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Electrophysiological properties of NG2⁺ cells: Matching physiological studies with gene expression profiles

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Abstract

NG2⁺ glial cells are a dynamic population of non-neuronal cells that give rise to myelinating oligodendrocytes in the central nervous system. These cells express numerous ion channels and neurotransmitter receptors, which endow them with a complex electrophysiological profile that is unique among glial cells. Despite extensive analysis of the electrophysiological properties of these cells, relatively little was known about the molecular identity of the channels and receptors that they express. The generation of new RNA-Seq datasets for NG2⁺ cells has provided the means to explore how distinct genes contribute to the physiological properties of these progenitors. In this review, we systematically compare the results obtained through RNA-Seq transcriptional analysis of purified NG2⁺ cells to previous physiological and molecular studies of these cells to define the complement of ion channels and neurotransmitter receptors expressed by NG2⁺ cells in the mammalian brain and discuss the potential significance of the unique physiological properties of these cells.

Keywords

oligodendrocyte progenitor; potassium channel; synapse; glutamate; gene expression; electrophysiology

1. Introduction

NG2⁺ cells comprise a widely distributed, dynamic population of precursor cells that remain abundant in the adult central nervous system (CNS). These cells have the ability to mature into oligodendrocytes and are critical for providing new oligodendrocytes to allow remyelination of axons following injury and in diseases such as multiple sclerosis, and are often referred to as oligodendrocyte precursor cells (OPCs). However, fate tracing studies

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indicate that most of these cells in the adult CNS do not differentiate into oligodendrocytes and these cells can be found in regions devoid of myelin, suggesting that they have other, as yet undefined, functions; for these reasons, they are termed NG2⁺ cells in this review. In mice, as well as humans, the generation of oligodendrocytes and the myelination of axons occurs over a protracted time period, beginning in early postnatal life and continuing well into adulthood (Waly et al., 2014). The extended period of myelination must be considered when discussing the physiological properties and behavior of NG2⁺ cells, as all physiological studies have been performed during this time. In such a dynamic cell population, time-, region-, and even individual cell-specific variability is to be expected, depending on the cell cycle stage, differentiation stage, and molecular and cellular milieu. Nevertheless, the numerous electrophysiological studies of NG2⁺ cells have provided critical insight into the conserved properties of these cells.

NG2⁺ cells are a unique glial population in that they display many characteristics typically associated with neurons. These include the expression of ‘neuronal’ genes, such as voltage-gated ion channels and ligand-gated ionotropic neurotransmitter receptors. Expression of these genes gives them a complex electrophysiological profile, in contrast to the linear profile of astrocytes and mature oligodendrocytes. Historically, this distinction has led to these cells being referred to as “complex astrocytes” or “complex glia” in the literature (Matyash and Kettenmann, 2010; Steinhäuser et al., 1994). Another unique feature of NG2⁺ cells is that they are the only glial cell type known to be capable of receiving synaptic connections from neurons (Bergles et al., 2000; Jabs et al., 2005; Lin and Bergles, 2004). At one time it was hypothesized that these cells could even differentiate into neurons (Aguirre and Gallo, 2004; Belachew et al., 2003; Goldman, 2003), although more recent studies have demonstrated that they are restricted to glial fates *in vivo* (see Akiko Nischiyama's chapter in this issue).

Much of our knowledge of the properties of NG2⁺ cells comes from whole-cell patch clamp recordings performed on cultured oligodendrocyte precursor cells, called O-2A cells due to their presumed ability to generate oligodendrocytes and type II astrocytes, and on NG2⁺ cells in acute brain slices. Physiological and pharmacological evidence has pointed to the expression of a vast array of channels and receptors in the membranes of these cells. Specific antagonists, RT-PCR, Western blot, and immunohistochemistry have provided some insight the molecular identity of the channels and receptors, but until recently there had been no systematic effort to catalog channel and receptor expression by NG2⁺ cells.

In recent years, two studies have used microarrays and RNA-Seq to systematically assay gene expression in a variety of cell types, including NG2⁺ cells (Cahoy et al., 2008; Zhang et al., 2014). The latter study used RNA-Seq to assess transcription levels of >22,000 genes within neurons, astrocytes, oligodendrocytes, NG2⁺ cells, microglia, endothelial cells, and pericytes (Zhang et al., 2014). NG2⁺ cells were harvested from P17 mouse cortex and depleted of microglia and endothelial cells by immunopanning on BSL1- and anti-CD45 antibody-coated plates. The remaining cells underwent immunopanning using an antibody specific for PDGFR α , a marker specific for NG2⁺ cells. This process resulted in a mostly pure NG2⁺ cell population, although the authors report that there is a remaining 5% contamination of microglia in the samples.

One obvious caveat to this data set is that it represents only a snapshot of gene expression at a particular time point (P17) and in one brain region (cortex), which cannot capture the full complexity of the population over time. Yet, it is still an invaluable resource to begin to understand the molecular basis for the physiological properties of NG2⁺ cells. Until now there has been no systematic effort to correlate these RNA-Seq findings with previous physiological and pharmacological studies. In this review, we will attempt to bring together these data sets and discuss the possible contributions of various channels and receptors to NG2⁺ cell behaviors. For consistency, the term “NG2⁺ cells” will be used when citing studies that examined OPCs/O-2A cells *in vitro* and *in vivo*, even if expression of the NG2 antigen was not confirmed. In cases where the oligodendroglial lineage of the cells was not established, terms such as “complex glia” or “glial progenitors” will be used, as appropriate.

The RNA-Seq transcriptome data is expressed in units of fragments per kilobase of transcript sequence per million mapped fragments (FPKM). Although a threshold of 0.1 FPKM was used to determine statistically significant expression (>99% confidence), due to space constraints, only more highly expressed channel and receptor genes will be considered in this review. A threshold of 2 FPKM was set for inclusion in summary tables. Genes with lower expression that have been identified in previous studies will be addressed in the text when appropriate. In addition, the discussion will be limited to plasma membrane ion channels and known neurotransmitter receptors.

2. Ion Channels

2.1 Passive membrane properties

NG2⁺ cells in acute brain slices isolated from juvenile animals typically have a capacitance of 15-30 pF, a membrane resistance of 100-500 MΩ, and a stable resting membrane potential (RMP) between -80 and -100 mV (Clarke et al., 2012; De Biase et al., 2010; Haberlandt et al., 2011; Kukley et al., 2008; Lin and Bergles, 2002; Maldonado et al., 2013). This RMP is near the calculated equilibrium potential for K⁺ (E_K), suggesting that K⁺ channels account for the majority of the resting membrane conductance. The predominant K⁺ channels open at rest (‘leak’ channels) are the inward-rectifier Kir4.1 and two-pore (K_{2p}) K⁺ channels.

Kir4.1 is an inward-rectifying channel expressed by astrocytes, oligodendrocytes, and NG2⁺ cells that has been implicated in setting the RMP. This channel also plays a prominent role in the buffering of K⁺ by Müller glia in the retina and astrocytes in the brain (Butt and Kalsi, 2006; Chever et al., 2010; Djukic et al., 2007; Kofuji et al., 2000; Neusch et al., 2006, 2001). The RNA-Seq transcriptome database shows that Kir4.1 (*Kcnj10*) mRNA is expressed at high levels in NG2⁺ cells (OPCs), higher than any other K⁺ channel subunit (See Table 1), although only at approximately half the level observed in astrocytes (Zhang et al., 2014). This is consistent with data from *Kir4.1-EGFPBAC*-transgenic mice, in which EGFP intensity, which reflects promoter activity, was observed to be approximately three-fold higher in GFAP⁺ astrocytes than in NG2⁺ cells (Tang et al., 2009). Kir4.1 subunits can form heteromeric channels with Kir5.1 (*Kcnj16*) subunits, resulting in channels with increased single-channel conductance and greater pH sensitivity (Butt and Kalsi, 2006; Hibino et al., 2004). mRNA for Kir5.1 is found at approximately two-fold higher levels in NG2⁺ cells than

in astrocytes, perhaps indicating a relatively greater role for heteromeric channels in these cells (Zhang et al., 2014).

Kir4.1 mediates the inward currents observed in whole cell recordings when the membrane potential of NG2⁺ cells is hyperpolarized from the RMP (Figure 1A-D). These inward currents are blocked by low concentrations of extracellular Ba²⁺, an inhibitor of Kir channels, and are also specifically abolished in “complex glia” of Kir4.1 glia-specific conditional knockout (cKO) animals (Djukic et al., 2007). “Complex glia” in Kir4.1 cKO animals are significantly depolarized to ~ -40 mV, have increased membrane resistance (~500 MΩ), and appear to be greatly reduced in number in the hippocampus. These observations, together with the severe disruption of myelin in Kir4.1 knockout animals (Djukic et al., 2007; Neusch et al., 2001), suggest an important role for Kir4.1 in the development, survival, or differentiation of NG2⁺ cells.

The membrane resistance of NG2⁺ cells remains low even in the presence of internal solution containing the K⁺ channel blockers Cs⁺ and tetraethylammonium (TEA) (D. Bergles, unpublished observations). This suggests the hypothesis that NG2⁺ cells must express additional ‘leak’ channels that are not sensitive to these blockers. Two-pore domain (K_{2p}) K⁺ channels fit this pharmacological profile (Felicangeli et al., 2014), and several studies have characterized the expression of K_{2p} channels by passive astrocytes (Seifert et al., 2009; Zhou et al., 2009), but relatively little is known about the expression and functional contribution of these channels in NG2⁺ cells. A major road-block is the lack of good pharmacological tools to specifically target these channels (Felicangeli et al., 2014). In a recent study, no change in NG2⁺ cell current properties was found in a TWIK1 knockout mouse, and no effect was observed on applying isoflurane, an activator of TREK1 (Maldonado et al., 2013). Yet, the RNA-Seq transcriptome database has identified several K_{2p} isoforms, notably TWIK1 (*Kcnk1*), TREK1 (*Kcnk2*), and *Kcnk10*, as highly-expressed in NG2⁺ cells (See Table 1) (Zhang et al., 2014). One possible explanation for the discrepancy between mRNA expression data and physiological data is that channels may not be heavily trafficked to the cell membrane, despite high levels of transcription and/or translation. This was found to be the case for TWIK1 in passive astrocytes, where the channel is largely sequestered in the intracellular compartment (Felicangeli et al., 2010; Wang et al., 2013). Better pharmacological and genetic tools will enable a more thorough investigation of the contribution of K_{2p} channels to the passive conductance of NG2⁺ cells.

In addition to their complement of K⁺ ‘leak’ channels, the RNA-Seq transcriptome database indicates that NG2⁺ cells express the Na⁺ leak channel *Nalcn* (See Table 2). This expression is at approximately the same level as that in neurons, and is five-fold higher than that of mature oligodendrocytes (Zhang et al., 2014). This slight baseline permeability to Na⁺ is another example of NG2⁺ cells displaying typically neuronal properties. But, due to the hyperpolarized RMP of these cells, it is clear that the K⁺ leak conductance dominates to a greater extent than in neurons.

It should be noted that NG2⁺ cell membrane properties are not constant during postnatal development. It has been shown that NG2⁺ cells undergo a dramatic change in their current-voltage relationship over the course of development from the first to the fifth postnatal week,

transitioning from outward rectification to an increasingly linear phenotype (De Biase et al., 2010; Maldonado et al., 2013). This change has been attributed to an increase in Kir4.1 expression, as the Ba²⁺-sensitivity of the currents increases over the same time period (Maldonado et al., 2013). These findings are in agreement with earlier studies of the changing electrophysiological properties of unidentified “complex glia” in hippocampus over time (Kressin et al., 1995; Zhou et al., 2006), as well as studies showing the broad upregulation of Kir4.1 in the CNS during postnatal development using sqRT-PCR, Western blot, and immunohistochemistry (Gupta and Kanungo, 2013; Kalsi et al., 2004; Nwaobi et al., 2014; Seifert et al., 2009). Such variability in channel expression must be taken into account when considering the results of the available RNA-Seq transcriptome database, which only captures NG2⁺ cells at one time in development (P17).

There is also regional variability in the passive membrane properties of NG2⁺ cells. In P5-P10 animals, a comparison of cortical and callosal NG2⁺ cells showed that white matter NG2⁺ cells have smaller capacitances (8 pF vs. 40 pF), higher membrane resistances (>2 GΩ vs. 500 MΩ), and a less negative RMP (−70 mV vs. −87 mV) than cells in gray matter (Chittajallu et al., 2004; De Biase et al., 2010). White matter NG2⁺ cells also have much smaller Cs⁺-sensitive Kir currents than gray matter NG2⁺ cells at this age. Although there are differences between white and gray matter early in postnatal development, these changes become less distinct with age as NG2⁺ cells dramatically increase their membrane conductance (De Biase et al., 2010).

2.2 Voltage-gated channels and excitability

2.2.1 Voltage-gated K⁺ channels—Upon depolarization, NG2⁺ cells display a non-linear current profile that is characteristic of A-type (K_A) and delayed-rectifier (K_{DR}) K⁺ channels (Figure 1A-D). These currents have been extensively characterized in culture and brain slice preparations (Barres et al., 1990; Berger et al., 1991; Borges et al., 1995; Kettenmann et al., 1991; Sontheimer et al., 1989; Williamson et al., 1997). A-type channels exhibit rapid activation and inactivation kinetics, contributing to the initial ‘peak’ depolarization; inactivation is so rapid that it gives the impression of a spike on the rising phase of the membrane response (see Figure 1D). Delayed-rectifiers, which activate more slowly and do not inactivate, contribute to the sustained ‘steady-state’ current. The delayed-rectifier current is blocked by internal Cs⁺ and external TEA, and the A-type current is blocked by 4-aminopyridine (4-AP) and internal Cs⁺ (Barres et al., 1990; Borges et al., 1995; Kettenmann et al., 1991; Sontheimer et al., 1989; Williamson et al., 1997). The relative proportions of the two current components vary by region. When compared to cortical NG2⁺ cells, white matter NG2⁺ cells in P5-P10 mice have higher *I*_{KDR} current densities, while *I*_{KA} density is the same, resulting in a higher *I*_{KDR}/*I*_{KA} ratio (Chittajallu et al., 2004).

The precise molecular identity of the channels responsible for these currents is not known. Most studies to date have focused on the *Shaker* family of K⁺ channels, K_v1.1-1.6. RT-PCR and immunocytochemical localization in cultured NG2⁺ cells have shown expression of K_v1.2, K_v1.4, K_v1.5, and K_v1.6 mRNA and protein, with K_v1.5 being the highest expressed (Attali et al., 1997). Another study using single-cell RT-PCR found that individual cultured

progenitors can express varying combinations of all six *Shaker* type channels, with $K_v1.2$, $K_v1.5$, and $K_v1.6$ being the most commonly expressed. However, only $K_v1.4$, $K_v1.5$, and $K_v1.6$ were found at the protein level using immunocytochemistry (Schmidt et al., 1999). A third study also using immunocytochemistry found expression of $K_v1.3$ through $K_v1.6$ protein in cultured progenitors (Chittajallu et al., 2002).

One consistent trend across multiple studies is that specific inhibition or knockdown of individual channel subunits (with the exception of $K_v1.5$ knockdown (Attali et al., 1997) and a partial effect of a toxin specific to $K_v1.3$ (Chittajallu et al., 2002)) has only minimal effects on the observed current properties of cultured NG2⁺ cells (Attali et al., 1997; Chittajallu et al., 2002; Schmidt et al., 1999). These observations, along with the heterogeneity of expression observed at the mRNA and protein levels, suggests that NG2⁺ cells express an assortment of heteromeric K⁺ channels that, in most cases, can compensate for missing individual subunits to maintain the overall current profile. Another important trend is that mRNA and protein levels of K_v channel subunits appear to be regulated independently, leading to disparities between mRNA and protein expression even within the same study (Attali et al., 1997; Chittajallu et al., 2002; Schmidt et al., 1999). This reinforces the need to exercise caution when using mRNA levels to make inferences about the abundance of membrane K⁺ channels.

With this caveat in mind, the RNA-Seq transcriptome database provides the most comprehensive analysis yet of K⁺ channel expression in NG2⁺ cells. Consistent with previous studies, mRNA for the delayed rectifier $K_v1.6$ is highly expressed (See Table 1). mRNA for the other previously identified *Shaker* type delayed rectifiers, however, is expressed only at lower levels: $K_v1.2$ and $K_v1.3$ are expressed at low but significant levels (See Table 1), while $K_v1.5$ is expressed at very low levels (0.8 FPKM). In addition, mRNA for several non-*Shaker* type delayed rectifier channels, $K_v7.2$ and $K_v2.1$, are expressed at high levels. $K_v7.2$ has been previously localized by RT-PCR and immunohistochemistry to at least a subset of cortical NG2⁺ cells (Wang et al., 2011). $K_v2.1$ has been found in many subtypes of neurons (Du et al., 1998; Trimmer, 1991) and in Schwann cells (Sobko et al., 1998), but to our knowledge has never been reported in NG2⁺ cells.

$K_v1.4$, the only *Shaker* type channel to display A-type properties, has only low expression in NG2⁺ cells in the database (0.7 FPKM). Other A-type channel subunits that are highly expressed are $K_v4.2$, $K_v4.3$, and $K_v3.3$. $K_v4.2$ and 4.3 have been found in neurons (Tsaur et al., 1997) and cultured astrocytes (Bekar et al., 2005), but never previously in oligodendrocyte lineage cells. $K_v3.3$ has been found in neurons, particularly in the cerebellum and brainstem (Chang et al., 2007), but not in glial cells. Of the K_v beta subunits, $K_v\beta_2$ was the most highly expressed, followed by $K_v\beta_3$ and $K_v\beta_1$.

The large conductance Ca²⁺-activated [BK] channel $K_{Ca}1.1$, along with its β_4 subunit, is highly expressed in NG2⁺ cells in the database (See Table 1). This confirms previous findings that BK channels, which are activated by both voltage and intracellular Ca²⁺, are expressed in cultured NG2⁺ cells (Buttigieg et al., 2011). There has also been some evidence of small conductance Ca²⁺-activated [SK] channel expression in cultured NG2⁺ cells (Barres

et al., 1990; Sontheimer et al., 1989); in the database, the SK channel subunits $K_{Ca2.1}$ and $K_{Ca2.2}$ are expressed at low but significant levels.

Voltage-gated K^+ channel expression by $NG2^+$ cells is known to play an important role in regulating cell behavior. $K_v1.3$ is upregulated during the G_1 phase of the cell cycle in $NG2^+$ cells, and blockade of this channel with specific toxins prevents the cells from entering the G_1/S transition (Chittajallu et al., 2002). Conversely, overexpression of $K_v1.3$ or $K_v1.4$ promotes the proliferation of cultured $NG2^+$ cells in the absence of mitogens, while overexpression of $K_v1.6$ inhibits proliferation in the presence of mitogens (Vautier et al., 2004). Knockdown and overexpression of $K_v1.5$ have not produced any effects on $NG2^+$ cell proliferation (Attali et al., 1997; Vautier et al., 2004). Differentiation of cultured $NG2^+$ cells into oligodendrocytes is not significantly affected by overexpression of K_v channel subunits, demonstrating that proliferation and differentiation of these cells can be regulated independently (Vautier et al., 2004).

2.2.2 Voltage-gated Na^+ channels—Like neurons, $NG2^+$ cells also express tetrodotoxin (TTX)-sensitive voltage-gated Na^+ (Na_v) channels (Barres et al., 1990; Berger et al., 1992a; Bergles et al., 2000; Borges et al., 1995; Maldonado et al., 2011; Sontheimer et al., 1989; Williamson et al., 1997). The expression density of these channels varies by region in the brain (Chittajallu et al., 2004), and decreases as $NG2^+$ cells differentiate into oligodendrocytes (De Biase et al., 2010; Sontheimer et al., 1989). Expression of Na_v channels together with K_v channels, in principle, endows $NG2^+$ cells with the necessary machinery to generate action potentials. Indeed, it has been observed that, in early postnatal animals, injection of depolarizing current into $NG2^+$ cells can result in spikes, somewhat similar to action potentials (Chittajallu et al., 2004; De Biase et al., 2010; Kárádóttir et al., 2008; Tong et al., 2009). However, these spikes have a high threshold for initiation, a smaller amplitude, slower kinetics, and are less stereotyped than neuronal action potentials. $NG2^+$ cells also exhibit a limited ability to sustain repetitive spiking in response to tonic depolarizing current injection, with most cells exhibiting a single spike (Chittajallu et al., 2004; De Biase et al., 2010) (but see (Kárádóttir et al., 2008)). The physiological relevance of this phenomenon is unclear, as synaptic inputs produce only minimal depolarization of $NG2^+$ cells (see Figure 1E-F) and spontaneous spiking has not been observed (De Biase et al., 2010). As the density of K^+ channels, and thereby the K_v/Na_v ratio, increases in adult animals, the ability of $NG2^+$ cells to generate spikes disappears (De Biase et al., 2010; Maldonado et al., 2011).

The molecular identity of the Na_v channels that support this form of excitability of $NG2^+$ cells has not been thoroughly investigated. One study analyzing the TTX-sensitivity of hippocampal $NG2^+$ cell Na^+ currents concluded that the IC_{50} of TTX (39.3 nM) of these currents was more compatible with an ensemble expression of multiple subunit types, rather than any individual subunit (Xie et al., 2007). This hypothesis is supported by the RNA-Seq database, which shows mRNA for $Na_v1.3$, $Na_v1.2$, $Na_v1.8$, and $Nav1.1$ (See Table 2). The beta subunits $Na_v\beta_1$, $Na_v\beta_3$, and $Na_v\beta_2$ are also expressed. So, it is likely that a heterogeneous mix of Na_v channel subunits contribute to the voltage dependent Na^+ currents.

2.2.3 Voltage-gated Ca^{2+} channels— Ca^{2+} signaling has been shown regulate many NG2^{+} cell and oligodendrocyte behaviors, including proliferation, migration, process extension, differentiation, and myelination (Cheli et al., 2015; Paez et al., 2009; Simpson and Armstrong, 1999; Soliven, 2001; Yoo et al., 1999). There are several potential mechanisms of Ca^{2+} elevation in NG2^{+} cells, including direct influx through plasma membrane voltage- or ligand-gated channels and release from internal Ca^{2+} stores. The presence of voltage-gated Ca^{2+} channels in NG2^{+} cells is particularly interesting, as they could directly link depolarization from synaptic activity to Ca^{2+} -triggered changes in cell behavior.

Electrophysiological recordings from “complex glia” in a high- Ba^{2+} , low- Na^{+} bath solution revealed voltage-dependent, divalent cation-mediated inward currents, indicative of Ca^{2+} channels (Akopian et al., 1996; Berger et al., 1992a). Later studies confirmed the presence of these currents in NG2^{+} cells and found that they were sensitive to inhibitors of L-type and T-type Ca^{2+} channels (Fulton et al., 2010; Haberlandt et al., 2011). However, the currents were small, indicating a low overall density.

The molecular identity of voltage-gated Ca^{2+} channel subunits in NG2^{+} cells has been examined *in situ* using single cell RT-PCR (Haberlandt et al., 2011). Consistent with pharmacological data, mRNA for L-type ($\text{Ca}_v1.2$ and $\text{Ca}_v1.3$), T-type ($\text{Ca}_v3.1$ and $\text{Ca}_v3.2$), P/Q-type ($\text{Ca}_v2.1$), and N-type ($\text{Ca}_v2.2$) α_1 subunits was detected. These findings are supported by the RNA-Seq transcriptome database, which yields a nearly identical list of highly-expressed α_1 subunits, along with a number of β , γ , and $\alpha_2\delta$ subunits (see Table 3). $\text{Ca}_v2.3$, an R-type subunit which was not detected by single-cell RT-PCR (Haberlandt et al., 2011) but was detected by RNA-Seq, is the only point of difference between the two datasets. $\text{Ca}_v1.2$ appears to be the principal pore-forming subunit, as siRNA knockdown of $\text{Ca}_v1.2$ in NG2^{+} cells eliminates ~75% of the Ca^{2+} elevation following depolarization (Cheli et al., 2015).

2.2.4 Voltage-gated Cl^{-} channels—In addition to cation channels, NG2^{+} cells express voltage-gated Cl^{-} channels. The RNA-Seq transcriptome database reveals that *Clc2* (*Clcn2*) is the only plasma membrane voltage-gated Cl^{-} channel gene expressed in NG2^{+} cells (See Table 4). This channel has inward-rectifying properties and a small unitary conductance (Walz, 2002), and can be activated by hyperpolarization, cell swelling, increased intracellular Cl^{-} concentration, or mild extracellular acidification (Jentsch, 2008).

Whole cell patch-clamp recordings from “complex” astrocytes in hippocampal and cortical brain slices have revealed a hyperpolarization-activated anion current that is absent in *Clc2*^{−/−} mice (Makara et al., 2003), supporting the expression of this channel by NG2^{+} cells. Immunohistochemistry and *in situ* hybridization have confirmed *Clc2* expression in GFAP⁺ astrocytes and CC1⁺ oligodendrocytes, although NG2^{+} cells specifically have not been addressed with these techniques (Blanz et al., 2007). *Clc2*^{−/−} mice display extensive and progressive vacuolation of myelin, supporting an important role in oligodendroglial cell function (Blanz et al., 2007).

2.3 Other Ion Channels

2.3.1 Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels—

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, part of the tetrameric voltage-gated K⁺ channel superfamily, are permeable to both Na⁺ and K⁺ (Wahl-Schott and Biel, 2009). They are activated by membrane hyperpolarization, giving rise to “anomalous rectification,” and many are open at the resting membrane potential in both neurons and glia (Shah, 2014). The biophysical properties of these channels, as well as the extent of modulation by cyclic nucleotides, varies with subunit composition (Santoro et al., 2000). There are four subunit isoforms, HCN1-4, and both homomeric and heteromeric channels can be formed *in vivo* (Wahl-Schott and Biel, 2009). These channels are responsible for the so-called *I_h* current in neurons, which is believed to contribute to maintenance of the RMP and to form part of the baseline membrane conductance, in addition to serving a pacemaking function in rhythmically-firing neurons (Santoro et al., 2000; Shah, 2014). Physiologically, NG2⁺ cells display evidence of anomalous rectification that is consistent with HCN channel expression, in which the membrane potential sags back to the resting potential upon hyperpolarization (Bergles et al., 2000).

The RNA-Seq transcriptome database reveals significant expression of two HCN channel subunits in NG2⁺ cells: HCN2 mRNA was present at higher levels than HCN3 (See Table 5). An early study using *in situ* hybridization in adult mice found HCN2 expression in a scattered population of cells in white matter tracts, suggesting its expression in glial cells in addition to neurons (Santoro et al., 2000). A later study in adult rat found that HCN2 antibody staining colocalized extensively with GST- π , a marker of mature oligodendrocytes, although not with NG2 (Notomi and Shigemoto, 2004). The transcriptome database shows that HCN2 mRNA expression in myelinating oligodendrocytes is four to five times higher than in NG2⁺ cells (Zhang et al., 2014); it is possible that the lower level of expression in NG2⁺ cells may not be sufficient for antibody detection. It is also possible that age differences between the studies could contribute to the discrepancy, as HCN2 protein levels are known to increase with age during postnatal development in the hippocampus (Surges et al., 2006).

2.3.2 TRP channels—The transient receptor potential (TRP) family of cation channels is very diverse, with a wide range of activation mechanisms, including ligand binding, G-protein coupled receptor (GPCR) activation, and physical stimuli such as temperature or pressure (Vennekens et al., 2012). The family can be divided into three subfamilies: the classic/canonical TRPs (TRPCs), the vanilloid receptor-related TRPs (TRPVs), and the melastatin-related TRPs (TRPMs). Several TRP channels are known to be expressed in glial cells: TRPC1 and TRPC3, nonselective cation channels activated by the phospholipase C signaling cascade, have been found in NG2⁺ cells and oligodendrocytes, respectively (Fusco et al., 2004; Paez et al., 2011; Weerth et al., 2007). TRPM3, a nonspecific cation channel activated by hypotonicity and sphingosine, has been found in both NG2⁺ cells and oligodendrocytes (Hoffmann et al., 2010).

According to the RNA-Seq transcriptome database, the most highly-expressed TRP channel subunit in NG2⁺ cells is TRPM7, a nonspecific cation-permeable channel that contains an

ion pore fused to a serine-threonine kinase domain (Fleig and Chubanov, 2014) (see Table 6). This channel is widely expressed and is likely important for the importation and homeostasis of trace metal ions (Monteilh-Zoller et al., 2003). It has also been demonstrated to play a role in proliferation, migration, differentiation, and death of a variety of cell types (Fleig and Chubanov, 2014). However, the role of TRPM7 has not been specifically examined in NG2⁺ cells. The TRP channels that have been previously linked to NG2⁺ cells, TRPC1 and TRPM3, are found at low but significant levels in the transcriptome database (see Table 6), along with TRPC2, a gene that is expressed in rodents, but is a pseudogene in humans (Clapham et al., 2005).

3. Receptors

3.1 Glutamate receptors

3.1.1 Ionotropic glutamate receptors—The ionotropic glutamate receptor family consists of four subfamilies: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, kainate receptors, N-methyl-D-aspartate (NMDA) receptors, and δ receptors. δ receptors are a poorly-understood class of ‘orphan’ receptors which bind peptides such as Cbln1, a member of the C1q tumor necrosis factor superfamily (Matsuda and Yuzaki, 2012), and play important roles in synaptogenesis (Hirano, 2012; Yasumura et al., 2012). These receptors are not activated by glutamate (Lomeli et al., 1993) and do not appear to function as ligand-gated ion channels *in situ*. In each of the other subfamilies, a variety of subunits assemble into tetrameric channels that are permeable to Na⁺, K⁺, and in some cases Ca²⁺, and currents through these receptors exhibit reversal potentials near 0 mV (Traynelis et al., 2010). AMPA receptor subunits are designated GluA1-GluA4, kainate receptor subunits are designated GluK1-GluK5, and NMDA receptor subunits are designated GluN1, GluN2A-GluN2D, and GluN3.

Studies of acutely isolated and cultured NG2⁺ cells revealed that application of the AMPA/kainate receptor agonists glutamate, quisqualate, or kainate elicited inward currents that were blocked by the antagonist CNQX (Barres et al., 1990; Borges et al., 1994; Steinhäuser et al., 1992; Wyllie et al., 1991). Concurrent studies of “complex glia” in brain slices confirmed the presence of such currents *in situ* (Berger et al., 1992b; Jabs et al., 1994; Steinhäuser et al., 1994). Analysis of the desensitization kinetics of these receptors (Patneau et al., 1994) and use of specific AMPA receptor antagonists (Bergles et al., 2000; Kukley and Dietrich, 2009) demonstrated that NG2⁺ cells in acute slices express both AMPA and kainate receptors. AMPA/kainate receptors are activated *in situ* at direct glutamatergic synapses formed with NG2⁺ cells (Bergles et al., 2000; Jabs et al., 2005). At present, NG2⁺ cells are the only glial cells that have been shown to form direct synapses with neurons, and in all cases, they are postsynaptic; NG2⁺ cells lack axon-like projections and EM analysis has not detected presynaptic specializations in their processes (Bergles et al., 2000; Ziskin et al., 2007).

RT-PCR of mRNA isolated from cultured NG2⁺ cells detected mRNA for all AMPA/kainate receptor subunits except GluK1, although GluA1 expression is very low (Holzwarth et al., 1994; Itoh et al., 2002; Patneau et al., 1994; Yoshioka et al., 1996). Single-cell RT-PCR from freshly isolated hippocampal “complex glia” revealed that GluA2, the subunit that renders

AMPA Rs Ca^{2+} -impermeable in its RNA-edited form, was expressed by all cells in heterogeneous combinations with the other subunits (Seifert et al., 1997a). Universal expression of GluA2 in cultured NG2 $^{+}$ cells has also been shown by immunocytochemistry (Deng et al., 2006). Moreover, >95% of the GluA2 mRNA in cultured NG2 $^{+}$ cells is edited to the Ca^{2+} -impermeable form (Itoh et al., 2000; Puchalski et al., 1994). Together, these data suggest that NG2 $^{+}$ cells express only Ca^{2+} -impermeable AMPARs. However, through analysis of the current-voltage relationship of agonist-induced responses, the use of antagonists specific to GluA2-containing AMPARs, and direct Ca^{2+} imaging of NG2 $^{+}$ cells in response to glutamate, it has been repeatedly shown that NG2 $^{+}$ cells express functional Ca^{2+} -permeable AMPARs (Bergles et al., 2000; W. P. Ge et al., 2006; Holzwarth et al., 1994; Itoh et al., 2002; Seifert et al., 1997b). Thus, it is likely that individual NG2 $^{+}$ cells express both Ca^{2+} -permeable and impermeable AMPARs within the same cell, a hypothesis supported by biochemical evidence (Deng et al., 2006; Itoh et al., 2002). The relative proportions of these receptors vary over time and even cellular domain, as Ca^{2+} -permeable AMPARs are more highly expressed in younger animals and at extrasynaptic sites (W. P. Ge et al., 2006), and their abundance can be affected by plasticity (W. P. Ge et al., 2006; Zonouzi et al., 2011).

The RNA-Seq transcriptome database is in good agreement with the findings of these previous studies (see Table 7). GluA2 is by far the most highly-expressed AMPA/kainate receptor subunit, followed by GluA3, GluK5, and GluA4. The remaining kainate receptor subunits and the AMPA receptor subunit GluA1 are expressed at lower, but still significant levels.

The function of AMPA/kainate receptors in NG2 $^{+}$ cells *in vivo* is not understood, but evidence from *in vitro* studies indicates that they likely regulate numerous NG2 $^{+}$ cell behaviors. AMPA/kainate receptor antagonists have been shown to stimulate NG2 $^{+}$ cell migration *in vitro* (Gudz et al., 2006), and application of agonists to NG2 $^{+}$ cells in dissociated culture or cultured brain slices has been shown to inhibit their proliferation and differentiation, likely by regulating the activity of K^{+} channels (Gallo et al., 1996; Yuan et al., 1998). Silencing of neuronal activity (and thereby glutamate release) with TTX or blockade of AMPARs was recently shown to increase NG2 $^{+}$ cell proliferation and differentiation in cerebellar slice cultures (Fannon et al., 2015). Notably, however, this increase in differentiation was not accompanied by an increase in myelination. Deciphering the involvement of this form of signaling in regulating NG2 $^{+}$ cell behavior *in vivo* will require direct manipulation of AMPA/kainate receptor expression within these cells, to exclude possible indirect effects on surrounding neurons.

The majority of initial studies of NG2 $^{+}$ cells in culture (Barres et al., 1990; Holzwarth et al., 1994; Patneau et al., 1994; Wyllie et al., 1991) and in acute brain slices (Berger et al., 1992b) found no evidence of NMDA receptor-mediated currents. However, one early study of cultured NG2 $^{+}$ cells reported that small inward currents were elicited in response to NMDA that were more prominent at depolarized potentials, were sensitive to extracellular Mg^{2+} , and were inhibited by the NMDAR antagonist MK-801 (Wang et al., 1996). Intracellular Ca^{2+} elevations, sensitive to the antagonist AP5, were also observed in response to NMDA application (Wang et al., 1996). mRNA for the GluN1 subunit has also been

detected by RT-PCR from cultured NG2⁺ cells (Wang et al., 1996; Yoshioka et al., 1996). Subsequent studies have extended these findings in brain slices and isolated optic nerves, showing with pharmacology, RT-PCR, and immunohistochemistry that functional NMDARs are expressed throughout the oligodendrocyte lineage (De Biase et al., 2010; Káradóttir et al., 2005; Micu et al., 2006; Salter and Fern, 2005; Ziskin et al., 2007). However, electrophysiological recordings have shown that NMDAR expression in NG2⁺ cells is not universal (only ~60% of cells in corpus callosum responded to NMDA), and the density of the receptors appears to be very low, with a maximal excitatory postsynaptic current (EPSC) of ~10 pA at 30 mV (Ziskin et al., 2007).

The discrepancy between early studies in dissociated or cultured cells and later studies *in situ* suggests either that cultured NG2⁺ cells downregulate their expression of NMDARs, or that NMDAR expression is disrupted by the papain used to isolate cells, as was found to be the case for retinal ganglion cells in similar conditions (Barres et al., 1990).

The RNA-Seq transcriptome database provides additional evidence of the expression of NMDARs by NG2⁺ cells (See Table 7). The most highly-expressed subunits are GluN3A, GluN1, and, at a lower level, GluN2C and GluN2D. Receptors containing GluN3A, GluN2C, and GluN2D are known to have reduced sensitivity to extracellular Mg²⁺, (Kuner and Schoepfer, 1996; Sasaki et al., 2002) and consistent with this observation, NMDAR-mediated currents in NG2⁺ cells have been shown to be less sensitive to Mg²⁺ than those in neurons (Káradóttir et al., 2005) (but see (Ziskin et al., 2007)).

Although mature oligodendrocytes express GluN1, GluN2C, and GluN3A subunits by immunohistochemistry and RT-PCR, (Káradóttir et al., 2005; Salter and Fern, 2005) physiological recordings in acute brain slices have shown that the density of NMDAR receptor expression is markedly reduced as NG2⁺ cells transition to the pre-myelinating stage (De Biase et al., 2010). The RNA-Seq database corroborates that expression of NMDARs declines upon NG2⁺ cell differentiation. For example, GluN1 mRNA expression is ~five-fold lower in newly formed oligodendrocytes than in progenitors (Zhang et al., 2014).

The function of NMDARs in NG2⁺ cells *in vivo* is not clear. NMDARs have been demonstrated to regulate the development and survival of neural progenitors (Ikonomidou et al., 1999; Jansson and Akerman, 2014; Nacher and McEwen, 2006). However, it has been shown that they are not required for NG2⁺ cell survival, formation of synaptic connections with neurons, or differentiation into oligodendrocytes *in vivo*, but may be important in regulating the expression of Ca²⁺-permeable AMPA receptors (De Biase et al., 2011). Recent studies indicate that, in the presence of neuregulin (NRG) or BDNF, glutamate signaling through NMDA receptors in oligodendroglia promotes activity-dependent myelination of axons in a co-culture system (Lundgaard et al., 2013). Addition of NRG or BDNF to culture media decreased the expression of the NR3A subunit and significantly increased the amplitude of NMDAR-mediated currents in NG2⁺ cells and oligodendrocytes. Addition of these factors also rendered myelination dependent on neuronal action potential firing and glutamate release. Interestingly, it was shown that remyelination after white matter damage *in vivo* is inhibited by NMDAR blockade, supporting the *in vivo* relevance of

this NMDAR-dependent myelination program (Lundgaard et al., 2013). The relative contributions of NG2⁺ cells and mature oligodendrocytes to this phenomenon need to be elucidated, but these results point to an important role of NMDARs in regulating myelination.

3.1.2 Metabotropic glutamate receptors—Studies using RT-PCR, immunohistochemistry, and Western blot have shown that cultured NG2⁺ cells express group I (mGluR1 and mGluR5), group II (mGluR3) and group III (mGluR4) metabotropic glutamate receptors (Deng et al., 2004; Luyt et al., 2006, 2003; Spampinato et al., 2015a). Functional studies have focused mainly on group I mGluRs, which elevate intracellular Ca²⁺ by activating phospholipase C (Niswender and Conn, 2010). In brain slices, the specific group I mGluR agonist 3,5-DHPG induces Ca²⁺ increases without accompanying current responses in hippocampal NG2⁺ cells, and this response is blocked by preincubation with the mGluR antagonist LY341495 (Haberlandt et al., 2011). The use of subunit-specific antagonists 3-MATILDA and MPEP revealed that the expression of mGluR1 and mGluR5 varies among individual NG2⁺ cells. Similar results had been previously observed in cultured NG2⁺ cells (Luyt et al., 2003).

The RNA-Seq transcriptome database is largely concordant with these findings (See Table 7). The most highly-expressed mGluR subunits are mGluR5, mGluR3, and mGluR4, while mGluR1 is expressed only at very low levels (0.5 FPKM).

Activation of group I mGluRs and subsequent elevation of intracellular Ca²⁺ in NG2⁺ cells has been implicated in promoting cell survival (Deng et al., 2004; Luyt et al., 2006) and in inducing plasticity of neuron-NG2⁺ cell synapses (Zonouzi et al., 2011). In particular, treatment with 3,5-DHPG leads to an increase in the single-channel conductance of AMPARs and the proportion of calcium-permeable AMPARs in cultured NG2⁺ cells. Such a mechanism may underlie the LTP-like synaptic potentiation that is observed at neuron-NG2⁺ cell synapses in the hippocampus (W. P. Ge et al., 2006). Additionally, activation of mGluR4, a group III mGluR, with the agonist L-AP4 was recently shown to accelerate the differentiation of cultured NG2⁺ cells (Spampinato et al., 2015b). Although *in vivo* studies to confirm these findings have not been performed, these results point to an important role for mGluRs in regulating NG2⁺ cell development and synaptic plasticity.

3.2 GABA receptors

3.2.1 Ionotropic GABA receptors—Cultured NG2⁺ cells have been shown to express GABA_A receptors, Cl⁻-permeable ion channels that are activated by the specific agonist muscimol and inhibited by bicuculline (Von Blankenfeld et al., 1991; Williamson et al., 1998). These receptors were subsequently found in “complex glia” and in physiologically-identified NG2⁺ cells in the hippocampus, cortex, and corpus callosum (Berger et al., 1992b; Lin and Bergles, 2004; Steinhäuser et al., 1994). GABA receptors in NG2⁺ cells can be activated *in situ* by direct synaptic connections from inhibitory interneurons or by spillover of GABA from nearby synapses (Kukley et al., 2008; Lin and Bergles, 2004; Vélez-Fort et al., 2010).

It has been consistently observed that application of GABA_AR agonists to NG2⁺ cells results in depolarization and elevation of intracellular Ca²⁺ (Kirchhoff and Kettenmann, 1992; Lin and Bergles, 2004; Pastor et al., 1995; Tanaka et al., 2009; Tong et al., 2009; Von Blankenfeld et al., 1991). This is in contrast to mature neurons, in which the opening of Cl⁻ channels typically causes membrane hyperpolarization. Experiments performed in the perforated patch configuration, which preserves the native internal Cl⁻ concentration, revealed that GABA_A-mediated currents in NG2⁺ cells have a reversal potential in the range of -30 to -40 mV, significantly more positive than the RMP or the reversal potential of neuronal GABA_ARs (~ -70 mV) (Lin and Bergles, 2004; Passlick et al., 2013; Tanaka et al., 2009; Tong et al., 2009). These findings led to the conclusion that NG2⁺ cells maintain higher internal Cl⁻ concentration than mature neurons. A similar phenomenon has been observed in other 'immature' proliferative cell populations in the neurogenic niches of the adult brain (S. Ge et al., 2006; Wang et al., 2003).

GABA_A receptors are heteropentamers typically composed of two α , two β , and one γ subunit (Olsen and Sieghart, 2008). Subunit composition affects the sensitivity, kinetics, pharmacological profile, and cell surface distribution of the receptor complex. The α subunit is the primary determinant of ligand sensitivity, with $\alpha 6$ showing the highest and $\alpha 3$ showing the lowest affinity (Farrant and Nusser, 2005; Gingrich et al., 1995). The α subunit is also an important determinant of response kinetics, with $\alpha 1$ associated with rapid decay time and $\alpha 5$ conferring a slow decay time (Burgard et al., 1996; Goldstein et al., 2002). The γ subunit, particularly $\gamma 2$, is important for receptor clustering and synaptic localization (Farrant and Nusser, 2005).

RT-PCR showed that mixed glial cultures containing 85% NG2⁺ cells contained mRNA for $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\gamma 2$, $\gamma 3$, and low levels of $\gamma 1$ (β subunits were not assessed) (Williamson et al., 1998). $\alpha 3$ is the most highly expressed subunit in culture, and $\alpha 6$ is absent, which is consistent with physiological data in culture and brain slices showing that NG2⁺ cells have low-affinity, slowly-activating receptors (Jabs et al., 2005; Lin and Bergles, 2004; Williamson et al., 1998). NG2⁺ cell GABAergic miniature EPSCs (mEPSCs) also have considerably slower decay kinetics than those of CA1 pyramidal neurons, consistent with expression of $\alpha 5$ and absence of $\alpha 1$ (Lin and Bergles, 2004).

Single-cell RT-PCR from genetically- and physiologically-identified NG2⁺ cells in hippocampus of P9-12 mice and cortex of P7-11 and P21-29 mice showed considerable heterogeneity of GABA_AR subunit expression (Balía et al., 2015; Passlick et al., 2013). Consistent with the *in vitro* studies, all α subunits except $\alpha 6$ were expressed to some extent; $\alpha 3$ and $\alpha 4$ were upregulated with age and $\alpha 5$ was downregulated. $\beta 2$ and $\beta 3$ were high at all ages while $\beta 1$ was low. $\gamma 1$ and $\gamma 3$ were high at both time points, while $\gamma 2$ subunit was dramatically downregulated with age. These findings were corroborated by physiological experiments using subunit-specific agonists and antagonists (Balía et al., 2015; Passlick et al., 2013). The finding that $\gamma 2$ subunit expression, which is associated with synaptic localization, decreases with age correlates with a decrease in the frequency of GABAergic synaptic contacts on cortical NG2⁺ cells as the animal matures (Balía et al., 2015; Vélez-Fort et al., 2010).

The RNA-Seq transcriptome database drawn from P17 animals lies in between the two time points measured by single cell RT-PCR (See Table 8). The concordance between the data sets is mixed: Among α subunits, $\alpha 3$ is the most highly expressed, while $\alpha 2$, $\alpha 4$, and $\alpha 1$ are expressed at lower levels. Surprisingly, $\alpha 5$ is expressed only at extremely low levels (0.6 FPKM). Among β subunits, in agreement with previous results, $\beta 3$ and $\beta 2$ are highly expressed while $\beta 1$ is expressed at lower levels. Among γ subunits, $\gamma 1$ is the most highly expressed while $\gamma 2$ and $\gamma 3$ are at low levels. It must be kept in mind that the transcription database is derived from dissociated cells, which no longer are in synaptic contact with neurons, which may alter their expression of receptor subunits. As GABA_A receptor activation induces depolarization, these receptors may work in concert with AMPA receptors to force greater membrane potential fluctuations and recruitment of other voltage-gated channels (e.g. Ca²⁺ channels).

3.2.2 Metabotropic GABA receptors—GABA_B receptors are metabotropic G-protein coupled receptors that typically hyperpolarize cells by opening K⁺ channels and inhibiting Ca_v channels, while also inhibiting cAMP production. They are formed by heterodimerization of two 7-transmembrane subunits, GABA_BR1 and GABA_BR2 (Benarroch, 2012). Despite extensive characterization of GABA_AR expression and GABAergic synaptic innervation of NG2⁺ cells, the question of whether GABA_B receptors are also expressed by these cells was not addressed until relatively recently. Immunohistochemical analysis of NG2⁺ cells in hippocampal slices, in addition to RT-PCR and Western blot in the NG2⁺ cell-like CG-4 cell line, demonstrated that these cells express GABA_BR1 and GABA_BR2 (Luyt et al., 2007). Functional expression of these receptors has not been demonstrated electrophysiologically, but it was shown that the specific GABA_BR agonist baclofen causes a reduction in cAMP levels and promotes the proliferation and migration of CG-4 cells, indicating that functional receptors are likely expressed (Luyt et al., 2007). These findings have not yet been extended to NG2⁺ cells *in situ*. In agreement with the findings from CG-4 cells, the RNA-Seq transcriptome database shows that GABA_BR1 and GABA_BR2 are among the most highly expressed GABAR subunits in NG2⁺ cells (see Table 8).

3.3 Receptors for neuromodulators

3.3.1 Adrenergic receptors—Adrenergic receptors (ARs) are G protein-coupled receptors that respond to norepinephrine (NE) and epinephrine and are divided into three broad classes: $\alpha 1$ receptors, which signal through G_q and activate phospholipase C, $\alpha 2$ receptors, which signal through G_i and inhibit cAMP production, and $\beta 1$ - $\beta 3$ receptors, which all signal through G_s and stimulate cAMP production (Bylund et al., 1994). These classes have diverse expression profiles, ligand affinities, and pharmacology, and each can be further divided into multiple isoforms.

The presence of $\alpha 1A$ receptors in cultured NG2⁺ cells was first inferred based on a measured increase in IP₃ production in response to application of NE or the $\alpha 1$ -specific agonist phenylephrine. This response was blocked by a specific $\alpha 1A$ antagonist, WB-4101, and not by antagonists to other subtypes (Cohen and Almazan, 1993). In a separate study, application of phenylephrine elevated intracellular Ca²⁺ levels in cultured NG2⁺ cells

(Kastritsis and McCarthy, 1993). When cultured NG2⁺ cells were assayed using RT-PCR and Western blot, they were found to express mRNA and protein for α_{1A} , α_{1B} , and α_{1D} receptor subtypes, while the use of specific antagonists pointed to α_{1A} as the key player promoting IP₃ production in response to norepinephrine (Khorchid et al., 2002). The development of transgenic mice expressing EGF under the control of the α_{1A} promoter, and another line overexpressing an α_{1B} -EGFP fusion protein, allowed for the localization of these proteins in the adult brain, and confirmed that both subtypes are expressed by NG2⁺ cells (Papay et al., 2006, 2004).

Application of the specific β -AR agonist isoproterenol to cultured NG2⁺ cells causes a significant accumulation of cAMP, indicating that functional β -ARs are also expressed (Ghiani et al., 1999). Activation of β -ARs with NE or isoproterenol, either in dissociated NG2⁺ cell cultures or in organotypic cerebellar slice cultures, leads to G₁ cell cycle arrest, inhibition of cell proliferation, and increased differentiation (Ghiani et al., 1999). However, an early study of cultured glial cells found no immunostaining for β -ARs on NG2⁺ cells, despite finding expression on astrocytes and oligodendrocytes (Ventimiglia et al., 1987). To our knowledge, no studies have assayed β -AR expression in NG2⁺ cells using RT-PCR.

The RNA-Seq transcriptome database shows only very low expression levels of adrenergic receptors by NG2⁺ cells (see Table 9); however, fewer receptors may be required, due to their high degree of intracellular signal amplification. β_2 and α_{1A} were the only subunits to surpass the 2 FPKM threshold, though β_2 , which is expressed highly in microglia (~100 FPKM), may be influenced by microglial contamination. β_1 , α_{1B} , and α_{2A} were expressed below the threshold (1.5, 1.4, and 1.4 FPKM, respectively). It may be that mRNA and protein levels are discordant, that the receptors are upregulated in culture, or that such low expression levels are still sufficient to mediate the observed responses. The functional significance of adrenergic signaling through these different receptor classes *in vivo* remains unexplored.

3.3.2 Acetylcholine receptors—Nicotinic acetylcholine receptors (nAChRs) are ionotropic receptors that pass Na⁺, K⁺, and sometimes Ca²⁺, and are activated by nicotine. They are pentamers composed of 16 possible subunits: α_1 - α_9 , β_1 - β_4 , γ , δ , and ϵ . Only certain combinations are found *in vivo*, and the most common combinations in the CNS are $\alpha_4\beta_2$, $\alpha_4\alpha_5\beta_2$, and α_7 homomers (Lukas et al., 1999).

Cultured cortical NG2⁺ cells exhibited inward currents in response to acetylcholine that were blocked by the nicotinic antagonist hexamethonium, (Belachew et al., 1998a) and RT-PCR and immunocytochemistry demonstrated that cultured NG2⁺ cells derived from corpus callosum expressed α_4 and β_2 nAChRs at high levels, and α_3 , α_5 , α_7 , and β_4 at lower levels (Rogers et al., 2001). Application of nicotine to the callosal cultures led to Ca²⁺ oscillations in NG2⁺ cells. In acute hippocampal slices of juvenile mice, recordings from electrophysiologically-identified NG2⁺ cells revealed that they express the highly Ca²⁺-permeable α_7 homomeric receptor, which is potentiated by PNU-120596 and inhibited by methyllycaconitine. In contrast to the RT-PCR data from culture, no evidence of DH β E-sensitive $\alpha_4\beta_2$ receptors was observed in these cells (Shen and Yakel, 2012; Vélez-Fort et al., 2009).

The RNA-Seq transcriptome database shows that the most highly-expressed nAChR subunit is α_4 , distantly followed by α_7 and β_2 (See Table 9). This list is shorter than, but consistent with, the list of subunits identified in cultured NG2⁺ cells by RT-PCR. It points to the expression of α_7 homomers or $\alpha_4\beta_2$ heteromers, although the expression level of α_4 is 17-fold higher than β_2 . The differences between the RT-PCR/RNA-Seq data and the pharmacological data could be due to differences between mRNA and protein expression, or regional differences between hippocampus and cortex. Pharmacological studies of cortical NG2⁺ cells in brain slices would help resolve this issue.

Muscarinic acetylcholine receptors (mAChRs) are metabotropic G protein-coupled receptors. There are five subtypes, designated M₁-M₅. M₁, M₃, and M₅ signal through the G_q protein, resulting in intracellular Ca²⁺ elevation, while M₂ and M₄ signal through the G_{i/o} protein, resulting in inhibition of cAMP production. To date, the only studies analyzing mAChR expression in NG2⁺ cells have been performed in culture, mainly by measuring cellular changes in response to the acetylcholine analog carbachol. A series of studies demonstrated that carbachol induces IP₃ generation, inhibits cAMP accumulation, causes an elevation of intracellular Ca²⁺, induces *c-fos* expression, and ultimately promotes cell survival and proliferation (Cohen and Almazan, 1994; Cohen et al., 1996; Cui et al., 2006; Kastritsis and McCarthy, 1993; Larocca and Almazan, 1997). (However, one study found no effect on proliferation, see (Gallo et al., 1996)). All of these effects were blocked by atropine, a nonspecific mAChR antagonist. RT-PCR analysis has provided further evidence of M₁ and M₂ receptor expression (Cohen and Almazan, 1994). In a follow-up study, more extensive RT-PCR analysis by the same group showed that M₃ is the most highly expressed isoform, followed by M₄, and M₁, M₂, and M₅ at lower levels (Ragheb et al., 2001). It was also demonstrated that the aforementioned effects on NG2⁺ cells are mainly mediated by M₃ receptors, as they were inhibited by the selective M₃ antagonist 4-DAMP (Ragheb et al., 2001).

Similar to that observed for adrenergic receptors, the RNA-Seq transcriptome database shows only very low levels of mAChR mRNA expression by NG2⁺ cells (See Table 9). Only one isoform, M₁, passed the threshold, at 2.1 FPKM. M₂-M₅ fell within the 0.1-1.9 FPKM range. Thus far, all studies of oligodendroglial mAChR expression have been performed on cultured cells, so it is possible that high mAChR expression is induced by the *in vitro* environment. Although mAChRs have been pharmacologically identified in astrocytes in acute hippocampal slices (Shelton and McCarthy, 2000), similar studies need be performed on NG2⁺ cells to determine if the expression pattern holds *in situ*.

Recent evidence for the functional importance of mAChRs in NG2⁺ cells comes from several high-throughput screening studies aimed at identifying therapeutic targets in demyelinating disorders. Benztropine, an antagonist of M₁ and M₃ receptors, strongly promoted expression of myelin basic protein (MBP) by differentiating NG2⁺ cells, and was effective at enhancing remyelination and reducing clinical severity in several animal models of multiple sclerosis (Deshmukh et al., 2013). A cluster of antimuscarinic compounds was also identified as promoting NG2⁺ cell differentiation in a study that assessed the formation of myelin-like wraps around small conical micropillars, and the muscarinic antagonist clemastine was shown to promote oligodendrocyte differentiation and remyelination after

demyelination *in vivo* (Mei et al., 2014). Finally, of particular relevance to humans, M₃ expression was identified in a microarray analysis of FACS-sorted fetal human forebrain OPCs (Abiraman et al., 2015). Treatment of cultured human OPCs with the muscarinic agonist oxotremorine-M prevented their differentiation to oligodendrocytes, while the specific M₃ antagonist darifenacin promoted differentiation. Moreover, treatment with the M₃ antagonist solifenacin increased differentiation of engrafted human OPCs in a demyelinated mouse model. These data provide additional evidence that muscarinic acetylcholine receptors play important roles in NG2⁺ cell biology, and are exciting targets for pharmacotherapy in demyelinating disorders.

3.3.3 Dopamine receptors—The dopamine receptor family consists of five G protein-coupled receptors that can be divided into two classes: D1 class (D1 and D5 receptors), which signal through G_s and stimulate cAMP production, and D2 class (D2, D3, and D4 receptors), which signal through G_{i/o} and inhibit cAMP production (Beaulieu and Gainetdinov, 2011).

D3 receptors, but not D2 receptors, were found by RT-PCR and Western blot in primary mixed glial cultures from newborn mice, while *in situ* hybridization and immunocytochemistry showed that the expression was restricted to NG2⁺ cells in the cultures (Bongarzone et al., 1998). In the same study D3 receptors were also found by immunohistochemistry in oligodendrocyte-like cells in the corpus callosum of juvenile, but not adult, mice. Stimulation of D3 receptors in NG2⁺ cells *in vitro* with quinpirole increases their differentiation (Bongarzone et al., 1998). A subsequent study confirmed D3 receptor mRNA expression in cultured NG2⁺ cells and showed that haloperidol, a D2/D3 inhibitor, promotes their proliferation and inhibits their differentiation (Niu et al., 2010).

Somewhat surprisingly given these results, the RNA-Seq database shows no significant expression of any dopamine receptors in NG2⁺ cells (Zhang et al., 2014). It is possible that the receptor expression previously observed may be unique to the culture environment, as there is limited evidence for their expression by NG2⁺ cells *in situ*. It is also possible that dopamine receptor expression *in vivo* is enriched in brain regions where dopamine is generated and released, such as the ventral tegmental area and nucleus accumbens, and is therefore not represented in available datasets from the hippocampus or cortex. If dopamine receptors are expressed by NG2⁺ cells *in vivo*, oligodendrogenesis and myelination within reward pathways may be affected by drugs of abuse that act by modulating the dopamine system (see Discussion).

3.3.4 Glycine receptors—Glycine receptors (GlyRs) are ionotropic receptors composed of five homo- or heteromERICALLY-assembled subunits. GlyRs conduct Cl[−] and are known to be mediators of synaptic inhibition in the brainstem and spinal cord (Lynch, 2009). Four α (α₁-α₄) and one β subunit have been identified. Unlike α subunits, the β subunit alone cannot form homomeric channels (Griffon et al., 1999) or produce glycine-gated currents (Bormann and Rundstrom, 1993) in heterologous systems. Application of glycine has been shown to induce an inward current and Ca²⁺ elevation in cultured cortical NG2⁺ cells, and these responses were inhibited by the GlyR antagonist strychnine and the Cl[−] channel blocker picrotoxin (Belachew et al., 2000, 1998a, 1998b). Similar currents have been observed in

unidentified glial precursor cells in rat spinal cord slices (Kirchhoff et al., 1996; Pastor et al., 1995).

RT-PCR and immunocytochemistry from cortical NG2⁺ cell cultures show expression of the α_2 and β subunits but not α_1 and α_3 (Belachew et al., 1998b). However, single cell RT-PCR of glial precursor cells from rat spinal cord slices found expression of the α_1 and β subunits, but not α_2 or α_3 (Kirchhoff et al., 1996). In contrast to both of these studies, the RNA-Seq database shows expression of only the GlyR β subunit, with no expression of any α subunits (see Table 9), a scenario that would not result in formation of functional receptors. Since glycinergic signaling is much more prominent in the spinal cord, it is possible that NG2⁺ cells in that region have much higher expression of GlyRs to enable these progenitors to be influenced by neuronal activity.

3.3.5 Purinergic receptors—Purinergic receptors are divided into two broad classes: P1 receptors, which are activated by adenosine, and P2 receptors, which are activated by ATP or other nucleotides.

P1 receptors are G protein-coupled receptors, of which there are four vertebrate homologs: A₁, A_{2A}, A_{2B}, and A₃. A₁ and A₃ are coupled to G_{i/o}, while A_{2A} and A_{2B} are coupled to G_s (Fredholm et al., 2001). By RT-PCR, cultured and freshly isolated cortical NG2⁺ cells have been shown to express all four adenosine receptors (Stevens et al., 2002), and expression of A₁ has been shown by immunocytochemistry (Othman et al., 2003). Application of specific adenosine receptor agonists to cultured NG2⁺ cells has been observed to increase their migration (Othman et al., 2003) and increase (Stevens et al., 2002) or decrease (Coppi et al., 2013) their differentiation, depending on the receptor subtype activated.

A₁ is the most highly expressed adenosine receptor in NG2⁺ cells in the RNA-Seq database, followed by A_{2B} (see Table 9). A_{2A} and A₃ are expressed only at very low levels (0.9-1.7 FPKM). Prior RT-PCR experiments were performed from NG2⁺ cells isolated from mice at P1, so the observed differences in expression may reflect changes in gene expression patterns with development. Further functional studies are needed to determine the impact of these distinct receptors on NG2⁺ cell behavior.

P2 receptors can be divided into two broad classes: P2X and P2Y. P2X receptors are ligand-gated ion channels. There are seven P2X receptor subunits, P2X₁-P2X₇, and they form oligomeric nonselective cation channels permeable to Na⁺, K⁺, and Ca²⁺ (Dal Ben et al., 2015; Khakh et al., 2001). Both homomeric and heteromeric subunit combinations are possible. P2Y receptors are G protein-coupled receptors, of which there are eight mammalian subtypes: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄. These vary in their expression pattern, ligand specificity, and second-messenger signaling. P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ couple to G_q, while P2Y₁₂, P2Y₁₃, and P2Y₁₄ couple to G_{i/o} (Abbracchio et al., 2006; Weisman et al., 2012).

NG2⁺ cells in culture and in brain slices respond to ATP with intracellular Ca²⁺ elevations (Agresti et al., 2005a; Bernstein et al., 1996; Hamilton et al., 2010; He and McCarthy, 1994). These elevations are not affected by changes in the external Ca²⁺ concentration but sensitive

to depletion of internal Ca^{2+} stores, supporting a role of the metabotropic P2Y receptors (Kirischuk et al., 1995). It should be noted that some studies did not detect Ca^{2+} elevations in the most immature cultured oligodendrocyte precursors (Kastritis and McCarthy, 1993) or in the majority of presumed oligodendrocyte precursors from early postnatal corpus callosum slices (Kirischuk et al., 1995). But, using the NG2-DsRed line to unambiguously identify NG2⁺ cells in the juvenile mouse corpus callosum, it was demonstrated that these cells respond to exogenous ATP with increases in intracellular Ca^{2+} (Hamilton et al., 2010). It was also shown that nearby astrocytes are a potential source of ATP, which release ATP response to action potential firing by axons (Hamilton et al., 2010). Use of specific agonists and antagonists showed that P2Y₁ and P2X₇ receptors are major contributors to this NG2⁺ cell response.

Using Western blot in NG2⁺ cell cultures, expression of P2X₁-P2X₄, P2X₇, P2Y₁, P2Y₂, and P2Y₄ receptors has been demonstrated (Agresti et al., 2005a, 2005b). Protein expression of P2Y₁ by NG2⁺ cells *in situ* has also been demonstrated by immunohistochemistry (Agresti et al., 2005a). Use of specific agonists and antagonists has confirmed that P2X₇ and P2Y₁ are the major purinergic receptors active in cultured NG2⁺ cells, and activation of P2Y₁ promotes their migration and inhibits their proliferation (Agresti et al., 2005a, 2005b).

The RNA-Seq transcriptome database largely confirms these findings (See Table 9), although the high expression of certain purinergic receptors by microglia, which are a 5% contamination in the NG2⁺ cell sample, may affect the interpretation of these results. The database shows the highest expression of P2Y₁₂ (67 FPKM), but this receptor is expressed at extremely high levels in microglia (~330 FPKM), so this may reflect mRNA from microglia. Similarly, P2Y₆ barely surpassed the threshold in NG2⁺ cells (2.7 FPKM), but it is expressed at much higher levels by microglia (~130 FPKM). Neither P2Y₁₂ nor P2Y₆ was found in cultured NG2⁺ cells by Western blot (Agresti et al., 2005a). Setting those results aside, the purinergic receptors with the highest expression in NG2⁺ cells are P2X₇, P2X₄, P2Y₁, and P2Y₂, which agrees with previous findings.

Recent studies indicate that NG2⁺ cells migrate to sites of focal injury and contribute to the formation of the glial scar (Hughes et al., 2013). This behavior is reminiscent of that exhibited by microglia, which are attracted to injured cells by ATP-dependent activation of P2Y₁₂ receptors (Haynes et al., 2006). It remains to be determined if NG2⁺ cells also use ATP as a chemoattractant signal to guide their movement to sites of injury to help promote repair.

3.3.6 Serotonin receptors—There are seven subtypes of serotonin receptors, 5-HT₁-5-HT₇. Other than 5-HT₃, which is ionotropic, all are metabotropic G protein-coupled receptors (Hoyer et al., 1994). Initial studies of cultured oligodendrocyte lineage cells (Belachew et al., 1998a; Karschin et al., 1994), glia in optic nerve explants (Kriegler and Chiu, 1993) and corpus callosum slices (Bernstein et al., 1996), and human embryonic stem cell-derived oligodendrocyte progenitors (Schaumburg et al., 2008) reported that they responded with depolarization or Ca^{2+} elevation upon serotonin application. These responses were often limited to subpopulations of cells, and the identities of the cells were often not defined, leaving it unclear whether NG2⁺ cells specifically express serotonin

receptors. Furthermore, several other studies failed to find evidence of serotonin receptor expression in oligodendroglia in culture or *in situ* (Kastritsis and McCarthy, 1993; Maxishima et al., 2001).

A recent study used immunohistochemistry and Western blot to assess 5-HT_{1A} and 5-HT_{2A} receptor expression in cultured rat oligodendrocyte lineage cells, and expression of both receptors was found throughout the lineage (Fan et al., 2015). Intracellular Ca²⁺ elevation was also detected in response to serotonin, and chronic serotonin or 5-HT_{2A} receptor agonist administration reduced differentiation of pure cultured NG2⁺ cells and reduced myelination of axons in co-cultures. However, at present there have been no analyses of the *in vivo* expression of serotonin receptors by NG2⁺ cells.

The RNA-Seq transcriptome database does not show significant expression of any serotonin receptor subunits in NG2⁺ cells (Zhang et al., 2014). The subtype with highest expression is 5-HT_{5A} receptor (1.4 FPKM, all other subunits < 0.3 FPKM). There are species differences (rat vs. mouse) and age differences (P1 vs. P17) between these studies, which could explain the discrepancy, or it is possible that serotonin receptors are upregulated in culture conditions. Nevertheless, the expression of serotonin receptors on NG2⁺ cells suggests that their behavior could be regulated by global changes in brain state, such as mood, appetite, and sleep. The potential effects of drugs affecting the serotonin system on NG2⁺ cells are discussed below (see Discussion).

4. Discussion

NG2⁺ cells are a dynamic population of glial cells that continue to divide, migrate and differentiate in the adult CNS, and perhaps not surprisingly, studies have revealed that there is significant variation in the physiological properties of these cells among different regions and between different developmental time points, and even between cells in the same brain region at the same age (Chittajallu et al., 2004; De Biase et al., 2011; W. P. Ge et al., 2006; Kressin et al., 1995; Maldonado et al., 2011). For example, white matter NG2⁺ cells have smaller capacitance, greater membrane resistance, and more depolarized RMP than gray matter NG2⁺ cells (Chittajallu et al., 2004). Both physiological studies and RNA-Seq analysis reveal that channel and receptor expression change dramatically as NG2⁺ cells differentiate into oligodendrocytes (De Biase et al., 2010; Zhang et al., 2014), and electrophysiological changes are associated with progression through the cell cycle in a variety of cell types (Blackiston et al., 2009; Sundelacruz et al., 2009), which may explain much of the observed variability. At present, the majority of physiological studies have been performed on cells *in vitro* or in tissue isolated from a small number of brain regions (e.g. hippocampus) from young animals, so the full extent of the variation in channel and receptor gene expression remain to be defined. Nevertheless, some general patterns have emerged from analysis of existing datasets. It is clear that NG2⁺ cells express a wide variety of ion channels, including voltage-gated channels that are used by neurons to promote excitability and shape the waveform of synaptic potentials and action potentials. Given that NG2⁺ cells exhibit limited excitability and have a narrow range of voltage excursion under physiological conditions, some obvious questions are raised: why do these cells express these channels, and under what conditions are they activated?

NG2⁺ cells express ligand-gated receptors for a wide variety of neurotransmitters, which may provide a means for adjusting their behavior in response to changing patterns of neuronal activity. In the case of glutamate and GABA, it is known that these receptors are activated at defined synaptic junctions, in addition to spillover from nearby synapses (Bergles et al., 2000; Jabs et al., 2005; Kukley et al., 2008; Lin and Bergles, 2004; Vélez-Fort et al., 2010). However, under normal conditions, excitatory postsynaptic potentials (EPSPs) and K⁺-mediated depolarizations result in small voltage changes at the cell body that are insufficient to induce substantial activation of voltage-gated channels (see Figure 1E-F). There are several possible mechanisms by which this seeming inconsistency may be explained. First, while significant depolarization is required to activate large numbers of voltage-gated channels, channel opening is a probabilistic phenomenon, and some channel opening will occur even during smaller depolarizations. It is possible that such low levels of channel opening are sufficient to trigger intracellular changes in ion concentration that induce relevant downstream effects. Indeed, while depolarization of the cell body is limited, NG2⁺ cells have a complex morphology and extend many thin, highly branched processes. Ion influx into the small volume of these processes could affect transporter function or kinase activity, or even induce local depolarizations and activation of voltage-gated channels. Second, analysis of membrane potential changes of NG2⁺ cells have been performed exclusively in culture or in slices. No truly *in vivo* physiological recordings of NG2⁺ cells have been performed. As NG2⁺ cells respond rapidly to tissue damage (Hughes et al., 2013), it is possible that their properties are altered following preparation of brain slices and that they are capable of experiencing a larger dynamic voltage range in the intact CNS.

NG2⁺ cells in all brain regions that have been examined increase their resting conductance to K⁺ as the animal matures, resulting in a lower membrane resistance and an I-V relationship that approaches linearity (Kressin et al., 1995; Maldonado et al., 2013; Zhou et al., 2006). This change has important implications for activation of voltage-gated conductances, as larger currents would be required to bring the membrane potential of the cell into a range where substantial gating is possible. However, if leak conductances are absent from the fine processes, they may experience much greater depolarization, as well as larger concentration changes. Such changes are not easily detectable by whole-cell patch clamp recording or the use of voltage-sensitive dyes, but new methods such as the use of genetically-encoded voltage and Ca²⁺ indicators may allow exploration of the voltage excursions experienced by these domains. NG2⁺ cells expressing fluorescent proteins in superficial cortical layers can be imaged through cranial windows (Hughes et al., 2013), potentially allowing analysis of NG2⁺ cell physiology *in vivo* and even correlation of their responses with animal behavior.

The expression of neuromodulator receptors has mainly been demonstrated by exogenous application of agonists in culture or in brain slices. Spontaneous activation of these receptors *in situ* or *in vivo* has not yet been reported. Unlike glutamate and GABA, neuromodulators such as norepinephrine and serotonin are typically released from varicosities by long-range projection neurons, potentially affecting all neurons and glia with relevant receptors in a broad area. One exception is ATP, which can be released locally by astrocytes and from synaptic vesicles (Hamilton et al., 2010; Jo and Role, 2002; Jo and Schlichter, 1999;

Pankratov et al., 2006). Although mRNAs encoding neuromodulatory receptors were present in low abundance, the high signal amplification exhibited by these receptors may enable a few receptors to exert profound alterations in NG2⁺ cells. Neuromodulator release is associated with global changes in brain state, such as mood, attention, and sleep. The regulation of NG2⁺ cell behavior by these state changes is largely unexplored, but the expression of neuromodulator receptors suggests that they have the potential to be affected and may contribute to the plastic changes in neural circuits induced by these neurotransmitters.

The RNA-Seq transcriptome database has provided the first comprehensive analysis of mRNA expression within this population of glial cells, enabling comparisons of gene expression between glial cell types and neurons. At present, this dataset is only a snapshot, revealing the properties of NG2⁺ cells from a single time point and brain region. Thus, referencing these values to physiological data acquired across a range of conditions, ages and brain regions is likely to reveal inconsistencies, as noted above. Furthermore, the RNA-Seq analysis only measures mRNA levels; it is possible that protein expression from some mRNAs may be tightly regulated and therefore may not correlate well with functional protein expression. Other methods such as immunohistochemistry and electrophysiology combined with specific pharmacology, which are not as amenable to high-throughput analysis, will be required to corroborate mRNA levels with the expression of functional channels and receptors.

NG2⁺ cells undergo dramatic morphological and physiological changes upon differentiation into oligodendrocytes. To determine how this maturation changes the complement of ion channels and receptors expressed, we identified 290 genes from the RNA-Seq database that encode subunits of plasma membrane ion channels or known neurotransmitter receptors (including all genes listed in Tables 1-9, in addition to other genes from the same categories expressed from 0.1-2 FPKM). The overall expression levels of these genes were significantly different among NG2⁺ cells, premyelinating oligodendrocytes, and mature oligodendrocytes (see Figure 2) (two-sample Kolmogorov–Smirnov test; NG2⁺ cells vs. premyelinating oligodendrocytes: $D = 0.17$, $Prob>|D| = 2.8 \times 10^{-4}$; NG2⁺ cells vs. mature oligodendrocytes: $D = 0.3$, $Prob>|D| = 6.1 \times 10^{-12}$; Premyelinating oligodendrocytes vs. mature oligodendrocytes: $D = 0.13$, $Prob>|D| = 0.01$). At any threshold of significance greater than 0.1 FPKM, NG2⁺ cells express a much larger group of channel and receptor genes than more differentiated cells. For example, 120/290 genes (41%) are expressed at ≥ 2 FPKM in NG2⁺ cells, while only 74/290 (26%) and 40/290 (14%) are expressed at that level in premyelinating and mature oligodendrocytes, respectively. This dramatic difference in the diversity of ion channel and receptor expression suggests that NG2⁺ cells are uniquely positioned within the oligodendrocyte lineage to be influenced by diverse forms of neural activity.

4.1 NG2⁺ cells as drug targets

The role of glial cells in neuropsychiatric disease is an area of increasing interest (for a comprehensive review, see (Di Benedetto and Rupprecht, 2013) and Joel Levine's chapter in this issue). Many therapeutics used to treat neuropsychiatric diseases target ion channels and

neurotransmitter receptors in the brain. Although, the intended targets are often neurons, NG2⁺ cells express many ‘neuronal’ genes that will also bind to these drugs; thus, these cells could contribute to both unexpected side effects and therapeutic benefits of these agents.

Among the most common targets of psychiatric drugs and drugs of abuse are the dopamine and serotonin systems. Olanzapine, an atypical antipsychotic, and haloperidol, a typical antipsychotic, have been shown to enhance cell proliferation and reduce differentiation of NG2⁺ cells *in vitro* (Kimoto et al., 2011; Niu et al., 2010). Antipsychotics are thought to function primarily by antagonizing D₂/D₃ and 5-HT_{2A} receptors, though they are able to bind to a wide variety of other neuromodulator receptors as well (Kusumi et al., 2015). The D₃ and the 5-HT_{2A} receptor have been found in cultured NG2⁺ cells, but not in the acutely isolated cortical NG2⁺ cells of the RNA-Seq database (see sections 3.3.3 and 3.3.6). In a concurrent *in vivo* study, it was found that administration of haloperidol to adult mice resulted in an increase in the density of NG2⁺ cells in the brain with no increase in the number of oligodendrocytes, supporting the *in vivo* relevance of these finding (Wang et al., 2010). It has also been shown that perinatal exposure to the selective serotonin reuptake inhibitor (SSRI) citalopram impairs oligodendrocyte development and myelination in the corpus callosum of rats, (Simpson et al., 2011) suggesting that the serotonin system is relevant to NG2⁺ cells *in vivo*.

In addition to therapeutics, drugs of abuse also frequently target ion channels and receptors, and may affect NG2⁺ cells in ways that are not well understood. One important example is nicotine: NG2⁺ cells express calcium-permeable nicotinic acetylcholine receptors *in vivo* (Vélez-Fort et al., 2009) (see section 3.3.2), and *in vitro* application of nicotine results in intracellular calcium oscillations in NG2⁺ cells (Rogers et al., 2001). At typical concentrations reached in the brain during smoking, application of nicotine to acute brain slices does not evoke an electrical response in NG2⁺ cells, but does desensitize the receptors, potentially interfering with their normal function (Vélez-Fort et al., 2009). However, the potential effects of most drugs on NG2⁺ cells have not been investigated, and dissociating the direct effects of these compounds on these cells versus indirect effects on neurons will require selective genetic manipulation of NG2⁺ cells, as in many cases their receptors cannot be selectively interrogated pharmacologically. As we increase our understanding of the contribution of glial cells to neuropsychiatric disease and develop new therapeutic strategies, it will be important to consider the potential effects on non-neuronal cells. NG2⁺ cells in particular, with their broad expression of ‘neuronal’ genes, are likely to be direct targets for both therapeutics and drugs of abuse.

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Highlights

- NG2⁺ cells display complex electrophysiological properties
- A rich complement of ion channels and receptors underlie these properties
- New RNA-Seq datasets allow a comprehensive analysis of NG2⁺ cell gene expression
- Diversity of ion channel and receptor expression declines as NG2⁺ cells differentiate
- Expression of ion channel and receptor genes makes NG2⁺ cells targets for many drugs

Figure 1. Membrane properties of NG2⁺ cells and response to glutamatergic synaptic input
A & B) Voltage clamp recordings from hippocampal NG2⁺ cells in acute brain slices isolated from a 5 week-old *PDGFRα-CreER; Rosa-CAG-EGFP(RCE)* mouse showing activation of voltage gated currents upon application of hyperpolarizing and depolarizing voltage steps from a holding potential of -90 mV. Voltage step induced artifacts at the beginning of the traces have been truncated for clarity. Cell in A: $C_m = 20.44$ pF, $R_m = 54.2$ MΩ, $R_a = 18.5$ MΩ; Cell in B: $C_m = 25.94$ pF, $R_m = 217.1$ MΩ, $R_a = 13.5$ MΩ. C & D) Current clamp recordings from the same cell as in A and B, respectively, showing the membrane potential changes induced by current injections from a resting membrane potential of -90.2 mV (C) and -91.9 mV (D). Arrow in D highlights inflection induced by rapid inactivation of A-type K⁺ channels. E) Examples of spontaneous EPSCs (sEPSCs) recorded from an NG2⁺ cell in the corpus callosum of the same animal. Average trace is of the four events shown. F) Examples of spontaneous EPSPs (sEPSPs) from the same cell as in (E), highlighting the small depolarization produced by these synaptic currents. Average trace is of the four events shown.

Figure 2. Expression of ion channel and neurotransmitter receptor genes at different stages in the oligodendrocyte lineage

Cumulative fraction of genes expressed at or below 0.1-100 FPKM in NG2⁺ cells (blue), premyelinating oligodendrocytes (green), and mature oligodendrocytes (red) of 290 genes identified from the RNA-Seq database (Zhang et al., 2014) representing ion channels and neurotransmitter receptors. This pool includes all genes listed in Tables 1-9, in addition to other genes from the same categories expressed from 0.1-2 FPKM. Bin size = 0.1 FPKM. The distributions are significantly different from one another in pairwise comparisons (two-sample Kolmogorov–Smirnov test; NG2⁺ cells vs. premyelinating oligodendrocytes: $D = 0.17$, $Prob>|D| = 2.8 \times 10^{-4}$; NG2⁺ cells vs. mature oligodendrocytes: $D = 0.3$, $Prob>|D| = 6.1 \times 10^{-12}$; Premyelinating oligodendrocytes vs. mature oligodendrocytes: $D = 0.13$, $Prob>|D| = 0.01$). The expression of ion channel and neurotransmitter receptor genes is significantly reduced as NG2⁺ cells differentiate into oligodendrocytes.

Table 1**K⁺ channels**

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Gutman et al., 2005)
<i>Kcnj10</i>	Kir4.1	82.6	Inward rectifier
<i>Kcnmb4</i>	K _{Ca} β ₄	45.5	BK (β subunit)
<i>Kcnd2</i>	K _v 4.2	27.0	A-type
<i>Kcna6</i>	K _v 1.6	24.1	Delayed rectifier
<i>Kcnk1</i>	K _{2p} 1.1/TWIK1	24.0	Inward rectifier (two pore)
<i>Kcnd3</i>	K _v 4.3	22.3	A-type
<i>Kcnk2</i>	K _{2p} 2.1/TREK1	19.6	Inward rectifier (two pore)
<i>Kcnq2</i>	K _v 7.2	10.9	Delayed rectifier
<i>Kcnc3</i>	K _v 3.3	8.9	A-type
<i>Kcnab2</i>	K _v β ₂	8.8	K _v (β subunit)
<i>Kcnk10</i>	K _{2p} 10.1	7.9	Inward rectifier (two pore)
<i>Kcnj16</i>	Kir5.1	7.0	Inward rectifier
<i>Kcnb1</i>	K _v 2.1	6.8	Delayed rectifier
<i>Kcnma1</i>	K _{Ca} 1.1	6.3	Large conductance calcium-activated (BK)
<i>Kcnh2</i>	K _v 11.1/HERG	5.5	Inward rectifier
<i>Kcnn1</i>	K _{Ca} 2.1	4.9	Small conductance calcium-activated (SK)
<i>Kcna2</i>	K _v 1.2	4.8	Delayed rectifier
<i>Kcnab3</i>	K _v β ₃	4.6	K _v (β subunit)
<i>Kcne4</i>	MiRP3	4.5	K _v (β subunit)
<i>Kcnab1</i>	K _v β ₁	4.5	K _v (β subunit)
<i>Kcnc1</i>	K _v 3.1	4.1	Delayed rectifier
<i>Kcnh8</i>	K _v 12.1	4.1	Slowly activating/inactivating voltage-gated channel
<i>Kcnn2</i>	K _{Ca} 2.2	3.7	Small conductance calcium-activated (SK)
<i>Kcna3</i>	K _v 1.3	3.6	Delayed rectifier
<i>Kcnh3</i>	K _v 12.2	3.6	Voltage gated
<i>Kcnj3</i>	Kir3.1	3.4	Inward rectifier
<i>Kcna1</i>	K _v 1.1	3.4	Delayed rectifier
<i>Kcnh5</i>	K _v 10.2	3.1	Outward rectifier, noninactivating
<i>Kcnd1</i>	K _v 4.1	2.9	A-type
<i>Kcnq3</i>	K _v 7.3	2.8	Delayed rectifier
<i>Kcnc4</i>	K _v 3.4	2.4	A-type
<i>Kcnf1</i>	K _v 5.1	2.3	Modifier of K _v 2 channels

Table 2**Na⁺ channels**

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Catterall et al., 2005a)
<i>Nalcn</i>	Nacln	19.3	Sodium leak channel
<i>Scn1b</i>	Na _v β ₁	14.5	Voltage-gated (β subunit)
<i>Scn3b</i>	Na _v β ₃	12.9	Voltage-gated (β subunit)
<i>Scn3a</i>	Na _v 1.3	11.4	Voltage-gated (TTX sensitive)
<i>Scn2a1</i>	Na _v 1.2	7.6	Voltage-gated (TTX sensitive)
<i>Scn8a</i>	Na _v 1.8	5.0	Voltage-gated (TTX resistant)
<i>Scn1a</i>	Na _v 1.1	4.9	Voltage-gated (TTX sensitive)
<i>Scn2b</i>	Na _v β ₂	4.8	Voltage-gated (β subunit)

Table 3
Ca²⁺ channels

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Catterall et al., 2005b)
<i>Cacng4</i>	TARP γ_4	275.8	γ subunit *
<i>Cacng7</i>	TARP γ_7	103.0	γ subunit *
<i>Cacnb3</i>	Cav β_3	25.7	β subunit
<i>Cacng8</i>	TARP γ_8	12.0	γ subunit *
<i>Cacng5</i>	TARP γ_5	11.7	γ subunit *
<i>Cacnb4</i>	Cav β_4	8.4	β subunit
<i>Cacna2d3</i>	Cav $\alpha_2\delta_3$	8.0	$\alpha_2\delta$ subunit
<i>Cacna1d</i>	Cav1.3	7.8	α_1 subunit, L-type
<i>Cacna1g</i>	Cav3.1	7.7	α_1 subunit, T-type
<i>Cacnb1</i>	Cav β_1	7.2	β subunit
<i>Cacna2d1</i>	Cav $\alpha_2\delta_1$	7.0	$\alpha_2\delta$ subunit
<i>Cacna1c</i>	Cav1.2	6.5	α_1 subunit, L-type
<i>Cacna1a</i>	Cav2.1	6.5	α_1 subunit, P/Q-type
<i>Cacna1e</i>	Cav2.3	6.0	α_1 subunit, R-type
<i>Cacna1h</i>	Cav3.2	4.1	α_1 subunit, T-type
<i>Cacna2d2</i>	Cav $\alpha_2\delta_2$	2.8	$\alpha_2\delta$ subunit
<i>Cacna1b</i>	Cav2.2	2.2	α_1 subunit, N-type

* Associates with both Ca²⁺ channels and AMPARs (Black, 2003)

Table 4**Cl⁻ channels**

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Walz, 2002)
<i>Clcn2</i>	Clc2	9.2	Hyperpolarization-activated Inward rectifying

HCN channels**Table 5**

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Wahl-Schott and Biel, 2009)
<i>Hcn2</i>	HAC1, BCNG2	14.8	Mixed Na ⁺ /K ⁺ current
<i>Hcn3</i>	HAC3, BCNG4	3.7	Mixed Na ⁺ /K ⁺ current

TRP channels**Table 6**

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Clapham et al., 2005)
<i>Trpm7</i>	CHAK-1, LTrpC-7	9.2	Contains a Ser-Thr kinase domain. Outward rectifier. Permeable to Na ⁺ , Ca ²⁺ , and Mg ²⁺
<i>Trpc1</i>	Trp1	4.2	Nonselective cation channel
<i>Trpc2</i>	mTrp2	2.6	Mouse pheromone channel, not expressed in humans
<i>Trpm3</i>	LTrpC-3	2.2	Nonselective cation channel

Table 7
Glutamate receptors

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Traynelis et al., 2010)
<i>Gria2</i>	GluA2, GluR2	108.4	AMPA
<i>Gria3</i>	GluA3, GluR3	54.4	AMPA
<i>Grik5</i>	GluK5, KA2	47.4	Kainate
<i>Gria4</i>	GluA4, GluR4	37.8	AMPA
<i>Grid1</i>	GluD1, $\delta 1$	30.1	Orphan
<i>Grin3a</i>	GluN3A	23.5	NMDAR
<i>Grik4</i>	GluK4, KA1	19.3	Kainate
<i>Grin1</i>	GluN1, NR1	16.3	NMDAR
<i>Grik3</i>	GluK3, GluR7	14.9	Kainate
<i>Grik2</i>	GluK2, GluR6	14.0	Kainate
<i>Grm5</i>	mGluR5	13.3	Metabotropic (Group I)
<i>Grik1</i>	GluK1, GluR5	12.7	Kainate
<i>Grid2</i>	GluD2, $\delta 2$	11.9	Orphan
<i>Gria1</i>	GluA1, GluR1	10.4	AMPA
<i>Grm3</i>	mGluR3	7.7	Metabotropic (Group II)
<i>Grin2c</i>	GluN2C, NR2C	5.5	NMDAR
<i>Grin2d</i>	GluN2D, NR2D	3.9	NMDAR
<i>Grm4</i>	mGluR4	2.1	Metabotropic (Group III)

Table 8
GABA receptors

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Benarroch, 2012; Olsen and Sieghart, 2008)
<i>Gabbr1</i>	GABA _B R1	55.4	Metabotropic
<i>Gabra3</i>	GABA _A R α 3	23.7	Ionotropic
<i>Gabrb3</i>	GABA _A R β 3	21.7	Ionotropic
<i>Gabbr2</i>	GABA _B R2	12.7	Metabotropic
<i>Gabrg1</i>	GABA _A R γ 1	12.2	Ionotropic
<i>Gabrb2</i>	GABA _A R β 2	10.9	Ionotropic
<i>Gabra2</i>	GABA _A R α 2	5.8	Ionotropic
<i>Gabra4</i>	GABA _A R α 4	3.9	Ionotropic
<i>Gabrg2</i>	GABA _A R γ 2	3.7	Ionotropic
<i>Gabra1</i>	GABA _A R α 1	3.6	Ionotropic
<i>Gabrb1</i>	GABA _A R β 1	3.5	Ionotropic
<i>Gabrg3</i>	GABA _A R γ 3	3.2	Ionotropic

Table 9
Receptors for neuromodulators

<i>Gene Name</i>	Also known as	Expression level in NG2 ⁺ cells (FPKM)	Additional Information (Abbracchio et al., 2006; Burnstock et al., 2011; Bylund et al., 1994; Caulfield and Birdsall, 1998; Fredholm et al., 2011; Khakh et al., 2001; Lukas et al., 1999; Lynch, 2009)
<i>P2ry12</i>	P2Y ₁₂	67.0 [*]	Metabotropic purinergic receptor
<i>Adora1</i>	A ₁	41.9	Adenosine receptor
<i>Chrna4</i>	nAChR α 4	37.7	Nicotinic acetylcholine receptor
<i>Glyrb</i>	GlyR β	36.8	Glycine receptor
<i>P2rx7</i>	P2X ₇	21.0	Ionotropic purinergic receptor
<i>Adora2b</i>	A _{2B}	8.8	Adenosine receptor
<i>P2rx4</i>	P2X ₄	5.6	Ionotropic purinergic receptor
<i>P2ry1</i>	P2Y ₁	5.0	Metabotropic purinergic receptor
<i>P2ry6</i>	P2Y ₆	2.7 [*]	Metabotropic purinergic receptor
<i>Adrb2</i>	β ₂ AR	2.4 [*]	β adrenergic receptor
<i>Chrna7</i>	nAChR α 7	2.2	Nicotinic acetylcholine receptor
<i>Chrm1</i>	mAChR1	2.1	Muscarinic acetylcholine receptor
<i>Adra1A</i>	α _{1A} AR	2.1	α adrenergic receptor
<i>Chrb2</i>	nAChR β 2	2.0	Nicotinic acetylcholine receptor
<i>P2ry2</i>	P2Y ₂	2.0	Metabotropic purinergic receptor

* These genes have very high expression in microglia, which are known to be a 5% contaminant in the NG2⁺ cell sample