the spaces between neurons, and their role remained largely enigmatic for an extended period. Recent research has revealed that astrocytes contribute to the homeostasis and plasticity of brain information processing through modifications of neural transmission, and they have been found to be deeply involved in various diseases. Consequently, they are garnering increasing attention as potential targets for drug development. For the efficient development of new pharmaceuticals to unlock therapeutic potential, identifying which subpopulations to target becomes a significant challenge. Therefore, it is anticipated that there will be an escalation in research elucidating the relationships between astrocyte subtypes and pathologies. As for the subset we have identified and

Explanation of figure 3:

Fig. 3: Olig2-astrocytes constitute a distinct subpopulation of astrocytes subsidiary to inhibitory GABAergic transmission: **a-b:** Immunohistochemical observations show that Olig2-astrocytes preferentially express GAT- 3, a GABA transporter (a), while they express less of GLT-1 and/or GLAST, glutamate transporters (b). **c-d:** YFP fluorescence of processes of Olig2-AS overlaps with vGAT (c) and vGluT1/2 immunoreactivity. **e-f:** Double immunoelectron microscopic images with DAB reactions for GFP antibody and for vGAT or vGluT1/2 antibody. GFP immunoreactive Olig2-astrocytic processes (green pseudo-color) make contact with inhibitory terminals with vGAT-immunoreactivity (e). But few processes contact excitatory terminals with vGluT1/2-immunoreactivity (f). **g:** Semi-quantitative analyses show that fine processes of Olig2-AS significantly contactvGAT-immunoreactive terminals than VGluT1/2 immunoreactive terminals. The difference of the ratios was statistically significant ($p = 0.016$, one-way ANOVA with post-hoc Turkey HSD test). Den, dendrite; Ax, axon.



For Explanation of figure 3 see page 7372:

are in the process of characterizing, Olig2-AS, our understanding of its functions and involvement in disease conditions is still at a preliminary stage.

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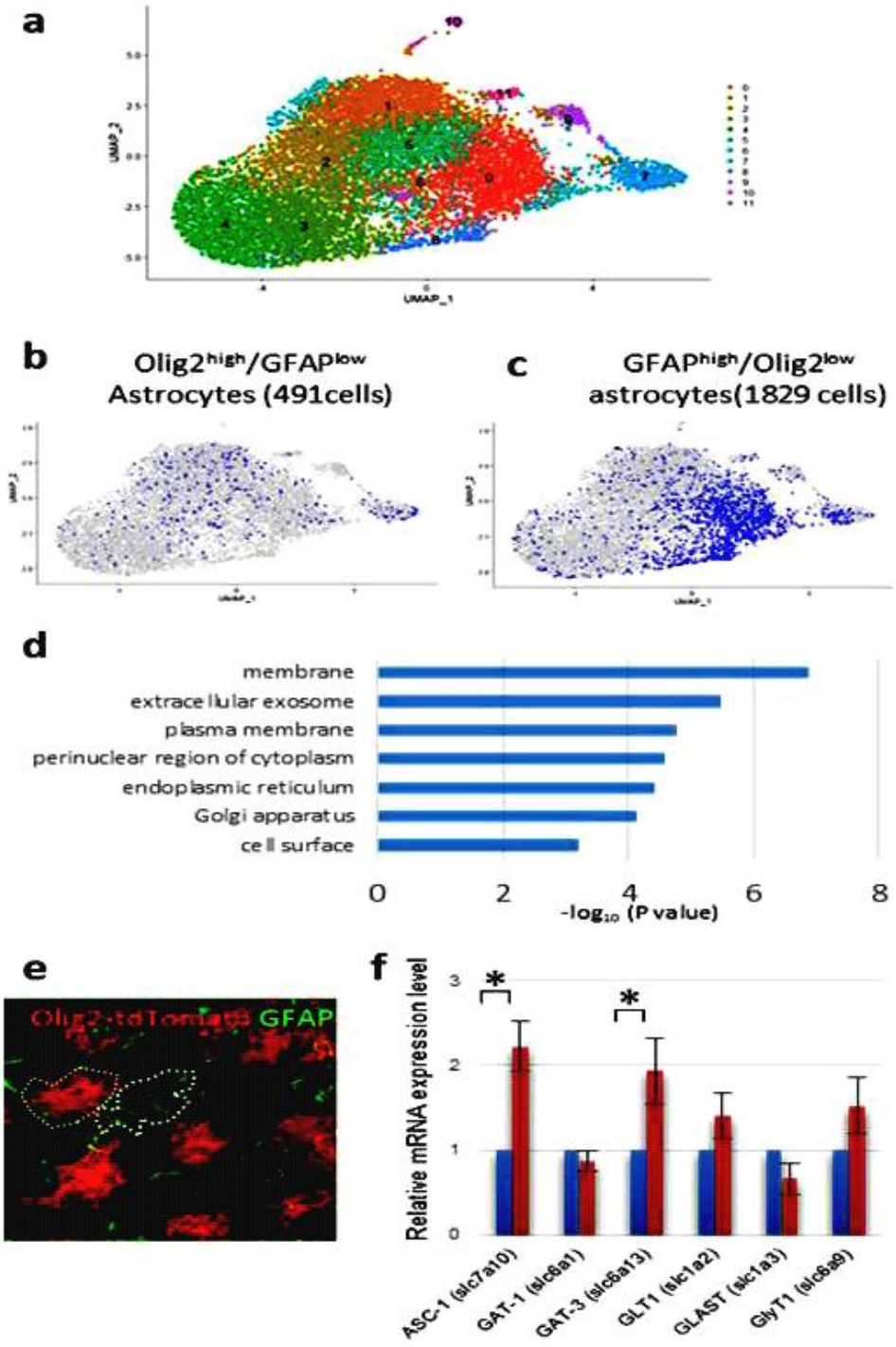

Fig. 4: Olig2-AS are enriched with GAT-3 (slc6a13) and ASC-1 (slc7a10) gene. **a:** The UMAP plot demonstrates that the astrocytes in the LGP were classified into twelve ASC clusters by unsupervised re-clustering. **b-c:** The distribution of Olig2^{high}/GFAP^{low}astrocytes (491 cells) (b) and GFAP^{high}/Olig2^{low} astrocytes (1,829 cells) (c) are depicted on UMAP plots. **d:** Gene Ontology (GO) analysis was performed on the Olig2^{high}/GFAP^{low} astrocytes gene set. The list displays the top seven GO terms obtained, ranked by p-value ($p<0.05$, adjusted p-value based on Bonferroni correction). **e:** Olig2-astrocytes (visualized by tdTomato red fluorescence) and GFAP-immunolabeled astrocytes are selectively dissected by laser microdissection. **f:** RT-qPCR analysis was conducted to determine mRNA expression levels of SLC membrane transporter genes. The expression of slc7a10 and slc6a13 mRNA was significantly higher in Olig2^{high}/GFAP^{low} astrocytes than in GFAP^{high}/Olig2^{low} astrocytes. Graphical data are presented as the mean ± SEM. A Student's t-test was used to compare mean values for unpaired data. Differences were considered significant when *$p < 0.01$.

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