TOPICAL REVIEW

Synapses on NG2-expressing progenitors in the brain: multiple functions?

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Progenitor cells expressing the proteoglycan NG2 represent approximately 5% of the total cells in the adult brain, and are found both in grey and white matter regions where they give rise to oligodendrocytes. The finding that these cells receive synaptic contacts from excitatory and inhibitory neurons has not only raised major interest in the possible roles of these synapses, but also stimulated further research on the developmental and cellular functions of NG2-expressing (NG2$^+$) progenitors themselves in the context of neural circuit physiology. Here we review recent findings on the functional properties of the synapses on NG2$^+$ cells in grey and white matter regions of the brain. In this review article we make an attempt to integrate current knowledge on the cellular and developmental properties of NG2$^+$ progenitors with the functional attributes of their synapses, in order to understand the physiological relevance of neuron–NG2$^+$ progenitor signal transmission. We propose that, although NG2$^+$ progenitors receive synaptic contact in all brain regions where they are found, their synapses might have different developmental and functional roles, probably reflecting the distinct functions of NG2$^+$ progenitors in the brain.

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The adult brain contains a significant reservoir of progenitor cells that can generate both neurons and glia (Emsley *et al.* 2005; Duan *et al.* 2008). Since its first identification (Stallcup & Beasley, 1987; Nishiyama *et al.* 1999), the progenitor population expressing the proteoglycan NG2 (referred here as NG2$^+$ progenitors or NG2$^+$ cells) has been extensively studied based on the following important attributes: (i) NG2$^+$ cells constitute a significant percentage (5%) of the total cells in the mammalian brain (Dawson *et al.* 2003); (ii) these cells are found as proliferative cells in the developing postnatal and in the adult brain, both in grey and white matter regions (Nishiyama *et al.* 2002; Dawson *et al.* 2003; Aguirre *et al.* 2004*a*; Ligon *et al.* 2006); (iii) NG2$^+$ cells generate white and grey matter oligodendrocytes of the brain and are therefore sometimes referred to as OPCs (Levine & Stallcup, 1987; Levine *et al.* 2001; Polito & Reynolds, 2005; Zhu *et al.* 2008); (iv) NG2$^+$ cells are often anatomically associated to neurons and receive excitatory and inhibitory synaptic contacts (Bergles *et al.* 2000; Lin & Bergles, 2004; Dayer *et al.* 2005; Karadottir *et al.* 2008; Mangin *et al.* 2008); and (v) NG2$^+$ cells are able to generate astrocytic and neuronal progenies under normal or pathological conditions (Belachew *et al.* 2003; Aguirre *et al.* 2007; Tamura *et al.* 2007; Zhu *et al.* 2008). These findings have understandably generated significant interest, particularly for the potential of endogenous pools of NG2$^+$ cells to be targeted in cell regeneration and cell repair therapies.

There is now extensive evidence that most if not all NG2$^+$ progenitors are contacted by functional synapses in the postnatal brain, both in grey and white matter areas. However, the cellular and physiological functions of these synaptic inputs are still unknown. Here, we review recent developments and different hypotheses that have been proposed since the initial discovery of direct synaptic transmission on NG2$^+$ cells by Bergles *et al.* (2000). Several review articles have been recently published on this topic (Lin & Bergles, 2002, 2004; Paukert & Bergles, 2006), and therefore rather than attempting a comprehensive review of the literature, we will focus the present article on specific questions that are currently pending on the possible function of synaptic transmission on NG2$^+$ progenitors in the brain. The recent work on the physiology of these neuron–NG2$^+$ progenitor synapses demonstrates that the existing dogma of the exclusivity of synapses between

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neurons needs to be revised, and that synapses between neurons and undifferentiated progenitor cells are likely to mediate a different type of information processing that is distinct from that occurring between neurons. We extensively discuss this notion and speculate on the specific functions that neuron–NG2$^+$ cell synapses might have in distinct brain regions.

Properties of neuron–NG2$^+$ cells synapses

It has been known for some time that various types of glial cells, including oligodendrocyte progenitor cells (OPCs) in white matter, express glutamate binding proteins and generate electrical currents in response to glutamate application, or in response to neuronal activity (e.g. Kriegler & Chiu, 1993; Mennerick & Zorumski, 1994; Bergles & Jahr, 1997). However, a conclusion common to all these studies is that, while glial cells are able to detect transmitter molecules that have accumulated in the extracellular space, quantal synaptic transmission is an exclusive property of neuronal communication. Therefore, the surprising finding that NG2$^+$ cells are able to discern single quanta of neurotransmitter released from individual neurons challenged one of the fundamental dogmas of synaptic transmission in the brain (Bergles *et al.* 2000; Lin & Bergles, 2004).

Quantal transmission on NG2$^+$ cells

Bergles and colleagues patch-clamped NG2-expressing glial cells in the hippocampal CA1 region, and recorded currents upon stimulation of CA3 pyramidal cells or local interneurons which were kinetically and pharmacologically indistinguishable from EPSCs and IPSCs typically recorded in neighbouring CA1 pyramidal neurons under identical conditions. Figure 1 shows a 3D reconstruction of an NG2-expressing cell from CA1 stratum radiatum. Consistent with the idea that currents on NG2$^+$ cells were due to synaptic events, it was demonstrated that they were due to action potential-mediated and calcium-dependent vesicular release of glutamate or GABA, and were not caused by spill-over from neighbouring synapses (Bergles *et al.* 2000; Lin & Bergles, 2004).

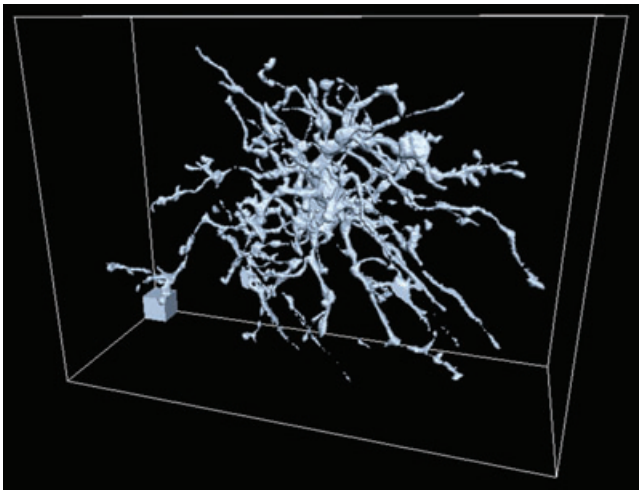

Figure 1. Three-dimensional reconstruction of a dye filled hippocampal NG2$^+$ cell from CA1 stratum radiatum
Note the parallel orientation of some processes in the direction of the Schaffer collaterals. The polygonal soma of the cell is hidden in the background. Isosurface rendering from a stack of confocal laser scans. Outer box, $30 \times 60 \times 80$ μm^3. Cube, $5 \times 5 \times 5$ μm^3.

The mechanism of transmitter release onto NG2$^+$ cells has been recently analysed in more detail and it was shown to consist of dedicated molecular machinery similar to that previously defined for classical synaptic transmitter release from neurons onto other neurons (Kukley *et al.* 2007), including (i) microdomain calcium signalling, (ii) calcium-dependent, cooperative and highly synchronous fusion events, (iii) release probability comparable to that observed in interneuronal communication, and (iv) a defined pool of readily releasable vesicles and a high rate of vesicle recycling allowing stable signalling even during enduring trains of action potential activity. Based on this mechanistic similarity to neuronal EPSCs and IPSCs, in the present review we will call these currents in NG2$^+$ cells 'synaptic'; however, this shall not imply a complete equivalence of the underlying ultra-structure or of the physiological role (see below).

Synaptic currents in NG2$^+$ cells have been described by several groups and in different brain regions, including cerebral cortex (Chittajallu *et al.* 2004; Kukley *et al.* 2008), cerebellar molecular layer (Lin *et al.* 2005), hippocampus (Bergles *et al.* 2000; Lin & Bergles, 2004; Ge *et al.* 2006; Kukley *et al.* 2008; Mangin *et al.* 2008) and corpus callosum (Kukley *et al.* 2007; Ziskin *et al.* 2007). What could be the physiological significance of detecting vesicular neurotransmitter release on NG2$^+$ cells, as compared to the activation of membrane receptors by ambient, extracellular neurotransmitter? A first advantage is that quantal transmission enables a given postsynaptic NG2$^+$ cell to determine the origin of this activity with excellent spatial resolution. A second advantage is the high degree of temporal resolution enabled by the high synchronicity of vesicle release with action potentials, and the rapid decay of synaptic currents. This allows NG2$^+$ cells to potentially discriminate frequencies of neuronal activity of up to several hundred hertz. In contrast, an elevation of the ambient transmitter concentration could equally be caused by high frequency firing of a population of remote neurons, or by low frequency firing of a few neighbouring cells.

The existence of quantal synaptic currents in NG2$^+$ progenitors in grey matter regions could be viewed as

misguided target selection during synaptogenesis, as the density of synapses in grey matter is generally very high. However, in white matter areas, the formation of specific synapses on $NG2^+$ cells from axon collaterals occurs in the absence of other synaptic targets, i.e. in brain regions devoid of neurons (Ziskin *et al.* 2007; Kukley *et al.* 2007; Karadottir *et al.* 2008). As white matter areas such as the optic nerve only consist of glial cells, axons and blood vessels, these findings raise the possibility that synaptic currents in $NG2^+$ cells represent an integral part of widespread signalling occurring between axons and $NG2^+$ progenitors. This view is also supported by *in vitro* findings demonstrating that, in coculture of hippocampal neurons with $NG2^+$ cells and oligodendrocytes, growing axons are targeting $NG2^+$ cells, while they avoid mature oligodendrocytes (Yang *et al.* 2006).

Different neurotransmitters are synaptically released onto $NG2^+$ cells

Experimental evidence accumulated so far demonstrates that the two major neurotransmitters in the brain, glutamate and GABA, mediate synaptic transmission onto $NG2^+$ cells. In some white matter areas, such as optic nerve and corpus callosum – which primarily contain glutamatergic fibres – $NG2^+$ progenitors display only glutamatergic currents (Kukley *et al.* 2007; Ziskin *et al.* 2007). Conversely, in cerebellar white matter $NG2^+$ cells display both glutamatergic and GABAergic currents, the latter most likely arising from Purkinje cell axons (Karadottir *et al.* 2008). Gray matter $NG2^+$ cells display both glutamatergic and GABAergic synaptic currents, consistent with the presence of axons of both excitatory principal neurons and inhibitory interneurons (Lin & Bergles, 2004; Kukley *et al.* 2008). The finding that two transmitter signalling systems converge onto individual $NG2^+$ progenitors in areas where they are both present (Lin & Bergles, 2004; Karadottir *et al.* 2008; Kukley *et al.* 2008) raises the question of how GABAergic and glutamatergic signals are integrated in individual $NG2^+$ progenitors and how these cells can discriminate between them.

Putative mechanisms of signal transduction in $NG2^+$ cells

By using perforated patch recordings on $NG2^+$ cells, Lin & Bergles (2004) showed that these cells do not possess the low intracellular chloride concentration that is typically observed in neurons and that, consistent with this observation, GABAergic currents in $NG2^+$ cells reverse at around –40 mV (Lin & Bergles, 2004). As $NG2^+$ cells display relatively negative resting potentials (approximately –80 mV) both glutamatergic and GABAergic synaptic inputs depolarize their membrane. Notably, $NG2^+$ cells can have a high input resistance in the range of several gigaohms, implying that even small synaptic inputs can cause strong depolarization of their membrane (Lin & Bergles, 2004). The consequences of this depolarization are currently unclear, although it could cause opening of voltage gated calcium channels present in $NG2^+$ cells (Berger *et al.* 1992), or changes in DNA synthesis, as reported for neuronal progenitors (LoTurco *et al.* 1995).

It has been recently reported that a subpopulation of $NG2^+$ progenitors in the rat white matter expresses a higher density of voltage-dependent Na^+ channels and can generate mature and fully developed action potentials (Karadottir *et al.* 2008). Immature action potentials were also observed in a subpopulation of cortical $NG2^+$ cells (Chittajallu *et al.* 2004). Therefore, it seems conceivable that membrane depolarization could activate Na^+ channels to generate action potentials in these $NG2^+$ cells. However, independent studies in mouse white and grey matter regions showed that $NG2^+$ progenitors do not spike, and express sodium currents which are consistently smaller than potassium currents (Berger *et al.* 1992; Bergles *et al.* 2000; Chittajallu *et al.* 2004; Ge *et al.* 2006; Ziskin *et al.* 2007; Kukley *et al.* 2007, 2008). Future comparative studies performed under the same recording conditions will further define whether subpopulations of $NG2^+$ progenitors can be identified based on these functional properties and will address this discrepancy.

As an alternative mechanism, the synaptic input may also induce a chemical rather than a solely electrical signal. The charge transferred by a glutamatergic or GABAergic quantal current is expected to produce tens of millimolar ion concentration changes in short segments of the thin ($\sim$200 nm) processes of $NG2^+$ cells. Such ion concentration changes may modulate sodium- or chloride-dependent processes, e.g. lead to intracellular Ca^{2+} accumulation via the Na^+/Ca^{2+} exchanger (Blaustein & Lederer, 1999), and would allow the cell to discriminate the type of transmitter released.

Finally, it has been suggested that synaptic release of glutamate might induce Ca^{2+} elevation in $NG2^+$ cells via Ca^{2+} permeable AMPARs (Bergles *et al.* 2000; Lin *et al.* 2005; Ge *et al.* 2006; Mangin *et al.* 2008). Therefore, glutamatergic synapses could influence $NG2^+$ cell physiology via Ca^{2+}-dependent intracellular signalling pathways. Additionally, synaptic release of transmitter could be transduced by metabotropic receptors on $NG2^+$ progenitors, as suggested by evidence that $NG2^+$ cells express mGluR3 and mGluR5a metabotropic glutamate receptors (Luyt *et al.* 2003). Whether these receptors are activated by synaptically released glutamate still remains to be established.

How widespread are neuron–NG2$^+$ cell synapses?

A crucial question is whether synaptic currents have been detected only in some NG2$^+$ progenitors owing to the use of electrophysiological methods that allow the measurement of very small currents, or whether synaptic inputs are a common feature of the entire NG2$^+$ cell population. This is an important question, as not all NG2$^+$ cells differentiate into oligodendrocytes; many persist in an undifferentiated state throughout life (Dawson *et al.* 2003; Reynolds & Hardy, 1997) and some differentiate into astrocytes or neurons (Zhu *et al.* 2008; Aguirre & Gallo, 2004; Belachew *et al.* 2003; Aguirre *et al.* 2004*a*). Therefore, it is important to define whether synaptic innervations may be restricted to a subpopulation of NG2$^+$ progenitors.

Directly addressing this question has been challenging, since synaptic currents in NG2$^+$ progenitors are generally small, due to tiny quantal amplitudes (Fig. 2) and a smaller number of input release sites per cell (although the surface density may be similar to that found in neurons). Therefore, spontaneously occurring synaptic currents are usually only detectable in a fraction of NG2$^+$ cells in which they appear at high frequency and display large amplitudes. However, where the frequency of spontaneous vesicles fusion is increased by a secretagogue (e.g. ruthenium red), and where ion concentrations and recording conditions are optimized, quantal glutamatergic and GABAergic currents have been demonstrated in practically all NG2$^+$ cells tested in grey and white matter regions (Lin *et al.* 2005; Kukley *et al.* 2007, 2008; Ziskin *et al.* 2007). Bearing in mind that the vast majority of oligodendrocytes are derived from NG2$^+$ cells (Zhu *et al.* 2008), this implies not only that synaptic currents are a consistent feature of NG2$^+$ cells, but also that most myelinating oligodendrocytes go through a stage of synaptic innervation. Even though most NG2$^+$ cells appear to receive at least one functional synaptic input, it is clear that synaptic density and efficacy vary between different developmental stages of NG2$^+$ cells (Ziskin *et al.* 2007; Kukley *et al.* 2008). It is at present unknown whether mature oligodendrocytes retain functional synapses, although it has been demonstrated that functional NMDA receptor channels are expressed in these cells (Karadottir *et al.* 2005; Salter & Fern, 2005; Micu *et al.* 2006). Therefore, it will be important to determine under which conditions glutamate activates NMDA receptors in mature oligodendrocytes.

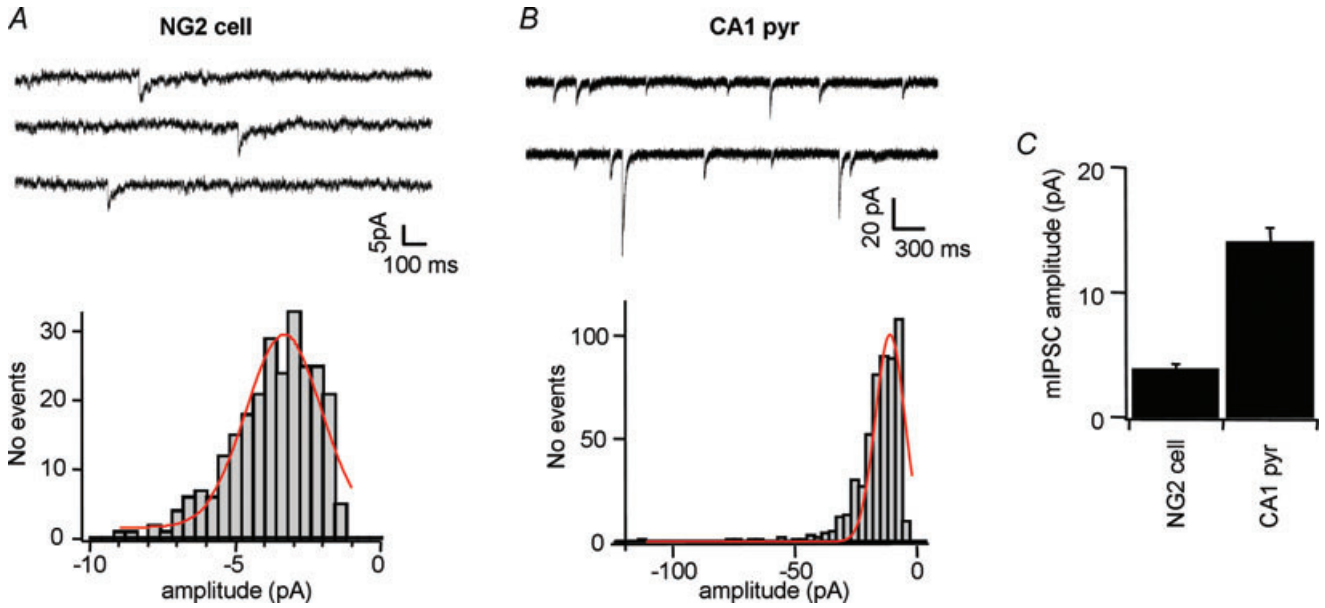

Figure 2. Quantal amplitude of GABAergic synaptic currents in NG2$^+$ cells is small when compared to neurons

A, top panel, whole-cell recording of an NG2$^+$ cell in the CA1 region of a murine hippocampal brain slice at postnatal day 10. TTX and glutamate receptor antagonists were present. Chloride reversal potential is –35 mV, and the cell was voltage clamped at –80 mV. Note the small amplitude and low frequency of unprovoked spontaneous synaptic currents. Bottom panel. Pooled histograms (from 4 NG2$^+$ cells) of peak current amplitudes. Most currents are smaller than 5 pA and it is likely that the smallest currents were lost in the recording noise. *B*, identical conditions as in *A*, but a whole-cell recording of a CA1 pyramidal cell is shown. Notice that the current scaling is different and that miniature synaptic currents are much more frequent and larger in amplitude than in NG2$^+$ cells. Histogram represents an average from 4 cells. *C*, summary bar graph of the mean miniature synaptic current amplitude in NG2$^+$ and in CA1 pyramidal cells under identical conditions, demonstrating that the quantal amplitude in NG2$^+$ cells is 3- to 4-fold smaller than in neurons.

Morphology of neuron–NG2$^+$ cell synapses

From the functional point of view, synaptic currents in NG2$^+$ cells do not differ from synaptic currents in neurons. However, it is less clear whether the underlying cellular structures that allow synaptic transmission between neurons and NG2$^+$ progenitors are also similar to those found in neuronal synapses. Electron micrographs of the ultrastructural features of synaptic contacts between neurons and NG2$^+$ cells in grey matter demonstrate a degree of specialization similar to that found in neuron–neuron synapses (Bergles *et al.* 2000; Lin & Bergles, 2004; Kukley *et al.* 2008). The presynaptic compartment appears as a bouton and contains a large number of vesicles. The pre- and postsynaptic membranes are well aligned, and submembraneous electron dense material is found pre- and postsynaptically at the putative sites of vesicle release. Notably, the same micrographs also indicate that NG2$^+$ cells frequently share presynaptic boutons, but not the release sites with neighbouring neuronal postsynaptic spines and dendrites.

The ultrastructural features of neuron–NG2$^+$ cell synapses appear to be different in distinct brain regions. In particular, in white matter regions the degree of synaptic specialization appears to be lower (Kukley *et al.* 2007). The alignment of the two membranes and the electron dense material is less prominent, the number of vesicles appears to be much smaller, and vesicles are contained either in axonal shafts or in inconspicuous varicosities. This may indicate that a sole axon–NG2$^+$ cell contact, i.e. when given NG2$^+$ cell does not share a presynaptic bouton with a neuron, is not sufficient to generate formation of a complete presynaptic specialization. It has been proposed that axons may release transmitters at discrete but arbitrary sites, which may be contacted by a process of an NG2$^+$ cell in a subsequent step. This speculative idea was generated by the finding that, in young optic nerve axons, small clusters of vesicles and fusion proteins are frequently found not to be associated with NG2$^+$ cells (Kukley *et al.* 2007). Similar findings of functional clusters of synaptic vesicles in the absence of an axodendritic contact were also reported in mature cultured neurons and were termed 'orphan release sites' (Krueger *et al.* 2003). Hence, it would be interesting to test whether glutamate, released either from axons or from existing neuronal synapses, can act as a chemoattractant for NG2$^+$ cell processes to initiate the formation of neuron–NG2$^+$ cell synapses.

Do cellular and developmental properties of NG2$^+$ cells suggest possible roles for their synapses?

It is now well established that glial cells are direct participants in synaptic transmission (Haydon & Carmignoto, 2006; Ni *et al.* 2007; Perea & Araque, 2007), and glial morphology appears to have evolved to fulfil this function. Astrocytes are morphologically specialized to modulate synaptic communication between neurons, as they extend their cellular processes into the synaptic cleft and participate in the control of the cellular microenvironment of synapses (Haydon, 2001; Haydon & Carmignoto, 2006). The majority of NG2$^+$ progenitors exhibit a stellate morphology with small cell body and many radial processes (Fig. 1) (Chittajallu *et al.* 2004, 2005; Polito & Reynolds, 2005; Kukley *et al.* 2007; Ziskin *et al.* 2007). These morphological features were originally determined based on immunostaining with anti-NG2 antibodies, and have been confirmed more recently after biocytin filling of electrophysiologically and immunocytochemically identified NG2$^+$ cells (Chittajallu *et al.* 2004, 2005; Kukley *et al.* 2007; Ziskin *et al.* 2007).

The presence of many cellular processes allows NG2$^+$ cells to receive multiple synaptic contacts and to rapidly detect neurotransmitter signals released by neurons through ligand-gated ionic channels (Belachew & Gallo, 2004). Furthermore, as NG2$^+$ progenitors also express a wide array of voltage-gated ionic channels (Lin & Bergles, 2002), neurotransmitter signalling most likely modulates the activity of other membrane channels in these cells. It is important to note that, although NG2$^+$ cells are equipped to receive a variety of signals and even display action potentials (Chittajallu *et al.* 2004; Karadottir *et al.* 2008), they possess no specialized functional outputs comparable to axons. Therefore, the cellular and physiological changes triggered by synaptic inputs on NG2$^+$ cells are not integrated to generate new signals that are forwarded to other cells through axon terminals.

Are neuron–NG2$^+$ cell synapses stable?

In vivo imaging of OPCs in the zebrafish demonstrate that the extension and retraction of their cellular processes is very dynamic and happens on the scale of several minutes (Kirby *et al.* 2006). This raises the question of whether synapses on NG2$^+$ cells are stable or undergo continuous dynamic changes. *In vivo* imaging analysis in rodents similar to that performed in zebrafish is not currently available; nevertheless there is conspicuous ultrastructural specialization frequently found at more differentiated neuron–NG2$^+$ cell synapses, which would unlikely fully develop during short-lived intercellular contacts. These structures have been called glial invaginations and consist of 50–100 nm wide, drop-like protrusions of axonal cytoplasm into the NG2$^+$ cell (Kukley *et al.* 2007). They are found in both grey and white matter regions, and can be identified in many electron micrographs (e.g. see Bergles *et al.* 2000 – Fig. 5a; Lin *et al.* 2005 – Fig. 7b). While the function of these structures is at present

unknown, their existence suggests that the neuron–NG2$^+$ cell contacts last longer than a few minutes.

Further evidence in support of the idea of stable neuron–NG2$^+$ cell synapses stems from the observation that synaptic currents appear to mature during the first few postnatal weeks (Mangin *et al.* 2008). The changes in the kinetics and amplitudes of quantal synaptic events suggest that the association of the pre- and post-synaptic membranes becomes tighter over that time frame. Based on the current ultrastructural evidence, it is too early to define the typical morphological substrate of neuron–NG2$^+$ cell synapses and the developmental sequence of events that culminates in their formation. Future studies employing a complete 3D reconstruction of these structures from serial sections are needed to address these issues. Analysis of synapse formation and structure *in vitro* could also provide interesting information concerning the clustering mechanism of glutamate and GABA receptors on NG2$^+$ cell postsynaptic membrane, and the dependence of this phenomenon on the presynaptic neurotransmitter input itself.

Synapses and NG2$^+$ cell proliferation

As NG2$^+$ progenitors represent the most proliferative cell population in the postnatal and adult bran, it is plausible that the synaptic contacts that they receive might regulate their proliferation potential *in vivo*. In the developing postnatal brain, the NG2$^+$ progenitor cell population is continuously expanding, as approximately 50% of the cells are actively dividing every 3 days (Kukley *et al.* 2008). Considering that newborn neurons require more than 7 days to acquire a synaptic input (Carleton *et al.* 2003; Ge *et al.* 2005), it may appear puzzling how all these immature newborn NG2$^+$ cells can get rapidly connected to functional release sites. The key difference may be that NG2$^+$ cells are actually born with synapses, i.e. that newly born NG2$^+$ cells are already 'wired' by new synaptic contacts while they divide and relocate (Kukley *et al.* 2008). This notion would imply that, when entering mitosis, the parent NG2$^+$ cell is able to keep its processes linked to the presynaptic release sites and to transfer these to the daughter cells upon cytokinesis. This mechanism would ascertain that synaptic contacts are established in all NG2$^+$ cells independently of their maturational stage, and would enable these cells to expand their pool and migrate in the brain while still monitoring synaptic activity arising in local networks.

Previous work performed in cultured cells and in organotypic slice cultures showed that glutamate inhibits NG2$^+$ cell proliferation by depolarizing the cell membrane and by blocking outward K$^+$ currents (Gallo *et al.* 1996), which are characteristically expressed only in dividing NG2$^+$ cells and not in differentiated oligodendrocytes (Gallo *et al.* 1996; Knutson *et al.* 1997). Endogenous sources of glutamate are themselves sufficient to inhibit the proliferation and lineage progression of NG2$^+$ cells via AMPAR activation in cerebellar slice cultures (Yuan *et al.* 1998). While this study did not define the exact mode of activation of their AMPAR, cerebellar NG2$^+$ are known to exhibit AMPAR-mediated synaptic currents at this stage (Lin *et al.* 2005; Karadottir *et al.* 2008). In order for glutamatergic inputs to regulate NG2$^+$ progenitor proliferation *in vivo*, EPSCs arising in different cellular compartments (e.g. cell body *versus* distant cellular processes) must be integrated in the cell soma, where patterns of activity are likely to depend not only on the number of synapses being activated, but also on their anatomical position in the NG2$^+$ cell. Moreover, the influence of glutamatergic inputs needs also to be integrated in concert with other signals that either promote or inhibit cell division, to modify NG2$^+$ cell proliferation rate. Previous findings that glutamate receptor activation in NG2$^+$ cells results in specific changes in the expression levels of cell cycle regulatory proteins, in particular p27 and p21 (Ghiani *et al.* 1999*a*,*b*), are consistent with the hypothesis that graded synaptic activation can be integrated with other cellular signals in NG2$^+$ progenitors to alter expression of regulators of cell cycle progression.

In conclusion, although the specific mechanism of EPSC integration in NG2$^+$ cells is still undefined, this is likely to depend on spatio-temporal summation, similar to the generation of action potentials in neurons. In other words, the influence of EPSCs on cell proliferation, as well as on other NG2$^+$ progenitor functions, would depend on their degree of synchronization, i.e. a higher degree of synchronization leading to a stronger summation. Thus, it remains to be determined whether synchronized activity actually inhibits or promotes NG2$^+$ cell proliferation, since it is debatable whether continuous application of agonists, as used in previous studies (Gallo *et al.* 1996; Yuan *et al.* 1998), is more likely to simulate synchronized over asynchronized stimulation.

Synapses and NG2$^+$ cell migration

NG2$^+$ progenitors are still migrating in the developing postnatal brain (Levison & Goldman, 1993; Zhu *et al.* 2008) while they exhibit synaptic activity (Kukley *et al.* 2008). Thus, NG2$^+$ progenitors are likely to migrate while they receive functional synaptic contacts, similar to interneurons in the molecular layer of the dentate gyrus (Morozov *et al.* 2006; Chittajallu *et al.* 2007). Synaptic release of neurotransmitter may act as a positional clue regulating the migration process, and glutamate has been shown to promote OPC migration via an α_v integrin–myelin proteolipid protein complex (Gudz *et al.*

2006). It has been recently shown in the mouse hilus that synaptic activity in $NG2^+$ cells is partially synchronized with local neurons (Mangin *et al.* 2008). Since $NG2^+$ cells are likely to integrate the activity arising in several neighbouring neurons, they could assess the degree of synchrony arising in any given set of contacted neurons while migrating. Since such synchrony is more likely to occur in neurons receiving inputs from the same areas and belonging to the same structure, it can be speculated that $NG2^+$ cells would be able to recognize functionally homogeneous group of neurons while migrating and that the neurotransmitter released from these neurons acts as a positional clue.

Synapses and $NG2^+$ cell differentiation

Functional synapses are maintained on $NG2^+$ progenitors also at non-migratory developmental stages, i.e. when they undergo cell cycle exit and initiation of cell differentiation (Kukley *et al.* 2008; J.-M. Mangin and V. Gallo, unpublished observations). Moreover, glutamatergic synapses on $NG2^+$ progenitors promote Ca^{2+} influx through the cell membrane, as a significant percentage of the AMPA channels on $NG2^+$ cells conduct Ca^{2+} ions (Bergles *et al.* 2000; Lin *et al.* 2005; Ge *et al.* 2006; Mangin *et al.* 2008). Permeability of AMPA channels to this cation appears to change with age (Itoh *et al.* 2002; Ge *et al.* 2006; Ziskin *et al.* 2007). Altogether, these observations suggest that synaptic activity might also play a role in coordinating cell cycle withdrawal and cell differentiation through Ca^{2+}-dependent events. Several transcription factors that play important roles in $NG2^+$ cell maturation along the oligodendrocyte lineage have been identified, including Yin Yang1 (YY1; He *et al.* 2007), Sox17 (Sohn *et al.* 2006), and Sox9 and 10 (Stolt *et al.* 2003, 2004; Wegner, 2008). In future studies, it will be important to determine whether different patterns of synaptic activity could regulate the signalling pathways that control expression of these transcription factors, and/or transcription of oligodendrocyte genes controlled by these proteins.

Putative roles of synapses on $NG2^+$ cells in grey matter

A difference in cell morphology is probably the more direct and less controversial distinction between grey and white matter $NG2^+$ cells (Chittajallu *et al.* 2004; Butt *et al.* 2005; Dayer *et al.* 2005). While white matter $NG2^+$ cells usually exhibit a fusiform soma and polarized processes aligned with axons bundles, grey matter $NG2^+$ cells will display a non-polarized, radial arborization (Butt *et al.* 2005). The main reason explaining these morphological differences may be the particular affinity that grey matter $NG2^+$ cells display for neurons present in their vicinity. Indeed, their soma and processes are frequently found associated to neuronal soma and neurites in cortex, hippocampus and cerebellum (Butt *et al.* 2005; Dayer *et al.* 2005), and some of the grey matter $NG2^+$ cells are so closely apposed to neurons that they could be considered as satellite cells (Dayer *et al.* 2005) (Fig. 3*A* and *B*). By analysing the spatial distribution of $NG2^+$ cells and neurons in the mouse hilus, it was recently demonstrated that $NG2^+$ cell somata are indeed statistically closer to neuronal somata than would be expected by a random distribution of both cell types (Mangin *et al.* 2008).

Since most $NG2^+$ cells exhibit glutamatergic and/or GABAergic synaptic currents in grey matter areas, one could expect that spatial proximity would facilitate synaptic connectivity between $NG2^+$ cell and their associated neurons. However, pair recordings of 'satellite' $NG2^+$ cells ($sNG2^+$ cells) associated to GABAergic interneurons in the mouse hilus revealed no functional connectivity between closely associated cells (Mangin *et al.* 2008). While this absence of connectivity may be specific to hilar interneurons, it clearly shows that anatomical proximity between $NG2^+$ cells and interneurons is serving other purposes than facilitating connectivity between cells.

In the same study, it was also shown that associated $NG2^+$ cell–interneuron pairs are more likely to receive glutamatergic inputs from the same presynaptic neuron (Fig. 3*C* and *D*), as compared to pairs separated by more than 200 μm (Mangin *et al.* 2008). However, the frequency of such shared inputs in a given $NG2^+$ cell–interneuron pair remains modest. On average, around 15% of all EPSCs observed in an $NG2^+$ cell are synchronized with an EPSC in a given associated interneuron. In a similar fashion, it has been previously shown in cerebellum that a small fraction (3/77) of $NG2^+$ cell–Purkinje cell pairs located in the same folia are connected by the same climbing fibres (Lin *et al.* 2005). Interestingly, while each Purkinje cell is contacted by only one climbing fibre, $NG2^+$ cells can receive inputs from several climbing fibres, providing them with the opportunity to monitor and integrate the activity arising in several neighbouring Purkinje cells. Similarly, $NG2^+$ cells in grey matter areas display a dense arborization contacting multiple neighbouring neurons (Butt *et al.* 2005), and are thus likely to integrate synaptic activity arising in neighbouring groups of neurons.

Do synapses inhibit the differentiation of grey matter $NG2^+$ cells into oligodendrocytes?

It has been reported that glutamatergic synapses on white matter $NG2^+$ cells are made by constitutively unmyelinated fibres in adult mice (Ziskin *et al.* 2007). Similarly, most grey matter $NG2^+$ cells are synaptically

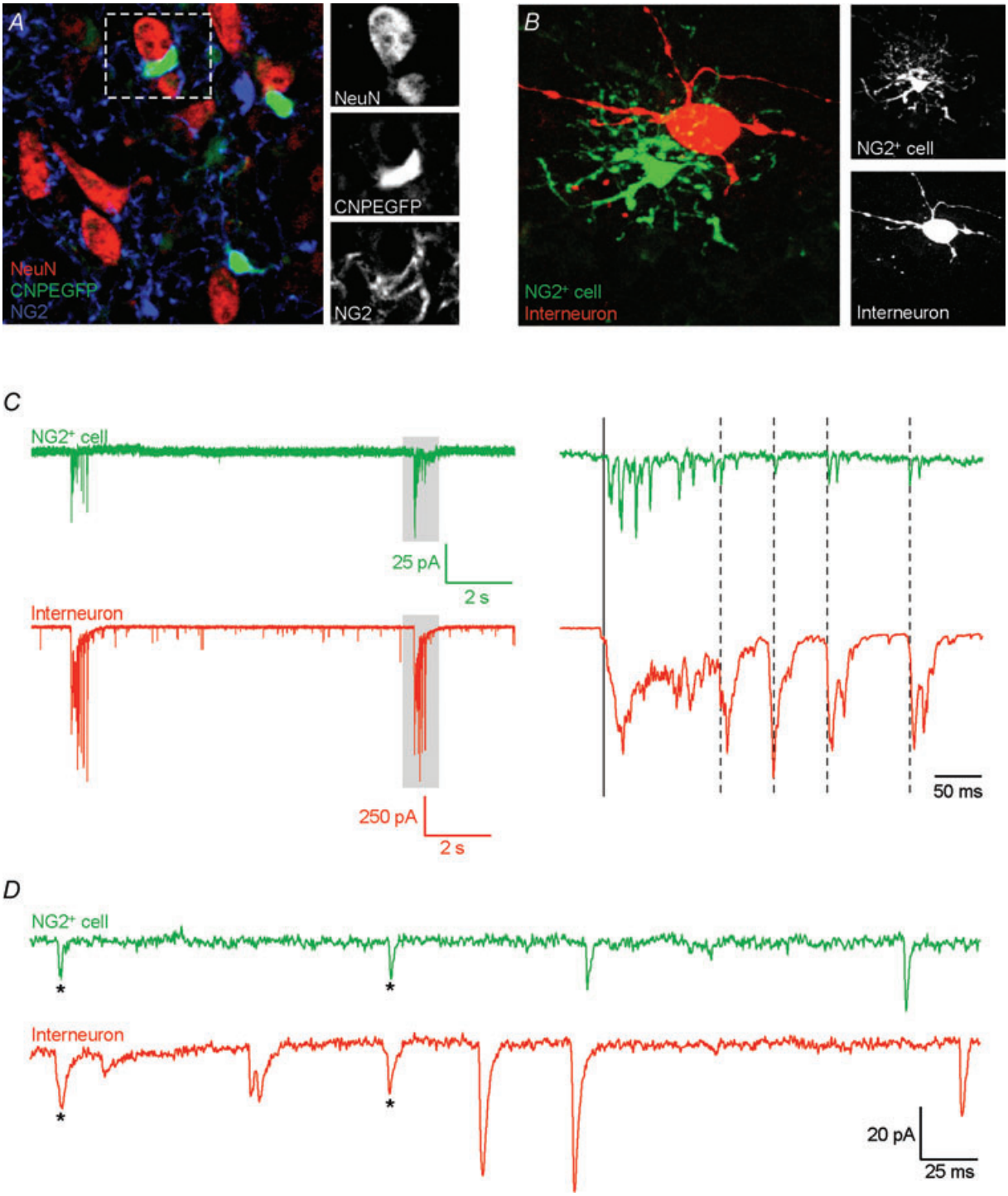

Figure 3. Anatomically associated pairs of hilar NG2$^+$ cell and interneurons exhibit synchronized synaptic activity

A, single-plane merged confocal image showing the dentate gyrus of a P15 CNP-EGFP (green) transgenic mouse immunostained for the proteoglycan NG2 (blue) and the neuronal marker NeuN (red). The dotted box indicates an NG2$^+$EGFP$^+$ cell associated to hilar neurons. Right panels show the individual staining for the area delimited by the dotted box. *B*, confocal images showing a hilar interneuron (red) and its sNG2$^+$ cell (green) filled with biocytin (red in B and grey in right lower panel) and tetramethylrhodamine dextran (green in B and grey in right upper panel), respectively. *C*, example of synchronized bursts observed in a hilar interneuron (V_h = –60 mV, upper trace) and its sNG2$^+$ cell (V_h = –80 mV, lower trace) recorded in a whole hippocampal slice preparation in the presence of 100 μM picrotoxin. The grey boxes indicate the interval of the trace in *A* magnified in the right panels. In the

connected, but do not differentiate into oligodendrocyte, myelination being minimal in grey matter areas. Moreover, since glutamate inhibits $NG2^+$ cell differentiation into oligodendrocyte (Gallo *et al.* 1996; Yuan *et al.* 1998), it is tempting to suggest that glutamate released from synapses acts as an antimyelination signal. One could even generalize that, since most GABAergic synapses are made by non-myelinated local interneurons, the synaptically released neurotransmitter acts as a myelination inhibitor. However, this hypothesis cannot explain why $NG2^+$ cells are found in poorly myelinated grey matter areas in the first place, if their lineage potential is limited to the generation of oligodendrocytes. Moreover, synaptic quantal transmission appears designed to carry information with a high degree of spatio-temporal resolution; it is thus hard to reconcile synaptic transmission on $NG2^+$ cells with such a broad function. Therefore, even if future studies demonstrate that neurotransmitters released onto $NG2^+$ cells regulate myelination *in vivo*, synaptic integration of $NG2^+$ cells in grey matter areas is likely to have additional roles than merely inhibiting myelination.

Are synapses predisposing $NG2^+$ cells to a neuronal fate?

$NG2^+$ progenitors are able to give rise to neurons *in vitro* (Kondo & Raff, 2000; Belachew *et al.* 2003) and *in vivo* after transplantation (Shihabuddin *et al.* 2000; Aguirre *et al.* 2004). There is also evidence that endogenous $NG2^+$ cells may give rise to interneurons in the hippocampus (Belachew *et al.* 2003; Aguirre *et al.* 2004), olfactory bulb (Aguirre & Gallo, 2004) and cortex (Dayer *et al.* 2005). It has also been shown that a subpopulation of $NG2^+$ cells express a density of Na^+ channels sufficient to generate immature single action potentials or, more rarely, bursts of spikes in the mouse cortex (Chittajallu *et al.* 2004). These findings support the hypothesis that oligodendrocyte and interneuron lineages are more tightly connected than one would initially expect. Such observations are themselves derived from studies showing that interneurons and oligodendrocytes share a common origin in the embryonic mammal telencephalon (He *et al.* 2001; Marshall & Goldman, 2002) as well as in the adult SVZ (Aguirre *et al.* 2004).

A recent study suggests that new interneurons could be generated from 'satellite' $NG2^+$ cells in the cortex (Dayer *et al.* 2005). In this context, it is particularly significant that satellite $NG2^+$ cells associated with interneurons in the mouse hilus (Mangin *et al.* 2008) are found to share common presynaptic inputs with these neurons, a property which would facilitate further differentiation steps towards an interneuron fate. However, a recent study using an NG2-Cre transgenic mouse model suggests that the ability of endogenous $NG2^+$ cells to generate interneurons during normal development *in vivo* is at best a marginal phenomenon (Zhu *et al.* 2008). In spite of these findings, the opportunity of manipulating endogenous $NG2^+$ cells to replace interneurons in order to strengthen inhibitory pathways damaged under pathological conditions may be less unrealistic than previously thought.

Does synaptic integration allow $NG2^+$ cells to modulate neuronal activity?

Because many $NG2^+$ cells persist in the adult brain in an apparently 'quiescent state', it has been proposed that these cells could actually correspond to a new class of mature glial cells whose exact physiological function(s) remain to be understood (Butt *et al.* 2005). $NG2^+$ cells are known to produce several extracellular matrix (ECM) molecules, such as hyaluronan, versican, phosphacan, neurocan and tenascins, which function to stabilize synapses by interacting with spectrin and ankyrin, two neuronal cytoskeleton components known to anchor ion channels and neurotransmitter receptors at synapses (Butt *et al.* 2005). Indeed, $NG2^+$ cell processes are known to interdigitate between glutamatergic pre- and postsynaptic elements (Ong & Levine, 1999; Bergles *et al.* 2000). Moreover, electron microscopy observations made in the CA1 stratum radiatum suggest that presynaptic boutons that make contacts with $NG2^+$ cells are frequently shared with a neighbouring neuron (Bergles *et al.* 2000). Indeed, it has been recently confirmed that anatomically associated pairs of $NG2^+$ cell–neuron tend to receive functional glutamatergic inputs from the same presynaptic neurons (Mangin *et al.* 2008). Taken together, these data suggest that $NG2^+$ cells can 'eavesdrop' on synapses made on neighbouring neurons via a dedicated presynaptic structure in which the presynaptic release site has been duplicated. Such a configuration would allow $NG2^+$ cells to monitor and regulate neuron–neuron synapses, either individually or by comparing their activity with other synapses contacted by $NG2^+$ cells.

right panels, dotted lines indicate recurrent EPSCs, which are usually observed after the initial discharge of EPSCs indicated by a continuous line. *D*, example of spontaneous activity recorded in a hilar interneuron ($V_h = -60$ mV, upper trace) and its $sNG2^+$ cell ($V_h = -80$ mV, lower trace) by using a specific caesium methanesulphonate internal solution to isolate EPSCs from GABAergic activity ($E_{Cl} = -80$ mV and $E_{cation} = 0$ mV). The application of 20 μM carbachol was used to increase the spontaneous EPSC activity in both cells. Asterisks indicate synaptic current that are synchronized between both cells.

Our understanding of neuron–NG2$^+$ cell synapses could also benefit from a parallel with cerebellar Bergmann glia. Similar to NG2$^+$ cells, Bergmann glia cell bodies are found tightly apposed to Purkinje cell somata and contact their dendrites via multiple processes. Bergmann glial cells receive multiple inputs from the same climbing fibres that innervate Purkinje cells, but do not share identical presynaptic release sites (Matsui & Jahr, 2003). Finally, in both Bergmann glia and NG2$^+$ cells, synaptic release of glutamate activates Ca^{2+}-permeable AMPA-Rs (Bergles *et al.* 2000; Matsui *et al.* 2007). The conversion of these receptors into Ca^{2+}-impermeable receptors by a viral transfection of GluR2 subunit (Iino *et al.* 2001) induces retraction of Bergmann glia processes, allowing climbing fibres to form aberrant multiple contacts onto Purkinje cells. Therefore, AMPAR-mediated Ca^{2+} entry appears to influence the morphology of glial cells, and by this means can regulate the connectivity of associated neurons. In the context of duplicated and shared presynaptic terminals mentioned above, it is possible that small changes in the morphology of NG2$^+$ cell postsynaptic processes may allow these cells to finely regulate the function of a neighbouring neuron–neuron contact. Indeed, it has been shown that the acquisition of faster kinetics that occurs in glutamatergic synapses during postnatal development is probably driven by modifications of the synaptic structure itself (Cathala *et al.* 2005). It can be also speculated that NG2$^+$ cells may influence synaptic activity by releasing neuromodulators in response to synaptic activation, as has been shown in astrocytes (Perea & Araque, 2007). Because NG2$^+$ cells are present in grey matter areas and receive synaptic contact as early as 4–5 postnatal day in mice (Bergles *et al.* 2000; Lin & Bergles, 2004; Mangin *et al.* 2008; Kukley *et al.* 2008), their ability to monitor individual neuron–neuron synapses would allow them to modulate the formation and the refinement of synaptic networks occurring during the first postnatal weeks. Moreover, by modulating the formation and stability of synapses received by neighbouring neurons during development, NG2$^+$ cells may regulate the emergence of synchronized and functionally equivalent groups of neurons.

Do synapses allow NG2$^+$ cells to detect 'pathological' patterns of neuronal activity?

The long-term consequences of epilepsy on NG2$^+$ cells and the physiological role of NG2$^+$ cells themselves in epilepsy are still largely unexplored. We have recently shown that hilar NG2$^+$ cells exhibit a distinct pattern of activity when presynaptic CA3 pyramidal neurons are pharmacologically synchronized by carbachol and by GABAergic antagonists (Mangin *et al.* 2008), two drugs which, respectively, induce gamma oscillations and epileptic-like ictal activity. Gamma oscillations are known to occur *in vivo* under physiological conditions (Fischer, 2004). In hilar NG2$^+$ cells, gamma oscillations are associated with a uniform fourfold increase in sEPSC frequency (0.15–0.65 Hz), without significant EPSC summation, as compared to basal conditions. By contrast, during epileptic-like ictal events, NG2$^+$ cells exhibit transient bursts of EPSCs at high frequency (40–100 Hz; Fig. 3*B*), which display significant summation and are separated by 20–60 s periods of low frequency activity (0.1–0.2 Hz) (see Fig. 3*B*). Such short bursts of high frequency activity might induce larger and transient increases of intracellular Ca^{2+}, which may be more likely to influence overall NG2$^+$ cell function (e.g. proliferation and differentiation) than the milder and uniform increase in EPSC frequency observed during gamma oscillations. Consistent with this hypothesis, it has been shown that hippocampal NG2$^+$ cells actively proliferate after electroconvulsive seizures (Wennström *et al.* 2003), suggesting that synaptic integration may allow NG2$^+$ cells to detect abnormal pattern of neuronal activity that will induce a functional response.

Putative roles of synapses on NG2$^+$ cells in white matter

Why have neurons developed dedicated release machinery at specific sites along their axons in the white matter, as these specialized structures are not used for synaptic communication with other neurons?

Oligodendrocytes are responsible for enwrapping axons in myelin sheaths to permit rapid action potential propagation by saltatory conduction. Since NG2$^+$ progenitors residing in white matter regions have been shown to generate only oligodendrocytes and other NG2$^+$ cells under normal physiological conditions (Zhu *et al.* 2008), we mainly consider in this section their function as OPCs. As highlighted in recent reviews, the process of myelination during development of the brain does not follow a rigid stereotypical programme, but is strongly modulated by neuronal activity (Fields, 2005; Fields, 2008). For example, raising animals in enriched environments has a supportive influence on the number of oligodendroglial cells and on the speed of myelination (e.g. Szeligo & Leblond, 1977; Sirevaag & Greenough, 1987; Juraska & Kopcik, 1988). At the cellular level (for review see Zalc & Fields, 2000), it has been reported that axonal activity is an important modulator not only of the generation of oligodendroglial cells, but also of the onset and speed of myelination (e.g. Barres & Raff, 1993; Demerens *et al.* 1996).

These results generate the question of how NG2$^+$ cells in white matter regions can detect that specific, electrically active neurons located at a considerable distance require myelination. Thus, there is a clear need

for a local signal with a high spatial and temporal resolution, which enables NG2$^+$ cells to recognize different firing frequencies of individual neighbouring axons. As reasoned above, vesicular glutamate release from axons would be ideally suited to fulfilling this task and guiding NG2$^+$ cells to axons requiring myelination. In fact, the findings in developing white matter that NG2$^+$ cells detect glutamate concentrations at lower levels than post-synaptic neurons and that there are putative 'orphan' release sites in unmyelinated axons (Kukley *et al.* 2007) suggest the hypothesis that NG2$^+$ cells might be in the process of approaching these axons along a concentration gradient of glutamate. Although a chemoattractant effect of axonally released glutamate on NG2$^+$ cells is as yet pure speculation, there is substantial evidence from culture systems that pharmacological activation of ionotropic glutamate receptors (AMPA/kainate type) influences their proliferation, migration and differentiation (e.g. Yuan *et al.* 1998; reviewed by Gallo & Ghiani, 2000).

Finally, the question of the final fate of the axon–NG2$^+$ cell junctions when NG2$^+$ progenitors differentiate is still pending. Are these structures lost, transformed into nodes of Ranvier, or used to initiate enwrapping of axons? Progressive loss of synapses on NG2$^+$ progenitors during cell differentiation would strongly suggest specific functions that relate to early developmental events, i.e. cell proliferation, migration and lineage progression to a committed oligodendrocyte stage.

Importantly, myelination is not only an early developmental phenomenon, but it continues until adulthood, when it may underlie certain forms of learning and memory (Fields, 2008; Fields, 2005). For example, concert pianists show an increased white matter signal in specific fibre tracts proportional to the amount of time spent practicing their instrument (Bengtsson *et al.* 2005). It is noteworthy that synaptic signals in white matter NG2$^+$ cells are present even in adult human brain (Fig. 4), suggesting that electrical activity in specific fibre tracts might be one of the mechanisms that regulate myelination during postnatal development and in adulthood also in humans.

The demonstration of axonal release of glutamate onto NG2$^+$ progenitors in white matter is likely to have important implications for our understanding of a variety of neurological and developmental disorders. Widespread glutamate release from axonal shafts is likely to be harmful and to cause significant cellular damage in a variety of pathological conditions, including ischaemia, vascular dementia, cerebral palsy and traumatic brain injury (Matute *et al.* 2001; Deng *et al.* 2003; Dewar *et al.* 2003; Follett *et al.* 2004; Stys, 2004). Interestingly, during ischaemia glutamate damages oligodendoglial cells not only by acting on AMPA receptors (McDonald *et al.* 1998; Tekkok & Goldberg, 2001), but also by activating NMDA receptors situated in myelin sheaths (Karadottir *et al.* 2005; Salter & Fern, 2005; Micu *et al.* 2006; Bakiri *et al.* 2008). The source of glutamate released in white matter under pathological conditions has been intensely debated, but it seems likely that vesicular release from axons plays a major role in contributing to the excessive extracellular levels of glutamate that cause damage in oligodendroglial cells.

Conclusion and future directions

As typically occurs after fundamental observations are made, the original finding that NG2$^+$ progenitors of the postnatal brain receive functional synapses similar to those existing between neurons (Bergles *et al.* 2000) has generated enormous interest and initiated a new field of research. Furthermore, the recent studies demonstrating that NG2$^+$ cells also respond to glutamate synaptically released from axons in white matter (Kukley *et al.* 2007; Ziskin *et al.* 2007) have not only further challenged existing dogmas on synaptic communication in the brain,

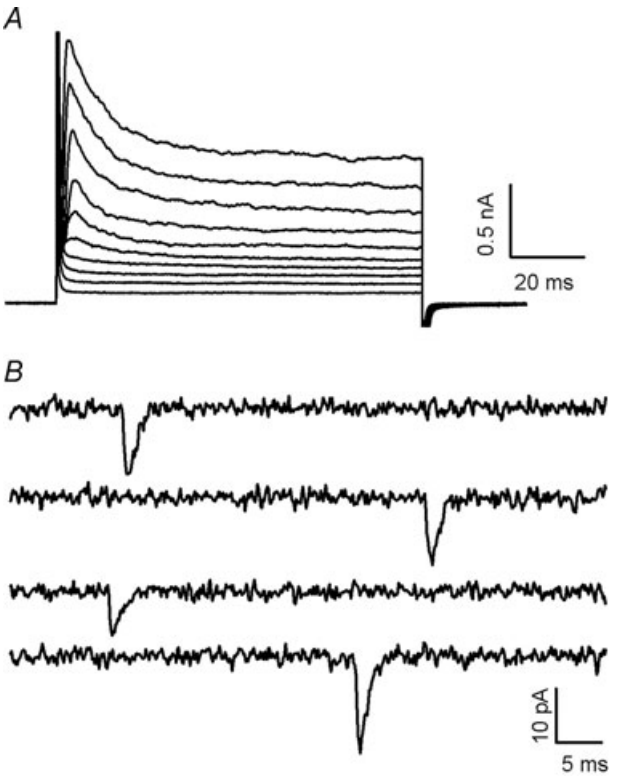

Figure 4. NG2$^+$ cells in adult human white matter display synaptic currents
Recording was performed in the fimbria of a human hippocampal brain slice prepared from a neurosurgical specimen. The hippocampus was removed for the treatment of medically resistant temporal lobe epilepsy (35 years of age). *A*, whole cell current pattern in response to depolarizing voltage steps ($\Delta V = 10$ mV) from a holding potential of –80 mV. The current pattern is very similar to rodent NG2$^+$ cells. *B*, spontaneous glutamatergic synaptic currents in the presence of Ruthenium Red (100 μM). Note the fast kinetics of currents, which are typical for glutamatergic synaptic currents in rodent NG2$^+$ cells.

but also opened up new perspectives on cellular interactions in white matter during normal development and in pathological conditions. In this review, we have summarized our view and speculated on future research directions that will likely move this exciting field forward. These directions are based on fundamental questions that relate to the function of neuron–$NG2^+$ cell synapses in the context of synaptic communication within specific neural networks, and in the developmental context of lineage fate decision of this progenitor cell population. Other important issues to be addressed in future studies might be more specific for $NG2^+$ cells found in white matter regions and relate to the interaction between $NG2^+$ cells and axons. What is the fate of synaptic inputs to $NG2^+$ cells when they differentiate to myelinating oligodendrocytes? What is the fate of axonal release sites/synapses contacting $NG2^+$ cells as axons become myelinated? Do unmyelinated axons that release glutamate on $NG2^+$ cells ever become myelinated? It will be interesting to find out whether a better understanding of the role of synapses in $NG2^+$ cells will result in elucidating the function of non-myelinating $NG2^+$ cells in the postnatal and adult brain.

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