Published in final edited form as:

Hippocampus. 2014 April ; 24(4): 383–395. doi:10.1002/hipo.22232.

Spatial Organization of NG2 Glial Cells and Astrocytes in Rat Hippocampal CA1 Region

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Abstract

Similar to astrocytes, NG2 glial cells are uniformly distributed in the central nervous system (CNS). However, little is known about the interspatial relationship, nor the functional interactions between these two star-shaped glial subtypes. Confocal morphometric analysis showed that NG2 immunostained cells are spatially organized as domains in rat hippocampal CA1 region and that each NG2 glial domain occupies a spatial volume of $\sim 178, 364 \mu\text{m}^3$. The processes of NG2 glia and astrocytes overlap extensively; each NG2 glial domain interlaces with the processes deriving from 5.8 ± 0.4 neighboring astrocytes, while each astrocytic domain accommodates processes stemming from 4.5 ± 0.3 abutting NG2 glia. In CA1 *stratum radiatum*, the cell bodies of morphologically identified glial cells often appear to make direct somatic-somata contact, termed as doublets. We used dual patch recording and post-recording NG2/GFAP double staining to determine the glial identities of these doublets. We show that among 44 doublets, 50% were NG2 glia-astrocyte pairs, while another 38.6% and 11.4% were astrocyte-astrocyte and NG2 glia-NG2 glia pairs, respectively. In dual patch recording, neither electrical coupling nor intercellular biocytin transfer was detected in astrocyte-NG2 glia or NG2 glia-NG2 glia doublets. Altogether, although NG2 glia and astrocytes are not gap junction coupled, their cell bodies and processes are interwoven extensively. The anatomical and physiological relationships revealed in this study should facilitate future studies to understand the metabolic coupling and functional communication between NG2 glia and astrocytes.

Keywords

NG2 glia; astrocyte; rat hippocampus; confocal microscopy; patch clamp

INTRODUCTION

NG2 (neuron-glia chondroitin sulphate proteoglycan 2) glial cells, also described as oligodendrocyte-type-2 astrocytes (O2A) (Raff et al., 1983), oligodendrocyte precursor cells (OPC) (Levine et al., 2001), “complex glia” (Schools et al., 2003; Steinhäuser et al., 1994; Zhou et al., 2000), or polydendrocytes (Nishiyama et al., 2002), have been recognized as the fourth member of glial cells in the mammalian CNS (central nervous system) (Peters, 2004), representing about 5–10% of the glial cell population in the developing and adult CNS

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(Trotter et al., 2010). While NG2 glial cells are morphologically similar to astrocytes, they do not express astrocytic marker GFAP and S100 β . Instead, they are so named because of their expression of chondroitin sulphate proteoglycan NG2 (Levine and Card, 1987; Stallcup, 1981), a membrane protein with a large extracellular domain and unknown function. NG2 glial cells are evenly distributed in both grey and white matter and proposed as the resident oligodendrocyte and astrocyte precursors; especially in the early developmental stages *in vivo* (Zhu et al., 2008a, b). NG2 glial cells remain populous in the adult brain after the completion of myelination (Levine et al., 2001) and are spatially interwoven with astrocytes (Wigley et al., 2007). Under various neuropathological states and after wound insults, NG2 glial cells proliferate to surround demyelination foci (Kang et al., 2010; Levine and Reynolds, 1999; McTigue et al., 2001). Physiologically, NG2 glial cells express ionotropic GABA_A and glutamate AMPA receptors and contact intimately with neurons to form conventional synapses with axons in the cerebellum, cortex and hippocampus (Bergles et al., 2000; Lin et al., 2005) and with non-myelinated axons in the corpus callosum (Kukley et al., 2007; Ziskin et al., 2007). These features indicate a complex role of NG2 glia in CNS function in contrast to the simplistic view of NG2 glia as precursor reservoirs for oligodendrocytes.

While NG2 glia can use the inwardly rectifying K⁺ channel Kir4.1 to sense change in extracellular K⁺ concentrations (Maldonado et al., 2013), it remains unclear whether this implies the involvement of NG2 glia in CNS K⁺ homeostasis, a role currently assigned to astrocytes. It is also unknown whether NG2 glia could functionally facilitate astrocytes in the roles of brain energy metabolism and neurotransmission (Gordon et al., 2007; Haydon, 2001; Kimelberg, 2010; Nedergaard et al., 2003; Rouach et al., 2008; Wang and Bordey, 2008).

NG2 glial cells in gray matter and cultured cerebellar slices are able to generate astrocytes (Dimou et al., 2008; Leoni et al., 2009; Zhu et al., 2008a, b). Functionally, ATP release from astrocytes initiates Ca²⁺ signals in NG2 glial cells in the optic nerve (Hamilton et al., 2010). Thus, investigating the lineage relationship and lineage interactions between NG2 glia and astrocytes are emerging to be new fronts of intensive research. To facilitate the research in this area, it is necessary to understand how NG2 glia and astrocytes are interspatially organized. We therefore examined the density and distribution pattern of NG2 glial cells as well as the spatial relationship between NG2 glia and astrocytes in rat hippocampal CA1 *striatum radiatum* by using confocal morphometric analysis and dual patch recording.

MATERIALS AND METHODS

Hippocampal slice preparation

Hippocampal slices were prepared from postnatal 21–28 day old Sprague-Dawley rats. The procedure was performed in accordance with a protocol approved by the Wadsworth Center, New York State Department of Health Institutional Animal Care and Use Committee. Rats were anesthetized with 100% CO₂ before decapitation and their brains were removed from the skull and placed in an ice-cold, oxygenated (5%CO₂/95%O₂, pH =7.4) slice preparation solution with the following contents (in mM): 26 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 10 MgCl₂, 10 glucose, 0.5 CaCl₂, and 240 sucrose. Final osmolality was 350 ± 2 mOsm. Coronal slices of 300 μ m thickness were cut with a Vibratome (Pelco 1500) and transferred to a nylon slice holder basket immersed in artificial cerebral spinal fluid (aCSF) with the following contents (in mM): 125 NaCl, 25 NaHCO₃, 10 glucose, 3.5 KCl, 1.25 NaH₂PO₄, 2.0 CaCl₂, 1 MgCl₂ (osmolality 295 ± 5 mOsm) at room temperature (20–22°C). The slices were kept in aCSF with continuous oxygenation for at least 60 minutes before recording.

Electrophysiology

Individual hippocampal slices were transferred to the recording chamber which was constantly perfused with oxygenated aCSF (2.0 ml/min). Astrocytes and NG2 glia located in the CA1 region were selected by infrared-differential interference contrast (IR-DIC) video microscopy (Olympus BX51W1) using a 40x water immersion objective and an IR-sensitive CCD camera to display the slice images on a monitor. Single and dual patch whole-cell currents were amplified by a MultiClamp 700A amplifier, sampled by a DIGIDADA 1322A Interface at 10–20 kHz, filtered at 1–2 kHz, and the data acquisition was controlled by PClamp 9.2 software (all from Molecular Devices., Sunnyvale, CA) installed on a Dell personal computer. The patch pipettes were fabricated from borosilicate capillaries (OD: 1.5 mm, Warner Instrument Corporation, Hamden, CT) using a Flaming/Brown Micropipette Puller (Model P-87, Sutter Instrument Co., Novato, CA). The patch pipettes had the resistance of 2.5–3.5 M Ω when filled with KCl-based pipette solution (see next section in the Methods). A minimum 2 G Ω seal resistance was required before rupturing the membrane for whole-cell recording. The membrane potential (V_M) was read in the “I=0” mode. Glial cells that showed a resting membrane potential that was more depolarized than –75 mV were not used. All the experiments were conducted at room temperature (20–22 °C).

The membrane capacitance (C_M), membrane resistance (R_M) and access resistances (R_a) were measured using the “Membrane test” protocol in the PClamp 9.2 program. In all the biophysical and pharmacological studies, the recordings were not used if the R_a was greater than 15 M Ω or varied greater than ± 5 M Ω .

Solutions and reagents

The composition of the standard aCSF used is given in the above section. The gap junction uncoupler meclofenamic acid (MFA, 100 μ M) was dissolved directly in aCSF without compensation for osmolality.

The standard electrode solution contained (in mM): 140 KCl, 0.5 Ca₂Cl, 1.0 MgCl₂, 5 EGTA, 10 HEPES, 3 Mg-ATP, and 0.3 Na-GTP (pH = 7.3, 290 $\pm$ 5 mOsm). The pH was adjusted to 7.25 with KOH. In some experiments, biocytin (0.1%), Alexa Fluor 568 (100 μ M) or Lucifer yellow CH (LY, 0.1%) was added to the electrode solution for the post-recording reconstruction of cell morphology and to identify the recorded cells for immunohistochemistry.

Intracellular tracer loading

Intracellular loading of Lucifer yellow (LY), Alexa Fluor 568 and biocytin was conducted as has been previously described by us and others (Karadottir and Attwell, 2006; Schools et al., 2006). In some experiments, LY and biocytin or LY and Alexa Fluor 568 containing electrode solutions were applied separately into the dual patch electrodes to determine the existence of cross-diffusion of dyes in the recording pairs, e.g., astrocyte-astrocyte, astrocyte-NG2 glia, NG2 glia-NG2 glia.

After patch recording, the slices were removed from the recording chamber and immediately fixed in phosphate-buffered 4% formaldehyde (pH 7.4) for one hour with gentle agitation at room temperature. The slices were washed in phosphate-buffered saline (PBS), and then stored at 4°C in PBS with 0.01% NaN₃ until histochemistry or immunohistochemistry was performed.

Histochemistry and immunohistochemistry procedures

Slices were permeabilized in 1% Triton X-100 in PBS for one hour. To visualize biocytin-filled cells, the slices were treated with 1:1,200 Cy2-streptavidin or 1:1,600 Cy3-streptavidin

(Jackson ImmunoResearch Labs) in PBS for 4 hours and subsequently washed in PBS. To block nonspecific binding of primary antibodies, the slices were incubated in 3% of normal goat serum (NGS) in PBS for four hours. Antibodies recognizing GFAP (mouse, Chemicon), GFAP (rabbit, Dako), and NG2 (rabbit, Chemicon) antigens were diluted in 3% NGS plus 0.1% Triton X-100 (NGS/TX). The slices were incubated for 18 hours at room temperature with gentle shaking. Slices were washed successively in NGS/TX, 0.1% Triton X-100 in PBS, and then in PBS. Cy3- and Cy5-conjugated secondary antibodies were selected based on the minimal emission wavelength overlap. NGS (3%) was used again to block nonspecific binding before incubation in the diluted secondary antibodies overnight. The specificities of all the secondary antibodies were tested with samples not exposed to primary antibodies, and no staining signals were seen. All steps were performed at room temperature and all incubations were done with gentle agitation in solutions containing 0.01% NaN_3 .

Confocal image acquisition and analysis

After histochemical and immunohistochemical procedures, the slices were transferred to a glass-bottom chamber containing PBS for confocal image acquisition. This was done with an inverted Carl Zeiss LSM510 META confocal microscope (Oberkochen, Germany) with $\times 25/0.8$ NA and $\times 63/1.4$ NA objectives. In an astrocytic syncytium disclosed by intracellular LY or biocytin loading, the patch electrode recorded cell always shows the highest fluorescent intensity, therefore can be readily distinguished from other cells in the network. The three-dimensional (3D) projections were made using Zeiss LSM Image Browser software. The lengths of processes were calculated from orthogonal section images with the function available in LSM510 software.

Estimation of NG2 glia density in CA1 *stratum radiatum*

Hippocampal slices were first immunolabeled with anti-NG2 antibody, then examined with an inverted Carl Zeiss LSM510 META confocal microscope under a $\times 25$ objective lens (NA 0.80) at zoom factor of 1.0. The scanned frame was set as $368.5\ \mu\text{m}$ for the x -axis, $368.5\ \mu\text{m}$ for the y -axis. The scanned depth in z -axis was in the range of $92.4\ \mu\text{m}$ – $107.2\ \mu\text{m}$. In each slice, a series of scanned optical stacks was obtained and the NG2 positive glial cells inside the stacks were carefully screened through by the Zeiss LSM Image Browser. The morphological criterion to differentiate NG2 glia from other NG2 positive cells, i.e. pericytes, lies in the existence of a stellate shape with numerous highly branched, thin and long processes extending radially from the cell body. As shown in Fig 2 C–E, although pericytes (arrows) are also positively stained by the anti-NG2 antibody, they encircle capillaries with broad, virtually continuous processes, and therefore can be readily differentiated from NG2 glia (Dore-Duffy and Cleary, 2011; Fröhlich et al., 2011; Hamilton et al., 2010; Wigley and Butt, 2009). Each optical stack image was printed out from a color-laser printer (HP LaserJet 200) for the following mapping work. To avoid double counting of the same NG2 glia, the counted NG2 glial cells were marked on the z -stack images printed on papers. The density of NG2 glia from each sample was then calculated from the following equation:

$$\text{NG2 glia density} = (\text{total number of NG2 glia}) / (\text{tissue volume}).$$

The tissue volume (μm^3) was calculated from $x \times y \times z$ (μm); these parameters have been defined above.

Data are presented as means $\pm$ SEM, unless indicated otherwise.

RESULTS

Morphological and electrophysiological characteristics of astrocytes and NG2 glial cells

To reveal the morphological complexity of individual astrocytes and NG2 glial cells in the CA1 *stratum radiatum*, biocytin was loaded into the recorded cell via patch electrode. To resolve the entire morphology of individual astrocytes, brain slices were pretreated with gap junction blocker 100 μ M MFA before biocytin loading. As shown in Fig. 1 A and C, several thick primary processes extend from the soma of each individual astrocyte, from which dense ramifications of fine processes emanate out to occupy a spatial domain (Fig. 1 A). NG2 glia also show a stellate morphology, but with relatively thin, long and highly branched processes that extend radially from cell body (Fig. 1 C).

Astrocytes and NG2 glia also differ in their electrophysiological phenotype. The whole-cell current profile of mature astrocytes is characterized by a linear current-voltage (I - V) relationship. The “passive” electrophysiological phenotype is due to the expression of leak K^+ conductance and extensive gap junctional coupling among astrocytes (Zhou et al., 2009) (Fig. 1 B). In contrast, NG2 glia exhibit small depolarization-induced voltage-gated inward Na^+ (I_{Na}) currents, outward transient K^+ current (I_{Ka}), and delayed rectifier K^+ current (I_{Kdr}). Combined expression of these voltage-gated ion conductance yields a strong outward rectification in the I - V relationship (Fig. 1 D). Based on this distinct whole-cell current profile, NG2 glia have been termed “complex” cells in the past (Schools et al., 2003; Steinhäuser et al., 1994; Zhou et al., 2000; Zhou and Kimelberg, 2000).

Spatial organization of NG2 glial cells in CA1 *stratum radiatum* region

Previous studies have shown that in the adult rat hippocampal CA1 region, astrocytes become repulsive in their spatial territories, and occupy domains such that their peripheral processes overlap less than 10% with neighboring astrocytes (Bushong et al., 2002; Ogata and Kosaka, 2002). To explore how NG2 glial cells are spatially organized in this brain region, biocytin was intracellularly loaded into individual NG2 glia to disclose the entire cell morphology and spatial occupancy. After biocytin loading, the slices were immunostained with NG2 antibody and then followed by confocal morphometric analysis of interspatial relationship among NG2 glia. A complete structure of an NG2 glial cell is shown in Fig. 2 B. The cell is characterized by a small cell soma with thin tortuous and ramified processes. Interestingly, NG2 glial cells are also spatially arranged as domains in this region (Fig. 2 A). Worth noting is that despite a close apposition between the biocytin loaded cell and other NG2 immunostained cells (arrowheads), tracer transfer was never observed among NG2 glia, which is consistent with the observations described in several previous reports (Bergles et al., 2000; Schools et al., 2006; Xu et al., 2010).

To explore the extent to which the NG2 glia domains overlap, the following experiments were carried out. First, we explored the density of NG2 glia in the CA1 *stratum radiatum* region. The density was quantified by counting the number of NG2 glial cells in each of five confocal z -stack images sets obtained from four rats. NG2 glia in all the samples appeared to be evenly distributed in this region with an estimated density of $(5.62 \pm 0.14) \times 10^3 / \text{mm}^3$ ($n=5$). According to this density, the average volume assigned to individual NG2 glia is $(178.36 \pm 4.3) \times 10^3 \mu\text{m}^3$. The radius for this assigned NG2 glia volume is $34.92 \mu\text{m}$ that is illustrated in the schematic drawing in Fig. 2 F marked as “ b ”.

We next sought to determine the actual spatial volume of NG2 glial domain by morphometric analysis of confocal z -stack projection images obtained from biocytin loaded NG2 glial cells. A representative NG2 glia resolved from this analysis is shown in the Fig. 2 B. From this analysis, the circle radius of each NG2 domain is $42.80 \pm 1.03 \mu\text{m}$ ($n = 71$)

("a" in Fig. 2 F), which was, as expected, greater than the radius that calculated from the density of NG2 glia noted above. This analysis also indicates an overlapping of peripheral processes among neighboring NG2 glial cells. The overlapping area between two neighboring NG2 glial cells is illustrated in Fig. 2 F in green color.

In the schematic drawing shown in Fig. 2 F, two interlaced NG2 glial cells are illustrated to show how the volume in which the processes of two neighboring NG2 domains are shared in their interfaces was calculated. The drawing assumed a constant spherical shape of NG2 glial domain. The inner spheres (purple circles, Fig. 2 F) represent the average cell volume that calculated from the density of NG2 glia, while the outer spheres (red circles, Fig. 2 F) represent the actual cell volume of NG2 glia. When the inner spheres of these NG2 glial cells come in contact with each other, the overlapping area of the outer spheres (green part in Fig. 2 F) was regarded as the shared cell volume. The overlapping portion between the two NG2 glial cells amounted to 4.77% of each outer sphere (the actual NG2 glial cell volume).

In summary, each NG2 glial domain shares around 5% of cell volume with other adjacent NG2 glial domain in their interwoven interface area. A caveat that should be noted in this calculation is that we have assumed a spherical shape for a NG2 glia. Although this is the closest assumption we could make, this assumption cannot simulate the actual shape of NG2 glia *in vivo* for a more precise volumetric analysis.

Organization of astrocyte and NG2 glial domains in CA1 *stratum radiatum* region

NG2 (Levine and Card, 1987; Stallcup, 1981) and GFAP (Nixdorf-Bergweiler et al., 1994; Schmidt-Kastner and Szymas, 1990) are known to be specific markers for NG2 glial cells and astrocytes, respectively. To determine the interspatial relationship between NG2 glial cells and astrocytes, we performed NG2/GFAP double immunostaining in hippocampal slices (Fig. 3 A–B). Similar to several previous observations, NG2 and GFAP staining was never found to be co-localized on the same glial cell, which is consistent with the notion that NG2 glia and astrocytes are distinct glial subpopulations. Also, NG2 glial cells and astrocytes showed a similar even distribution pattern throughout the hippocampal CA1 region.

It appeared that both NG2 glia and astrocytes defined their own domains, because the domains of the same glial type overlap only slightly in their interface areas, while the domains of different glial types overlap extensively (Fig. 3 A–B). It is also noticeable that, while the processes of both NG2 glia and astrocytes extend to blood vessels, only astrocytic processes form perivascular end-feet (Fig. 3 C).

As GFAP stained cytoskeleton intermediate filaments represent only about 15% of the total astrocytic volume (Bushong et al., 2002), we next sought to use biocytin intracellular loading and confocal scan images to reconstruct the entire structure of an individual astrocyte and to reveal the interspatial relationship between a single astrocyte with its neighboring NG2 glial cells. We have previously shown that inhibition of gap junctions allows better disclosure of the full structure of individual astrocyte with biocytin intracellular loading (Xu et al., 2010). Therefore, the brain slices were first pretreated with gap junction inhibitor 100 μ M MFA for one hour prior to biocytin loading via patch electrode. High resolution images obtained from the confocal microscopy showed an extensive interdigitation of the processes deriving from astrocytes and NG2 glia. In single plane image (Fig. 3 E) and orthogonal z-stack image projection in the same region (Fig. 3 e), the fine processes of NG2 (green) and astrocytes (red), characteristically shown as irregular shaped dots, were closely associated. Often, two glial cell bodies were intimately associated

as a doublet. Shown in Fig. 3 F–H is an NG2 glia-astrocyte doublet, where an NG2 glial domain and an astrocytic domain can be completely overlapped (Fig. 3 H).

Overall, morphometric analysis of confocal images from orthogonal view revealed an individual astrocyte interlace with 4.5 ± 0.3 NG2 glia ($n=5$) and each NG2 glia interact with 5.8 ± 0.4 astrocytes within its domain ($n=5$).

Electrical coupling only exists among astrocytes

We have recently studied the electrical coupling between CA1 astrocytes in rat hippocampus (Xu et al., 2010). To answer further whether electrical coupling occurs between different glial subtypes, dual patch recording was conducted *in situ*. To allow tracer transfer to be measured simultaneously in the same experiment, one of the following tracers, Alexa Fluor 568, biocytin or LY, was included in one of the electrode solutions for dual patch recording. The slices were stained post-recording to determine the existence of tracer coupling. As shown in the Fig. 4 A and B, electrical coupling and tracer coupling could always be detected from paired astrocyte recordings. To measure electrical coupling, voltage steps were delivered through one astrocyte at a time, while the membrane potential of the second astrocyte in the pair was held at -80 mV to monitor the transjunctional conductance. Change in holding currents, required to maintain the cell at -80 mV, equaled the amount of transjunctional currents (Xu et al., 2010).

In contrast, neither electrical nor tracer coupling could be detected from NG2 glia-NG2 glia or astrocyte-NG2 glia pairs (Fig. 4 C, E). Interestingly, in some recorded NG2 glia-NG2 glial pairs, while the two cell bodies attached closely to one another, their processes extended in the opposite directions (Fig. 4 C).

As for the spatial relationship between NG2 glia and astrocyte, a typical example is shown in the Fig. 4 E, where an astrocyte and an NG2 glia cell shared their spatial territories extensively. Additionally, NG2 glia stretched out its long and fine processes to make contact with the soma of its neighboring astrocyte. Likewise, the fine astrocytic processes also reached out to NG2 glia.

In summary, the domains of NG2 glia and astrocytes are interwoven extensively, though they are not electrically coupled through gap junction channels.

The identity of glial doublet in CA1 *stratum radiatum* region

A close somato-somatic apposition of glial cells has been reported previously (Bushong et al., 2002; Ge et al., 2009; Ogata and Kosaka, 2002; Ong and Levine, 1999; Peters et al., 1991; Wigley and Butt, 2009). In acute rat hippocampal slices, the somas of glial cells that make direct contact, termed as doublets, were encountered frequently in CA1 *stratum radiatum* region as shown in Fig. 5 B. In the present study, we refer the glial doublets to two glial somas that contact each other directly. The glial soma in brain slice can be morphologically recognized by its irregular shape and small size, typically less than $10 \mu\text{m}$ in diameter. We verified direct somatic-somata contact by viewing the pairs in *z*-axis under DIC.

To understand the glial identity of doublets, dual patch recording was applied to determine the existence of intercellular electrical coupling, and NG2/GFAP double staining was followed to correlate the recorded cells with NG2 glia or astrocytes. From 44 dual patch recordings, we found that the doublets comprised of the pairs of astrocyte-astrocyte, NG2 glia-NG2 glia or astrocyte-NG2 glia (Fig. 5). Quantitatively, 50% the doublets were NG2 glia-astrocyte pairs (22/44 pairs), and another 38.6% (17/44) and 11.4% (5/44) were astrocyte-astrocyte and NG2 glia-NG2 glia pairs, respectively (Fig. 6).

DISCUSSION

To address the interspatial relationship between NG2 glial cells and astrocytes, we examined the distribution pattern of NG2 glia, the spatial relationship between NG2 glia and astrocytes and potential electrical coupling between the same and different glial subtypes. We show that NG2 glial cells are like astrocytes in that they space out as distinct domains with little interdigitation in the interfaces of their domains. In contrast, NG2 glial and astrocytic domains are intimately associated. The anatomical relationship revealed in the present study should serve as an essential anatomic basis for the metabolic coupling and functional communication between these two glial subtypes.

Arrangement of NG2 glia in rat hippocampal CA1 *stratum radiatum*

To understand how NG2 glial cells are organized in the adult brain, we quantitatively examined the distribution pattern and density of NG2 glial cells in CA1 *stratum radiatum*. We show that the evenly distributed NG2 glial cells occupy exclusive territories in a tiled manner in this region. Between the two adjacent NG2 glial cells, the two domains overlap only in ~ 5% at their interface territories. A high density of NG2 glia is maintained through local NG2 glia proliferation (Hughes et al., 2013), possibly to maintain a high self-renewal capacity for generating oligodendrocytes under physiological conditions and for tissue repair under pathological states.

Spatial relationship between NG2 glia and astrocytes in CA1 *stratum radiatum*

We show that NG2 glia and astrocytes are intimately spatially associated in adult rat hippocampus. Specifically, the processes of NG2 glial cells overlap extensively with those processes stemming from astrocytes. On many occasions, these two glial subtypes may even make direct cell body to cell body contact, termed doublets, and under this condition the two cell domains appear to overlap completely (Fig. 3 F–H). Functionally, underlying such spatial organization should be an essential anatomic basis that subserves signaling communication between the two glial subtypes. For example, recent evidence indicates that astrocytes signal to NG2 glial cells via release of ATP and glutamate that evokes a Ca^{2+} rise in NG2 glia (Hamilton et al., 2009; Hamilton et al., 2008). This newly appreciated signaling pathway may modulate the activity of NG2 glia and the intimate contacts both on astrocyte-NG2 glia somas and along their processes where astrocyte-NG2 glia communication occurs (Hamilton et al., 2010). Moreover, the same study also showed NG2 glia expression of synaptophysin, indicating the ability of NG2 glia to release neurotransmitters and communicate bidirectionally with astrocytes in their overlapping domains.

A more recent study has revealed a highly dynamic nature of NG2 glia processes; the motile filopodia located at the tips of the advancing processes survey the local environment constantly (Hughes et al., 2013). It remains to be determined whether and how the intimately associated astrocytic processes can regulate the dynamic movement of the fine processes of NG2 glial cells via release of gliotransmitters, such as ATP and glutamate, and how the responses of NG2 glial cells to these astrocyte derived signals affect NG2 glial migration, proliferation, differentiation and death. NG2 glia can also release modulatory substances, such as brain derived neurotrophic factor (BDNF), to coordinate the activity of the neuron-glia network (Tanaka et al., 2009). Therefore, it is reasonable to infer that the interaction occurring in this type of the shared NG2 glia-astrocytes domain may be the strongest. It would be intriguing to know in the future to what extent within the zone of overlap the processes from adjacent NG2 glia and astrocytes overlap. A great deal of insight into this issue should direct future hypotheses regarding the nature of signals that regulate NG2 glia density and the molecules that regulate repulsive and attractive behaviors between the homotypic and heterotypic neighboring glial cells.

The nature of glial doublets in CA1 *stratum radiatum*

The presence of glial doublets in hippocampus has been described previously (Bushong et al., 2002; Ogata and Kosaka, 2002; Ong and Levine, 1999; Wigley and Butt, 2009). NG2 glial cells represent the majority of mitotically active cells in the brain (Dawson et al., 2003) with lineage potential to give rise to oligodendrocytes, astrocytes, and neurons (Nishiyama et al., 2009). Such a multiple lineage potential of NG2 glia led to the hypothesis of the existence of multiple cell types in glial doublets as the results of offspring of the post-dividing NG2 glial cells. While generation of oligodendrocytes becomes a general consensus for the role of NG2 glia, evidence in support of additional precursor potential of NG2 glia for astrocytes (Zhu et al., 2008a, b; Guo et al. 2009; Leoni et al., 2009), or of a restricted lineage potential as committed oligodendrocyte precursors in the postnatal life (Kang et al., 2010; Simon et al., 2011; Zhu et al., 2011; Hughes et al., 2013) is an issue to be resolved in the future.

The division and self-renewal of NG2 glial cells has been elegantly revealed by two *in vivo* time-lapse confocal microscopy studies (Ge et al., 2009; Hughes et al., 2013). A recent study has shown that another dividing cell pool in postnatal brain comprises of astrocytes; although, the amount of dividing astrocytes decreases considerably in the adult brain (Ge et al., 2012). In this study, the local generation of astrocytes has been shown as the major astrocyte source in postnatal cortex. Although we did not find evidence to link glial doublets to the mitotic NG2 glia or astrocyte division in the developing hippocampus, we could not fully rule out this possibility that glial doublets may still be the “snapshots” of those post-cell division moments either from NG2 glial cells or astrocytes.

In our study, we found 38.6% of glial doublets were astrocyte-astrocyte pairs in CA1 *stratum radiatum*. Therefore, it seems sensible to infer that these doublets may represent the very early stage of post cell division. We show that NG2 glia-astrocyte pairs comprise of the highest percentage of doublets (50%). A recent study reveals that the fate of NG2 glial cells is likely to be age dependent and that NG2 glial cells in the postnatal brain generate only NG2 glial cells or oligodendrocytes, whereas NG2 glial cells in the embryonic brain generate protoplasmic astrocytes in the gray matter in addition to oligodendrocytes and NG2 glial cells (Zhu et al. 2011). However, we have no direct evidence to suggest that asymmetric NG2 glia division is the underlying mechanism for a high percentage of NG2 glia-astrocyte doublets. Alternatively, such a unique spatial relationship may favor a hypothesis of extensive communications occurring between NG2 glia and astrocytes in the brain.

Acknowledgments

Grant sponsor: Chinese Scholarship Council; Grant number: 2007101798 (GX)

Grant sponsor: National Natural Science Foundation of China; Grant number: 81000491 (GX), 81030021 (WW)

Grant sponsor: National Science Foundation; Grant number: IOS0641828 (MZ)

Grant sponsor: National Institute of Neurological Disorders and Stroke; Grant number: RO1NS062784 (MZ)

The authors thank Dr. Gary P. Schools for assistance and discussion of confocal image analysis and Ms. Catherine Alford for critical reading and editing of the manuscript. Dr. Guangjin Xu is a recipient of a scholarship from the Chinese Scholarship Council and of a Travel Award from the journal of *Glia* for the 10th European meeting on Glial Cells in Health and Disease (2011).

References

- Bergles DE, Roberts JD, Somogyi P, Jahr CE. Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. *Nature*. 2000; 405:187–191. [PubMed: 10821275]
- Bushong EA, Martone ME, Jones YZ, Ellisman MH. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci*. 2002; 22:183–192. [PubMed: 11756501]
- Dawson MR, Polito A, Levine JM, Reynolds R. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. *Mol Cell Neurosci*. 2003; 24:476–488. [PubMed: 14572468]
- Dimou L, Simon C, Kirchhoff F, Takebayashi H, Gotz M. Progeny of Olig2-expressing progenitors in the gray and white matter of the adult mouse cerebral cortex. *J Neurosci*. 2008; 28:10434–10442. [PubMed: 18842903]
- Dore-Duffy P, Cleary K. Morphology and properties of pericytes. *Methods Mol Biol*. 2011; 686:49–68. [PubMed: 21082366]
- Fröhlich N, Nagy B, Hovhannisyan A, Kukley M. Fate of neuron-lia synapses during proliferation and differentiation of NG2 cells. *J Anat*. 2011; 219:18–32. [PubMed: 21592101]
- Ge WP, Miyawaki A, Gage FH, Jan YN, Jan LY. Local generation of glia is a major astrocyte source in postnatal cortex. *Nature*. 2012; 484:376–380. [PubMed: 22456708]
- Ge WP, Zhou W, Luo Q, Jan LY, Jan YN. Dividing glial cells maintain differentiated properties including complex morphology and functional synapses. *Proc Natl Acad Sci U S A*. 2009; 106:328–333. [PubMed: 19104058]
- Gordon GR, Mulligan SJ, MacVicar BA. Astrocyte control of the cerebrovasculature. *Glia*. 2007; 55:1214–1221. [PubMed: 17659528]
- Guo F, Ma J, McCauley E, Bannerman P, Pleasure D. Early postnatal proteolipid promoter-expressing progenitors produce multilineage cells in vivo. *J Neurosci*. 2009; 29:7256–7270. [PubMed: 19494148]
- Hamilton N, Hubbard PS, Butt AM. Effects of glutamate receptor activation on NG2-glia in the rat optic nerve. *J Anat*. 2009; 214:208–218. [PubMed: 19207982]
- Hamilton N, Vayro S, Kirchhoff F, Verkhratsky A, Robbins J, Gorecki DC, Butt AM. Mechanisms of ATP- and glutamate-mediated calcium signaling in white matter astrocytes. *Glia*. 2008; 56:734–749. [PubMed: 18293404]
- Hamilton N, Vayro S, Wigley R, Butt AM. Axons and astrocytes release ATP and glutamate to evoke calcium signals in NG2-glia. *Glia*. 2010; 58:66–79. [PubMed: 19533604]
- Haydon PG. GLIA: listening and talking to the synapse. *Nat Rev Neurosci*. 2001; 2:185–193. [PubMed: 11256079]
- Hughes EG, Kang SH, Fukaya M, Bergles DE. Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nat Neurosci*. 2013; 16:668–676. [PubMed: 23624515]
- Kang SH, Fukaya M, Yang JK, Rothstein JD, Bergles DE. NG2+ CNS glial progenitors remain committed to the oligodendrocyte lineage in postnatal life and following neurodegeneration. *Neuron*. 2010; 68:668–681. [PubMed: 21092857]
- Karadottir R, Attwell D. Combining patch-clamping of cells in brain slices with immunocytochemical labeling to define cell type and developmental stage. *Nat Protoc*. 2006; 1:1977–1986. [PubMed: 17487186]
- Kimelberg HK. Functions of mature mammalian astrocytes: a current view. *Neuroscientist*. 2010; 16:79–106. [PubMed: 20236950]
- Kukley M, Capetillo-Zarate E, Dietrich D. Vesicular glutamate release from axons in white matter. *Nat Neurosci*. 2007; 10:311–320. [PubMed: 17293860]
- Leoni G, Rattray M, Butt AM. NG2 cells differentiate into astrocytes in cerebellar slices. *Mol Cell Neurosci*. 2009; 42:208–218. [PubMed: 19616628]
- Levine JM, Card JP. Light and electron microscopic localization of a cell surface antigen (NG2) in the rat cerebellum: association with smooth protoplasmic astrocytes. *J Neurosci*. 1987; 7:2711–2720. [PubMed: 3305798]

- Levine JM, Reynolds R. Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. *Exp Neurol*. 1999; 160:333–347. [PubMed: 10619551]
- Levine JM, Reynolds R, Fawcett JW. The oligodendrocyte precursor cell in health and disease. *Trends Neurosci*. 2001; 24:39–47. [PubMed: 11163886]
- Lin SC, Huck JH, Roberts JD, Macklin WB, Somogyi P, Bergles DE. Climbing fiber innervation of NG2-expressing glia in the mammalian cerebellum. *Neuron*. 2005; 46:773–785. [PubMed: 15924863]
- Maldonado PP, Velez-Fort M, Levavasseur F, Angulo MC. Oligodendrocyte precursor cells are accurate sensors of local K^+ in mature gray matter. *J Neurosci*. 2013; 33:2432–2442. [PubMed: 23392672]
- McTigue DM, Wei P, Stokes BT. Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. *J Neurosci*. 2001; 21:3392–3400. [PubMed: 11331369]
- Nedergaard M, Ransom B, Goldman SA. New roles for astrocytes: redefining the functional architecture of the brain. *Trends Neurosci*. 2003; 26:523–530. [PubMed: 14522144]
- Nishiyama A, Watanabe M, Yang Z, Bu J. Identity, distribution, and development of polydendrocytes: NG2-expressing glial cells. *J Neurocytol*. 2002; 31:437–455. [PubMed: 14501215]
- Nishiyama A, Komitova M, Suzuki R, Zhu X. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nat Rev Neurosci*. 2009; 10:9–22. [PubMed: 19096367]
- Nixdorf-Bergweiler BE, Albrecht D, Heinemann U. Developmental changes in the number, size, and orientation of GFAP-positive cells in the CA1 region of rat hippocampus. *Glia*. 1994; 12:180–195. [PubMed: 7851987]
- Ogata K, Kosaka T. Structural and quantitative analysis of astrocytes in the mouse hippocampus. *Neuroscience*. 2002; 113:221–233. [PubMed: 12123700]
- Ong WY, Levine JM. A light and electron microscopic study of NG2 chondroitin sulfate proteoglycan-positive oligodendrocyte precursor cells in the normal and kainate-lesioned rat hippocampus. *Neuroscience*. 1999; 92:83–95. [PubMed: 10392832]
- Peters, A.; Palay, SL.; de Webster, HF. The fine structure of the nervous system. 3. New York: Oxford University Press; 1991. p. 295p
- Peters A. A fourth type of neuroglial cell in the adult central nervous system. *J Neurocytol*. 2004; 33:345–357. [PubMed: 15475689]
- Raff MC, Miller RH, Noble M. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature*. 1983; 303:390–396. [PubMed: 6304520]
- Rouach N, Koulakoff A, Abudara V, Willecke K, Giaume C. Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science*. 2008; 322:1551–1555. [PubMed: 19056987]
- Schmidt-Kastner R, Szymas J. Immunohistochemistry of glial fibrillary acidic protein, vimentin and S-100 protein for study of astrocytes in hippocampus of rat. *J Chem Neuroanat*. 1990; 3:179–192. [PubMed: 2363851]
- Schools GP, Zhou M, Kimelberg HK. Electrophysiologically “complex” glial cells freshly isolated from the hippocampus are immunopositive for the chondroitin sulfate proteoglycan NG2. *J Neurosci Res*. 2003; 73:765–777. [PubMed: 12949902]
- Schools GP, Zhou M, Kimelberg HK. Development of gap junctions in hippocampal astrocytes: evidence that whole cell electrophysiological phenotype is an intrinsic property of the individual cell. *J Neurophysiol*. 2006; 96:1383–1392. [PubMed: 16775204]
- Simon C, Gotz M, Dimou L. Progenitors in the adult cerebral cortex: cell cycle properties and regulation by physiological stimuli and injury. *Glia*. 2011; 59:869–881. [PubMed: 21446038]
- Stallcup WB. The NG2 antigen, a putative lineage marker: immunofluorescent localization in primary cultures of rat brain. *Dev Biol*. 1981; 83:154–165. [PubMed: 7016634]
- Steinhäuser C, Jabs R, Kettenmann H. Properties of GABA and glutamate responses in identified glial cells of the mouse hippocampal slice. *Hippocampus*. 1994; 4:19–35. [PubMed: 7914797]
- Tanaka Y, Tozuka Y, Takata T, Shimazu N, Matsumura N, Ohta A, Hisatsune T. Excitatory GABAergic activation of cortical dividing glial cells. *Cereb Cortex*. 2009; 19:2181–2195. [PubMed: 19131437]

- Trotter J, Karram K, Nishiyama A. NG2 cells: Properties, progeny and origin. *Brain Res Rev.* 2010; 63:72–82. [PubMed: 20043946]
- Wang DD, Bordey A. The astrocyte odyssey. *Prog Neurobiol.* 2008; 86:342–367. [PubMed: 18948166]
- Wigley R, Hamilton N, Nishiyama A, Kirchhoff F, Butt AM. Morphological and physiological interactions of NG2-glia with astrocytes and neurons. *Anat J.* 2007; 210:661–670.
- Wigley R, Butt AM. Integration of NG2-glia (synantocytes) into the neuroglial network. *Neuron Glia Biol.* 2009; 5:21–28. [PubMed: 19785922]
- Xu G, Wang W, Kimelberg HK, Zhou M. Electrical coupling of astrocytes in rat hippocampal slices under physiological and simulated ischemic conditions. *Glia.* 2010; 58:481–493. [PubMed: 19795502]
- Zhou M, Kimelberg HK. Freshly isolated astrocytes from rat hippocampus show two distinct current patterns and different $[K^+]_o$ uptake capabilities. *J Neurophysiol.* 2000; 84:2746–2757. [PubMed: 11110805]
- Zhou M, Schools GP, Kimelberg HK. GFAP mRNA positive glia acutely isolated from rat hippocampus predominantly show complex current patterns. *Brain Res Mol Brain Res.* 2000; 76:121–131. [PubMed: 10719222]
- Zhou M, Xu G, Xie M, Zhang X, Schools GP, Ma L, Kimelberg HK, Chen H. TWIK-1 and TREK-1 are potassium channels contributing significantly to astrocyte passive conductance in rat hippocampal slices. *J Neurosci.* 2009; 29:8551–8564. [PubMed: 19571146]
- Zhu X, Bergles DE, Nishiyama A. NG2 cells generate both oligodendrocytes and gray matter astrocytes. *Development.* 2008a; 135:145–157. [PubMed: 18045844]
- Zhu X, Hill RA, Dietrich D, Komitova M, Suzuki R, Nishiyama A. Age-dependent fate and lineage restriction of single NG2 cells. *Development.* 2011; 138:745–753. [PubMed: 21266410]
- Zhu X, Hill RA, Nishiyama A. NG2 cells generate oligodendrocytes and gray matter astrocytes in the spinal cord. *Neuron Glia Biol.* 2008b; 4:19–26. [PubMed: 19006598]
- Ziskin JL, Nishiyama A, Rubio M, Fukaya M, Bergles DE. Vesicular release of glutamate from unmyelinated axons in white matter. *Nat Neurosci.* 2007; 10:321–330. [PubMed: 17293857]

Figure 1. Morphology and electrophysiology of astrocytes and NG2 glia in rat hippocampal CA1 region

Morphology of an astrocyte (A) and an NG2 glia (C) revealed by confocal scan images from intracellular biocytin loading via recording electrode. In order to disclose the entire structure of the individual astrocyte, brain slices were pretreated with the gap junction blocker, 100 μ M MFA. (A) Confocal projection image of an astrocyte; the cell characteristically shows several thick primary processes and dense ramifications extended from cell soma.

Comparatively, NG2 glial cell also shows a stellate morphology, but with thin, long, and highly branched processes extending outwards radially from the soma (C). The electrophysiological phenotypes in (B) and (D) were measured from the astrocyte and NG2 glia shown in (A) and (C), respectively. The electrophysiological phenotype of an astrocyte is characterized by a linear current-voltage (I - V) relationship "passive" current profile (B). In contrast, NG2 glia exhibit small depolarization-induced voltage-gated inward Na^+ (I_{Na}) currents, outward transient K^+ (I_{Ka}), and delayed rectifier K^+ (I_{Kdr}) channel currents. Combined expression of these voltage-gated ion conductance yields a strong outward rectification in the I - V relationship (D). The low density Na^+ channel currents in recording (D) were disclosed after subtraction of leak and capacitive currents and presented in expanded scale below D. Voltage steps were in the range of -180 to $+20$ mV at 20 mV increments and a duration of 50 ms. Scale bars represent 10 μ m (A) and 20 μ m (C).

Figure 2. Spatial arrangement of NG2 glia in CA1 stratum radiatum region

(A) The spatial relationship of an NG2 glial domain and its neighboring NG2 glia. The biocytin filled NG2 glia is shown in white and the boundary of the cell domain is marked by a yellow line. In the vicinity of the NG2 domain, eight neighboring NG2 cells are marked by red arrowheads and their processes are tangled with each other. Note that no tracer coupling was observed among the neighboring NG2 cells. (B) The morphology of a biocytin-filled NG2 glial cell was visualized in high resolution. Subsequent confocal analysis and 2D maximum projection shows the extensive arborization of their processes. Note that numerous nodules appeared along the fine processes. (C–E) Pericytes are morphologically distinct NG2 immunoreactively positive cells compared to NG2 glia. Both NG2 glia and pericytes are positively stained for anti-NG2 antibody (red). However, pericytes show a distinct perivascular location and a relatively large soma directly apposed to the blood vessels (arrows), whereas NG2-glia are stellate shape cells with numerous processes that are

always away from the blood vessels (asterisks). **(F)** Schematic drawing summarizes the spatial volume and domain overlapping of NG2 glia in CA1 *stratum radiatum*. The concentric circles represent two territories of an NG2 glia revealed by morphometric analysis: the radius “*a*”, 42.80 μm , was revealed by biocytin image that yields a full spatial volume of 328,246 μm^3 . Radius “*b*”, 34.92 μm , was calculated from the volume density of NG2 glia in this region, $5.62 \times 10^3/\text{mm}^3$, which corresponds to an average spatial volume of 178,364 μm^3 . The overlapping portion between the two NG2 glia cells (green part in Fig. 2 F) amounted to 4.77% of each outer sphere. The scale bars represent 20 μm in A and B, and 50 μm in C, D, E.

Figure 3. Organization of astrocyte and NG2 glia domains in rat hippocampal slices
(A) Confocal projection image of NG2/GFAP double immunofluorescence reveals that NG2 glia and astrocytes are arranged in overlapping domains in the CA1 *stratum radiatum* region. **(B)** A high resolution projection image reveals more details of spatial organization of NG2 glia and astrocytes. **(C)** An astrocyte (green) and an NG2 cell (red) both extended processes to a blood vessel, but only the astrocyte makes perivascular contacts (arrowheads). Also note that the processes from the NG2 glia and astrocyte are intimately interlaced. **(D)** A confocal projection image shows the morphological complexity of an astrocyte and its spatial relationship with other neighboring NG2 glia. **(E and e)** Single plane **(E)** and orthogonal projections images **(e1 and e2)** revealed from the same region shown in **(D)**; images **e1** and **e2** show the fine processes from an NG2 glia (green) and an astrocyte (red) that are interwoven considerably **(F–H)** A single plane high power optical section shows a direct soma-soma contact between an NG2 glial cell (red) and an astrocyte (green). An astrocyte was identified by whole-cell passive current profile (not shown) and the diffusion of biocytin from this recorded cell to its coupled syncytium **(G)**. The scale bars represent 50 μm in **A**, 10 μm in **C** and 20 μm in **B** and **D–H**.

Figure 4. Analysis of electrical coupling between astrocyte-astrocyte, NG2-NG2 glia and NG2 glia-astrocyte recording pairs

(A) Confocal projection image shows an astrocytic syncytium; LY (green) and biocytin (red) were loaded separately via two recording electrodes into two neighboring astrocytes. The cross diffusion of LY and biocytin yielded yellow color in some of the coupled astrocytes in the syncytium (white arrows). The astrocytic identity of the recorded cells and the associated syncytium was confirmed by colocalization of LY or biocytin with anti-GFAP immunoreactivity (blue). Note that both LY and biocytin diffused to the same blood vessel (arrowheads). The dual patch recordings from this astrocyte-astrocyte pair are shown in (B). Voltage steps were delivered to one of the astrocytes in the pair at a time, while the membrane potential of the second cell was constantly held at -80 mV to monitor the transjunctional conductance. Change in holding currents, required to maintain the cell at -80 mV, equal to the amount of transjunctional currents. Whole-cell voltage steps were sequentially delivered to the two cells, respectively. The voltage command steps were 50 ms in duration and -180 to $+20$ mV in voltage range with 20 mV increments. Tracer and electrical coupling could be detected in paired astrocyte-astrocyte recordings. (C) Confocal projection image of CA1 *stratum radiatum* containing a recorded NG2-NG2 glia pair. The somas of the two recorded NG2 cells were directly in contact. Two NG2 glial cells were separately filled with LY (green) and the Alexa Fluor 568 (red), and both cells were confirmed to be NG2 glia based on positive NG2 staining (blue). Dual patch recordings from this NG2 pair are shown in (D). Neither tracer nor electrical coupling could be detected in this NG2 glia pair. (E) Confocal projection image contains a recorded NG2-astrocyte pair; astrocyte was filled with biocytin (red) and NG2 glia was filled with LY (blue) positively stained for NG2 (green). (F) Dual patch recording detected no electrical coupling from this NG2-astrocyte pair. The voltage pulses in (D) and (F) were 200 ms long from -180 to $+20$ mV in 20 mV increments. The scale bars represent $10\ \mu\text{m}$ in C and $20\ \mu\text{m}$ in A and E.

Figure 5. Direct soma-soma contact between NG2-NG2 glia, NG2-astrocyte and astrocyte-astrocyte

(A) Single plane confocal image of Cy5-fluorescence, showing a pair of NG2 glia (blue) in the CA1 *stratum radiatum*. Noticeably, the somas of two neighboring NG2 glia are directly in contact, giving an appearance of twins (P22 rat). (B) The same NG2 glia pair is shown in DIC. The overlay of NG2 staining (A) and DIC (B) is shown in (C). (D–F) z-stack projection images of triple immunofluorescence for NG2 (green in E), GFAP and biocytin (D), and merged (F). Biocytin (red) loading via recording electrode disclosed the somas of the coupled astrocytes and GFAP (blue) revealed the skeleton of astrocytes (D). In merged image of triple staining for NG2, GFAP, and biocytin (F), the arrowhead indicates the soma-soma contact between an NG2 glia (green) and an astrocyte (red/blue). (G) Analysis of a z-stack sections in high resolution (G) and orthogonal projections in x- (g2) and y- (g1) planes of the region confirm the direct soma contact between the neighboring NG2 glia and astrocyte. (H) A z-stack projection image shows an astrocytic syncytium; intracellularly loaded biocytin is shown in green, and GFAP staining is shown in red. Note that all biocytin stained cells were also GFAP+. The arrowhead points to the somas of a pair of coupled astrocytes that are closely in contact. (I) Single z-plane image in high power shows the somas of two apposed astrocytes in detail and the orthogonal projections in x-(i2) and y-(i1) planes of this region indicates a direct contact of these cells. The scale bars represent 20 μm in A–C, G and I, and 50 μm in D–F and H.

Figure 6. Summary of glial doublets in hippocampal CA1 region

Dual patch recording and GFAP/NG2 post-recording double staining were performed to determine the nature of these unique glial pairs. Among the 44 studied doublets, 50% were NG2-astrocyte pairs (22/44 pairs), and another 38.6% (17/44) and 11.4% (5/44) were astrocyte-astrocyte and NG2-NG2 glia pairs, respectively.