Published in final edited form as:

Dev Neurobiol. 2012 June ; 72(6): 805–820. doi:10.1002/dneu.20977.

Isolation of a Novel Rat Neural Progenitor Clone that Expresses *Dlx* Family Transcription Factors and Gives Rise to Functional GABAergic Neurons in Culture

Hedong Li^{1,2,4}, Anna T. Hader², Yu R. Han², Joseph A. Wong¹, Joanne Babiarz^{1,2}, Christopher L. Ricupero^{1,2}, Sasha Blue Godfrey¹, John P. Corradi³, Myles Fennell³, Ronald P. Hart^{1,2}, Mark R. Plummer², and Martin Grumet^{1,2,*}

¹W.M. Keck Center for Collaborative Neuroscience, Rutgers Stem Cell Research Center, Rutgers, State University of New Jersey, 604 Allison Rd, Piscataway, NJ 08854-8082

²Department of Cell Biology & Neuroscience, Rutgers, State University of New Jersey, 604 Allison Rd, Piscataway, NJ 08854-8082

³Bristol-Myers Squibb Company, 5 Research Parkway, Wallingford, CT 06492

⁴West China Developmental & Stem Cell Institute, West China 2nd University Hospital, Sichuan University, Chengdu 610041, P.R. China

Abstract

GABAergic interneurons are lost in conditions including epilepsy and CNS injury, but there are few culture models available to study their function. Towards the goal of obtaining renewable sources of GABAergic neurons, we used the molecular profile of a functionally-incomplete GABAergic precursor clone to screen 17 new clones isolated from GFP⁺ rat E14.5 cortex and ganglionic eminence (GE) that were generated by viral introduction of v-myc. The clones grow as neurospheres in medium with FGF2, and after withdrawal of FGF2 they exhibit varying patterns of differentiation. Transcriptional profiling and qPCR indicated that one clone (GE6) expresses high levels of mRNAs encoding *Dlx*1, 2, 5 and 6, glutamate decarboxylases, and presynaptic proteins including neuropeptide Y and somatostatin. Protein expression confirmed that GE6 is a progenitor with restricted differentiation giving rise mostly to neurons with GABAergic markers. In co-cultures with hippocampal neurons, GE6 neurons became electrically excitable and received both inhibitory and excitatory synapses. After withdrawal of FGF2 in cultures of GE6 alone, neurons matured to express β III-tubulin, and staining for synaptophysin and vesicular GABA transporter (VGAT) were robust after 1-2 weeks of differentiation. GE6 neurons also became electrically excitable and displayed synaptic activity, but synaptic currents were carried by chloride and were blocked by bicuculline. The results suggest that the GE6 clone, which is ventrally derived from the GE, resembles GABAergic interneuron progenitors that migrate into the developing forebrain. This is the first report of a relatively stable fetal clone that can be differentiated into GABAergic interneurons with functional synapses.

Keywords

neuronal progenitor; v-myc; GABA; GABAergic development

*Correspondence should be addressed to: Dr. Martin Grumet or Dr. Hedong Li W. M. Keck Center for Collaborative Neuroscience Rutgers, State University of New Jersey 604 Allison Rd, Piscataway, NJ 08854-8082 Tel. 732/445-6577 Fax. 732/445-2063 mgrumet@rci.rutgers.edu.

INTRODUCTION

GABAergic interneurons are critical components of neural circuitry that modulate synaptic activation by secreting gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain. GABAergic inhibitory interneurons synapse on glutamatergic projection neurons and modulate their functions in the forebrain. During development, glutamatergic projection neurons migrate radially from the ventricular zone to the forebrain (Englund et al., 2005; Hevner, 2006). Most GABAergic neurons originate in the subpallium of the forebrain, and migrate tangentially into the developing cortex in rodents (Anderson et al., 1997; Lavdas et al., 1999; Pleasure et al., 2000; Anderson et al., 2001; Wichterle et al., 2001; Xu et al., 2004; Cobos et al., 2005; Cobos et al., 2007). Ventrally derived interneurons and their precursors express *Dlx-1* and *Dlx-2* transcription factors, which are essential for their tangential migration into the dorsal cortex (Anderson et al., 1997). Additionally, *Dlx-1* and *Dlx-2* mRNAs are expressed in the medial GE (MGE) and lateral GE in a restricted pattern (Bulfone et al., 1993; Eisenstat et al., 1999) and their expression is critical for normal patterns of dendritic branching, axon growth, apoptosis, and cortical layering in vivo (Cobos et al., 2005; Cobos et al., 2007).

Interneurons are particularly sensitive to brain injury resulting from trauma and their loss results in decreased GABAergic tone and hyperexcitability (Fritsch et al., 2009). This loss is believed to exacerbate seizures in the traumatized brain and transplantation of cells that secrete GABA has been shown to reduce seizures in models of temporal lobe epilepsy (Lowenstein et al., 1992). While cell transplantation holds promise for future treatments, much needs to be learned about GABAergic interneurons and their progenitors so that they can be used effectively as long-term treatments for disease processes such as epilepsy. Mature GABAergic interneurons exhibit variable features and can be subclassified by their morphologies (Monyer and Markram, 2004; Ascoli et al., 2008; Gelman and Marin, 2010), by their expression of calcium-binding proteins (calretinin, calbindin and parvalbumin) and specific neuropeptides (neuropeptide Y, somatostatin, neurotensin and vasoactive intestinal peptide) (Marin et al., 2000; Xu et al., 2004). Importantly, certain subtypes of GABAergic interneurons have been correlated with neurological diseases such as Schizophrenia (Woo and Lu, 2006) and transplantation of GABAergic neurons after spinal cord injury alleviates allodynia (Mukhida et al., 2007; Eaton and Wolfe, 2009).

To overcome the limited intrinsic capacity of the central nervous system (CNS) for neuronal replacement, it is critical to identify appropriate sources of stem or progenitor cells that will integrate and differentiate in adult regions of interest to restore function after disease or trauma. It is becoming increasingly clear from transplantation research that simply supplying neurons that replace a deficiency in a neurotransmitter like dopamine (Allan et al., 2010; Rhee et al., 2011) or GABA (Eaton et al., 1999; Becerra et al., 2007; Mukhida et al., 2007) may not provide a long-term solution. One must overcome the challenges that mature neurons do not integrate effectively in the adult CNS and that neuronal progenitors may not differentiate sufficiently to obtain long-term survival with functional synapses. Human embryonic stem cells have been induced to give rise to GABAergic neurons (Nat and Dechant, 2011) and this approach or the use of induced pluripotent stem cells (iPSCs) (Zhang et al., 2010) may provide sufficient sources of potential cells. However, there is little understanding of the developmental signals including epigenetic modifications that will yield highly enriched populations of neurons capable of differentiation. The GE has been identified as the source of different GABAergic progenitors during mammalian development in vivo but these cells are difficult to isolate in a pure form. Isolation of such cells would allow detailed transcriptional profiling to describe molecular changes that accompany, and are responsible for, development of GABAergic neurons as well as physiological studies of the purified cells.

One approach to limit heterogeneity among the GABAergic progenitors is to isolate stabilized clones of cells. The expression of myc in neural stem/progenitor cells has yielded stabilized clones of cells that can be expanded in the presence of growth factors such as FGF2, and after withdrawal, the cells differentiate into different types of mature neural cells from mouse and rat (Whittemore and Snyder, 1996; Vescovi and Snyder, 1999; Li et al., 2004; Hasegawa et al., 2005; Li et al., 2008). The v-myc-transformed mouse clone C17.2 (Snyder et al., 1992) has been used extensively for transplantation in mammalian models of neural disease. Our expression profiling on undifferentiated rat progenitors shows robust differences among different clones that correlate with their developmental fates after differentiation in vitro. These expression profiles of progenitors may be used to predict cell fates and may be useful for identifying candidate cells that will acquire particular properties and functions. In this study, we have used an expression profile for the L2.2 clone (Li et al., 2008), which is a GABAergic progenitor, to identify a new clone isolated from the GE called GE6. In contrast to L2.2 that developed sufficiently to support action potentials but did not show synaptic activity, GE6 cells matured further to form synapses in co-cultures with hippocampal neurons as well as in culture by themselves. Expression profiling revealed significant differences between these two clones of GABAergic progenitors in expression of gene products involved in neuronal maturation and synaptogenesis.

METHODS

Generation, cell culture and differentiation of neural progenitor clones

Generation and culture of progenitor clones from embryonic rat embryonic cortical cultures after viral infection to induce v-myc expression were described previously (Li et al., 2004). Following the same protocol, we isolated 17 v-myc transduced clones from E14.5 dorsal (Ctx) and ventral (GE) forebrains from GFP⁺ Fischer rats (Marano et al., 2008). The progenitor cell differentiation protocol has been described previously (Li et al., 2008). Briefly, immortalized clones were cultured overnight on poly-lysine/laminin-coated substrates in FGF2 containing DMEM/F12 culture medium (proliferation medium). For differentiation, the medium was removed and replaced with DMEM/F12/B27 medium lacking FGF2 (DMEM/F12) (differentiation medium) and 0.5% fetal bovine serum (FBS) was added to promote cell survival. To optimize for more rapid differentiation of GE6 into neurons, we developed a procedure involving a pre-differentiation step in which cells were grown on poly-lysine-coated dishes in differentiation medium for 3 days. Then the supernatant containing dead cells was removed and the attached cells that began to exhibit morphological changes were removed by treatment with trypsin and replated on poly-lysine-coated substrates in differentiation medium on glass (with laminin) coverslips or 35 mm dishes (without laminin) for immunostaining or electrophysiological recordings.

Progenitor clone GE6 in hippocampal neuronal co-cultures

Hippocampal neuronal cultures were prepared as previously described (Magby et al., 2006). The proliferating GE6 cells were prepared in single cell suspension and added to the center of 7-day cultures of E17 hippocampal cells seeded at 10,000 cells/dish. Whole-cell patch clamp recordings on GE6 cells were performed after additional incubation for 7 to 14 days in co-cultures as indicated. GE6 cells were identified by their intrinsic GFP signal under a fluorescent microscope. After recording, selected cultures were fixed with 4% paraformaldehyde in PBS and stained with pre- and post-synaptic markers indicated.

Gene expression analysis

Progenitor cells were cultured on laminin-coated 35 mm dishes in DMEM/F12 serum free medium containing FGF2 (10 ng/ml) at 3×10^5 cells per dish. The next day, differentiation was initiated by changing to differentiation medium lacking FGF2 and including 0.5% FBS.

Triplicate cultures were harvested at day 0 (prior to FGF2 withdrawal), and 3 or 7 d after differentiation. RNA was prepared with Trizol lysis buffer (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. All target-labeling reagents were purchased from Affymetrix (Santa Clara, CA). Double-stranded cDNAs were synthesized from 1.2 µg total RNA through reverse transcription with an oligo-dT primer containing the T7 RNA polymerase promoter and double strand conversion using the cDNA Synthesis System. Biotin-labeled cRNA was generated from the cDNA and used to probe Affymetrix HT_RAT-FOCUS arrays. All cDNA and cRNA target preparation steps were processed on a Caliper GeneChip Array Station from Affymetrix. Array hybridization, washing and scanning were performed according to the manufacturer's recommendations. Scanned images were subjected to visual inspection and a chip quality report was generated by the Affymetrix's Expression console. The data quality control was performed by Partek Genomic Suite (Partek, Inc., St. Louis, MO). The image data was processed utilizing the Affymetrix GCOS algorithm and the Robust Multiarray Average (RMA) method to determine the specific hybridizing signal for each gene (Irizarry et al., 2003). Principal component analysis of the samples was carried out using all of the microarray data. A 2-way ANOVA using clone and time in differentiating conditions as factors identified differentially expressed genes. Heatmaps were generated with z-scored data to enable direct comparison of profiles. Microarray data have been deposited with NIH GEO (Accession number GSE31635).

Quantitative RT-PCR (qPCR)

qPCR was performed as described previously (Li et al., 2003). *GAPDH* was used to normalize the expression levels of each sample. Primers for detecting genes are as described previously (Li et al., 2008) or as shown in Table 1.

Immunocytochemistry

The methods for immunocytochemistry were described previously (Li et al., 2004). Antibodies used in this study were mouse IgGs: anti-vimentin (1:10, DSHB), anti-GFAP (1:200, Beringher), anti-nestin (1:20, DSHB), anti-β-III tubulin (1:500, TuJ1, Covance), anti-GalC (1:50, McKinnon lab), anti-parvalbumin (1:200, Chemicon), and anti-calbindin (1:200, Sigma), anti-Gephyrin (1:200, Synaptic Systems), anti-VGAT (1:200, Synaptic Systems), anti-VGlut1 (1:200, Synaptic Systems); rabbit IgGs: anti-BLBP (1:1000, Chemicon), anti-GFAP (1:200, Dako), anti-GAD65/67 (1:200, Chemicon), anti-calretinin (1:1000, Chemicon), anti-neuropeptide Y (1:500, Chemicon), and anti-somatostatin (1:200), anti-Synaptophysin (1:200, Synaptic Systems); chicken IgY: anti-β-III tubulin (1:500, Aves). Secondary antibodies included Oregon-Green-, AMCA- or Rhodamine-Red-conjugated antibodies against appropriate species (1:200, Molecular Probes). DAPI (10 µg/ml, Sigma) was included in the secondary antibody incubations to label nuclei.

Western blot analysis

Western blot analysis was carried out following methods previously described (Li et al., 2008). The blots were developed using ECL plus detection system (GE Healthcare Amersham). Anti-GAPDH (mouse IgG, 1:1000, Chemicon) was used to normalize the sample loading.

Electrophysiological techniques

Whole-cell patch-clamp and current-clamp recordings were performed following methods previously described (Li et al., 2008). After establishing a gigohm seal and rupturing the cell membrane (break-in), the holding potential was set to −70 mV. A series of test potentials was given to measure the amplitude of the voltage-gated Na current. Ongoing

synaptic activity was characterized using voltage-clamp mode for 7–8 min post-break-in. Using break-in as the time point zero, analysis was initiated at 2–3 min post-break-in depending on cell stability. This resulted in ~5 min of analysis per recording. Evoked synaptic activity was measured using extracellular field stimulation with a fine-tipped electrode (Maximov et al., 2007). The recording mode was subsequently changed to current-clamp to assess action potential amplitude and time course. Between 1 and 4 recordings were made from each dish of cells. Signals were recorded with an Axoclamp 200 amplifier, digitized at 2.9 kHz, and filtered at 2 kHz with acquisition and analysis controlled with custom-written software. The bath solution, called neuron recording solution or NRS, consisted of (in mM): 1.67 CaCl₂, 1 MgCl₂, 5.36 KCl, 137 NaCl, 17 glucose, 10 HEPES, and 13.15 sucrose, pH 7.5 (NaOH). The pipette solution contained (in mM): 105 K-methanesulfonate, 17.5 KCl, 10 HEPES, 0.2 EGTA, 8 NaCl, and freshly added 2 Mg-ATP, 2 Na₂-ATP, and 20 phosphocreatine, pH 7.3 (KOH). All reagents were purchased from Sigma.

RESULTS

Isolation and analysis of neural stem/progenitor clones from dorsal and ventral forebrain

A goal of this study was to isolate progenitor clones for GABAergic neurons that could develop functional synapses. Clone L2.2 was found previously to differentiate into neurons that exhibited GABAergic properties but they were unable to form synapses (Li et al., 2008). Therefore, we hypothesized that the distinct molecular profile of undifferentiated L2.2 would be useful for identifying additional GABAergic progenitor clones prior to differentiation. The resulting clones could then be differentiated and tested for formation of functional synapses. To screen the clones obtained prior to differentiation, we prepared RNA and performed qPCR analysis comparing the selected genes. The target genes (Fig. 1A) included several that are differentially expressed between the neuronal progenitor clone L2.2 and the multipotential clone L2.3 including *BLBP* (*FABP7*), a marker for radial glial neural stem cells (NSC) (Li et al., 2008). Transcription factors *Dlx1* and *Dlx2* were weakly expressed in all but one clone, GE6, which expressed these at high levels. Many clones showed strong signals for *BLBP* suggesting they are multipotential NSC (Anthony et al., 2004) and many also expressed the transcription factors *Olig-2* (Petryniak et al., 2007) and *Pax6* (Gomez-Lopez et al., 2011) found in neural progenitors. Clustering based on the expression of these seven genes among various clones clearly segregated GE6 with L2.2 into a subgroup indicating that they are the most closely related among these clones (Fig. 1B).

To analyze their developmental potentials, each clone was cultured for 6 days in the absence of FGF2 and then assessed by immunofluorescence staining using anti-TuJ1 and anti-GFAP to identify neurons and astroglia, respectively. GE6 cultures had primarily TuJ1⁺ neurons and showed little staining for GFAP. GE2 cultures had only astroglia and Ctx8 cultures showed the presence of both neurons and astroglia (Fig. 2A). Expression patterns of most of the other clones resembled Ctx8, except glial-like clones Ctx7 and GE4, 5, and 8. For additional characterization we focused on GE6 and compared it to Ctx8 and GE2 as exemplars of multipotential and gliogenic progenitors, respectively. Immunoblotting with antibodies from extracts of the differentiated clones confirmed expression of TuJ1 in GE6 and Ctx8, and GFAP in GE2 and Ctx8 (Fig. 2B). The combined results indicate that GE6 gives rise primarily to neurons, Ctx8 is multipotential, and GE2 gives rise to astrocytes but not to neurons. Similar immunological analyses of the remaining clones suggested that the major cluster in the dendrogram correspond to 12 multipotential clones including Ctx8. The cluster of four clones including GE2 all were gliogenic and had high levels of *Pax6* expression, which is expressed in mature astrocytes (Sakurai and Osumi, 2008).

GE6 cells differentiate into distinct GABAergic interneuron subtypes

The properties of GE6 being derived from the ventral forebrain, expressing *Dlx1/2*, and differentiating into neurons, suggested this clone is a GABAergic interneuron progenitor. We therefore analyzed the phenotype of GE6 when induced to differentiate simply by removing FGF2. After 6 days of differentiation, neurons were detected by immunostaining for TuJ1 and nearly every positive cell also expressed glutamic acid decarboxylase (GAD65/57) suggesting they are GABAergic (Fig. 3A). Additional immunostaining for interneuron subtype markers revealed that nearly all GE6 neurons also express neuropeptide Y (Npy) and somatostatin (Sst), and only a small subset were positive for calretinin (Fig. 3A). The differentiation phenotype of clone GE6 contrasts with GABAergic progenitor clone L2.2, which gives rise to mostly calretinin⁺ cells but to very few Npy- and Sst-positive interneuron subtypes (Li et al., 2008). Parvalbumin (PV) expression was also detected in GE6 neurons after differentiation for one (Fig. 3B) and two-weeks of differentiation (data not shown). These differences in interneuron phenotype between the L2.2 and GE6 GABAergic progenitor clones was also confirmed by western blot analysis (Fig. 3B) where calretinin expression was high in L2.2 and low in GE6. Conversely, PV, Npy and Sst expression were higher in GE6 than in L2.2. These patterns of expression in GE6 cells are characteristic for interneurons derived from the MGE (Wonders and Anderson, 2006)

Electrophysiological interactions of GE6 cells in co-cultures with hippocampal neurons

To determine whether GE6 neurons can interact functionally with other neurons they were co-cultured with hippocampal neurons. Hippocampal neurons were chosen because cultures prepared from E18 embryos have been well-characterized both electrophysiologically and immunocytochemically (Craig et al., 1993; Craig et al., 1994; Craig et al., 1996). The cultures contain both glutamatergic and GABAergic neurons and display ongoing synaptic activity that can be eliminated almost completely by TTX indicating that it is action potential-evoked (Magby et al., 2006). Thus the potential exists for synaptically competent hippocampal neurons to connect with the differentiated GE6 cells.

The GE6 neurons were identified in these cultures by their intrinsic GFP fluorescence that was also visible in multiple processes (Fig. 4A). GFP⁺ cells showed depolarization-induced action potential firing when recorded in current-clamp mode (Fig. 4B). In voltage clamp mode, we detected ongoing synaptic activity in GE6 cells including EPSCs (circles) and IPSCs (triangles) at a holding potential of -70 mV (Fig. 4C, top trace). Setting the holding potential to -20 mV illustrated the different reversal potentials for the two types of synaptic currents (Fig. 4C, bottom trace). To analyze the IPSPs, we utilized bicuculline, a competitive antagonist for GABA_A receptors. Under baseline conditions (NRS bath solution, upper 2 traces) mostly IPSCs and occasional EPSCs were seen (Fig. 4D). Following bath perfusion with 50 mM bicuculline, IPSCs were no longer present and bursts of EPSCs became evident (middle 2 traces). Upon perfusion of both bicuculline and 10 mM 6,7-dinitroquinoxaline-2,3-dione (DNQX), an AMPA and Kainate receptor antagonist, ongoing synaptic activity was virtually eliminated (Fig. 4D, bottom 2 traces). These results suggest that GE6 neurons can propagate action potentials and receive both excitatory and inhibitory synapses.

The timing of synapse formation was analyzed after different periods of GE6 co-culture with hippocampal neurons. Nearly 80% of GE6 cells exhibited action potentials within 5-9 days but less than 10% of these had functional synapses. By 12-15 days over 90% exhibited action potentials and the majority of these cells had functional synapses. One week later all cells tested exhibited action potentials and had functional synapses (Fig. 4E), demonstrating that differentiated GE6 neurons function for extended times in culture.

Expression Profiling of GE6 and Differentiation

The results above show that undifferentiated GE6 cells acquire GABAergic properties after differentiation, becoming electrically excitable and forming synapses. The GE6 cells were chosen based on a limited molecular expression profile found in L2.2 cells. Considering that both GE6 and L2.2 showed GABAergic differentiation but only GE6 formed synapses, we reasoned that unbiased molecular profiling comparing these two GABAergic progenitors may reveal sets of genes that promote maturation of GABAergic neurons and synapse formation. To broaden the scope of the comparison against other types of progenitors, we tested other clones that were prepared by similar methods, including the multipotential clones Ctx8 and L2.3 (isolated concurrently with L2.2) (Li et al., 2004) and the gliogenic clone GE2. Cultures for each clone were used to prepare mRNA for expression profiling under identical conditions at 3 and 7 days after withdrawal of FGF2 and compared to the non-differentiated cells (Day 0). Principal component analysis showed similar patterns for the two multipotential clones Ctx8 and L2.3 as well as with the L2.2 clone but the GE6 clone segregated from this cluster (Fig. 5A). This indicated that there are substantial differences between the GABAergic progenitor clones L2.2 and GE6. Remarkably, the GE2 clone was very different from the rest, consistent with its divergent gliogenic fate.

We next analyzed microarray expression results for individual genes that are widely used as cell type markers. As expected, levels of the neural stem marker *nestin* decreased with increasing times of differentiation, although levels were low in L2.2 even prior to differentiation (Fig 5B). Dramatic and rapid increases were observed in the β *III-tubulin* in GE6, L2.2, and L2.3 that are consistent with the generation of neurons in these clones with differentiation. The glial clone GE2 as well as the multipotential clones showed early increases in *GFAP* and *S100B* during differentiation, indicating gliogenesis. However, these astroglial markers showed only weak and delayed expression in GE6 (Fig. 5B), consistent with the weak detection of astroglia in culture by GFAP staining (Fig. 2A).

To understand further the maturation of these different clones, we selected sets of transcription factors, neurotransmitters and synaptic proteins from the microarray results for qPCR analysis of mRNA levels (Fig. 5C). Undifferentiated GE6 cells initially expressed *Olig1/2* and *Ascl1* but their levels declined rapidly with differentiation and were nearly undetectable by 3 days. At this point levels of *Dlx1* and *Dlx5* were high and remained elevated through day 7. This transition suggests a neuronal GABAergic phenotype and was accompanied by substantial increases in expression of mRNAs for several neurotransmitters (*Npy* and *Sst*) and presynaptic proteins (*Snap25*, *Stxbp1*, and *Syt1*, 4, 5, 6) that were highest 7 days after differentiation. By contrast, L2.2 showed partially overlapping but much weaker expression of some of these genes including *Dlx1/5*, *Npy* and several synaptic proteins (Fig. 5C). The inability of L2.2 to express robust levels of these mRNAs may result in low levels of critical proteins that may explain why L2.2 could not form synapses. In contrast to these neuronal progenitor clones, there was stark contrast with the gliogenic GE2 clone, which expressed *Pax6* but not the other transcription factors. Additional differences were observed vs. the two multipotential clones Ctx8 and L2.3 that showed some similarity with each other in initial expression of *Olig1/2* and *Ascl1*, but there was little similarity to the other clones.

Given the importance of *Dlx5* and *Dlx6* in addition to *Dlx1* and *Dlx2* in differentiation of GABAergic neurons, we performed qPCR analysis because *Dlx5* and *Dlx6* probes were not present on the microarray (Fig. 5D). We compared relative expression among GE6, GE2, and Ctx8 for these genes and several other genes. As expected, the GABAergic GE6 clone expressed increasing mRNA levels from all the *Dlx* genes (1, 2, 5, and 6) that were tested but there was little or no expression in the other two clones. The GABAergic GE6 clone also expressed increasing levels of *GAD-2* mRNA with differentiation, consistent with the GABAergic phenotype of GE6. We also tested for the expression of *v-myc* that was used to

stabilize the clones and found low expression in Ctx8, and decreasing expression in GE2 and GE6 with differentiation.

We further compared expression profile differences between GE6 and L2.2 that may provide a better understanding for their differences in maturation (Fig. 5E). The transcription factor *NeuroD1* was expressed transiently in L2.2 but not detected in GE6. *NeuroD1* is a transcription factor that inhibits expression of *Ascl1*, thereby allowing GABAergic differentiation to proceed (Roybon et al., 2010). In contrast to GE6 that expressed *Ascl1* prior to differentiation and downregulated it within 3 days, undifferentiated L2.2 did not express *Ascl1*, and upregulated it transiently at 3 days of differentiation (Fig. 5C). The expression at 3 days of *NeuroD1* is expected to inhibit expression of *Ascl1* (Roybon et al., 2010), which was no longer observed by 7 days (Fig. 5C and E). However, the delayed appearance of *Ascl1* may decrease the ability of L2.2 cells to differentiate into mature GABAergic neurons. Another gene product that regulates neural development, Neuregulin-1 (*Nrg1*), was expressed at 3-7 days after GE6 differentiation but was not detected in L2.2 cells. *Nrg1* promotes excitatory synapse development and function of GABAergic interneurons (Ting et al., 2011) and perhaps its absence from L2.2 cells contributes to their incomplete maturation.

Electrophysiological and histochemical maturation of GE6 alone

Given the robust ability of GE6 cells to mature after withdrawal of FGF2 to express neurotransmitters and synaptic proteins, we decided to study its maturation alone under the same culture conditions that were favorable for synapse formation with the hippocampal co-cultures. After 14 days of differentiation, spontaneous postsynaptic currents were detected (Fig. 6A). Based upon time course, all events from complete recordings in which synaptic activity could be analyzed were judged to be IPSCs. In only one recording did we observe any sign of potentially excitatory synaptic currents, and in this recording the events were infrequent and possibly non-synaptic. Thus within the limits of our detection, we consider the GE6 clone as producing a virtually pure population of inhibitory neurons under our culture conditions. To confirm this, we used extracellular field stimulation to obtain I-V relationships made by eliciting synaptic responses at different holding potentials (Fig. 6B). Consistent with a GABAergic phenotype, the responses reversed at a voltage close to the calculated chloride equilibrium potential (~-45 mV).

The timing of synapse formation was analyzed for early and late time periods (Fig. 6C, “nonprediff”). During the first 7-13 days in culture, GE6 cells exhibited action potentials but functional synapses were not detected. By 14-19 days over 90% of GE6 neurons exhibited action potentials only 36% of these cells had functional synapses. In no case did we see any evidence for autaptic transmission, suggesting that all the synaptic connections formed were intercellular. In parallel experiments with Ctx8 we detected some neurons with action potentials but no synaptic activity (data not shown).

In these differentiation conditions that contained 0.5 % FBS, the small number of astrocytes present expanded at longer incubation times. The presence of astrocytes per se has been reported to promote synapse formation but their overgrowth may limit cell-cell interaction among neurons. Thus, to limit growth of the astrocytes, we pre-differentiated GE6 cells in the absence of FGF2 and FBS for three days and then passed the cells into tissue culture dishes for incubation in differentiation medium with 0.5% FBS. In these pre-differentiated cultures there was a higher density of neurons and we detected synapses in >80% of the cells as early as 10 days after FGF2 withdrawal (3 days of pre-differentiation + 7 days in vitro after re-plating) and the cells continued to exhibit activity for 15-17 days (Fig 6C, “prediff”).

Analysis of the neurons in the GE6 cultures showed that nearly every TuJ1⁺ cell was double labeled with synaptophysin in a punctate pattern characteristic of neurons. The extensive overlap of TuJ1 with VGAT indicates these are GABAergic neurons (Fig. 7A). In contrast, the GE6 culture did not show staining for the glutamatergic VGlut-1 indicating that glutamatergic neurons were not derived from GE6 at least under the various culture conditions that we have tested. We also determined localization in the GE6 cultures of gephyrin since it found postsynaptically on GABAergic but not glutamatergic synapses (Craig et al., 1996). Gephyrin staining was detected in neurons that expressed synaptophysin most intensely in the perinuclear region and in punctate accumulations in the processes (Fig. 7B). The combined results indicate that neurons differentiated from the GE6 clone form GABAergic synapses.

DISCUSSION

We describe the generation and characterization of a panel of immortalized neural progenitor clones derived from E14.5 rat brains that proliferate in the presence of FGF2 and differentiate upon its withdrawal. Most of these clones are multipotential, representing NSC and a small group of clones gave rise to astrocytes upon differentiation. However, one clone, called GE6, isolated from the GE, gives rise primarily to GABAergic neurons. A combination of morphological and immunological assays demonstrated that individual clones have different fates, and molecular analyses demonstrated that clones with apparently similar fates can have major differences in gene expression patterns after differentiation. Moreover, mRNA profiling of different clones revealed dramatic differences in expression of transcription factors before differentiation that may predict different fates upon differentiation. GE6 exhibited a robust pattern of gene expression suggesting differentiation into GABAergic neurons that is consistent with morphological and electrophysiological analysis of the differentiated cells.

The origins of different types of GABAergic neurons have been studied extensively based on the expression of different neurotransmitters. GE6 cells express PV robustly, suggesting their origin is the MGE where NPY and SST are also expressed (Wonders and Anderson, 2006). In contrast, L2.2 did not express PV but did express CR, indicating that it did not originate from the MGE. The strong expression of calretinin (CR) in L2.2 suggests it may have arisen from the dorsal caudal GE (Wonders and Anderson, 2006). In any case, the complementary patterns of expression for PV and CR suggest L2.2 and GE6 originate from different regions of the GE.

The isolation of the GABAergic progenitor clone GE6 was guided by expression of a panel consisting mostly of transcription factors to distinguish among various undifferentiated clones by qPCR. This panel that included *Dlx* and *Olig* family genes clearly distinguished GE6 from the other 16 clones described here (Fig. 1). Remarkably, GE6 exhibited more robust GABAergic differentiation than the L2.2 clone that was used to generate the panel of markers used to isolate it. Moreover, this panel of 7 genes clearly distinguished the remaining 16 clones into subgroups of 4 gliogenic and 12 multipotential clones. Microarray analyses of representative clones from each of the three groups derived in this study and including two from a previous study (Li et al., 2004; Li et al., 2008) confirmed several expected expression changes during development for neural markers including nestin, β III-tubulin, and GFAP. Interestingly, the combined gene expression profiles for *Olig1/2*, *Ascl1*, *Dlx1/5* and *Pax6* were sufficient to distinguish among these five clones even before differentiation (Fig. 5C). The results suggest that these and perhaps additional transcription factors can be used to distinguish among populations of enriched cells before differentiation. In addition, clonal populations as described here may be particularly useful in identifying cell surface proteins that can then be used to identify and segregate cells from heterogeneous

populations that are typically obtained without clonal selection. This approach should be useful for a wide range of sources of NSC including those derived from primary fetal tissue, embryonic stem cells and iPSC. Recent progress demonstrates the ability to transfect genes involved in neuronal development (including *Ascl1*, *Brn2*, *Myt1*, and *NeuroD1*) into ESC and fibroblasts to generate mixtures of neurons but few report enrichment in GABAergic neurons (Vierbuchen et al., 2010; Pang et al., 2011). These panels of genes did not include those critical for development of GABAergic neurons such as *Dlx* family and perhaps their inclusion would yield much higher proportions of GABAergic neurons. Preliminary results demonstrate that transient overexpression of *Ascl1*, *Dlx1*, and *Dlx5* is sufficient to drive enhanced neurogenesis and synapse formation in Ctx8 (CLR and RPH, unpublished).

The newly analyzed clones GE2, GE6 and Ctx8 showed robust differences in gene expression that distinguished them clearly whereas the previously-identified clones L2.2 and L2.3 did not exhibit strong expression for most genes analyzed. The latter two were isolated by a similar method except they were treated with LIF for 2 days (Li et al., 2004). LIF treatment results in changes in gene expression (Li and Grumet, 2007) that preserve radial glia and favors astrogliogenesis (e.g. L2.3) (Du et al., 2006) but also yielded the neuronal progenitor clone L2.2. By comparison to GE6, L2.2 exhibits weak and delayed expression of key transcription factors, such as *Ascl1* that is critical for neurogenesis (Roybon et al., 2010) (Roybon et al., 2010) and *Dlx* genes that are critical for GABAergic differentiation (Petryniak et al., 2007). Thus it is not surprising that the maturation of L2.2 neurons is incomplete by comparison to GE6. The results suggest that early treatment of NSC with LIF may compromise their ability to yield mature neurons and it was not used in derivation of the 17 clones described here.

The observation that *Dlx1* and *Dlx2* expression increased in GE6 cells during development confirms in vivo results that these genes are found to down-regulate *Olig2* expression (Petryniak et al., 2007). Microarray and qPCR results confirm this transition and continued expression of *Ascl1* in these precursors has been reported to direct development of oligodendrocytes (Petryniak et al., 2007). However, we saw a dramatic decrease in *Ascl1* by 3 days of differentiation that was accompanied by only transient detection of small numbers of oligodendrocytes in our cultures that expressed galactocerebroside in a characteristic staining pattern (HL and MG unpublished observations). The failure of oligodendrocytes to survive development from GE6 may be due to inhibition by *Dlx1* and *Dlx2* (Petryniak et al., 2007), which are expressed robustly by day 3 of differentiation and maintain high levels at least through day 7. While the pattern of mRNA expression in undifferentiated Ctx8 showed similarities to undifferentiated GE6, qPCR and microarrays demonstrated differences in *Dlx-1*, -2, -5, and -6, *GAD-2*, *Npy* and *Syt6* as early as after 3 days of differentiation and subsequently that clearly distinguish these clones. The similarity in the undifferentiated cells may reflect that both are NSC and we suspect that subtle differences in these genes in combination with differences in other genes (e.g. *Nrg1* and *NeuroD1*, Fig. 5E) may be able to distinguish these related neural progenitors. Finally, robust expression of *Pax6*, which is important for gliogenesis (Sakurai and Osumi, 2008), was expressed strongly even in undifferentiated GE2 cells and it increased during differentiation. *Pax6* expression has been associated with radial glial cells and neurogenic determination but it has also been associated with a gliogenic fate depending on dosage and developmental stage-specific interactions with transcription factors partners (Gomez-Lopez et al., 2011).

Ventrally derived interneurons and their precursors express *Dlx* transcription factors, which are essential for their tangential migration into the dorsal cortex (Anderson et al., 1997). The expression in the GE6 clone of mRNAs for *Dlx-1*, -2, -5, and -6 as well as other mRNAs including *Sst* and *calbindin* (data not shown) is consistent with the ventral origin of cortex-invading interneuron precursors to the developing forebrain (Welagen and Anderson, 2011).

The relative deficiencies in expression of these and other synaptogenic genes in L2.2 cells by comparison to GE6 are likely to be contributing factors in the inability of L2.2 to establish synapses. Among these it is noteworthy that mutations in *Stxbp1* causes infantile epilepsy, suggesting the functional importance of this syntaxin binding protein (Saito et al., 2008) in GABAergic neurons.

Neural stem/progenitor cell clones generated by v-myc immortalization have been used in culture (Neville et al., 2009) and in vivo (Bakshi et al., 2006) as models for certain types of neural cells from both rodents (Li et al., 2004; Hasegawa et al., 2005; Li et al., 2008) and humans (De Filippis et al., 2008). The isolation and molecular characterization of 17 new clones and the detailed analysis of three of them in this report suggests that each clone is different but groups of clones can be segregated based on their fates after differentiation and patterns of gene expression even prior to differentiation. Potential interference from expression of v-myc is probably mitigated because its expression decreases after differentiation in culture for GE6 (Fig. 5D) as was observed for other myc-expressing clones (De Filippis et al., 2007) and it was undetectable 24-48 hours after engraftment into rodent brains (Flax et al., 1998). Although myc-expressing clones have not been reported to form tumors, there is concern of potential abnormal growth or tumorigenesis as in the case of myc-expressing iPS cells. Given that l-myc can be used to prepare iPS cells but has little transformation activity compared to other forms of myc (Nakagawa et al., 2010), it may be more useful for generating future clones. In any case, myc-expressing clones are useful for preparing homogenous populations of cells for a wide range of molecular and physiological studies.

It is now well established that differentiated ES cells, neuronal progenitors, and cells reprogrammed to adopt a neuronal fate develop the capability to express ion channel proteins, show decreased membrane resistance, and become electrically excitable, firing action potentials with distinctive characteristics (Li et al., 2008). Less often observed, however, is the development of synaptic connectivity, particularly with some sign of subtype specificity. In the majority of cases, formation of synaptic connections requires an additional trigger, such as co-culturing with primary neurons or astrocytes, or transfection of multiple transcription factors (Dodla et al., 2010). A good example of this dichotomy can be seen in two studies of reprogrammed astrocytes, in which initial attempts utilizing the proneural genes *Neurog2* and *Ascl1* produced neurons from glia that were able to fire action potentials, but showed no evidence of synaptic connectivity when cultured alone even though they could receive synaptic inputs when cultured with cortical neurons (Berninger et al., 2007). A subsequent study, however, in which transcription factor expression strength and persistence were increased by utilizing silencing-resistant retroviral vectors, did result in reprogrammed astrocytes that both received and formed synaptic connections (Heinrich et al., 2010). Moreover, the nature of the synaptic connection could be influenced by the molecules expressed. Use of neurogenin-2 resulted in excitatory glutamatergic connections whereas infection with *Dlx2* produced neurons that made inhibitory GABAergic connections (Heinrich et al., 2010). This is an interesting finding, especially when compared to results obtained from a study of two human ES cell lines both of which yielded glutamatergic and GABAergic neurons after differentiation but with cell line-specific biases in the relative proportions of inhibitory versus excitatory neurons (Wu et al., 2007).

Differentiated GE6 cells are unusual in that they form synapses when maintained in vitro, and do so with a relatively rapid time course. Unlike differentiated ES cells, they form GABAergic connections without requiring co-culturing with neurons or astrocytes, transfection of additional transcription factors, or treatment with neurotrophins. GE6 produced nearly all IPSCs, consistent with our conclusion that it becomes an inhibitory neuron. GE6 does give rise slowly to some astroglia, which may provide support for

neuronal maturation. However, based upon the genetic profiling and in vivo function (Petryniak et al., 2007), the maturation of GE6 neurons is likely due in large part to the substantial expression of *Dlx* transcription factors. Comparison of clones GE6 to L2.2 indicates that the latter has lower expression of *Dlx* genes and did not form synapses in vitro even in the presence of added astrocytes (Li et al., 2008).

With regard to use as implantable cells, the GE6 clone has two potential advantages. First, the subtype specificity means that cells can be used for the specific purpose of promoting inhibitory connections without the complication of also producing a significant population of excitatory neurons, which was not observed when GE6 cells were cultured alone. Second, the connections observed to date are intercellular and not predominantly autaptic as has been seen in other studies (Heinrich et al., 2010). Because autapses are generally only formed under conditions in which neurons are isolated from all potential targets, this finding brings up the important issue of whether promotion of synapse formation is occurring because the normal self-avoidance that prevents synapse formation among processes of the same cell (Schmucker, 2007) has been disabled, or whether the synapses being made are subject to the normal regulatory parameters that govern the patterns of connectivity seen in the native tissue. The absence of autaptic connections suggests the latter, although future studies in which GE6 neurons are introduced into cultured brain slices or in vivo will be needed to address this question. Preliminary lumbar transplantation of undifferentiated GE6 cells following thoracic rat spinal cord contusion showed that the GFP⁺ GE6 cells migrated to the injury site after one week (Jae H. Kim and MG). Further studies are needed to study the fate of the GE6 in sites of CNS trauma and their functional consequences.

Acknowledgments

This work was supported by grants from NIH, the New Jersey Commission on Spinal Cord Research, the New Jersey Commission on Science & Technology, Bristol-Myers Squibb Co. and Invitrogen, Inc. CLR was a fellow of the NSF IGERT Stem Cell training program (NSF DGE 0801620).

References

- Allan LE, Petit GH, Brundin P. Cell transplantation in Parkinson's disease: problems and perspectives. *Curr Opin Neurol.* 2010; 23:426–432. [PubMed: 20489615]
- Anderson SA, Eisenstat DD, Shi L, Rubenstein JL. Interneuron migration from basal forebrain to neocortex: dependence on *Dlx* genes. *Science.* 1997; 278:474–476. [PubMed: 9334308]
- Anderson SA, Marin O, Horn C, Jennings K, Rubenstein JL. Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development.* 2001; 128:353–363. [PubMed: 11152634]
- Anthony TE, Klein C, Fishell G, Heintz N. Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron.* 2004; 41:881–890. [PubMed: 15046721]
- Ascoli GA, Alonso-Nanclares L, Anderson SA, Barrionuevo G, Benavides-Piccione R, Burkhalter A, Buzsaki G, Cauli B, Defelipe J, Fairen A, Feldmeyer D, Fishell G, Fregnac Y, Freund TF, Gardner D, Gardner EP, Goldberg JH, Helmstaedter M, Hestrin S, Karube F, Kisvarday ZF, Lambolez B, Lewis DA, Marin O, Markram H, Munoz A, Packer A, Petersen CC, Rockland KS, Rossier J, Rudy B, Somogyi P, Staiger JF, Tamas G, Thomson AM, Toledo-Rodriguez M, Wang Y, West DC, Yuste R. Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. *Nat Rev Neurosci.* 2008; 9:557–568. [PubMed: 18568015]
- Bakshi A, Shimizu S, Keck CA, Cho S, LeBold DG, Morales D, Arenas E, Snyder EY, Watson DJ, McIntosh TK. Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following traumatic brain injury. *Eur J Neurosci.* 2006; 23:2119–2134. [PubMed: 16630059]
- Becerra GD, Tatko LM, Pak ES, Murashov AK, Hoane MR. Transplantation of GABAergic neurons but not astrocytes induces recovery of sensorimotor function in the traumatically injured brain. *Behav Brain Res.* 2007; 179:118–125. [PubMed: 17324477]

- Berninger B, Costa MR, Koch U, Schroeder T, Sutor B, Grothe B, Gotz M. Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. *J Neurosci*. 2007; 27:8654–8664. [PubMed: 17687043]
- Bulfone A, Puelles L, Porteus MH, Frohman MA, Martin GR, Rubenstein JL. Spatially restricted expression of *Dlx-1*, *Dlx-2* (*Tes-1*), *Gbx-2*, and *Wnt-3* in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J Neurosci*. 1993; 13:3155–3172. [PubMed: 7687285]
- Cobos I, Borello U, Rubenstein JL. *Dlx* transcription factors promote migration through repression of axon and dendrite growth. *Neuron*. 2007; 54:873–888. [PubMed: 17582329]
- Cobos I, Calcagnotto ME, Vilaythong AJ, Thwin MT, Noebels JL, Baraban SC, Rubenstein JL. Mice lacking *Dlx1* show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nat Neurosci*. 2005; 8:1059–1068. [PubMed: 16007083]
- Craig AM, Banker G, Chang W, McGrath ME, Serpinskaya AS. Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. *J Neurosci*. 1996; 16:3166–3177. [PubMed: 8627355]
- Craig AM, Blackstone CD, Hugarir RL, Banker G. The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. *Neuron*. 1993; 10:1055–1068. [PubMed: 7686378]
- Craig AM, Blackstone CD, Hugarir RL, Banker G. Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc Natl Acad Sci U S A*. 1994; 91:12373–12377. [PubMed: 7809044]
- De Filippis L, Ferrari D, Rota Nodari L, Amati B, Snyder E, Vescovi AL. Immortalization of human neural stem cells with the c-myc mutant T58A. *PLoS ONE*. 2008; 3:e3310. [PubMed: 18836552]
- De Filippis L, Lamorte G, Snyder EY, Malgaroli A, Vescovi AL. A novel, immortal, and multipotent human neural stem cell line generating functional neurons and oligodendrocytes. *Stem Cells*. 2007; 25:2312–2321. [PubMed: 17556596]
- Dodla MC, Mumaw J, Stice SL. Role of astrocytes, soluble factors, cells adhesion molecules and neurotrophins in functional synapse formation: implications for human embryonic stem cell derived neurons. *Curr Stem Cell Res Ther*. 2010; 5:251–260. [PubMed: 20214556]
- Du ZW, Li XJ, Nguyen GD, Zhang SC. Induced expression of *Olig2* is sufficient for oligodendrocyte specification but not for motoneuron specification and astrocyte repression. *Mol Cell Neurosci*. 2006; 33:371–380. [PubMed: 17035043]
- Eaton MJ, Plunkett JA, Martinez MA, Lopez T, Karmally S, Cejas P, Whittemore SR. Transplants of neuronal cells bioengineered to synthesize GABA alleviate chronic neuropathic pain. *Cell Transplant*. 1999; 8:87–101. [PubMed: 10338278]
- Eaton MJ, Wolfe SQ. Clinical feasibility for cell therapy using human neuronal cell line to treat neuropathic behavioral hypersensitivity following spinal cord injury in rats. *J Rehabil Res Dev*. 2009; 46:145–165. [PubMed: 19533527]
- Eisenstat DD, Liu JK, Mione M, Zhong W, Yu G, Anderson SA, Ghattas I, Puelles L, Rubenstein JL. *DLX-1*, *DLX-2*, and *DLX-5* expression define distinct stages of basal forebrain differentiation. *J Comp Neurol*. 1999; 414:217–237. [PubMed: 10516593]
- Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF. *Pax6*, *Tbr2*, and *Tbr1* are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci*. 2005; 25:247–251. [PubMed: 15634788]
- Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billingham LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol*. 1998; 16:1033–1039. [PubMed: 9831031]
- Fritsch B, Qashu F, Figueiredo TH, Aroniadou-Anderjaska V, Rogawski MA, Braga MF. Pathological alterations in GABAergic interneurons and reduced tonic inhibition in the basolateral amygdala during epileptogenesis. *Neuroscience*. 2009; 163:415–429. [PubMed: 19540312]
- Gelman DM, Marin O. Generation of interneuron diversity in the mouse cerebral cortex. *Eur J Neurosci*. 2010; 31:2136–2141. [PubMed: 20529125]

- Gomez-Lopez S, Wiskow O, Favaro R, Nicolis SK, Price DJ, Pollard SM, Smith A. Sox2 and Pax6 maintain the proliferative and developmental potential of gliogenic neural stem cells In vitro. *Glia*. 2011
- Hasegawa K, Chang Y-W, H. L, Berlin Y, Ikeda O, Kane-Goldsmith, Grumet M. Embryonic radial glia bridge spinal cord lesions and promote functional recovery following spinal cord injury. *Exp Neurol*. 2005; 193:394–410. [PubMed: 15869942]
- Heinrich C, Blum R, Gascon S, Masserdotti G, Tripathi P, Sanchez R, Tiedt S, Schroeder T, Gotz M, Berninger B. Directing astroglia from the cerebral cortex into subtype specific functional neurons. *PLoS Biol*. 2010; 8:e1000373. [PubMed: 20502524]
- Hevner RF. From radial glia to pyramidal-projection neuron: transcription factor cascades in cerebral cortex development. *Mol Neurobiol*. 2006; 33:33–50. [PubMed: 16388109]
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003; 4:249–264. [PubMed: 12925520]
- Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG. The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J Neurosci*. 1999; 19:7881–7888. [PubMed: 10479690]
- Li H, Babiarz J, Woodbury J, Kane-Goldsmith N, Grumet M. Spatiotemporal heterogeneity of CNS radial glial cells and their transition to restricted precursors. *Dev Biol*. 2004; 271:225–238. [PubMed: 15223331]
- Li H, Berlin Y, Hart RP, Grumet M. Microtubules are critical for radial glial morphology: possible regulation by MAPs and MARKs. *Glia*. 2003; 44:37–46. [PubMed: 12951655]
- Li H, Chang YW, Mohan K, Su HW, Ricupero CL, Baridi A, Hart RP, Grumet M. Activated Notch1 maintains the phenotype of radial glial cells and promotes their adhesion to laminin by upregulating nidogen. *Glia*. 2008; 56:646–658. [PubMed: 18286610]
- Li H, Grumet M. BMP and LIF signaling coordinately regulate lineage restriction of radial glia in the developing forebrain. *Glia*. 2007; 55:24–35. [PubMed: 17001659]
- Li H, Han YR, Bi C, Davila J, Goff LA, Thompson K, Swerdel M, Camarillo C, Ricupero CL, Hart RP, Plummer MR, Grumet M. Functional differentiation of a clone resembling embryonic cortical interneuron progenitors. *Dev Neurobiol*. 2008; 68:1549–1564. [PubMed: 18814314]
- Lowenstein DH, Thomas MJ, Smith DH, McIntosh TK. Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. *J Neurosci*. 1992; 12:4846–4853. [PubMed: 1464770]
- Magby JP, Bi C, Chen ZY, Lee FS, Plummer MR. Single-cell characterization of retrograde signaling by brain-derived neurotrophic factor. *J Neurosci*. 2006; 26:13531–13536. [PubMed: 17192436]
- Marano JE, Sun D, Zama AM, Young W, Uzumcu M. Orthotopic transplantation of neonatal GFP rat ovary as experimental model to study ovarian development and toxicology. *Reprod Toxicol*. 2008; 26:191–196. [PubMed: 18848623]
- Marin O, Anderson SA, Rubenstein JL. Origin and molecular specification of striatal interneurons. *J Neurosci*. 2000; 20:6063–6076. [PubMed: 10934256]
- Maximov A, Pang ZP, Tervo DG, Sudhof TC. Monitoring synaptic transmission in primary neuronal cultures using local extracellular stimulation. *J Neurosci Methods*. 2007; 161:75–87. [PubMed: 17118459]
- Monyer H, Markram H. Interneuron Diversity series: Molecular and genetic tools to study GABAergic interneuron diversity and function. *Trends Neurosci*. 2004; 27:90–97. [PubMed: 15102488]
- Mukhida K, Mendez I, McLeod M, Kobayashi N, Haughn C, Milne B, Baghbaderani B, Sen A, Behie LA, Hong M. Spinal GABAergic transplants attenuate mechanical allodynia in a rat model of neuropathic pain. *Stem Cells*. 2007; 25:2874–2885. [PubMed: 17702982]
- Nakagawa M, Takizawa N, Narita M, Ichisaka T, Yamanaka S. Promotion of direct reprogramming by transformation-deficient Myc. *Proc Natl Acad Sci U S A*. 2010; 107:14152–14157. [PubMed: 20660764]
- Nat R, Dechant G. Milestones of directed differentiation of mouse and human embryonic stem cells into telencephalic neurons based on neural development in vivo. *Stem Cells Dev*. 2011; 20:947–958. [PubMed: 21166522]

- Neville CM, Huang AY, Shyu JY, Snyder EY, Hadlock TA, Sundback CA. Neural precursor cell lines promote neurite branching. *Int J Neurosci*. 2009; 119:15–39. [PubMed: 19116829]
- Pang ZP, Yang N, Vierbuchen T, Ostermeier A, Fuentes DR, Yang TQ, Citri A, Sebastiano V, Marro S, Sudhof TC, Wernig M. Induction of human neuronal cells by defined transcription factors. *Nature*. 2011
- Petryniak MA, Potter GB, Rowitch DH, Rubenstein JL. Dlx1 and Dlx2 control neuronal versus oligodendroglial cell fate acquisition in the developing forebrain. *Neuron*. 2007; 55:417–433. [PubMed: 17678855]
- Pleasure SJ, Anderson S, Hevner R, Bagri A, Marin O, Lowenstein DH, Rubenstein JL. Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. *Neuron*. 2000; 28:727–740. [PubMed: 11163262]
- Rhee YH, Ko JY, Chang MY, Yi SH, Kim D, Kim CH, Shim JW, Jo AY, Kim BW, Lee H, Lee SH, Suh W, Park CH, Koh HC, Lee YS, Lanza R, Kim KS. Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. *J Clin Invest*. 2011; 121:2326–2335. [PubMed: 21576821]
- Roybon L, Mastracci TL, Ribeiro D, Sussel L, Brundin P, Li JY. GABAergic differentiation induced by Mash1 is compromised by the bHLH proteins Neurogenin2, NeuroD1, and NeuroD2. *Cereb Cortex*. 2010; 20:1234–1244. [PubMed: 19767311]
- Saitou H, Kato M, Mizuguchi T, Hamada K, Osaka H, Tohyama J, Uruno K, Kumada S, Nishiyama K, Nishimura A, Okada I, Yoshimura Y, Hirai S, Kumada T, Hayasaka K, Fukuda A, Ogata K, Matsumoto N. De novo mutations in the gene encoding STXBP1 (MUNC18-1) cause early infantile epileptic encephalopathy. *Nat Genet*. 2008; 40:782–788. [PubMed: 18469812]
- Sakurai K, Osumi N. The neurogenesis-controlling factor, Pax6, inhibits proliferation and promotes maturation in murine astrocytes. *J Neurosci*. 2008; 28:4604–4612. [PubMed: 18448636]
- Schmucker D. Molecular diversity of Dscam: recognition of molecular identity in neuronal wiring. *Nat Rev Neurosci*. 2007; 8:915–920. [PubMed: 18026165]
- Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwig EA, Cepko CL. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell*. 1992; 68:33–51. [PubMed: 1732063]
- Ting AK, Chen Y, Wen L, Yin DM, Shen C, Tao Y, Liu X, Xiong WC, Mei L. Neuregulin 1 promotes excitatory synapse development and function in GABAergic interneurons. *J Neurosci*. 2011; 31:15–25. [PubMed: 21209185]
- Vescovi AL, Snyder EY. Establishment and properties of neural stem cell clones: plasticity in vitro and in vivo. *Brain Pathol*. 1999; 9:569–598. [PubMed: 10416994]
- Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*. 2010; 463:1035–1041. [PubMed: 20107439]
- Welagen J, Anderson S. Origins of neocortical interneurons in mice. *Dev Neurobiol*. 2011; 71:10–17. [PubMed: 21154906]
- Whittemore SR, Snyder EY. Physiological relevance and functional potential of central nervous system-derived cell lines. *Mol Neurobiol*. 1996; 12:13–38. [PubMed: 8732538]
- Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A. In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development*. 2001; 128:3759–3771. [PubMed: 11585802]
- Wonders CP, Anderson SA. The origin and specification of cortical interneurons. *Nat Rev Neurosci*. 2006; 7:687–696. [PubMed: 16883309]
- Woo NH, Lu B. Regulation of cortical interneurons by neurotrophins: from development to cognitive disorders. *Neuroscientist*. 2006; 12:43–56. [PubMed: 16394192]
- Wu H, Xu J, Pang ZP, Ge W, Kim KJ, Blachi B, Chen C, Sudhof TC, Sun YE. Integrative genomic and functional analyses reveal neuronal subtype differentiation bias in human embryonic stem cell lines. *Proc Natl Acad Sci U S A*. 2007; 104:13821–13826. [PubMed: 17693548]
- Xu Q, Cobos I, De La Cruz E, Rubenstein JL, Anderson SA. Origins of cortical interneuron subtypes. *J Neurosci*. 2004; 24:2612–2622. [PubMed: 15028753]

Zhang N, An MC, Montoro D, Ellerby LM. Characterization of Human Huntington's Disease Cell Model from Induced Pluripotent Stem Cells. PLoS Curr. 2010; 2:RRN1193. [PubMed: 21037797]

A. **B.**

Figure 1. Quantitative RT-PCR comparison of clones isolated from E14.5 Fischer rat brains with the neuronal restricted progenitor L2.2

A. RNAs were isolated from individual cultures of undifferentiated clones derived from cortex (CTX) and ganglionic eminence (GE) grown in FGF2. Mean relative expression levels assayed in triplicate for each gene were normalized first to GAPDH as a loading control, and then to the signals observed for L2.2 cells for comparisons. **B.** Hierarchical clustering analysis based on the expression profiles of this set of genes divided the clones into three major clusters. The smallest cluster contained two clones indicating that GE6 (marked with an asterisk) is most closely related to L2.2, which has previously been identified as an interneuronal progenitor clone.

Figure 2. Differentiation of clones in culture

A. After withdrawal of FGF2, cultures of GE6, Ctx8 and GE2 clones were maintained for 6 days before fixation. Immunostaining with antibodies to TuJ1 and GFAP showed neurons in GE6, both neurons and astroglia in Ctx8 and only astroglia in GE2. **B.** Comparable cultures extracted for protein and analyzed by Western blotting confirmed the same expression patterns; GAPDH was used to control for protein loading. Note the predominance of TuJ1 in GE6 in contrast to the predominance of GFAP in GE2, while both are expressed in Ctx8.

Figure 3. Differentiated GE6 neurons display GABAergic phenotypes

A. GE6 cells were differentiated for 6 days by withdrawal of FGF2 and then analyzed by immunostaining. Nearly every cell was recognized by antibodies against TuJ1 and GAD65/67, suggesting they are GABAergic neurons. These neurons also stained for Npy and Sst, but a small fraction of the neurons stained for calretinin. **B.** Lysates of GE6 and L2.2 cultures after 6-day differentiation were compared by Western immunoblot analysis using markers for interneurons and their subtypes. Lysate of E18 rat cortical tissue was used as a reference for antibodies and GAPDH was used as sample loading control. GE6 and L2.2 differentiation showed relative differences in expression for the neurotransmitters calretinin, PV, Npy, and Sst.

Figure 4. GE6 cells acquire neuronal electrophysiological features and receive synaptic inputs when co-cultured with hippocampal neurons

A. Co-culture of GFP⁺ GE6 cells with dissociated hippocampal cells. GE6 cells were prepared as described in the Materials and Methods and were added to 7-days cultures of hippocampal neurons. A phase contrast image of a typical field is shown on the left and the center panel is an inverse fluorescence image that clearly shows the GFP⁺ GE6 cells in black. The right panel is a combined image of the same field with the red arrows indicating a GE6 cell. **B.** Example trace of depolarization-induced action potential firing obtained with whole-cell current-clamp recording from a GE6 cell. **C.** Example traces of ongoing synaptic activity. Note presence of EPSCs (circles) and IPSCs (triangles) at a holding potential of

–70 mV (trace 1). Setting the holding potential to –20 mV illustrates the different reversal potentials for the two types of synaptic currents (trace 2). **D.** Example traces showing the effect of pharmacologic block of neurotransmitter receptors. Under baseline conditions (NRS bath solution, upper 2 traces) mostly IPSCs and occasional EPSCs were seen. Following bath perfusion with 50 mM bicuculline, IPSCs were no longer present and bursts of EPSCs became evident (middle 2 traces). Upon perfusion of both bicuculline and 10 mM DNQX, ongoing synaptic activity was virtually eliminated (bottom 2 traces). **E.** Acquisition of action potential firing (AP) or synaptic activity (SA) at different times in culture. The number of recordings is indicated on the bars. All results were obtained from at least two separate platings.

Figure 5. Expression profiling of selected clones with diverse patterns of neural differentiation.
A. Principal component analysis for GE6, GE2 and CTX8 compared to the interneuronal progenitor clone L2.2 and the multipotential clone L2.3. Cell lines are identified by color and time of differentiation (0, 3, or 7 d) by size of circles. Note the tight clustering of replicate samples indicating reproducibility among parallel cultures and microarrays. **B.** Expression profiling of *nestin*, *βIII-tubulin*, *GFAP*, and *S100B*, using results from Affymetrix microarray results. The mean antilog-RMA intensities are plotted $\pm$ SEM. **C.** Heat map of expression profiles of selected transcription factors, neurotransmitters and

synaptic proteins. Z-scored data are indicated by colors, with red being larger positive values and violet being smaller negative values, relative to the overall mean.

D. Quantitative RT-PCR (qPCR) of additional *Dlx* family members, *GAD-2* and *v-myc* mRNAs. Aliquots of the same preparations of total cellular RNA used in the microarray study were assayed by SYBR green qPCR. Results are expressed relative to the mean of the Day 0 Ctx8 group for comparison (plotted as mean $\pm$ SEM; n=3). A two-way ANOVA (Days, Cell) was run for each assay. Tukey's post-hoc tests found that the differentiation time point indicated by asterisks were significantly different from Day 0 (*p<0.05; **p<0.001).

E. Expression profiling of GABAergic differentiation modifiers *NeuroD1* and *Nrg1*. Results are taken from Affymetrix data as described in panel B.

Figure 6. GE6 cells made and received inhibitory synaptic connections when cultured alone
A. Example traces of spontaneous IPSCs. **B.** Inhibitory synaptic current evoked by extracellular field stimulation (inset; arrow indicates blanked stimulus artifact). Example I-V relationship made by eliciting synaptic responses at different holding potentials. The response reversed at -44.7 mV, very close to the calculated chloride equilibrium potential. **C.** Acquisition of action potential firing (AP) or synaptic activity (SA) at different times in culture. “Div” refers to the number of days in vitro following final plating. The number of recordings is indicated on the bars. All results were obtained from at least two separate platings. Note that cells obtained using the pre-differentiation protocol (“prediff”) form synapses earlier in vitro than cells prepared with the standard protocol (“nonprediff”).

Figure 7. Differentiated GE6 neurons display only GABAergic phenotypes

GE6 cells were pre-differentiated for 3 days by withdrawal of FGF2 and then passed onto 35 mm dishes and cultured for one week before being analyzed by electrophysiology and then fixed in 4 % paraformaldehyde. Neurons were identified by immunostaining with chick anti- β III tubulin (Aves), rabbit anti-Synaptophysin, mouse anti-Gephyrin, anti-Vesicular Glutamate 1 (VGlut-1) and anti-Vesicular GABA Transporter (VGAT). Note staining for VGAT but not for VGlut-1 indicating the presence of GABAergic but not glutamatergic neurons.

Table 1

Primers used for qPCR.

Name	Forward	Reverse
<i>Dlx1</i>	CCTACGTCCCCAGCTACACG	GAAGCGGGTGAGTGCGAA
<i>Dlx2</i>	CATGGGCTCCTACCAGTACCA	CGTAGGAAGGTGTACGCGGC
<i>Dlx5</i>	CGGCCACCGATTCTGACTAC	CTGGTATGGGTGAGCGCTT
<i>Dlx6</i>	GGGAAATCAGGTTCAACGGA	AGTCTGCTGAAAGCGGTGGT
<i>GAD-2</i>	CTCCAACATGTACGCCATGC	CTGACGTGAATGCGATGAGC
<i>v-myc</i>	GTCAGGGTCATCCCCATCAA	AGTGGGCTGTGTGGAGGTTT