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EXPRESSION AND FUNCTION OF REST IN OLIGODENDROCYTE LINEAGE CELLS

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Lisa Marie Evans DeWald

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Lisa Marie Evans DeWald

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Joel M. Levine, Ph.D. – Dissertation Advisor
Professor, Neurobiology and Behavioral Sciences

Holly Colognato, Ph.D. – Chairperson of Defense
Assistant Professor, Pharmacological Sciences

David A. Talmage, Ph.D.
Associate Professor, Pharmacological Sciences

Rolf Sternglanz, Ph.D.
Distinguished Professor, Biochemistry and Cell Biology

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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` The RE1-silencing transcription factor (REST) regulates the expression of neural specific genes by acting as a transcriptional repressor in non-neuronal cells. REST binds to repressor elements (RE1s) in the DNA and recruits histone deacetylases (HDACs) and other histone modifying and chromatin remodeling proteins through its corepressors mSin3 and CoREST. RE1s are located on a large number of genes that are required for the development and maintenance of the neuronal phenotype and are normally repressed in non-neural cells. By regulating gene expression, REST participates in the orderly developmental transition from a neuroepithelial precursor or stem cell to a functional neuron. Glial cells also develop from neuroepithelial stem cells but little is known about the role of REST in the development of glia.

Here, I examine the expression and function of REST in glial cells, with a focus on oligodendrocytes and their precursors. Oligodendrocytes are the myelin forming cells of the central nervous system. They develop from an identified precursor known as oligodendrocyte progenitor cells (OPCs). The differentiation of an OPC into a mature oligodendrocyte occurs in a step or stage-wise progression and is regulated by both genetic and epigenetic mechanism. OPCs are highly plastic cells that share a common lineage with some classes of neurons,

have some properties usually associated with neurons, and are capable of being reprogrammed to act as neural stem-like cells that can develop into functional neurons. In this thesis, I demonstrate that REST is an important regulator in oligodendrocyte differentiation and OPC cell fate determination.

REST is expressed in glial cells and functions as a transcriptional repressor in OPCs. REST transcript and protein expression increase 4-fold during the first 48hrs of oligodendrocyte differentiation. During this maturation period, expression of REST regulated genes decreases as oligodendrocyte specific genes are activated. Perturbing REST function by overexpressing dominant negative REST (DnREST) or REST-VP16 (a chimeric protein containing the DNA binding domain of REST fused to the activation domain of VP16) results in a decrease in oligodendrocyte formation as demonstrated by immunofluorescence, qRT-PCR, immunoblot, and clonal analysis. Additionally, REST loss of function inhibits the ability of OPCs to develop into process bearing GFAP-positive type II astrocytes. Perturbing REST function in differentiating OPCs results in a significant increase in neuronal-like cells at the expense of glia. Through this loss-of-function approach, I demonstrated that REST has 2 functions in the oligodendrocyte lineage: it is required for the timely and complete differentiation of OPCs into oligodendrocytes and it prevents the expression of neuronal properties, suggesting that REST is an important regulator of OPC lineage plasticity.

Dedicated to my husband, Kyle,
for all of his love, support, and patience

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LIST OF ABBREVIATIONS

2A	Type II astrocyte
ASTRO	Astrocyte
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BDNF	Brain-derived neurotrophic factor
CGN	Cerebellar granule neuron
ChIP	Chromatin immunoprecipitation assay
CNS	Central nervous system
DnREST	Dominant negative REST
ES	Embryonic stem (cells)
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ID2	Inhibitor of DNA binding 2 / Inhibitor of differentiation 2
ID4	Inhibitor of DNA binding 4 / Inhibitor of differentiation 4
LOF	Loss of function
MBP	Myelin basic protein
MS	Multiple sclerosis
NRSF	Neuron-restrictive silencer factor
OLIGO	Oligodendrocyte
ON	Optic Nerve
OPC	Oligodendrocyte progenitor cell
PCR	Polymerase chain reaction
PDGFαR	Platelet-derived growth factor alpha receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element

RE1	Repressor / response element 1
REF	Rat embryonic fibroblast
REST	RE1 silencing transcription factor
rGFP	GFP expressing retrovirus (pMXsIG)
rDnREST	DnREST expressing retrovirus (pMXsIG-DnREST)
SCLC	Small cell lung cancer
SCM	Stem cell media
TH	Thyroid hormone
TR	Thyroid hormone receptor
TSA	Trichostatin A
YY1	Yin Yang 1

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CHAPTER I
GENERAL INTRODUCTION

Introduction to oligodendrocytes and their precursors

The central nervous system (CNS) is composed of two major classes of cells: neurons and glia. Originally, neurons were the primary focus of study in the nervous system while glial cells were simply classified as the glue that functions to support these neurons. It is now evident that glial cells have important and distinct roles within the CNS and are a major area of research. The glial cells of the mammalian central nervous system include astrocytes, microglia, oligodendrocytes, and oligodendrocyte progenitor cells (OPCs). Astrocytes provide structural and functional support for neurons and are involved in modulating synaptic transmission whereas microglia, the immune cells of the CNS, are phagocytic cells that are mobilized after injury, infection, and disease. The focus of this thesis is on oligodendrocytes and their precursors.

Oligodendrocytes are the myelin producing cells in the CNS and are responsible for insulating axons to enable the rapid salutatory conduction of action potentials. Myelin also functions to maintain axon integrity and loss or destruction of this myelin is associated with various demyelinating diseases including multiple sclerosis (MS). Oligodendrocytes develop from unique identified precursors known as oligodendrocyte progenitor cells (OPCs). OPCs undergo a complex temporally regulated program of proliferation, migration, and differentiation following their appearance at mid-gestation. OPCs comprise approximately 8% of the glia population in the central nervous system and can be identified by cell-type specific markers including the chondroitin sulfate proteoglycan NG2, the lipid antigen A2B5, and the platelet derived growth factor alpha receptor (PDGFαR). During their differentiation into mature oligodendrocytes, the cells lose the expression of these progenitor marker proteins. Differentiating OPCs transition through an O4-positive pre-oligodendrocyte stage to a mature myelinating stage where they express myelin proteins including the myelin associated glycoprotein (MAG) and myelin basic protein (MBP) (Nishiyama et al., 2009). OPCs are not

only the primary source of oligodendrocytes during development, they can also generate oligodendrocytes in the mature CNS and are the primary source of remyelinating cells in demyelinated MS lesions (Nishiyama et al., 2009).

OPCs constitute the major dividing population of cells within the adult brain and make up about 70% of the cells labeled following a pulse injection of bromodeoxyuridine (Dawson et al., 2003). Not all OPCs develop into oligodendrocytes and a large number remain as progenitors in the mature white and grey matter. In fact, many more OPCs remain in the adult CNS than would be required for the replacement of oligodendrocytes. This suggests that OPCs may have other important functions within the CNS and exactly what those functions are remains somewhat controversial. OPCs express voltage-dependent ion channels and neurotransmitter receptors, proteins usually associated with neurons (Sontheimer et al., 1989, Barres et al., 1990, Berger et al., 1992). Furthermore, there is a synaptic association between OPCs and neurons in the developing and adult CNS (Bergles et al., 2000, Jabs et al., 2005, Gallo et al., 2008). These synaptic contacts between OPCs and axons have been described for neurons in the cerebellum, hippocampus, and corpus callosum (Bergles et al., 2000, Lin et al., 2005, Kukley et al., 2007, Ziskin et al., 2007). Excitation of NG2-positive cells can occur via neuronal release of GABA or glutamate which can act on OPC GABAA or AMPA receptors respectively and invoke a calcium signal (Paukert and Bergles, 2006, Gallo et al., 2008, Hamilton et al., 2010). In addition to having some neuronal properties, recent evidence suggests that OPCs have multipotent stem cell properties and are not restricted to the oligodendrocyte lineage (Liu and Casaccia, 2010, Trotter et al., 2010).

OPC plasticity

It is well established that OPCs in culture can give rise to neurospheres, neural stem-like cells, neurons, oligodendrocytes, and process bearing GFAP-positive type-II astrocytes depending on their external environment (Kondo and Raff, 2000, Marin-Husstege et al., 2002, Belachew et al., 2003, Kondo and Raff, 2004, Liu et al., 2007a, Liu and Casaccia, 2010). These type II-astrocytes (2A's)

differ from the typical astrocytes (type-I) normally found in-vivo and discussed above. Research studies performed by Kondo and Raff suggest that type II astrocytes are an intermediate step between an OPC and a neuronal stem-like cell. Once reverted to a neural stem-like cell, these OPCs can develop into neurons, type-1 astrocytes, as well as oligodendrocytes (Kondo and Raff, 2000). OPCs are also capable of forming neurospheres in tissue culture, a hallmark of multipotent neural stem cells. These OPC derived neurospheres can differentiate into astrocytes, oligodendrocytes, as well as electrically excitable neurons (Kondo and Raff, 2000, Belachew et al., 2003). Additionally, OPCs themselves can directly give rise to the different cell types (excluding type I astrocytes) (Belachew et al., 2003). NG2+/CNP-GFP+ cells isolated from CNP-GFP transgenic mice generated neurons when grafted into neurogenic regions of both embryonic and early postnatal brain (Belachew et al., 2003). Furthermore, in vivo evidence also suggests that NG2-positive cells give rise to GABAergic interneurons in the hippocampus (Belachew et al., 2003, Aguirre and Gallo, 2004, Aguirre et al., 2004, Windrem et al., 2004, Dayer et al., 2005). Contrary to these studies, Akiko Nishiyama's laboratory found that OPCs do not give rise to neurons but do develop into more than 40% of the protoplasmic astrocytes in the gray matter of the ventral posterior forebrain through the use of double transgenic mice expressing NG2-Cre and the Cre reporter lacZ/EGFP (Novak et al., 2000, Zhu et al., 2008b). Therefore, although the in vitro neuronal potential of these OPCs is well established, how these cells behave in vivo remains controversial.

Having insight into factors responsible for regulating OPC differentiation will help to better understand remyelination in demyelinating diseases as well as regeneration after spinal cord injury. OPCs are rapidly generated at sites of spinal cord injuries and if a way to reprogram the fate of these cells in vivo was understood, OPCs could potentially participate in regeneration and repair (Jones et al., 2002). This thesis focuses on two aspects of understanding the molecular mechanisms that regulate OPC differentiation. First, because of the multipotent stem cell-like nature of OPCs many questions arise as to what factors contribute to determining their cell fate. In culture the fate of an OPC can be influenced and

altered depending on external cues in the media. In this thesis I concentrate on the intracellular mechanisms that participate in regulating this fate choice. Second, because of the involvement of oligodendrocytes in demyelinating diseases such as MS, it is important to understand how oligodendrocyte differentiation is regulated. Acutely demyelinated lesions are remyelinated efficiently but this often fails in chronically demyelinated lesions (Nishiyama et al., 2009, Bradl and Lassmann, 2010). Remyelination in fresh lesions may be due, in part, to growth factors supplied by infiltrating macrophages that are not present to the same extent at the chronic stage (Diemel et al., 1998, Kotter et al., 2001, Kotter et al., 2005). In addition to environmental factors, failure to remyelinate is likely the result of intrinsic signals as well. Therefore, it is important to understand the molecular mechanisms that regulate OPC differentiation.

Oligodendrocyte differentiation: Intrinsic factors

Ultrastructural studies in oligodendrocytes show that the chromatin appears to be compact, a phenotype typically associated with transcriptional repression (Marin-Husstege et al., 2002, Menn et al., 2006, Liu and Casaccia, 2010). Inhibiting this repression prevents the development of oligodendrocytes (Marin-Husstege et al., 2002, Menn et al., 2006, Liu and Casaccia, 2010). The differentiation of an OPC to a mature oligodendrocyte requires crosstalk between transcription factors and epigenetic modulators to regulate gene expression. Epigenetics refers to heritable changes in gene activity that control gene expression due to information carried by the genome rather than changes in the DNA itself. The proteins responsible for these epigenetic changes have enzymatic activities that modify histones or chromatin thereby affecting histone-histone contacts or the interaction of histones with DNA. These changes can alter the accessibility of transcription factors to bind the DNA. Furthermore, both the modifications and the modifying enzymes can recruit or repulse other proteins or complexes with enzyme activities to further regulate gene expression. This section will introduce some of the enzymes involved in epigenetic regulation, how they are involved in OPC differentiation, and finally, transcription factors

responsible for regulating gene expression and OPC differentiation through the recruitment of these enzymes.

Introduction to Transcriptional and Epigenetic Regulation

Many histone modifying enzymes have been identified that have the ability to acetylate, methylate, phosphorylate, ubiquitinate, sumoylate, and ADP-ribosylate amino acids in histones as well as enzymes that remove these modifications such as deacetylases and demethylases (Sterner and Berger, 2000, Zhang and Reinberg, 2001, Cuthbert et al., 2004, Nowak and Corces, 2004, Hassa et al., 2006, Nathan et al., 2006, Nelson et al., 2006, Shilatifard, 2006, Wang et al., 2006a, Kouzarides, 2007). Of these modifications, the most studied is histone acetylation/deacetylation and methylation (Liu and Casaccia, 2010). Histone acetyltransferases (HATs) are responsible for acetylating lysine residues particularly on the N-terminal tail of nucleosomal histones. This neutralizes the basic charge and allows the chromatin to unfold and therefore is generally associated with transcriptionally active chromatin. Histone deacetylases (HDACs) on the other hand, are responsible for removing these acetyl groups resulting in a more positive charge on the tails of the histones which can then interact with the negatively charged DNA. This results in chromatin compaction making it difficult for transcriptions factors to bind and activate genes located within these regions.

Methylation by methyltransferases can occur on both arginine and lysine residues. Methylation of H3K4 (lysine 4 of histone 3), H3K36, and H3K79 are associated with active transcription whereas H3K9, H3K27, and H4K20 are connected to transcriptional repression (Kouzarides, 2007). Demethylation of these lysine residues is associated with the opposite effect. These modifications can impact transcription through the recruitment of proteins such as the chromatin condensing heterochromatin protein 1(HP1) (Ballas and Mandel, 2005). However, this simplified version of histone methylation is much more complicated in reality. For instance, H3K9 methylation, although generally associated with repression, may participate in gene activation when positioned in the coding region of a gene as opposed to the promoter region (Vakoc et al., 2005,

Kouzarides, 2007). Arginine methylation can also result in either gene activation or repression and methylation of both lysines and arginines can be mono-, di- or trimethylated adding additional complexity. The multi-functions of demethylases (the enzymes that remove these methyl groups from histones), further complicate the picture. For instance, LSD1 can demethylate H3K4 to repress transcription or H3K9 to activate transcription (Shi et al., 2004, Metzger et al., 2005, Kouzarides, 2007).

In addition to histone methylation, the DNA itself can also be methylated at the C-5 position of cytosine residues at CpG dinucleotides. Methyl-CpG binding proteins, such as the repressor MeCP2, can bind methylated DNA and in turn recruit additional modifying enzymes such as HDACs (Nan et al., 1998, Jaenisch and Bird, 2003, Ballas and Mandel, 2005). DNA methylating enzymes are highly expressed in embryonic stem cells and are required for differentiation but not for self-renewal, implicating the importance of epigenetic mechanisms during differentiation (Jackson et al., 2004, Tsumura et al., 2006, Liu and Casaccia, 2010). Enzymes that regulate these different epigenetic modifications, transcription factors and other proteins that affect the recruitment of these modifying enzymes, and availability of each of these proteins at the transcriptional and translational level, all regulate the activation or repression of a single gene.

Epigenetic Regulation in OPCs

Chromatin remodeling and histone modifying enzymes are important regulators of OPC differentiation. Members of the SWI/SNF chromatin remodeling complex are required for OPCs to be converted to neural stem like cells through a type II astrocyte dependent pathway (Kondo and Raff, 2004). HDACs in particular have a key role in oligodendrocyte development. Marin-Husstege et al., (2002) first showed that blocking HDAC activity in OPCs with the pharmacological inhibitor trichostatin A (TSA) prevents their differentiation into mature oligodendrocytes (Marin-Husstege et al., 2002). A later study from the same laboratory demonstrated that inhibition of HDACs promotes lineage

plasticity on OPCs. The cells development into GFAP-positive astrocytes and electrically excitable TUJ1-positive neuronal cells at the expense of oligodendrocytes both in vitro and in transplantation studies (Liu et al., 2007a). In addition to being required for the development of oligodendrocytes in vitro and in vivo, HDACs also have a role in repair following demyelination (Marin-Husstege et al., 2002, Shen et al., 2005, Cunliffe and Casaccia-Bonnefil, 2006, Shen et al., 2008b, Ye et al., 2009, Liu and Casaccia, 2010). HDAC activity is not required for the progression of OPCs into type II astrocytes, similar to the neuronal differentiation of neural progenitor cells (Marin-Husstege et al., 2002, Hsieh et al., 2004). In fact, histone acetylation rather than deacetylation enhances both neuronal and astrocyte gene expression (Hsieh et al., 2004, Song et al., 2004, Liu et al., 2007a). Therefore, the recruitment of HDACs and other epigenetic modulators to the DNA by transcription factors is a key determinant in whether an OPC will develop into an oligodendrocyte.

Regulators of oligodendrocyte differentiation

For differentiation to occur, the transcription of oligodendrocyte differentiation inhibitors must be repressed whereas positive regulators of oligodendrocyte development need to be transcriptionally activated. Numerous transcription factors have been implicated in promoting the differentiation of OPCs including the basic helix-loop-helix proteins Olig1 and Olig2 (Ligon et al., 2006). Olig1/2 double knockout mice lack oligodendrocyte lineage cells and oligodendrocytes fail to develop properly in both Olig1 and Olig2 null mice (Lu et al., 2002, Zhou and Anderson, 2002, Xin et al., 2005). Other transcription factors including Yin Yang 1 (YY1), Zfp388, myelin transcription factor 1 (Myt1), Mash1 (Ascl1), Nkx2.2, and Sox10 are involved in regulating OPC differentiation, some of which are directly involved in activating myelin genes (Kim et al., 1997, Berndt et al., 2001, Nielsen et al., 2004, Gokhan et al., 2005, Xin et al., 2005, Wang et al., 2006b, Wissmuller et al., 2006, Li et al., 2007, Sugimori et al., 2008). For example, co-expression of Ascl1 with Nkx2.2 or Olig2 in vitro can activate myelin genes and induce oligodendrocyte differentiation

(Gokhan et al., 2005, Sugimori et al., 2008). Transcriptional regulation of myelin genes during differentiation is a complex process. Some transcription factors can have both repressor and activator functions, some are regulated through positive or negative feedback loops (Zhou et al., 2001, Liu et al., 2007b), and others have additional developmental roles in the CNS, such as Olig2 and Myt1in neuronal development (Bellefroid et al., 1996).

Inhibitors of oligodendrocyte differentiation including Id2, Id4, and Hes5, are present in proliferating OPCs but decrease as the cells differentiate into mature oligodendrocytes (Liu et al., 2006, Shen et al., 2008b). Many of these inhibitors are transcription factors themselves, such as Hes5 (Liu et al., 2006). Several well identified pathways have key roles in the transcriptional repression of oligodendrocyte development including the Notch, and canonical Wnt pathways (figure I-1) (Wegner, 2008, Li et al., 2009, Fancy et al., 2010, Liu and Casaccia, 2010). The Notch signaling pathway is generally believed to repress oligodendrocyte development, partially due to the activation of its downstream target Hes5 (Genoud et al., 2002, Givogri et al., 2002, Liu et al., 2006, Zhang et al., 2009b). Hes5 represses myelin genes directly by recruiting HDACs to their promoters. Hes5 also indirectly regulates myelin gene expression by sequestering and inhibiting the functions of the activators Ascl1 and Sox10 mentioned above (Liu et al., 2006). Hes5 itself is regulated by HDAC activity and a decrease in Hes5 expression correlates with an increase in myelin gene expression (Shen and Casaccia-Bonnefil, 2008).

The canonical Wnt signaling pathway and its downstream effector, the transcription factor Tcf4, can prevent the development of oligodendrocytes (Leung et al., 2002, He et al., 2007, Ye et al., 2009, Fancy et al., 2010). Wnt signaling stabilizes β-catenin which then translocates into the nucleus and binds Tcf4. Tcf4 can then activate the expression of the differentiation inhibitors Id2 and Id4 as well as repress Olig2 and MBP gene expression. Id2 and Id4 form heterodimers with oligodendrocyte activating factors including Olig1, Olig2, and Ascl1 to inhibit their function and therefore prevent oligodendrocyte maturation (Samanta and Kessler, 2004, Li et al., 2009).

The effects of Wnt signaling on the differentiation of OPCs are thought to be independent of the Notch signaling pathway (Ye et al., 2009). For oligodendrocytes to differentiate, inhibitory pathways need to be repressed. HDACs can counter the negative effect of Wnt signaling by converting Tcf4 from a repressor of myelination to an activator (Ye et al., 2009). This is achieved through the competition of HDACs and β-catenin for the binding of Tcf4. The Tcf4/HDAC complex represses Id2 and Id4 rather than increases their expression (Ye et al., 2009, Liu and Casaccia, 2010). The transcription repressor YY1 provides additional support to overcome this inhibitory pathway by complexing with HDACs to repress transcription of both Id4 and Tcf4 (He et al., 2007). In this dissertation, I will discuss a possible mechanism by which notch mediated inhibition can be overcome during oligodendrocyte development.

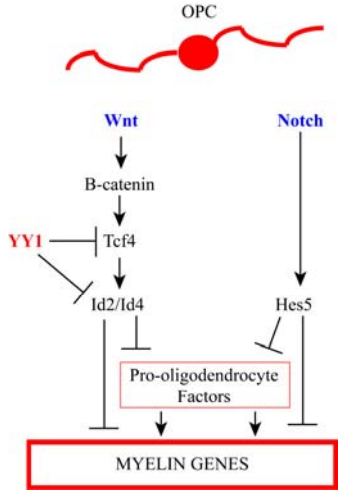


FIGURE I-1. Inhibitors of oligodendrocyte development

Considering that OPCs have multiple cell fates and can give rise to neurons, it is possible that in addition to activating myelin genes and repressing inhibitors of oligodendrocyte differentiation, the decision of an OPC to become an oligodendrocyte also requires factors to repress neuronal specific genes. One of the major DNA binding proteins that recruits HDACs and other chromatin and histone modifying enzymes to regulate neuronal gene expression is the RE-1 silencing transcription factor, REST (also known as neural restricted silencing

factor or NRSF). No functional studies have been carried out to identify a role for REST in oligodendrocyte development. However, a very recent study discovered that REST is required during the BMP-induced astrocytic differentiation of embryonic neural progenitor cells, suggesting that REST may have a role in the development of other glia (Kohyama et al., 2010).

Introduction to REST regulation

The transcriptional repressor REST was first identified as a negative regulator of neuronal differentiation through its ability to repress the expression of neuronal genes in non-neuronal cells (Chong et al., 1995, Schoenherr and Anderson, 1995). Since then, REST has been shown to regulate a large number of genes required for the neuronal phenotype in non-neuronal cells. These genes include those that code for ion channels, synaptic vesicle proteins, transcription factors, signaling molecules, growth factors, structural proteins, neurotransmitters and neuropeptide synthesizing enzymes (Afar et al., 1996, Lonnerberg et al., 1996, Schoenherr et al., 1996, Bessis et al., 1997, Kallunki et al., 1997, Mieda et al., 1997, Bai et al., 1998, Mu and Burt, 1999, Brene et al., 2000, De Gois et al., 2000, Abderrahmani et al., 2001, Andria and Simon, 2001, Seth and Majzoub, 2001, Lunyak et al., 2002, Mbikay et al., 2002, Quinn et al., 2002, Gurrola-Diaz et al., 2003, Lietz et al., 2003, Kim et al., 2006). REST functions by interacting with a repressor element in the DNA known as the Repressor Element 1 (RE1; also known as Neuron-Restrictive Silencer Element, NRSE) (Chong et al., 1995, Schoenherr and Anderson, 1995). RE1s can be located within promoters, introns, exons, and downstream of transcriptional stop sites (Otto et al., 2007). Although REST was first identified as a regulator of neuronal genes outside of the nervous system, it is now understood that REST has other roles such as regulating both heart (Kuwahara et al., 2003) and pancreatic development (Martin et al., 2003).

REST is a 116-kDa zinc finger protein related to the Gli-Kruppel family of zinc finger proteins (Chong et al., 1995). Eight of the nine C2H2 type zinc fingers of REST bind to the DNA through the interaction with a 21-23-base pair element in the DNA, the RE1 (Chong et al., 1995, Schoenherr and Anderson,

1995, Shimojo et al., 2001). In addition to this DNA binding domain, REST also contains repressor domains and proline and lysine rich regions that are thought to be involved in proper protein folding (Chong et al., 1995). REST mediated gene repression is achieved through the recruitment of specific corepressors to its repressor domains, ultimately leading to the formation of a repressor complex. The repressive functions of this complex are multifaceted and are mediated through the combinatorial action of enzymes discussed above including histone deacetylases, histone demethylases and methylases, and chromatin modifying proteins (Andres et al., 1999, Huang et al., 1999, Naruse et al., 1999, Grimes et al., 2000, Roopra et al., 2000, Humphrey et al., 2001, You et al., 2001, Roopra et al., 2004, Ooi and Wood, 2007). The REST protein contains two separate repressor domains that interact with different corepressor complexes, one located on the amino- terminus and one on the carboxy- terminus. The C-terminal repressor domain contains the ninth zinc finger that is required for the repressive activity through the recruitment of a corepressor, CoREST (Tapia-Ramirez et al., 1997, Andres et al., 1999). CoREST is only found in organisms with a nervous system but is expressed by both neurons and non-neuronal cells (Dallman et al., 2004, Ballas and Mandel, 2005). The CoREST complex contains many proteins including the histone deacetylases HDAC1 and HDAC2 (Andres et al., 1999, You et al., 2001), the histone H3K4 demethylase LSD1 (Shi et al., 2004), and the histone H3K9 methyltransferases G9a (Shi et al., 2003, Roopra et al., 2004) and SUV39H (Lunyak et al., 2002). In addition to these histone modifying proteins, the CoREST complex also interacts with components of the chromatin remodeling SWI-SNF complexes including BRG, BAF1701, and BAF57 (Humphrey et al., 2001, You et al., 2001, Battaglioli et al., 2002), the methyl DNA-binding protein MECP2 (Lunyak et al., 2002, Ballas et al., 2005), and c-terminal binding proteins (CtBP) (Zhang et al., 2002, Garriga-Canut et al., 2006), although both MECP2 and CtBP can also be recruited by REST directly (Roopra et al., 2004, Garriga-Canut et al., 2006, Ooi and Wood, 2007).

The N-terminal repressor domain of REST, located within the first 83 residues of the N-terminus, recruits the corepressor protein mSin3 (Tapia-Ramirez

et al., 1997, Huang et al., 1999, Leichter and Thiel, 1999, Naruse et al., 1999, Grimes et al., 2000, Roopra et al., 2000). While CoREST is only found in organisms with a nervous system, mSin3 is present in all eukaryotes (Dallman et al., 2004, Ballas and Mandel, 2005). The REST/mSin3 complex interacts with the histone deacetylases HDAC1, HDAC2, HDAC 4, and HDAC5 (Huang et al., 1999, Naruse et al., 1999, Grimes et al., 2000, Roopra et al., 2000). The mSin3/REST complex can also interact with MCP2 and with the retinoblastoma-associated proteins RbAp48 and RbAp46 (Zhang et al., 1998, Alland et al., 2002, Fleischer et al., 2003, Ballas and Mandel, 2005). Both CoREST and mSin3 repressor complexes are important for REST function. Transcriptional repression of some genes requires both complexes whereas only the N- or C- terminal complex may be sufficient to repress others (Bingham et al., 2007). Altogether, REST interacts with a wide array of proteins that have various functions. By associating with these and other regulatory cofactors, REST regulates epigenetic changes responsible for differential gene expression in a context-dependent manner and allows for both long-term silencing and transient repression. This will become more evident when neuronal differentiation is discussed later.

The RE1

In addition to the complex nature of REST mediated repression through the recruitment of multiple histone and chromatin modifying proteins, variations in the RE1 site itself adds an additional obstacle in studying REST regulated genes. The RE1 was actually identified a few years prior to the discovery of the REST protein by the same two groups that later identified REST (Maue et al., 1990, Mori et al., 1992). In 1990, Gail Mandel's group discovered a negative regulatory element upstream of the type II sodium channel gene that was responsible for restricting its expression to neuronal cells (Maue et al., 1990). Two years later, David Anderson's group found that a similar negative regulatory region consisting of 21 base pairs was required for repressing the neuron-specific gene SCG10 in non-neuronal cells (Mori et al., 1992). Soon after, RE1 sites were found to restrict the expression of other genes including the brain derived

neurotrophic factor (Timmusk et al., 1993), Synapsin I (Li et al., 1993), and dopamine-β-hydroxylase (Ishiguro et al., 1993) to neurons. Following the discovery of REST as the transcription factor responsible for regulating genes containing this repressor element, several groups performed *in silico* studies in an attempt to identify potential REST regulated genes. They used the original 21bp canonical RE1 sequence identified in SCG10 and the type II sodium channel genes to generate algorithms for the genome-wide analysis of RE1 sites (Lunyak et al., 2002, Bruce et al., 2004, Johnson et al., 2006, Mortazavi et al., 2006, Wu and Xie, 2006). Although there were some variations in these studies, they all found that a large percent (ranging from 40-90%) of potential targets identified were expressed solely in the nervous system. A small number of genes that have no obvious function in neurons or neuronal development were also identified as potential REST targets, suggesting that REST may be involved in regulating other cellular functions in addition to neuronal gene expression. Interestingly, about 10% of the genes whose expression is highly reduced during the OPC to oligodendrocyte transition contain potential RE1s, suggesting that REST may regulate OPC differentiation (Bruce et al., 2004, Cahoy et al., 2008).

Because these initial studies only identified possible RE1 sites and therefore potential REST regulated genes, several groups performed genome-wide binding experiments including serial analysis of chromatin occupancy (SACO or ChIPSAGE) (Otto et al., 2007), large-scale chromatin immunoprecipitation assays with ultrahigh-throughput DNA sequencing (ChipSeq) (Johnson et al., 2007, Jothi et al., 2008), and array-based chromatin immunoprecipitation microarray (ChIPchip) (Johnson et al., 2007, Abrajano et al., 2009) to identify RE1 sites that are physically occupied by REST (Johnson et al., 2007, Otto et al., 2007, Johnson et al., 2008, Jothi et al., 2008). In these studies, non-canonical RE1 sequences were identified that differed from the 21bp canonical RE1 motif. These novel RE1s include a compressed RE1 lacking one of the two nucleotides at positions 10 and 11 and expanded RE1s containing an insertion of 3-9 nucleotides between positions 11 and 12 (Johnson et al., 2007, Otto et al., 2007). Discovery of these non-canonical RE1 sites identified new REST target genes that were not

previously associated with having an RE1. Out of 1314 RE1 sites identified in the mouse genome by SACO, 893 were the 21bp canonical RE1, 27 were compressed RE1s, and 394 were expanded RE1s (Otto et al., 2007).

RE1 binding selection of REST is cell type specific and both REST binding affinity and clearance of REST from its binding site vary with the RE1 motif (Sun et al., 2005, Johnson et al., 2008, Bruce et al., 2009). The non-canonical sites tend to have a weaker interaction with REST compared to canonical sequence motifs (Bruce et al., 2009). Furthermore, the non-canonical sites tend to be associated with cell or tissue specific genes whereas canonical RE1s are generally associated with REST targets common to all cell types (Bruce et al., 2009). One of the genes containing an expanded RE1, Hes5, inhibits oligodendrocyte differentiation (Liu et al., 2006). If REST is a functional repressor in oligodendrocyte progenitor cells, it is possible that it could use this weakly interacting non-canonical RE1 site to selectively repress Hes5 during the onset of oligodendrogenesis.

In addition to regulating genes that code for proteins, computational analyses and genome-wide binding studies have identified a large number of genes that code for noncoding RNAs, such as macroRNAs and microRNAs (miRNAs), as REST targets (Conaco et al., 2006, Mortazavi et al., 2006, Wu and Xie, 2006, Otto et al., 2007, Johnson et al., 2008, Jothi et al., 2008). One of the first REST regulated miRNAs to be identified in mice was the proneural *miR-124a* (Conaco et al., 2006, Visvanathan et al., 2007). In differentiated neurons, the lack of REST expression allows for the activation of *miR-124a* which can negatively regulate many non-neuronal transcripts (Conaco et al., 2006). Because REST has the ability to regulate the expression of both transcription factors and miRNAs, REST has the ability to indirectly regulate many genes that do not contain RE1s. MicroRNAs have an important role during oligodendrocyte differentiation (Kawase-Koga et al., 2009, Shin et al., 2009, Dugas et al., 2010, Zhao et al., 2010). One of the highest expressed miRNAs in the oligodendrocyte lineage, *miR-338*, contains a potential RE1 sequence, is capable of binding REST,

but is not negatively regulated by REST in TCMK1 cells as demonstrated by a luciferase reporter assay (Otto, S. and Conaco, C., unpublished data).

Understanding REST regulation

Despite that a large number of genes contain functional RE1s, perturbing REST function alone is not sufficient to derepress every REST regulated gene and therefore REST is not a global switch for their expression (Chen et al., 1998). Knockout studies demonstrated that mice homozygous null for REST display widespread apoptotic cell death, malformation in the telencephalic vesicles, and expression of the neuron-specific protein, Beta III tubulin, in non-neural tissues (Chen et al., 1998, Jones and Meech, 1999). REST knock-out mice die by embryonic day 11.5, precluding studies to understand REST regulation postnatally and throughout development (Chen et al., 1998). Despite the derepression of Beta III tubulin, embryos failed to express other identified neuronal REST targets such as Synapsin I, Calbindin, or SCG10. This is likely a result of a lack of appropriate activators or expression of additional repressors during this stage of development. To look at a slightly later stage, the same group infected chick embryos with a virus encoding a dominant negative form of REST (DnREST) containing the DNA binding domain but lacking the N and C-terminal repressor domains. DnREST was sufficient to derepress the REST regulated target genes Beta III tubulin, SCG10, and N-Cam but not Middle Neurofilament (Chen et al., 1998).

Over expression of REST-VP16, a chimeric transactivator protein that contains the DNA binding domain of REST fused to the activation domain of the herpes simplex viral protein VP16, can promote the differentiation of C2C12 myoblasts into cells with a mature neuronal phenotype (Watanabe et al., 2004). This phenotype included depolarization-dependent calcium influx, synaptic vesicle recycling, and the expression of the REST regulated terminal neuronal differentiation genes Beta III tubulin, SCG10, BDNF, Synapsin, and the Leucine-rich repeat neuronal protein, Lrrn1. The in vitro differentiated neurons derived from the C2C12 myoblasts were injected into the mouse brain where they

survived and incorporated into the normal brain without producing tumors. Similarly, activation of REST target genes in neural stem cell lines by over expressing the REST-VP16 chimeric protein is sufficient to induce neuronal differentiation (Su et al., 2004).

In addition to gene repression, REST has also been associated with transcriptional activation (Bessis et al., 1997, Kallunki et al., 1997, Armisen et al., 2002, Kuwabara et al., 2004). Mutation of the RE1 site in the mouse L1cam gene resulted in the loss of its expression in some neuronal cells (Kallunki et al., 1997). Furthermore, inhibiting REST function in developing *Xenopus laevis* embryos decreased the expression of some RE1-containing genes including Scn2a2, Stmn2, and Tubb3 in ganglia and cranial neurons (Armisen et al., 2002). Otto et al., (2007) reported a class of REST targets that show a decrease rather than an increase in their transcriptional activity when REST function is perturbed. However, construction of a luciferase reporter assay containing the RE1 site for one of these genes, Syt2, lead them to conclude that the activation functions of REST are indirect because DnREST was able to derepress luciferase activity (Otto et al., 2007).

The role of REST in neuronal development

REST regulation of neuronal gene expression is necessary for proper nervous system development. During the transition from an embryonic stem cell (ES) to a neuron, the REST complex differentially regulates gene expression depending on the developmental stage to allow for the timely activation of genes required for neuronal differentiation and the neuronal phenotype (Ballas et al., 2005). Unlike fully differentiated post-mitotic cells, neuronal gene chromatin in embryonic stem cells and neuronal progenitor must be repressed but not silenced so that they are ready for expression upon neuronal differentiation. To do this, REST recruits CoREST, mSin3, HDAC, and MeCP2 to neuronal gene chromatin in ES cells, but the DNA within and surrounding the RE1 remains unmethylated (Ballas et al., 2005). There is also a reduction in the association of the REST complex with the histone H3-K9 methyltransferase G9a in ES cells (Ballas et al.,

2005). Instead, neuronal gene chromatin in ES cells are di- and tri- methylated on H3-K4 and the chromatin is globally enriched in H3-K4 acetylation (Ballas et al., 2005). These modifications are normally associated with actively transcribed genes, although the neuronal gene chromatin in ES cells are repressed, physically associated with REST, and the REST complex interacts with the histone H3-K4 demethylase LSD1 (Ballas et al., 2005). Therefore the REST complex represses neuronal genes in ES cells primarily through HDACs and these genes remain inactive but poised for activation. Blocking HDAC activity is sufficient to induce the neuronal differentiation of adult hippocampal neural progenitor cells (Hsieh et al., 2004), similar to the effect of blocking HDACs in OPCs.

The differential recruitment of proteins to the REST complex in stem cells and differentiated cells is accompanied by variations in REST targets (Johnson et al., 2008). Genome-wide binding experiments performed in ES cell, neuronal stem cells, and fibroblasts demonstrated that the most genes occupied by REST in neural stem cells are also bound in ES cells (Johnson et al., 2008). However, a large set of sites bound by REST in ES cells were unique to that cell type.

The transition from embryonic stem cells to neural progenitors requires posttranslational degradation of REST to levels that are just sufficient to keep neuronal genes inactive (Ballas et al., 2005). REST protein can be degraded by the E3 ubiquitin ligase, β-TrCP (Westbrook et al., 2008). However, the proteasomal degradation of REST in neural progenitors is suggested to be ubiquitin-independent since polyubiquitinated REST was not detected (Ballas et al., 2005). As neural progenitors differentiate into mature neurons, REST dissociates from the neuronal gene chromatin and becomes transcriptionally repressed (Ballas et al., 2005). In post-mitotic cortical neurons, REST transcription is repressed by the recruitment of a complex containing the retinoic acid receptor (RAR), N-CoR, mSIN3A, CoREST, HDAC, and MeCP2 to a retinoic acid receptor element (RARE) 400 bp upstream of the REST transcriptional start site (Ballas et al., 2005).

In addition to posttranslational degradation and gene repression, REST can be negatively regulated by other mechanisms. First, a brain specific truncated

isoform of REST, REST4, has been identified. REST4 may act as a negative regulator of REST by competing with endogenous REST for binding to the RE1 therefore preventing REST mediated repression (Tabuchi et al., 2002). Both REST and REST4 interact with RILP, a LIM domain protein, for nuclear translocation (Shimojo and Hersh, 2003). In some neurons, the huntingtin protein interacts with REST and RILP to sequester REST in the cytoplasm (Shimojo, 2008). Also, a small modulatory double stranded RNA (smRNA) has been identified that can interact with the REST transcriptional machinery through a dsRNA/protein interaction and may convert REST from a repressor to an activator during the differentiation of adult hippocampal stem cells (Kuwabara et al., 2004). Lastly, there is also an RE1 sequence in the REST promoter itself suggesting some type of autoregulation (Johnson et al., 2008).

Although REST regulates neuronal development, it is somewhat controversial whether REST has a role in the maintenance of embryonic stem cell pluripotency (Singh et al., 2008, Buckley et al., 2009, Jorgensen et al., 2009a, Jorgensen et al., 2009b, Yamada et al., 2010). Overexpression of Oct4, Sox2, Nanog, and either Klf4 or c-myc reversed differentiated fibroblasts back to an embryonic stem cell state indicating that differentiation may be reversible (Takahashi and Yamanaka, 2006, Wernig et al., 2007, Mikkelsen et al., 2008, Deng et al., 2009). The reprogrammed cells exhibited histone modification and DNA methylation patterns identical to ES cells (Wernig et al., 2007, Huangfu et al., 2008a, Huangfu et al., 2008b, Mikkelsen et al., 2008, Shi et al., 2008, Deng et al., 2009, Liu and Casaccia, 2010). One study observed that knocking down REST expression in mouse ES cells causes a loss of self-renewal and a decrease in the expression of the self-renewal/pluripotency markers Oct4/Sox2/Nanog (Singh et al., 2008) whereas other groups claim that REST-deficient ES cells maintain the ability to self-renew and the transcript levels of Oct3/Sox2/Nanog do not change (Buckley et al., 2009, Jorgensen et al., 2009b). A more recent study suggests that REST is involved in suppressing self-renewal genes during the early differentiation of ES cells and that REST is not required for the maintenance of pluripotency (Yamada et al., 2010). Either way, REST is a part of the

Oct4/Sox2/nanog regulatory network and they share a large number of gene targets including Nanog itself which contains an RE1 (Johnson et al., 2008). REST is a target of Oct4/Sox2/Nanog and knockdown of either Nanog or Oct4 decreases REST expression in mouse ES cells (Boyer et al., 2005). REST was also identified as a target in the Wnt pathway. Expression of Wnt1 in chick embryonic spinal cord induces the expression of REST (Nishihara et al., 2003). Like Nanog, RE1 sequences were also identified in the regulatory regions of Wnt genes (Johnson et al., 2008).

REST and disease

As discussed above, REST regulation is a complicated and complex event. REST is an important regulator in development and perturbing this regulation can have serious and life threatening consequences. Normal REST function is disrupted in many diseases including cancer, Huntington's disease, epilepsy, Rett syndrome, and X-linked mental retardation (Palm et al., 1998, Palm et al., 1999, Coulson et al., 2000, Paquette et al., 2000, Calderone et al., 2003, Coulson et al., 2003, Gurrola-Diaz et al., 2003, Zuccato et al., 2003, Neumann et al., 2004, Coulson, 2005, Fuller et al., 2005, Tawadros et al., 2005, Westbrook et al., 2005, Majumder, 2006, Su et al., 2006, Ooi and Wood, 2007, Tahiliani et al., 2007, Ding et al., 2008). Although REST is transcriptionally repressed during neurogenesis and is typically not thought to be expressed by neuronal cells, some studies have reported REST expression in differentiated neurons. REST protein can be detected in rat hippocampal and cortical neurons where it is upregulated in response to seizure and ischemia and most likely contributes to neuronal death (Palm et al., 1998, Calderone et al., 2003). Zuccato et al., (2003) demonstrated that REST protein is expressed and normally sequestered in the cytoplasm of some neuronal cells by indirectly interacting with the wild type huntingtin protein. In Huntington's disease, the mutant huntingtin protein loses the ability to interact with REST, allowing it to enter the nucleus where it can disrupt normal gene expression by repressing genes such as BDNF (brain derived neurotrophic factor) (Zuccato et al., 2003).

REST was first linked to cancer in studies from Tonis Timmusk's laboratory (Palm et al., 1999). Overexpression, deletion, depletion, mutation, and alternative splicing of REST have all now been associated with different types of cancer (Coulson, 2005). Timmusk's laboratory found that a neuron specific truncated isoform of REST containing a weakened DNA binding domain and lacking the C-terminal repressor domain was overexpressed in neuroblastoma cells. Soon after, loss of REST function was linked to small cell lung cancer (SCLC) (Coulson et al., 2000, Lawinger et al., 2000). A variety of human tumors, including SCLC, activate the expression of neuronal genes and exhibit a neuroendocrine phenotype. Several SCLC cell lines and primary samples from human SCLC contain high levels of a truncated isoform of REST that is not expressed in normal bronchial epithelial cells (Coulson et al., 2000). This truncated isoform of REST likely functions as dominant negative, consistent with the expression of neuronal markers in neuroendocrine tumors such as SCLC. Other studies showed a lack of REST protein in SCLC cell lines as opposed to an overexpression of the truncated isoform (Gurrola-Diaz et al., 2003, Neumann et al., 2004, Majumder, 2006). Loss of REST function was also identified in prostatic LNCaP cancer cells which contain a neuroendocrine phenotype (Tawadros et al., 2005). In 2005, REST was confirmed as a tumor suppressor in a RNA interference (RNAi) library screen that identified genes required to suppress human mammary epithelial cells from transforming (Westbrook et al., 2005). The same studied showed that one third of 38 colon cancer cell lines and 42 primary human colon tumors contained different sized chromosomal deletions encompassing the REST locus (Westbrook et al., 2005). Transduction of a colon cancer cell line containing this deletion, SW1417, with a retrovirus expressing REST resulted in a greater than 50 fold reduction in colony formation, suggesting that the proliferation of these cancer cells is dependent on the loss of REST function (Westbrook et al., 2005).

In contrast to its role as a tumor suppressor, REST can also function as an oncogene in human medulloblastoma tumors and cell lines (Lawinger et al., 2000, Fuller et al., 2005). Abnormally high levels of both Myc and REST are expressed

in many human medulloblastomas (Fuller et al., 2005, Su et al., 2006). High levels Myc result in proliferation of the neural stem cells whereas high levels of REST prevent the neural stem cells from activating the neuronal genes required for differentiation and therefore the cells remain in an undifferentiated proliferative state (Majumder, 2006). Overexpression of Myc or REST alone does not appear to be sufficient to cause tumorigenesis (Paquette et al., 2000, Fleischer et al., 2003, Su et al., 2006). However, activation of REST target genes by expressing REST-VP16 in the REST overexpressing medulloblastoma tumor cells blocked their tumorigenic potential and inhibited tumor growth in mice (Lawinger et al., 2000, Paquette et al., 2000, Fuller et al., 2005, Su et al., 2006).

REST and glia

REST regulation is a complex process that can be regulated at multiple levels including variations in RE1s, levels of REST expression, differential gene regulation between cell types, negative regulators of REST, and whether REST functions as an activator or repressor in a particular setting. REST regulates neuronal differentiation by recruiting HDACs and other proteins to repress neuronal genes. Because OPCs have neurogenic potential and require HDAC repression to differentiate into mature oligodendrocytes, I wanted to examine the role of REST in OPC cell fate determination and differentiation. Understanding oligodendrocyte differentiation will help to understand remyelination in demyelinating diseases such as MS.

Prior to the completion of these studies, nothing was known about the expression or function of REST in glia or during glial development. However, within the past few months two groups identified a potential role for REST in gliogenesis. The first group performed a genome-wide binding experiment using ChIP on chip assays to identify REST and CoREST binding sites during astrocyte and oligodendrocyte lineage specification and oligodendrocyte maturation (Abrajano et al., 2009). In a different study, Kohyama et al., (2010) examined the role of REST in BMP induced astrocyte differentiation. They discovered that

REST is upregulated during the neural progenitor cell to astrocyte transition and that REST regulation is required for BMP induced astrocyte differentiation.

These recent studies examined REST expression and function in glia. However, neither group investigated the function of REST during oligodendrocyte differentiation or its influence on the fate choice of an OPC. In this dissertation, I will demonstrate that REST is a functional transcriptional repressor in oligodendrocyte lineage cells and is involved in regulating OPC cell fate determination and differentiation.

CHAPTER II
EXPERIMENTAL PROCEDURES

Cell culture

Primary cultures of rat glia

Rat glial cultures were prepared using the mechanical dissociation or shaking method with some modifications (McCarthy and de Vellis, 1980). Cerebral cortices from postnatal day 1 to 3 Sprague Dawley rats were dissected, minced, and digested using 0.25% trypsin (Worthington Biochemical Corporation) in phosphate buffered saline (PBS) and 80μg/ml Deoxyribonuclease I (Dnase, Sigma) for 20 minutes at 37°C. Cells were further dissociated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gemini) using a series of flamed end Pasteur pipettes. Cells were washed 3x's with Leibovitzs L-15 Medium (L15, Gibco) and plated in 25μg/ml poly-L-lysine (PLL, Sigma) coated 75cm^2 tissue culture flasks in DMEM containing 1% penicillin-streptomycin (P/S) and 10% FBS, 1 pup per flask. Cells were grown at 37°C for 10 days with a media change every 2-3 days. On day 10, 2-3hrs post media change, the flasks were sealed with Parafilm and shaken at 37°C at 250rpm overnight (16-20hrs) followed by an additional 30 minutes at 300rpm. Nonadherent cells were filtered through a 20 micron nylon mesh filter (Spectrum Laboratories, Inc.) to remove astrocyte aggregates. The remaining cells underwent two rounds of differential adhesion on tissue culture dishes (Corning, NY) for 30 minutes each at 37°C (with a brief, gentle agitation after 15min by carefully swirling the dishes) to remove contaminating astrocytes and microglia. Halfway through (15min) each differential adhesion, dishes were briefly and gently swirled a few times to prevent OPCs from adhering to the bottom. The nonadherent cells (OPCs) were plated on 25μg/ml PLL coated tissue culture dishes or 50 μg/ml PLL coated glass coverslips and grown in Neurobasal A Medium supplemented with B27 containing 1%P/S, 1mM L-Glutamine (all from Gibco), 10ng/ml platelet-derived growth factor AA ($PDGF_{AA}$) and 10ng/ml

fibroblast growth factor basic (bFGF) (both from PreproTech). This media promotes OPCs to proliferate without differentiating (Pringle et al., 1989, McKinnon et al., 1990, Engel and Wolswijk, 1996). OPCs remained in proliferation media or were switched to oligodendrocyte differentiation media (DMEM supplemented with N2 (Gibco) containing 1%P/S, 1% FBS, 40ng/ml thyroxine (T4) (Sigma), and 30ng/ml tri-idothyronine (T3) (Sigma)), 2A differentiation media (DMEM containing 1%P/S and 10% FBS), or stem cell media (NBA/B27 containing 1% P/S, 1% FBS and 1mM L-Glutamine). Type I astrocytes were prepared by removing the adherent cells (astrocytes) from the 75cm^2 tissue culture flasks following the overnight shake using 0.05% trypsin-EDTA (Gibco). Astrocytes were grown in DMEM containing 1%P/S and 10% FBS. These methods resulted in a 90-95% pure population of each cell type. For some experiments OPCs were further purified using magnetic bead cell separation as per the manufacturer's instructions (MACS, Miltenyi Biotec). Briefly, following differential adhesion, OPCs were incubated at 4°C with anti-A2B5 IgM antibody for 20 minutes, washed 2x's in degassed buffer (PBS pH 7.2 containing 0.5% BSA and 2mM ethylenediaminetetraacetic acid (EDTA)), and incubated with rat anti-mouse IgM MicroBeads (MACS, Miltenyi Biotech) at 4°C for 20 minutes with occasional, gentle agitation. Cells were washed 2x's with buffer and separated using positive selection MS separation columns (MACS, Miltenyi Biotec).

Primary cultures of rat optic nerve glia

Optic nerves were dissected from postnatal day 7 rats, minced, and resuspended in 0.25% trypsin in PBS, 200µg/ml collagenase , and 80µg/ml Dnase for 25 minutes at 37°C. Cells were resuspended in DMEM+10%FBS and a series of different sized flamed end Pasteur pipettes were used to mechanically dissociate the cells. After 4 washes with L15, cells were plated on 50µg/ml PLL coated glass coverslips in DMEM containing 1%P/S and 10% FBS.

Primary cultures of cerebellar granule neurons

Cerebellar granular neurons (CGN) were purified using a discontinuous Percoll gradient (Hatten, 1985). Postnatal day 5-6 rat cerebella were dissected, minced, and digested using 0.25% trypsin in PBS plus 80µg/ml Dnase for 20 minutes at 37°C. Following several washes in L15, the neurons were placed on top of a step gradient of 35%/60% Percoll (Sigma) and centrifuged. Cells that maintained at the 35/60% interface (CGNs) were collected, washed, resuspended in DMEM containing 1%P/S, 10% FBS and 20mM KCl, and plated on 25µg/ml PLL coated tissue culture dishes.

Cell Lines

Rat embryonic fibroblast (REF) and HEK293 cell lines were grown in DMEM supplemented with 1% P/S and 10% FBS. Rat pheochromocytoma PC12 cells were maintained in DMEM containing 1% P/S, 5% FBS, and 10% horse serum. Plat-E cells were grown in DMEM containing 10% FBS, 1% P/S, 1µg/ml puromycin dihydrochloride (Sigma) and 10µg/ml Blasticidin S HCl (Invitrogen). All cell lines were passed 1:5 every 3-5 days.

Nucleofections

Transfections were performed using the Nucleofector electroporation system as per the manufacturer's instructions (Amaxa). Cells were nucleofected with 1-3µg of DNA depending on the experiment and $3x10^6$ cells were used per nucleofection. For cell lines, cell line V solution (Amaxa) and program U-29 were used for PC12 cells and cell line T solution (Amaxa), program O-17 for REFs. PC12 cells and REFs were plated on 25µg/ml PLL coated 60mm tissue culture dishes for Luciferase assays. OPCs were transfected using the Nucleofector electroporation program O-17 with the rat oligodendrocyte transfection reagent (Amaxa). Following the second differential adhesion during the OPC preparation/purification (see above), cells were rinsed and plated overnight (37°C) in OPC proliferation media with high growth factors (20ng/ml $PDGF_{AA}$ and 20ng/ml bFGF) in uncoated Petri dishes at a concentration of 4-

$6x10^6$ cells per dish. OPCs were harvested by gentle pipeting, centrifuged, and resuspended in DMEM containing 1% P/S and 10% FBS (about $3x10^6$ cells/ml media) for 1hr at 37°C prior to nucleofection. Following nucleofection, OPCs were plated on 25µg/ml PLL coated 60mm tissue culture dishes or 50µg/ml PLL coated glass coverslips.

Immunocytochemistry

A list of all primary antibodies can be found at the end of Materials and Methods. Secondary antibodies used for immunocytochemistry include Alexafluor594 donkey anti-rabbit, Alexafluor488 donkey anti-rabbit, Alexafluor594 donkey anti-mouse, and Alexafluor594 donkey anti-rabbit (all from Molecular Probes, Inc). Live cell staining of cell surface antigens was performed using antibodies against NG2, A2B5, and O4. Cells were rinsed once and blocked with L15/NDS (L15 containing 5% normal donkey serum, NDS) for 20 minutes at room temperature. Primary and secondary antibodies were diluted in L15/NDS and cells were incubated with the primary antibody for 30-45 minutes. Cells were washed 3x's (all washes are 3min each) with L15/NDS and incubated for 30min with secondary antibody followed by 2x L15/NDS and 1x PBS washes. Cells (unless being double labeled to visualize REST expression, see below for REST detection) were fixed with 4% paraformaldehyde (PFA) for 10-15min and washed 3x's in PBS. To visualize the expression of endogenous proteins using antibodies against MBP, GFAP, GFP and TUJ1, cells were fixed in PFA and rinsed as above (in some cases cells were first live stained for NG2, A2B5, or O4) and incubated in blocking solution (PBS containing 5% NDS, 0.01% sodium deoxycholate, and 0.02% NP40) for 30 minutes at room temperature. Primary and secondary antibodies were diluted in blocking solution and staining was performed as above. For immunofluorescence staining to detect REST protein, cells had to undergo microwave fixation (in some cases, cells were first live stained for NG2, A2B5, or O4). For microwave fixation, individual coverslips in a 35mm dish were rinsed 2x's in PBS, covered in 4% PFA and placed on a glass plate over ice (one dish at a time) for 20 seconds in a microwave

containing a cooling system to help minimize heating (Pelco 3451 Lab Microwave System with a Pelco 3420 Microwave Load Cooler and a Pelco 3430 Microwave Power Controller). Cells were rinsed 3x's with PBS, blocked and permeabilized in blocking solution (PBS containing 5% NDS and 0.1% Triton X-100) for 30 minutes and incubated with a REST specific antibody diluted in blocking solution (alone or in combination with antibodies against MBP or GFAP) overnight at 4°C. Cells were rinsed 3x's in blocking solution and incubated with secondary antibody diluted in blocking solution for 30 minutes followed by 3 washes with PBS. For some experiments, DAPI staining was performed by diluting DAPI to 1μg/ml in PBS and covering the cells for 5 minutes after all other primary and secondary staining was complete. Cells were washed 4x's with PBS and mounted onto slides.

Proliferation assays

Retroviral infected OPCs were grown in proliferation media for 3d followed by a media switch to either oligodendrocyte, stem cell, or 2A growth media for 44hrs. 10uM 5-Bromo-2'-deoxyuridine (BrdU, Sigma) was added to the culture media for 4 hours at 37°C. Cells were rinsed in PBS and fixed in 4% PFA for 10 minutes followed by 3 washes in PBS. Immunofluorescence staining to detect GFP using a rabbit anti-GFP antibody was performed as described previously. Following the secondary antibody and several washes, cells were fixed a second time in 4% PFA for 5 minutes, washed 3x's in PBS, and covered with cold 100% methanol for 15min at -20°C. After 3 washes in PBS, cells were incubated in 2N HCL for 10min at room temperature followed by 10min at 37°C and washed 3x 5min in 100mM Sodium Borate (pH 8.5). Cells were washed with PBS 3x's and incubated in blocking solution (PBS containing 5% NDS and 0.01% Triton X-100) for 30 minutes. Mouse anti-BrdU and secondary antibodies were diluted individually in blocking solution. Cells were covered with primary antibody for 30 minutes, washed in blocking buffer 3x's 3min, followed by 30min incubation with both secondary antibodies and 3 washes in PBS. DAPI staining and mounting was performed as described previously.

Clonal analysis

OPCs were purified and plated in 25µg /ml PLL coated 100mm tissue culture dishes in proliferation media for 1-2 days followed by pMXsIG or pMXsIG-DnREST retroviral infection (see below). OPCs were grown for 5d post infection in proliferation media. One dish of control or DnREST infected cells were passed onto a large number of 50µg /ml PLL coated 18mm glass coverslips using 0.05% trypsin-EDTA diluted 1:1 with PBS (about 1,000 cells per coverslip) so that all infected cells in each condition originated from the same population. OPCs were grown for 2d in proliferation media, washed with L15, and plated in proliferation, oligodendrocyte, stem cell, or 2A differentiation media.

Cell extracts and immunoblot analysis

To isolate optic nerve protein, whole rat optic nerves were dissected and placed in PBS on ice. Following centrifugation, optic nerves were homogenized in buffer (2% sodium dodecyl sulfate (SDS), 10mM Tris (pH 7.5), 5mM EDTA (pH8.0), 200uM phenylmethanesulfonyl fluoride (PMSF), 1µg /ml aprotinin, and leupeptin) using a Wheaton overhead stirrer (Wheaton Instruments) and tissue grinder (Duall 20, Kontes Glass Co). Samples were centrifuged at 30,000 rpm for 15 minutes at 20°C in an Optima TLX ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was collected and a Pierce BCA Protein Assay (Micro BCA Protein Assay Kit, Thermo Scientific) was performed to measure protein concentration. For primary cells and cell lines, whole cell lysis was performed by rinsing the cells 2x in cold PBS and incubating them on ice in NP-40 Lysis buffer (25mM Tris (pH 7.5), 125mM NaCl, 1.0% NP-40, 1mM EDTA, 200uM PMSF, 1µg /ml aprotinin, and leupeptin) for 30 minutes. Samples were centrifuged at 14K for 10 minutes at 4°C and the supernatant containing the cellular protein was collected. Nuclear extracts were prepared using a modified Dignam method (Grimes et al., 2000). Cells were washed and centrifuged in cold PBS. Cell pellets were resuspended in lysis buffer (20mM HEPES (pH 7.9), 1.5 mM MgCl2, 10mM KCl, 0.5mM DTT, and complete Mini protease inhibitor

cocktail (ROCHE)) and passed 5 times through a 25 gauge needle using a 1ml syringe. Following centrifugation the supernatant (containing the cytoplasmic fraction) was saved and the nuclei were resuspended in nuclear lysis buffer (20mM HEPES pH 7.9, 1.5mM MgCl2, 0.2mM EDTA, 20% glycerol, 0.42M NaCl, 0.5mM DTT, and complete Mini protease inhibitor cocktail) and rotated for 20 minutes at 4°C. Samples were centrifuged and the nuclear protein was isolated. Protein concentrations were measured using a standard Bradford assay (Bio-Rad). All samples were boiled for 3 minutes in 6X SDS-loading buffer. SDS-polyacrylamic gel electrophoresis and Western blotting were performed using standard techniques. Proteins were electrophoresed on 6 to 8% polyacrylamide gels, electrophoretically transferred to nitrocellulose (HybondTM-ECL, Amersham Biosciences) and blocked in TBS or TBST containing 5% milk. Primary antibodies used are listed at the end of Materials and Methods and were used with ECL™ anti-rabbit IgG horseradish peroxidase linked whole antibody (from donkey) or ECL™ anti-mouse Ig horseradish peroxidase linked whole antibody (from sheep) secondary antibodies (Amersham Biosciences).

Chromatin immunoprecipitation assays (ChIP)

ChIP assays were performed as described previously (Ballas et al., 2001) for OPCs and REFs. Briefly, 75% confluent cells were treated at room temperature with 1% formaldehyde for 10min to crosslink protein to the DNA. Following three washes with ice cold PBS, cells were collected and lysed in a cell lysis buffer (5mM HEPES (pH8.0), 85mM KCL, 0.5% Triton-X-100, and complete Mini protease inhibitor cocktail (Roche)). Isolated nuclei were resuspended in a nuclear lysis buffer (50mM Tris-HCL pH 8.0, 10mM EDTA, 1% SDS, and complete Mini protease inhibitor cocktail) and sonicated on ice using a Branson Sonifier to generate chromatin fragments with an average length of 300-800 base pairs. Nuclear extracts were precleared using recombinant protein-G agarose beads (Invitrogen) coated with yeast tRNA (20mg/ml) and Salmon Sperm DNA (10mg/ml) for 1 hour at 4ºC. The chromatin was immunoprecipitated by diluting the cleared chromatin 1:10 in an IP buffer (0.01% SDS, 1.1% Triton X-

100, 1.2mM EDTA, 16.7mM Tris-HCL (pH 8.0), 167mM NaCL, and complete Mini protease inhibitor cocktail) and incubated with 4µg /ml of either rabbit anti-REST-P73 (Chong et al., 1995), rabbit anti-REST-C (Ballas et al., 2001), or a non-specific rabbit IgG antibody (Santa Cruz) for 12-16 hours at 4ºC. Immunocomplexes were collected using recombinant protein-G agarose beads preadsorbed with yeast tRNA (20mg/ml) and Salmon Sperm DNA (10mg/ml) for 4hrs at 4ºC. Samples were washed 2x with dialysis buffer (2mM EDTA, 50MM Tris-HCl (pH 8.0), and 0.2% Sarkosyl), 1x with TSE-500 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.0), and 500mM NaCl), and 5x with LiCl Detergent/Wash (100mM Tris-HCl (pH 8.0), 500mM LiCl, 1% Triton X-100, and 1% deoxycholic acid). Following elution, formaldehyde crosslinks were reversed by heating at 65ºC for 12 hours and samples were purified using a PCR purification kit (Qiagen) and resuspended in 50ul of TE. The following primers were used to amplify DNA regions containing an RE1 sequence:

Scn2a1	Left CCAAGATTATATGTCAGCTCGCAG
	Right CAACTTCGTCAAGCAGGGTCAGG
NF-M	Left GTTCATTGTGCCAACTCTGCAGTGC
	Right GGACATCTGAAGCTTCTAGCAGGTAAGG,
SCG10	Left ACAGTCCGAAGTGCAAATGCCAG
	Right CTGCCGTTCAGCAAATCATCCAG
NeuroD2	Left GACTGGAGGCATAGGTCACCTTTCTAC
	Right GGAAGTCACCACTAGCACATTTCGCG
Grik3	Left CCTGTTAGGAGGGTCGCCCAG
	Right CCGCCACAGAAGGCAGAAGACC

Primers were also built around a random region of the NG2 gene (*CSPG4*) that does not contain a RE1 as a control (left CTTCTCTGGACCCCACTCAC, right TGGCCCTCAAATATGTCCAC). A 50ul PCR reaction contained 2ul of eluted chromatin, 5ul 10X PCR Buffer (Roche), 5ul 10X PCR Enhancer (Invitrogen), 200mM dNTPs (Roche), 200nM forward and reverse primers, and 0.5ul Taq Polymerase (Roche). DNA was amplified using a Gene AMP PCR System 9700 (Applied Biosystems) with an initial denaturation at 94ºC for 4min, 40 cycles of 94ºC for 30sec, 56-58ºC for 30sec, and 72ºC for 1min, ending with a final 7min incubation at 72ºC. Samples were analyzed on a 1.5% agarose (Invitrogen) gel containing ethidium bromide (Sigma).

Real-time RT-PCR

Total RNA from primary cells, cell lines, and minced whole optic nerve was isolated by TRIZOL (Invitrogen) RNA extraction and treated with Rnase-free DNase (TURBO DNA-*free*, Ambion) following both manufacturers' protocols. RNA concentrations were measured using a NanoDrop 1000 spectrophotometer (ThermoScientific) and 250-300ng of total RNA was used for reverse transcription with Superscript II Reverse Transcriptase (Invitrogen) via manufacturer's protocol. Quantitative real-time PCR was performed using FastStart Universal SYBR Green Master Mix (Roche) in an Applied Biosystems 7300 Real-Time PCR System under the cycling conditions: 95°C /10 minutes followed by 40 cycles of 95°C/15sec and 57°C /1min (Otto et al., 2007). The relative abundance of the specific mRNA being examined was normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH). All primers were designed using Massachusetts Institute of Technology's Primer3 software and include:

Calb1:	Left CCGAACAGATCTTGCCCTTA
	Right GCGCACAGTTATGGTTTTAGA
CelsR3:	Left CCATCCTTGCCTCTTTCAAC
	Right TGGAAGGTCCAAGTGCAGA
Ctnnb1:	Left TTTCACTCTGGTGGATACGG
	Right GCAGCCCATCAACTGGATA
CNP:	Left AGTGCAAGACGCTGTTCATC
	Right AAGCATCAGCAGACACCATCT
DCX:	Left AGCACAGTGGATTCCTGGTC
	Right ATGGATACCATGCGGTTCA
DCX:	Left ACAGAACCAGAACCTTGCAG
	Right AGCGTACACAATCCCCTTGA
GAPDH	Left AAGTATGATGACATCAAGAAGGTGGT
	Right AGCCCAGGATGCCCTTAGT
GFAP:	Left AGTTACCAGGAGGCACTCG
	Right CGATGTCCAGGGCTAGCTTA
GFAP:	Left AGTTACCAGGAGGCACTCG
	Right CGATGTCCAGGGCTAGCTTA
GFAP:	Left GGAGCTCAATGACCGCTTT
	Right CTCCTTGGCTCGAAGCTG
GluR2:	Left GGGCGCTGATCAAGAATACA
	Right GCGAAACTGTTGGCTACCTC

Hes1:	Left CTGACGGCCAATTTGCTT
	Right GTTAGGACCCACCGAGGTC
Hes5:	Left GAGATGCTCAGTCCCAAGGA
	Right GCAGTTTCAGCTGCTCAATG
Hes5:	Left AAGAGCCTGCACCAGGACTA
	Right CGCTGGAAGTGGTAAAGCAG
Id2:	Left CTCCAAGCTCAAGGAACTGG
	Right CGATCTGCAGGTCCAAGATA
Id4:	Left GTGCGATATGAACGACTGCT
	Right CTGCAGGTCCAGGATGTAGTC
MAG:	Left GTGGGCTCCCTTTCTCTTG
	Right ATGTTGGCAGAGAGGAGCAG
Mash1/Acl1:	Left GCCCAGCACTCTCTCACTTC
	Right TGGGATTATTTGGCTGAACC
MBP	Left CACAAGAACTACCCACTACGG
	Right GGGTGTACGAGGTGTCACAA
MOBP:	Left CCCACCCTTCACCTTCCT
	Right CCAGTCCTCCTCCTTCTTCTG
Myt1L:	Left CGTGACTACTTTGACGGAAATG
	Right AATTCCTCTCACAGCCTGCTT
Myt1 predicted:	Left TCATTGCAACTTCCCTCCTAA
	Right GGTCCTTTTCACCCTCATCA
Nav1.2:	Left GATCCTCATCTGCCTCAACA
	Right TGAACAGGACAATGAACACCA
NF-M:	Left AGCCTGGAGAAAGACACCAA
	Right TTGTCACCCACTTCCTCCTC
NF-M:	Left GAGAGCAGCCTCGACTTCAG
	Right CCCTGCAGCTGCTCTTTCT
NG2:	Left ACCCAGCAGGATGGCTTC
	Right CTCATTCACGTCCCTCACAG
NeuroD1:	Left CCAAATCATACAGCGAGAGC
	Right CTCCTTCTTGTCTGCCTCGT
NeuroD1:	Left AGGAACACGAGGCAGACAAG
	Right TCTTGGGCTTTTGATCATCC
Ngn3 :	Left GAAAGGGAGGGAGTCAGAGC
	Right GAACTGTGCGAATCCTGGTT
Nkx2.2:	Left GTGATCATCGTTGCCAAATG
	Right AAACCGATGCAACTCAAACA
PDGFαR:	Left CACACCGGATGGTACACTTG
	Right GCAGAATCGTCCTCTTCCAC
Olig1:	Left AACTCCTTGGACCAGTGCAT
	Right CGCCAGTTAAATTCGGCTAC
Olig2:	Left GTGCATCACCCCATCCTC
	Right CCGATGGAGACTTGAGCAG
PLP:	Left GCACTGTTCTGTGGATGTGG
	Right GAAGAAGAAAGAGGCAGTTCCA

PLP:	Left GCTGTGCTAGATGTCTGGTAGG
	Right GCTTCATGTCCACATCCACA
Pou4F3:	Left GCTCAAGCCAGTCCTCCA
	Right GTTTGCGCTTACGCTCACT
Pou4F3:	Left AGAGAAGCGCTCACTCGAAG
	Right GGACAGCAGAGTACTTCATTCG
REST	Left GACGGTCAGCGAATACCACT
	Right CGCATGTGTCGAGTTAGATGA
SCG10:	Left GGCATTTGTGCCACTGTAAG
	Right CAGCAACCTTCAGGAGTTGG
Sox2:	Left ATGGGCTCTGTGGTCAAGTC
	Right CTGATCATGTCCCGGAGGT
Sox10:	Left AACCTCATCCCTCACCTAACTG
	Right GCAGTGCTAACTGAGGCTGA
SYN1:	Left GAAGCCAGACTTTGTGCTGA
	Right ACTGGGGATCCCAGCATAC
SYTII:	Left GATCTTCGTAGGCAGCAACG
	Right CCTCAGGCTTCAGAGAGTGC
TrkC:	Left ACTATGTGGGCTCCGTGCT
	Right CCAGGAGGGGAAAGAGGTT
Tubb3:	Left CATGAACGACCTGGTGTCTG
	Right CTGGGCTTCCGACTCCTC
YY1:	Left ATGAGAAAGCATCTGCACACC
	Right CCAGCTGGTGGTCGTTTTAGC

Luciferase assay

PC12 cells, REFs, and purified OPCs were nucleofected (see above) with 1.0µg of either pGL3-TK-GAD1+ RE1 (reporter plasmid with flanking sequence containing the RE1 site of the glutamic acid decarboxylase (GAD1) gene upstream of a TK promoter and the firefly luciferase gene; (Conaco et al., 2006) or pGL3-TK-GAD1 ΔRE1 (similar reporter plasmid containing the same region of the GAD1 gene but with the RE1 site deleted) (Conaco et al., 2006), along with 100ng of pRL-TK vector encoding the Renilla luciferase gene (Promega). For some experiments, cells were co-nucleofected with 1.0µg of pcdna1-DnREST expression vector (Chong et al., 1995, Otto et al., 2007), pcDNA3-REST-VP16, or an empty vector (pcDNA, Invitrogen). Following nucleofection, cells were plated on 25µg /ml PLL coated 60mm tissue culture dishes in their individual growth media (proliferation media for OPCs) for 48 hours and the ratio of firefly

verses Renilla luciferase activity was measured using the dual luciferase reporter assay system (Promega) following the manufacturer's protocol.

Adenoviral vectors and infection

Adenoviral vectors (pAdTrackCMV vector containing GFP) expressing dominant negative REST (DnREST, lacks the sequences coding for the amino- and carboxy- terminal repressor domains), REST-VP16 (which contains the same region of the REST gene as DnREST fused to the activation domain of the herpes simplex viral protein VP16), or expressing GFP alone have been described previously (Chong et al., 1995, Ballas et al., 2005). HEK293 cells were used for adenovirus production. Cells were infected, collected after 2 days, and the virus was extracted by several freeze/thaw cycles in dry ice/EtOH and a 37° water bath followed by centrifugation. Adenovirus from the supernatant was isolated by a cesium chloride (CsCl) density gradient (1.25g/ml:1.4g/ml CsCl) spun at 32,000rpm in an ultracentrifuge at 4°C. OPCs were purified and infected after 2-3 days in proliferation media. For oligodendrocyte and 2A infection, OPCs were purified, grown in proliferation media for 2-3d, and switched to either oligodendrocyte or 2A differentiation media for 3 days prior to infection. Type I astrocytes were isolated as described previously and were infected 3-6 days post purification. All cells were infected for 8-16 hours with adenovirus at an MOI (multiplicity of infection) of 10-25.

Retroviral vectors and infection

DnREST (from pcDNA1) (Chong et al., 1995) was cloned into the pMXs-IRES-GFP (pMXsIG) vector between the EcoRI and XhoI sites. PMXsIG and pMXsIG-DnREST retrovirus were assembled in Plat-E cells (Morita et al., 2000). Puromycin dihydrochloride, Blasticidin S HCl, and P/S were removed from Plat-E cell media 24hrs prior to transfection and retroviral vectors were transfected into Plat-E cells using *Trans*IT-293 transfection reagent (MirusBio) following manufactures protocol. After 24, 36, and 48 hours the supernatant was collected and stored at -80°C and cells received fresh media (DMEM/10%FBS/1%P/S). To

concentrate the retrovirus the supernatant collected from the different time points was thawed, combined, filtered through a 0.45um filter to remove cells and debris, centrifuged for 90min at 4°C at 25,000rpm and resuspended in a small volume of PBS. OPCs were purified and infected after 1-2 days in proliferation media. Cells were infected to 60-80% efficiency by incubating the cells with virus for 5-8hrs with 3µg /ml Polybrene (Sigma) to increase infectivity (retroviral concentrations were at least 10^6 colony forming units (CFU/ml).

Tissue processing and immunofluorescence staining of optic nerve

Cryostat sections of developing and adult optic nerve were processed and stained as described previously (Tan et al., 2006).

OPC transplantations

Purified OPCs were infected with control or DnREST retrovirus as described above, and grown in proliferation media in culture for about 3 days. On the day of transplantation, OPCs were harvested by trypsin and gentle pipeting, centrifuged, and resuspended in DMEM containing 1% P/S and 10% FBS (about $3x10^6$ cells/ml media) for 1hr at 37°C to recover. OPCs were centrifuged and resuspended at a concentration of $2\text{-}5x10^5$ cells per 1µl of Hanks balanced salt solution and were kept on ice (HBSS). P2 rat pups were anesthetized by hypothermia by placing them on ice for 3-5 minutes and placed on the stereotactic device. Cells were transplanted using a Hamilton syringe (2.5µl, #62) with a 28 gauge needle (1.25”). The syringe was placed in the stereotactic device positioned above the animal. Stereotactic coordinates from bregma: lateral, 1.5mm, anterior 1.4mm, depth 2.0mm. 1.5 µl of the cell suspension was slowly injected over the period of 2-3 minutes. The needle remained in the animal for 1-2 minutes and was slowly withdrawn. After suturing, the pup was placed on a heated blanket and covered in mineral oil before being returned to the mother. After 2 and 9 days the animals were anesthetized using Isoflurane (Baxter) and perfused with 4% PFA. The brains were removed, postfixed in 4% PFA for an hour, and cryopreserved. Cryostat sections processed and stained as described previously (Tan et al., 2006).

Electrophysiological techniques

Whole-cell patch-clamp recordings were performed on control, DnREST, or REST-VP16 adenoviral or retroviral infected OPCs after 4-6 days in either 2A media, stem cell media, or stem cell media plus FGF-basic as described previously (Liu et al., 2007a) using a pipette solution of 105mM K-methanesulfonate, 17.5mM KCL, 10mM HEPES, 0.2mM EGTA, 8mM NaCl, 2mM Mg-ATP, 2mM Na_2-ATP, 0.3mM Na_3-GTP, and 20mM phosphocreatinine. Infected cells were identified by GFP expression.

Primary antibodies

Antibodies listed were used for: immunocytochemistry (IC), immunohystochemistry (IH), Western Blot analysis (WB), cell isolation/purification (CP) and chromatin immunoprecipitation assay (ChIP).

Antibody List:

Mouse anti-A2B5 (ATCC) IC, CP
Mouse anti-Beta actin (Sigma) WB
Mouse anti-BrdU (Sigma) IC
Rabbit anti-Cleaved Caspase-3 (Cell Signaling) IC
Mouse anti-CNPase (Sigma) WB
Mouse anti-GFP (Molecular Probes) IC, IH
Mouse anti-GFAP (Sigma) IC
Mouse anti-NeuN (Millipore) IH
Mouse anti-Neurofilament (DSHB) WB, IC
Mouse anti-NG2 (Chemicon) IC
Mouse anti-O4 (a gift from R. Bansal, U of Conn Health Science Center) IC
Mouse anti-MBP (Millipore) IC
Mouse anti-TUJ1 (Covance) IC, WB
Rabbit anti-CoREST (Gail Mandel) IC, WB
Rabbit anti-GFP (Molecular Probes) IC, IH
Rabbit anti-Histone H3 (Cell Signalling) WB
Rabbit anti-REST-C (Gail Mandel) IC, IH, WB, ChIP
Rabbit anti-REST P73 (Gail Mandel) ChIP
Rabbit anti-SCG10/Stathmin-2 (UC Davis/NIH Neuromab Facility) WB, IC
Rabbit anti-Snap25 (Abcam) WB
Rabbit anti-Synapsin I (Millipore) WB, IC

CHAPTER III
REST EXPRESSION AND FUNCTION AS A TRANSCRIPTIONAL REPRESSOR IN GLIA

INTRODUCTION

Gene repression is required for OPCs to develop into mature oligodendrocytes (Marin-Husstege et al., 2002, Liu and Casaccia, 2010). Some of these genes include those that code for myelin inhibitors and inhibitors of oligodendrocyte differentiation such as Id proteins and Hes5 (Samanta and Kessler, 2004, Liu et al., 2006, Li et al., 2009). Because OPCs are highly plastic cells that can also give rise to neurons, it's likely that neuronal genes also need to be repressed for the initiation of oligodendrocyte maturation. Bioinformatics and genome-wide studies of protein-DNA interactions have demonstrated that many of the genes repressed during the OPC to oligodendrocyte transition contain REST binding sites (Bruce et al., 2004, Johnson et al., 2006, Wu and Xie, 2006, Johnson et al., 2007, Otto et al., 2007, Johnson et al., 2008, Jothi et al., 2008, Abrajano et al., 2009). REST represses a large set of neuronal genes in non-neuronal cells and participates in regulating the developmental transition of a neural stem cell into a neuron (Chong et al., 1995, Schoenherr and Anderson, 1995, Ballas et al., 2005, Otto et al., 2007). Glial cells develop from the same neuroepithelial stem cells as neurons do but the role of REST in glial development has been mostly unexplored.

Before determining if REST has a functional role in OPC specification and differentiation, it is important to first examine REST expression in glial cells and determine if it functions as a transcriptional repressor. REST repression can be negatively regulated by the brain specific isoform REST4 (Tabuchi et al., 2002), proteasomal degradation (Ballas et al., 2005), cytoplasmic sequestering (Shimojo and Hersh, 2003), autoregulation (Johnson et al., 2008), and by a small modulatory double stranded RNA (Kuwabara et al., 2004). Therefore, if REST is expressed in

oligodendrocyte lineage cells it cannot be assumed that it interacts with RE1 sequences and functions to repress RE1 containing genes.

In this chapter, I will demonstrate that REST is expressed in the different types of glia and functions as a transcriptional repressor in oligodendrocyte lineage cells. I will also provide evidence that REST differentially regulates gene expression in the different glia types and that REST function is cell-type dependent.

RESULTS

REST is expressed in glial cells

To examine REST expression in primary glial cells, OPCs and astrocytes were isolated from p2-p4 rat cortices using a modification of the shaking method (Yang et al., 2005). Oligodendrocytes and 2As were obtained by switching purified OPCs from proliferation media to media that promotes their differentiation (see Experimental Methods). The cell purity of the different glial cultures was measured after 5 days in their specified media by immunofluorescence staining using antibodies against cell specific antigens (NG2 and A2B5 for OPCs, O4 for immature oligodendrocytes, MBP for mature oligodendrocytes, and Glial Fibrillary Acidic Protein (GFAP) for astrocytes and 2A's). In OPC, astrocyte, and 2A cultures, 90-95% of the cells expressed the proper cell marker antigens by immunofluorescence. In the OPC cultures grown under oligodendrocyte differentiation conditions, about 90% of the cells expressed the cell marker antigen O4 and 40-50% expressed MBP. High levels of transcripts for cell specific genes including *NG2* in OPCs, *MBP* in oligodendrocytes, and *GFAP* in astrocytes were detected by qRT-PCR (figure III-1a). Only trace amounts of *GFAP* and *MB*P were detected in OPCs, *MBP* and *NG2* in astrocytes, and *NG2* and *GFAP* in oligodendrocytes, providing additional evidence that the glial cultures contained minimal contamination and were differentiating properly.

To determine if any cultures contained neuronal cells, mRNA expression of the neuronal genes *SCG10*, *NF-M*, and *Scn2a1* was measured by qRT-PCR (figure III-1b). Relative to cerebellar granular neurons (CGNs) the glial cultures expressed only a trivial amount of *SCG10*, *NF-M*, and *Scn2a1* transcripts. Lack of neuronal cell contamination in the different glial cultures was confirmed by immunofluorescence staining using an antibody against the immature neuronal marker TUJ1 (beta tubulin III). Less than 2-3% of cells in any of the purified cell populations expressed TUJ1. To further examine the OPC cell population, I measured the mRNA levels of different glial, neuronal, and stem cell genes by

qRT-PCR in OPCs relative to rat E14 brain and P12 cortex (figure III-1c). As expected, genes such as *NG2* and *PDGFaR* showed a higher level of expression in OPCs than in either P12 cortex or E14 brain whereas *neuroD1* and *neuroD2* had significantly lower levels of expression. *MBP* expression was higher in OPCs than E14 brain due to a lack of oligodendrocyte maturation at embryonic day 14. *MBP* expression in the P12 cortex was significantly higher than in either OPCs or E14 brain. These data show that the OPC, 2A, oligodendrocyte, and astrocyte cultures used in the following experiments were highly purified cultures with minimal cell (glial and neuronal) contamination.

REST expression and subcellular localization were examined in the different glial cell types after 4-5days in their specified media. I performed immunofluorescence staining using a polyclonal antibody against the C-terminal domain of REST (REST-C) in combination with antibodies against cell specific proteins. Nuclear REST expression was detected in OPCs, O4 and MBP expressing oligodendrocytes, 2A's, and type 1 astrocytes (figure III-2). REST protein expression in OPCs, oligodendrocytes, and astrocytes was confirmed by immunoblot analysis using rat embryonic fibroblasts (REFs) as a positive control and PC12 cells, which express trace amounts of REST, as a negative control (figure III-3a). In addition to expressing REST, glial cells also expressed the co-repressors CoREST (figure III-3a) and mSin3a (Ballas et al., 2009) suggesting that a functional repressor complex could exist in these cells. Figure III-3b verifies nuclear compartmentalization of REST protein in glia. I isolated nuclear and cytoplasmic proteins and detected REST expression by immunoblot analysis. C6 cell line nuclear extracts were used as a positive control and cerebellar granular neurons as a negative control. In addition to expressing REST protein, OPCs, oligodendrocytes, 2As, and astrocytes express similar levels of REST transcripts relative to REFs (figure III-3c).

Because it took 12-15 days to purify and differentiate the different glia, I wanted to verify that REST expression was not induced by the relatively long cell culture period. To test this, I examined REST expression in glia from short term optic nerve cultures and within the intact postnatal optic nerve. First, I confirmed

that REST is expressed in OPCs originating from the optic nerve. Dissociated p7 rat optic nerve cultures containing mixed glia were grown in OPC proliferation media for 3 days and cells were immunostained with anti-REST and anti-NG2 antibodies (figure III-4a). Consistent with cortical derived OPCs, OPCs originating from the optic nerve expressed nuclear REST after a few days in culture. To confirm that REST expression was not induced from long culture periods in the cortical bulk glia, short term cultures of freshly dissociated p7 rat optic nerve cells were examined. Following dissociation, cells were plated onto coverslips and grown for three hours in DMEM containing 10% FBS to allow adhesion. REST protein was detected in greater than 90% of OPCs, oligodendrocytes, and astrocytes expressing the trypsin resistant, cell specific antigens A2B5, O4, and GFAP respectively after only 3 hours in culture (figure III-4b).

To examine REST expression in developing glia *in vivo*, I performed immunofluorescence staining of postnatal day 12 rat optic nerve sections. As shown in figure III-5a, P12 optic nerve showed strong immunoreactivity for REST with a staining pattern typical for oligodendrocyte lineage cells. REST protein (figure III-5c) and mRNA (figure III-5d) were detected in p0, p7, and adult whole optic nerve by immunoblot and qRT-PCR analysis, although REST expression declined with age. Together these data show that oligodendrocyte lineage cells and astrocytes express REST and its decreased expression in adult animals suggests that REST may have a functional role during development.

REST is a functional transcription repressor in OPCs

The presence of nuclear REST in oligodendrocyte lineage cells suggests that REST could be functioning as a transcription repressor, similar to its functions in other cell types. However, REST can be negatively regulated by a truncated form of the REST protein as well as by a small noncoding dsRNA (Tabuchi et al., 2002, Kuwabara et al., 2004). To rule out the possibility that REST is negatively regulated in OPCs and is functioning as a repressor of transcription, a luciferase reporter assay was performed (Otto et al., 2007). OPCs,

REFs, and PC12 cells were nucleofected with a plasmid containing a region of the GAD1 gene with or without an RE1 site upstream of a minimal TK promoter capable of driving *Photalis pyralis* (firefly) luciferase expression (figure III-6a). Cells were co-nucleofected with a vector encoding the *Renilla* luciferase gene for normalization. After 48hours, changes in luciferase activity were measured and compared. If cells express functional REST protein, luciferase activity should be repressed when an RE1 is present. As shown in figure III-6b, luciferase activity increased 13.4 fold in OPCs expressing the RE1 negative construct relative to OPCs expressing the RE1-containing construct. Similarly, luciferase activity was derepressed in rat embryonic fibroblasts when the RE1 was removed. This derepression was significantly lower in PC12 cells which express only trace amounts of REST.

To confirm that a REST/RE1 interaction was responsible for the decreased luciferase activity, I co-nucleofected the OPC cultures with an additional plasmid expressing either DnREST or REST-VP16 (figure III-6c). Displacing endogenous REST from the RE1 by expressing DnREST was sufficient to derepress the luciferase gene. REST-VP16 further activated luciferase expression compared to cells expressing only the plasmid lacking an RE1. These results demonstrate that REST can interact with canonical RE1 sites in OPCs and can function as a transcriptional repressor.

I performed chromatin immunoprecipitation assays to determine whether REST physically associates with an RE1 element in known REST regulated genes. REST protein/DNA complexes were immunoprecipitated with a polyclonal antibody against the DNA binding domain of REST (P73) or with the REST-C antibody. A non-specific rabbit IgG antibody was used as a negative control. In OPCs, PCR amplification of the immunoprecipitated DNA showed that REST was bound to the RE1 elements in several genes that code for neuronal proteins including *NF-M*, *SCG10*, *Grik3*, *Scn2a1*, *NeuroD2*, and *L1cam* (figure III-7a). However, REST was not bound to a randomly chosen site lacking an RE1 upstream of the NG2 gene. Quantitative real-time PCR demonstrated that the genes bound by REST in my chromatin immunoprecipitation assays were

transcriptionally repressed in OPCs compared to CGNs or p12 rat cortex (figure III-7b). Conversely, OPCs expressed high levels of transcript for NG2 which was barely detected in the CGN cultures.

If REST is responsible for the transcriptional repression of RE1-containing neuronal genes in glia then perturbing REST function should increase their expression. To examine this, I infected OPCs with adenoviruses expressing either DnREST, REST-VP16, or GFP alone for 72 hours and measured mRNA levels by qRT-PCR. Gene expression was normalized to GAPDH and fold change was measured in cells expressing DnREST or REST-VP16 relative to cells infected with the control GFP adenovirus. Expression of DnREST or REST-VP16 was sufficient to derepress or activate the transcription of numerous RE1-containing genes in OPCs, including *NF-M*, *SCG10*, *Scn2a1*, and *NeuroD2* (figure III-8 and III-9). Expression of RE1 containing genes also increased in both oligodendrocytes and 2A's when DnREST or REST-VP16 proteins were expressed (figure III-9a). Changes in gene expression were analyzed for additional genes in REST-VP16 infected cultures including several transcription factors involved in neurogenesis or gliogenesis (figure III-9b) and cell specific proteins (figure III9c). These changes may be direct or indirect since RE1s have been identified for only some of these genes such as *Hes5* and *Myt1*, but not for others. Gene expression changes varied between cell types but more genes were derepressed or activated in OPCs than in the other types of glia. This suggests that gene expression in OPCs may be subject to a greater degree of epigenetic regulation than in either oligodendrocytes or 2As.

DISCUSSION

Rest Expression in glia

In this chapter I demonstrated that astrocytes and OPCs isolated from rat cortices, as well as OPCs induced to differentiate into oligodendrocytes or type II astrocytes, all express REST. Furthermore, REST expression is localized to the nuclear compartment along with its corepressors where they form a functional repressor complex to regulate gene expression. REST was also detected in rodent optic nerve glia both in culture and in vivo. Within the rat optic nerve, mature MBP-positive oligodendrocytes begin to appear around postnatal day 4 (Colello et al., 1995). By day 7, MBP-positive cells can be detected along the length of the optic nerve (Colello et al., 1995). Interestingly, both REST protein and transcript levels were highest in newborn rats and they declined as the animals matured. At postnatal day 7, when many OPCs have developed into MBP-expressing oligodendrocytes, REST expression declined 1.5 fold compared to newborn optic nerve. This progressive decline in REST expression may be the result of a decrease in all glia or a loss of expression in a subset of cells. The high levels of REST detected in newborn rat glia suggest that REST may have an important function during development. If REST is required for the initiation of oligodendrocyte differentiation it could explain the high levels of REST expression at birth and the progressive decrease expression following myelination within the optic nerve.

HDAC repression is required for OPCs to develop into mature oligodendrocytes (Marin-Husstege et al., 2002, Liu and Casaccia, 2010). The efficiency of HDAC recruitment to myelin inhibitory factors such as Hes5 and Id4 declines in OPCs as animals age (Shen et al., 2008a). This could be explained by a decrease in the expression of both HDACs and transcription factors like YY1 that recruit HDACs to these genes. If REST is required to repress oligodendrocyte inhibitors during differentiation, such as the RE1containing Hes5 gene, then the decrease in HDAC recruitment in adults may partially be explained by a decrease in REST expression.

REST as a transcription factor in OPCs

In addition to expressing nuclear REST protein, I demonstrated that REST functions as a transcriptional repressor in OPCs. Furthermore, it was bound to several identified RE1 sites located in neuronal genes including the pro-neural transcription factor, NeuroD2. Overexpression of NeuroD2 protein in a mouse neuroblastoma cell line is sufficient to induce neuronal differentiation (Noda et al., 2006). Considering that OPCs can develop into neurons, it is probable that genes such as NeuroD2 need to be tightly regulated for the progression of an OPC into an oligodendrocyte. Using a loss of function technique, I demonstrated that REST is responsible for regulating this pro-neural transcription factor in OPCs. Perturbing REST function by expressing DnREST or REST-VP16 resulted in an increase in NeuroD2 gene expression along with several other neuronal genes. REST LOF had a larger effect on some RE1 containing genes compared to others. This could be due to the presence or absence of other activators or repressors, the conformation of the chromatin affecting protein recruitment to specific genes, or the type or location of the RE1 which varies on a gene by gene basis (Otto et al., 2007). Some RE1s have a weak interaction with REST and may require higher levels of REST expression for repression (Otto et al., 2007, Bruce et al., 2009). REST regulates gene expression in OPCs on a gene by gene basis and does not function as a master switch that simply turns a set of genes on or off. This complex and differential gene regulation by REST is likely affected by amount of REST present in OPCs at a given time.

REST-dependent gene regulation: variations between glial cell types

There are also variations in REST regulated gene expression among different cell types. For example, REST gene regulation and binding patterns differ between neuronal stem cells, embryonic stem cells, and differentiated fibroblasts (Johnson et al., 2008). This cell type specific regulation by REST is demonstrated in glia when the results from the REST LOF approach are compared between OPCs and their differentiated cell types. RE1 containing genes are

affected differently by REST LOF in OPCs, oligodendrocytes, and 2As. While the expression of some genes increase in one cell type, their expression may decrease or not change in a different cell type. For example, Calbindin gene expression increases in the absence of REST repression in all glial types except for 2As expressing REST-VP16, in which case its expression decreases. This is most likely an indirect effect in which REST-VP16 may be activating a negative regulator of Calbindin in 2As. Similarly, Syt2 is differentially affected by REST LOF in the different glia. In most cases, it is expression either increases or does not change. However, Otto et al., (2006) reported that Syt2 is indirectly repressed in the absence of REST rather than activated in TCM1 kidney cells. This further demonstrates the variation in REST gene regulation among different cell types.

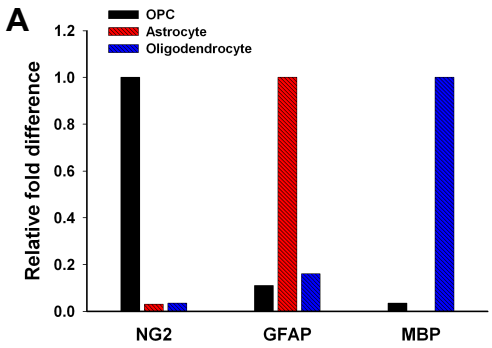
Summary

In this chapter I demonstrated that REST regulates gene expression in oligodendrocyte lineage cells. Furthermore, perturbing REST function can alter the expression of a large number of genes in OPCs, many of which are generally only associated with neurons. In the following chapters I will examine the importance of REST function in OPC fate specification and differentiation with an emphasis on the transition of an OPC to a mature oligodendrocyte.

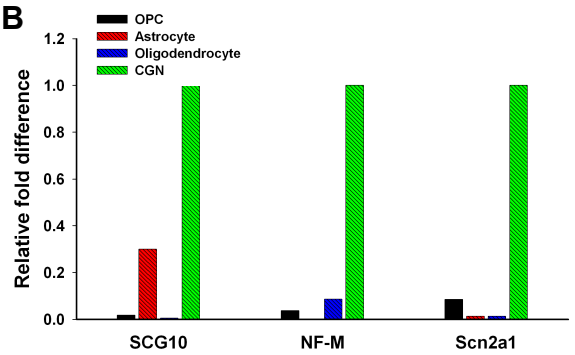
Figure III-1. Isolation of primary glial cultures with minimal cell contamination. OPCs, oligodendrocytes, 2As, and astrocytes were isolated as described in Experimental Procedures. **(A)** Transcript levels of *NG2*, *GFAP*, and *MBP*, were analyzed by real-time RT-PCR in the various purified glial cultures. Fold difference in mRNA expression for each gene is relative to the specific cell type (*NG2* for OPCs, *GFAP* for astrocytes, and *MBP* for oligodendrocytes) after normalization to *GAPDH*. The cell specific genes are expressed in the proper cell type with minimal contamination in other cultures. **(B)** Transcript levels for the neuronal genes *SCG10*, *NF-M*, and *Scn2a1* are minimal in the glial cultures relative to cerebellar granular neurons (CGNs) after normalization to *GAPDH*. **(C)** OPC cell purity was further analyzed by real-time RT-PCR. Glial (*MBP*, *GFAP*, *NG2*, *Nkx2.2*, *Olig2*, and *PDGFaR*), neuronal (*DCX*, *NeuroD1*, and *NeuroD2*), and stem cell (*nestin* and *sox2*) gene transcript levels are normalized to *GAPDH* and fold difference in OPCs is shown relative to E14 rat brain (column 1) or P12 rat cortex (column 2). The fold difference in P12 cortex relative to E14 brain is also shown (column 3). Relative fold differences are representative of at least 3 independent experiments.

FIGURE III-1

A



B



C

GENE	Fold Change: OPC / E14 brain	Fold Change: OPC / P12 cortex	Fold Change: P12 cortex / E14 brain
DCX	0.52	1.62	0.32
MBP	309.76	0.23	1365.30
GAPDH	1.00	1.00	1.00
GFAP	26.45	0.12	214.52
Mash1	1.27	14.42	0.09
Nestin	1.05	16.85	0.06
NeuroD1	0.04	0.05	0.84
NeuroD2	0.35	0.07	5.33
NG2	17.21	4.27	4.03
Nkx2.2	9.71	8.63	1.13
OLIG2	1.65	0.84	1.98
PDGFaR	46.05	9.75	4.72
Sox2	2.39	4.81	0.50

Figure III-2. Nuclear localization of REST expression in glia. (A) Immunofluorescence staining showing nuclear localization of REST protein in OPCs, oligodendrocytes, 2As, and astrocytes. Purified bulk glial cultures were grown in their specified media for 5 days and immunostained with a REST specific antibody (green), cell-type specific antibodies (red, top to bottom, OPCs=NG2, pre-oligodendrocytes=O4, mature oligodendrocytes=MBP, type II astrocytes=GFAP, astrocytes=GFAP), and DAPI to recognize nuclei (blue). **(B)** Lower power image showing REST expression in NG2-positive OPCs. All scale bars indicate 50μm.

FIGURE III-2

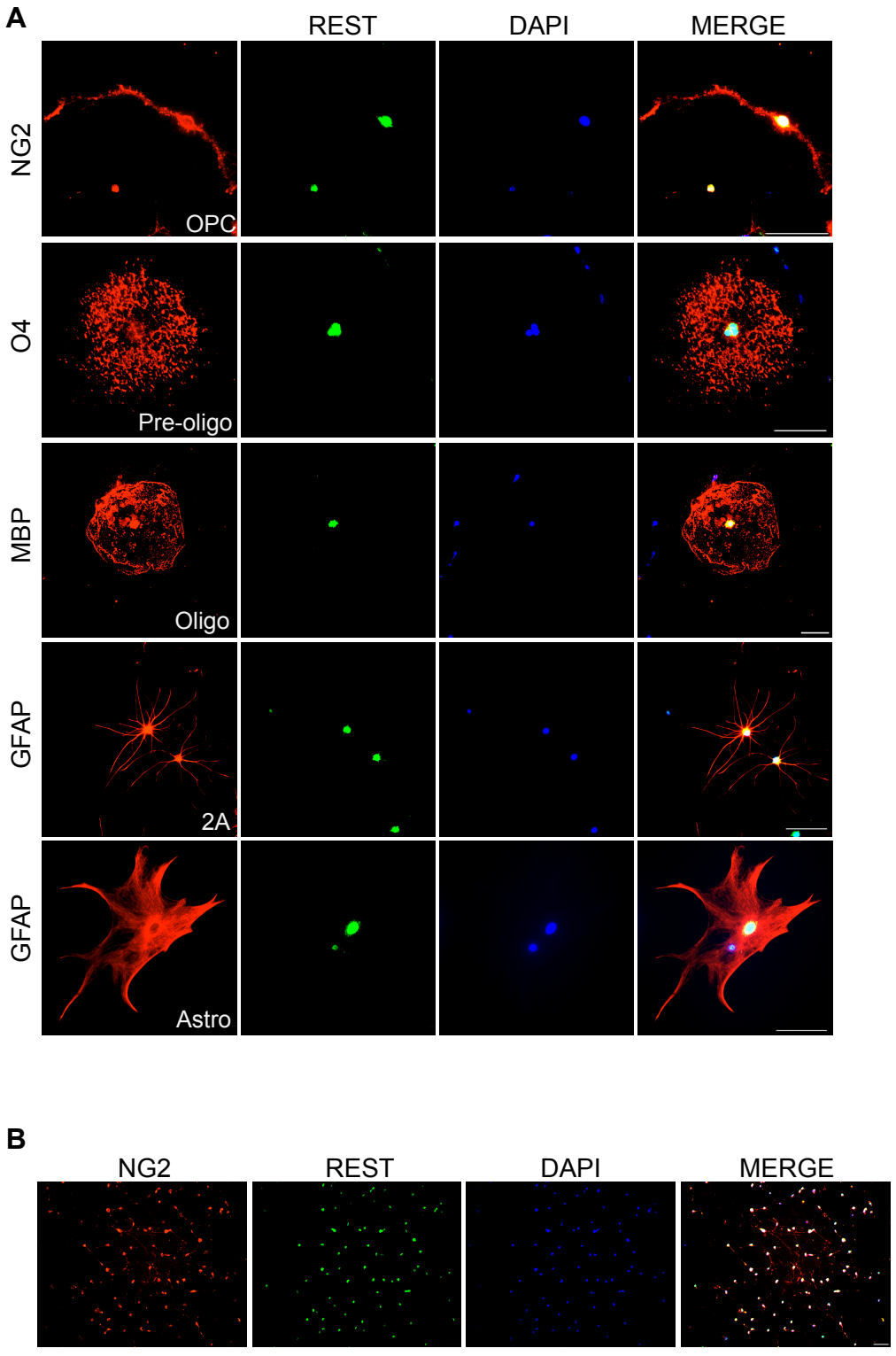

A
REST
DAPI
MERGE
NG2
OPC
O4
Pre-oligo
MBP
Oligo
GFAP
2A
GFAP
Astro
B
NG2
REST
DAPI
MERGE

Figure III-3. Levels of REST expression in glia. Purified OPCs, astrocytes, and oligodendrocytes were grown in their specified media for 5 days and REST protein and transcript levels were measured in each of the glia. **(A)** Immunoblot showing REST and CoREST protein expression in nuclear extracts from OPCs, astrocytes (astro), and oligodendrocytes (oligo). REFs (rat embryonic fibroblasts) are used as a positive control and PC12 cells as a negative control for REST expression. **(B)** REST expression is limited to the nuclear compartment in the different glia as shown by immunoblot. Nuclear proteins (N) and cytoplasmic proteins (C) were isolated from each cell type. C6 glioma cells are used as a positive control and cerebellar granular neurons (CGNs) as a negative control for REST expression. All immunoblot data are representative of 3 independent experiments. **(C)** Real-time RT-PCR showing the fold difference in REST mRNA levels in OPCs, oligodendrocytes, type II astrocytes (2As), astrocytes, PC12's and CGNs relative to REFs after normalization to *GAPDH*. REST transcript is expressed at comparable levels in the different glia but is significantly lower in PC12's and CGNs. Error bars represent the standard deviation from three independent experiments. *P-value $\leq$0.001, Student's t-test.

FIGURE III-3

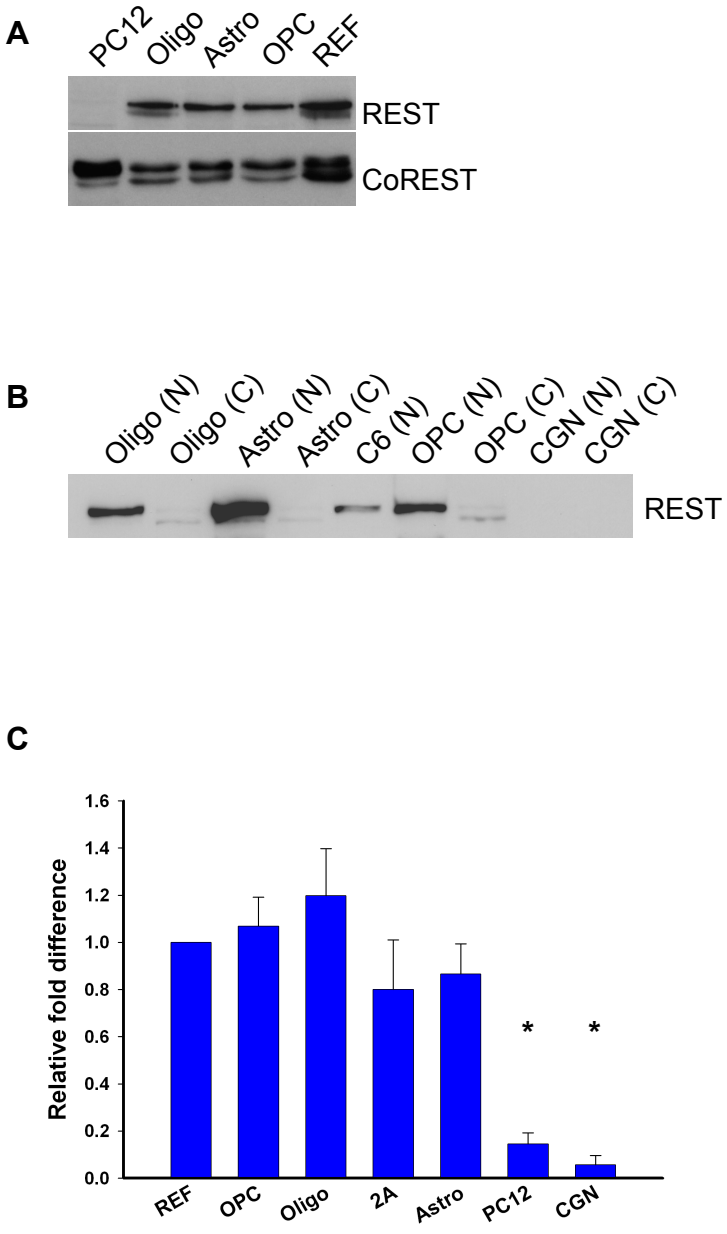

A
PC12
Oligo
Astro
OPC
REF
REST
CoREST
B
Oligo (N)
Oligo (C)
Astro (N)
Astro (C)
C6 (N)
OPC (N)
OPC (C)
CGN (N)
CGN (C)
REST
C
Relative fold difference
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
REF
OPC
Oligo
2A
Astro
PC12
CGN

Figure III-4. REST is expressed in dissociated optic nerve glia. P7 rat optic nerves were dissociated and grown in culture for 3 days **(A)** or only three hours **(B)**. **(A)** After three days in culture, immunofluorescence staining shows that NG2-positve (red) OPCs originating from the optic nerve express nuclear REST protein (green). Data is representative of at least 4 independent experiments. **(B)** Immunostaining showing REST protein expression (green) in dissociated optic nerve glia after only 3 hours in culture. Antibodies against trypsin resistant cell specific proteins were used to recognize the various glia (red, top to bottom; OPC=A2B5, pre-oligodendrocytes=O4, astrocytes=GFAP) and cell nuclei are shown by DAPI staining (blue). Data is representative of at least 2-3 independent experiments. All scale bars indicate 50μm.

FIGURE III-4

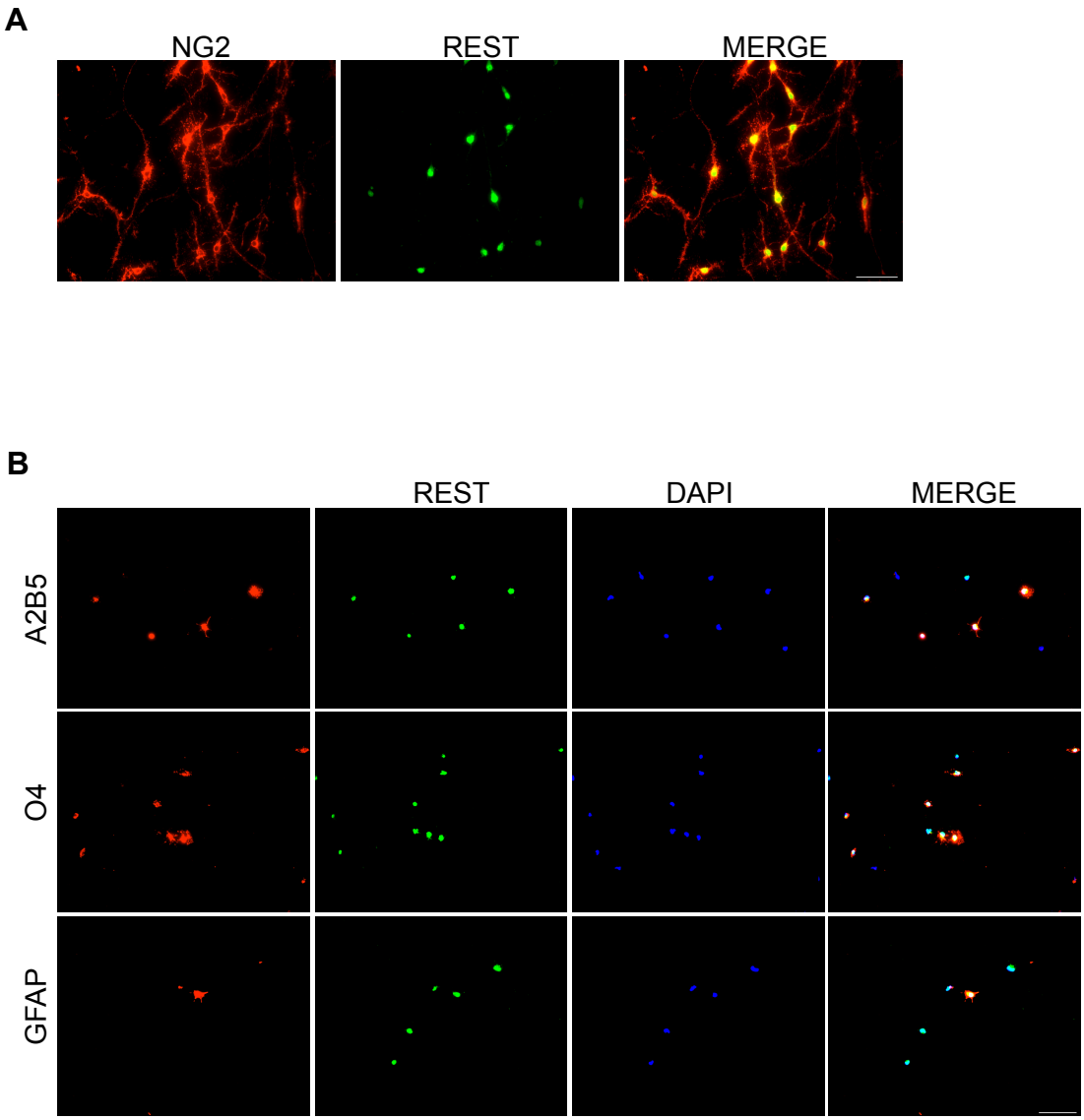


Figure III-5. In vivo analysis of REST expression in optic nerve. Immunofluorescence staining of p12 rat optic nerve sections for REST protein (green) **(A)** or secondary antibody alone as a control **(B)**. The scale bar represents 50μm. **(C)** Immunoblot analysis showing detection of REST in cell protein extracts from p0, p7, and adult rat whole optic nerve (50ug protein per lane). Cerebellar granular neurons (CGNs) are used as a negative control. **(D)** Real-time RT-PCR showing the fold difference in REST expression in p7, p15, and adult rat whole optic nerve relative to p0 optic nerve after normalization to *GAPDH*. Both REST protein **(C)** and transcript **(D)** levels decrease as animals mature to adulthood. Error bars represent the standard deviation from six PCR runs from at least two independent experiments.

FIGURE IV-5

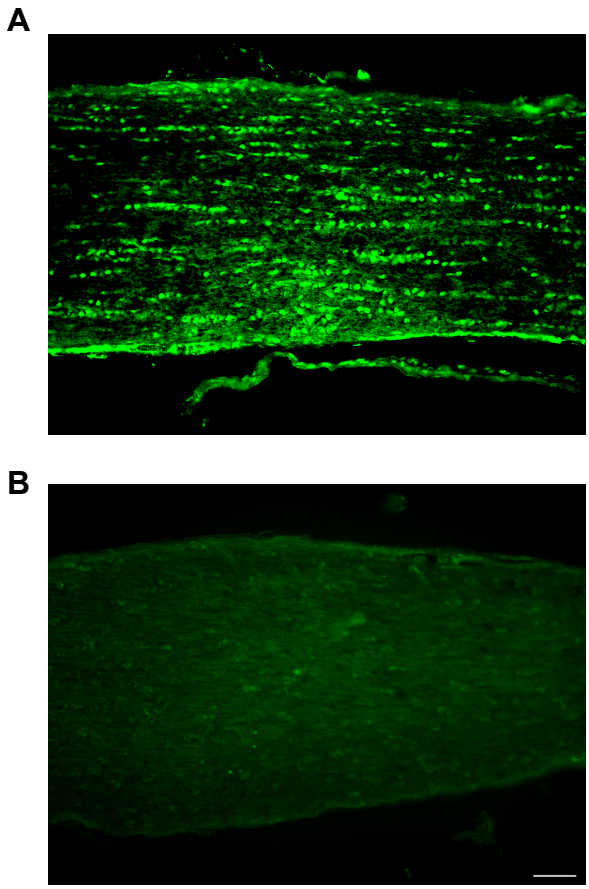

A
B
C
CGN
P0
P7
Adult
REST
D
Relative fold difference
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P0
P7
P15
Adult
*
*
*

Figure III-6. REST is a functional transcription repressor in OPCs. Luciferase reporter assays show that REST represses RE1 containing genes in oligodendrocyte progenitor cells. **(A)** OPCs, rat embryonic fibroblasts (REFs), and PC12s were nucleofected with a plasmid either containing (+RE1) or lacking (-RE1) a canonical RE1 site from the GAD1 gene upstream of the firefly luciferase gene and grown for 48hrs. Cells were co-nucleofected with a plasmid expressing the *renilla* luciferase gene for normalization. **(B)** –RE1 luciferase activity is shown relative to +RE1 for each cell type. Luciferase activity increases in OPCs and REFs when the RE1 is absent. This increase is minimal in PC12 cells which contain only trace amounts of REST. Error bars represent the standard deviation of the averages from three independent experiments. *P-value ≤0.007, Student's t-test. **(C)** Luciferase reporter assay showing that OPCs co-nucleofected with DnREST derepress luciferase gene expression in the presence of an RE1. Co-nucleofection with REST-VP16 further activates that expression. Fold change in luciferase activity is relative to +RE1 nucleofected OPCs. Error bars represent the standard deviation of the averages from three independent experiments. *P-value ≤0.005, Student's t-test.

FIGURE III-6

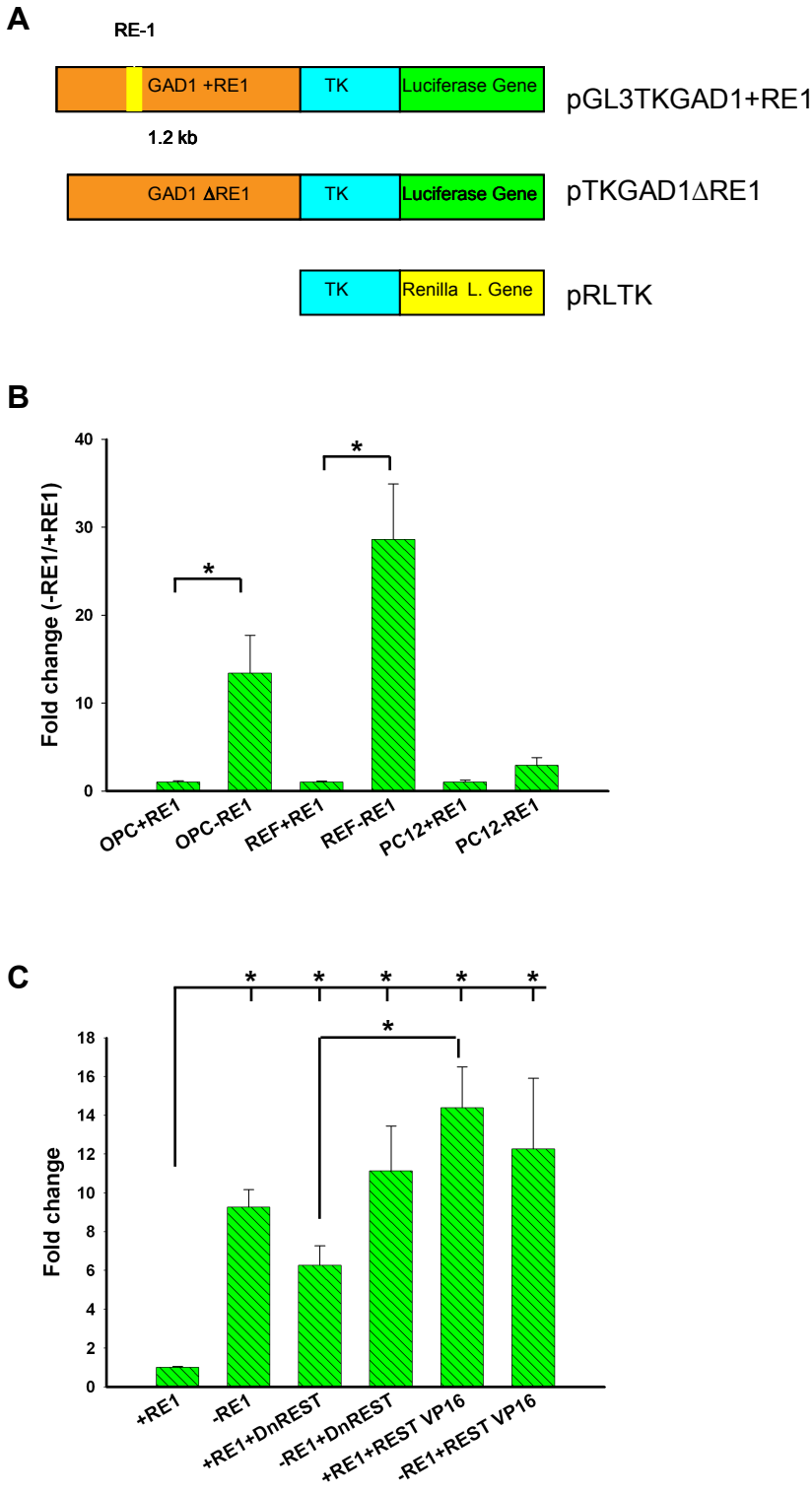
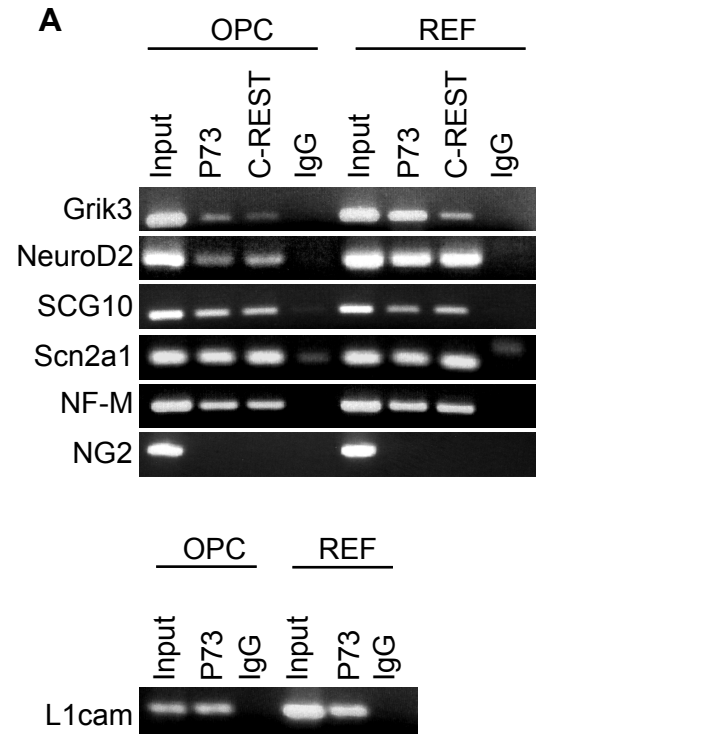

A
RE-1
GAD1 +RE1
TK
Luciferase Gene
pGL3TKGAD1+RE1
1.2 kb
GAD1 ΔRE1
TK
Luciferase Gene
pTKGAD1ΔRE1
TK
Renilla L. Gene
pRLTK
B
Fold change (-RE1/+RE1)
40
30
20
10
0
*
*
OPC+RE1
OPC-RE1
REF+RE1
REF-RE1
PC12+RE1
PC12-RE1
C
Fold change
18
16
14
12
10
8
6
4
2
0
*
*
*
*
*
+RE1
-RE1
+RE1+DnREST
-RE1+DnREST
+RE1+REST VP16
-RE1+REST VP16

Figure III-7. REST interacts with RE1s in neuronal genes in OPCs and these genes are transcriptionally repressed. (A) Chromatin immunoprecipitation assays showing that REST binds to an RE1 element in *Grik3*, *NeuroD2*, *SCG10*, *Scn2a1*, *NF-M*, and *L1cam*, but not to a randomly chosen site upstream of the *NG2* gene in both OPCs and control rat embryonic fibroblasts (REFs). REST protein/DNA complexes were immunoprecipitated with anti-P73 or anti-REST-C polyclonal antibodies. A non-specific rabbit IgG antibody was used as a negative control and input containing total DNA as a positive control. Data is representative of 2-4 independent experiments. **(B)** Real-time RT-PCR showing the fold difference in *NF-M*, *SCG10*, and *Scn2a1* gene expression in OPCs relative to cerebellar granular neurons (CGNs), *NG2* relative to OPCs, and *NeuroD2* relative to P12 rat cortex after normalization to *GAPDH*. The neuronal genes bound by REST in figure **A**, but not *NG2*, are repressed in OPCs compared to CGNs or P12 cortex. Error bars represent the standard deviation from the averages of three independent experiments.

FIGURE III-7


A
OPC
REF
Input
P73
C-REST
IgG
Input
P73
C-REST
IgG
Grik3
NeuroD2
SCG10
Scn2a1
NF-M
NG2
OPC
REF
Input
P73
IgG
Input
P73
IgG
L1cam

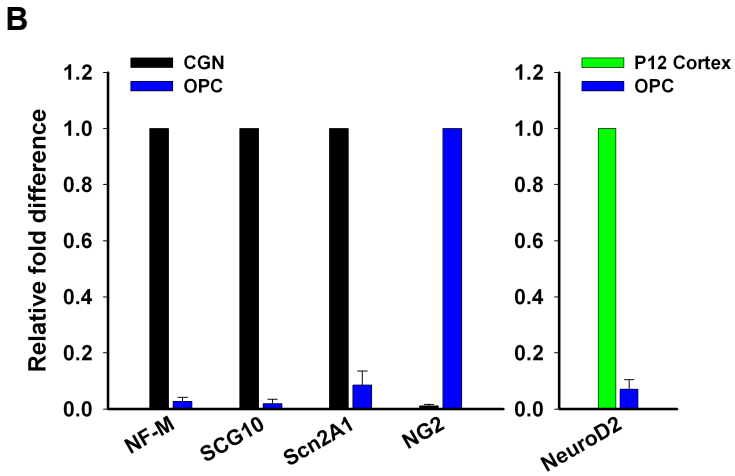

B
CGN
OPC
P12 Cortex
OPC
Relative fold difference
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NF-M
SCG10
Scn2A1
NG2
NeuroD2

Figure III-8. REST regulates neuronal gene expression in OPCs. Purified OPCs were infected with either DnREST, REST-VP16, or GFP control adenoviruses and grown in proliferation media for 72hrs. Total RNAs were extracted and transcript levels of several known REST regulated neuronal genes were analyzed by real-time RT-PCR. The relative fold change in gene expression is shown for DnREST and REST-VP16 infected OPCs relative to control infected cells after normalization to *GAPDH*. Perturbing REST function results in the derepression or activation of numerous neuronal genes in OPCs. Error bars represent the standard deviation from the averages of multiple PCR runs from at least 2-3 independent experiments. All gene expression changes with REST loss of function have a P value < 0.01 using the Students t-test.

FIGURE III-8

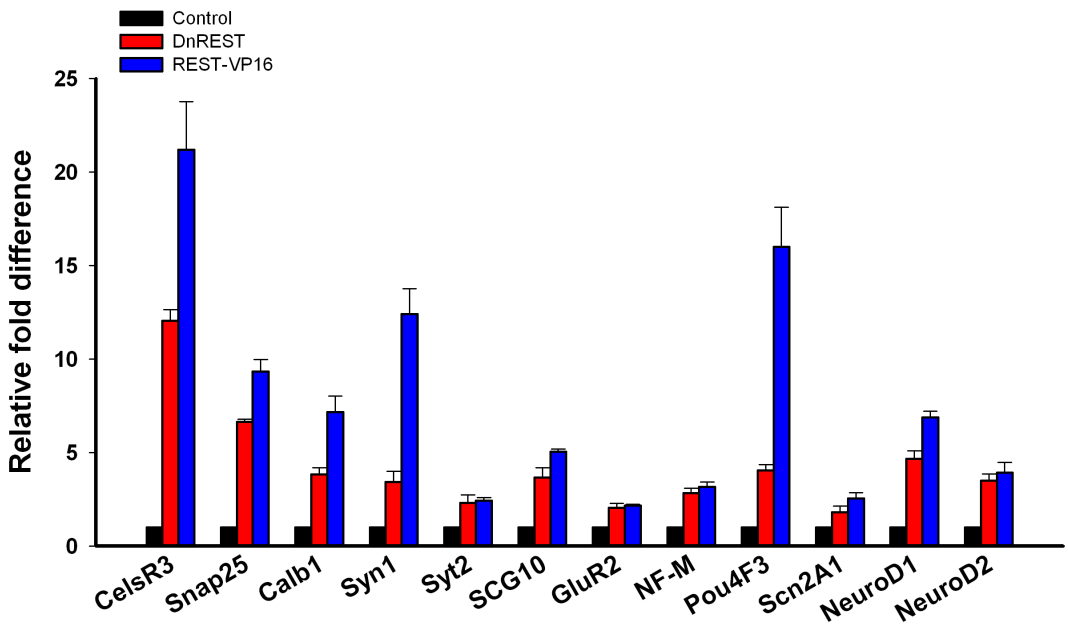

Control
DnREST
REST-VP16
Relative fold difference
25
20
15
10
5
0
CelsR3
Snap25
Calb1
Syn1
Syt2
SCG10
GluR2
NF-M
Pou4F3
Scn2A1
NeuroD1
NeuroD2

Figure III-9. Loss of REST repression induces multiple gene changes in glia. The table shows changes in gene expression when REST function is perturbed in OPCs and in differentiated oligodendrocytes (oligos) and type II astrocytes (2As). Purified OPCs were grown in proliferation media or differentiated into oligodendrocytes or 2A's. Each cell type was infected with adenoviruses expressing either DnREST, REST-VP16, or GFP as a control. After 72hrs, transcript levels were measured by real-time RT-PCR. Transcript levels are normalized to *GAPDH* and fold changes in gene expression are relative to control infected cells for each cell type. Data is representative of 3 independent experiments. The list of genes includes **(A)** genes known to be directly regulated by REST, **(B)** pro-neural and/or pro-glial transcription factors that may or may not be REST regulated, and **(C)** cell specific markers.

FIGURE III-9

		AdREST-VP16			AdDnREST		
		2A	Oligo	OPCs	2A	Oligo	OPCs
A	CelsR3	+++	+++	+++	++	+++	++
	Snap25	+++	+++	+++	+	++	++
	Calbindin	-	+	++	+	+	++
	Synapsin	++	++	++	++	++	+
	Synaptotagmin	+	NC	+++	NC	NC	+
	SCG10	++	++	+	++	+	+
	GluR2	NC	+	++	NC	+	+
	NFM	++	NC	+	+	NC	+
	Pou4F3	+++	++	+++	++	++	++
	Type II Na	NC	NC	+	NC	NC	NC
	TrkC	-	NC	NC	-	NC	NC
	NeuroD1	+++	+++	++	+	++	+
	NeuroD2	+++	++	+	NC	++	+
B	Myt1	+	++	++			
	Myt1L	+	NC	+			
	Olig1	NC	NC	NC			
	Olig2	NC	+	++			
	Hes1	NC	+	NC			
	Hes5	+	+	NC			
	Mash1	-	+	+			
	Sox2	NC	NC	NC			
	Nkx2.2	-	-	NC			
C	DCX	NC	++	++			
	Nestin	NC	+	+			
	PDGFaR	NC	NC	++			
	PLP	NC	NC	+			
	GFAP	-	-	-			

+++ = > 15 fold increase

++ = 4 - 15 fold increase

+ = 1.5 - 4 fold increase

NC = No change

- = > -1.5 fold decrease

CHAPTER IV
REST REGULATES OPC CELL FATE SPECIFICATION AND IS REQUIRED FOR PROPER OLIGODENDROCYTE DEVELOPMENT

INTRODUCTION

Neurons, astrocytes, and oligodendrocytes are generated sequentially from the same neural progenitor cells (Gage, 2000, Temple, 2001). The differentiation of a neural progenitor into a specific cell type requires the activation of genes required for that transition and the repression of genes involved in alternative fate choices. Not all neural progenitors that develop into OPCs will give rise to oligodendrocytes. Many of the cells remain as OPCs while others may develop into astrocytes and neurons (Kondo and Raff, 2000, Belachew et al., 2003, Dawson et al., 2003, Aguirre et al., 2004, Zhu et al., 2008a). Both intrinsic and extrinsic factors are involved in regulating the fate choice of an OPC. Oligodendrocyte maturation requires transcription factors to activate myelin genes and repress myelin gene inhibitors (figure I-1). In this chapter I will examine the role of the transcription factor REST in oligodendrocyte development.

In the previous chapter, I demonstrated that REST is a functional transcription factor in OPCs that interacts with RE1 sites in several genes to represses their expression. REST regulates a large number of neuronal genes and is involved in regulating neuronal development. Since OPCs are highly plastic cells that have the ability to develop into neurons, I wanted to determine if REST has a role in determining their cell fate and if REST repression is required for oligodendrocyte maturation.

REST can regulate different sets of genes depending on the type of cell and developmental stage of the cell. Because of OPC plasticity and REST's cell type specificity, I examined the role of REST in OPCs grown under various conditions including those that promote proliferation or differentiation. In this chapter I will demonstrate that REST expression increases during the onset of oligodendrocyte differentiation and is required for oligodendrocyte maturation.

Perturbing REST function in OPCs prevents the development of both oligodendrocytes and GFAP-positive type II astrocytes. Rather, the cells begin to display neuronal properties suggesting that REST repression may be required for the glial fate choice of an OPC.

RESULTS

Loss of REST function inhibits oligodendrocyte and 2A maturation and induces neuronal properties

The data in the previous chapter demonstrate that REST is expressed by glia and functions as a transcriptional regulator in oligodendrocyte lineage cells. REST regulation of neuronal gene expression is necessary for proper nervous system development and has important regulatory roles during the development of neurons from embryonic stem cells (Ballas and Mandel, 2005). Because OPCs are highly plastic and develop from the same neuroepithelial stem cells as neurons, we wanted to determine whether REST also regulates the development of oligodendrocyte lineage cells.

First, REST expression was closely examined during the differentiation of OPCs into oligodendrocytes. OPC cultures were expanded in proliferation media for 3 days and then switched to oligodendrocyte or 2A differentiation media. Total RNA was isolated from OPCs at time zero and every 12 to 24 hours for 5 days after the media was changed and REST mRNA expression was measured by qRT-PCR. After normalization to GAPDH, changes in gene expression were measured at each time point relative to time zero (OPCs). As shown in figure IV-1a, REST mRNA expression increased 4.0 fold (±0.6, n=3) after 36 hours in oligodendrocyte differentiation media and returned to baseline as the cells matured into MBP expressing oligodendrocytes. This increase in REST mRNA level was accompanied by a 3-4 fold increase in REST protein expression 45hrs after oligodendrocyte induction compared to OPCs (figure IV-1b). Consistent with an increase in REST transcript and protein during the initiation of oligodendrocyte differentiation, the expression of some RE1 containing genes decreased after 3 days in differentiation media relative to OPCs (figure IV-1d) whereas the expression of myelin-specific genes increased and genes associated with OPCs including NG2 and PDGFαR decreased (figure IV-1c). REST mRNA levels also slightly increased during the initiation of 2A differentiation (P-value <0.05), but this increase was significantly less than in cells induced to develop into oligodendrocytes (P-value <0.001) (figure IV-1a). The increase in REST expression when oligodendrocytes begin to differentiate suggests

that REST mediated repression may have a functional role during the initiation and maintenance of oligodendrocyte maturation.

A loss-of-function (LOF) approach was used to determine if REST function is required for proper oligodendrocyte development. OPCs were co-nucleofected with a plasmid expressing GFP and one expressing either DnREST, REST-VP16, or an empty vector and analyzed after 3 and 5 days in oligodendrocyte media. Differentiation was monitored by the appearance of the immature (O4, figure IV-4b) and mature (MBP, figures IV-2 and IV-3a) oligodendrocyte markers in GFP-positive cells by immunofluorescence staining. After 3 days, the percent of GFP-positive cells expressing O4 was higher in control cells compared to cells expressing DnREST or REST-VP16 (figure IV-4b). REST LOF also resulted in a significant decrease in the number of cells expressing the mature oligodendrocyte marker MBP compared to control when measured after 3 days (figure IV-3a). By day 5, 68.4% (±7.6) of the control cells expressed MBP whereas only 50.3% (±2.9) of DnREST and 40.5% (±7.6) of REST-VP16 nucleofected cells were MBP-positive (figure IV-3a).

These decreases in O4 and MBP expression could be due to either an inhibition of differentiation and/or a temporal delay in that differentiation. Since protein expression after nucleofection of plasmids declines after 3-4 days, I constructed retroviruses expressing GFP and DnREST (pMXsIG-DnREST, hereafter referred to as rDnREST) or REST-VP16 (pMXsIG-REST-VP16) to further investigate REST LOF. Purified OPCs were grown in proliferation media for 2 days, infected with a control retrovirus expressing GFP (pMXsIG, hereafter referred to as rGFP) or rDnREST, and grown for an additional 5-6 days in proliferation media to allow for the expression of the virally encoded proteins. rGFP and rDnREST infected cells were plated onto coverslips in OPC media for 2 days and then switched to oligodendrocyte differentiation media. Differentiation was assayed by staining with antibodies against MBP and O4 at time 0, and 2, 4, and 6 days post media change. Time zero, or OPCs, refers to the time in which the OPCs were switched from proliferation media to differentiation media. Consistent with the nucleofection findings, the development of O4 and MBP-positive oligodendrocytes was significantly reduced with REST LOF compared to control (figures IV-3b, IV-4a, and IV-4c). MBP-positive cells were slower to appear and, after 6

days in oligodendrocyte-inducing media, the percentage of MBP-positive cells was significantly lower in the rDnREST infected cells relative to control rGFP infected cells (figure IV-3b). As shown in figure IV-4c, immediately after initiating oligodendrocyte differentiation, the control infected OPCs began to express the pre-oligodendrocyte antigen O4. Cells infected with rDnREST also expressed O4; however this expression occurred more slowly than in control cultures so that after 6 days of culture, fewer total cells were O4-positive.

To confirm a decrease in oligodendrocyte maturation in the retroviral cultures, I performed quantitative real-time PCR to compare oligodendrocyte specific gene transcript levels between rDnREST and rGFP infected cells. *MBP* mRNA expression was measured every day for 4 days after oligodendrocyte induction and the fold change in *MBP* expression at each time point was compared relative to control OPCs at time zero. Consistent with the decrease in MBP immunostaining, OPCs expressing DnREST had lower levels of MBP transcripts throughout differentiation compared to control cells (figure IV-3c). I also measured transcript levels of other oligodendrocyte genes including *MOBP*, *MAG*, and *CNP* after 3 days in oligodendrocyte media. Similar to *MBP*, transcript levels of these oligodendrocyte genes significantly decreased in rDnREST infected cells relative to control (figure IV-3d).

The decrease in oligodendrocyte maturation observed above suggests that REST LOF could be causing either a temporal delay in differentiation or an inhibition of differentiation. If oligodendrocyte maturation was being inhibited rather than delayed, it is possible that OPCs that did not develop into oligodendrocytes after expression of DnREST were differentiating along other lineages. Given that the inhibition of HDAC activity can promote the neuronal differentiation of OPCs (Liu et al., 2007a, Lyssiotis et al., 2007), nucleofected and infected cells were stained with antibodies identifying young neurons (TUJ1). 12.9±3.2% of total DnREST nucleofected and 16.4±2.2% of total rDnREST infected cells were TUJ1-positive whereas only 1.89±0.42% and 1.42±0.75% of cells expressed TUJ1 in control nucleofected and control rGFP infected cultures respectively (figures IV-5). OPCs nucleofected with a plasmid expressing REST-VP16 had a higher percent of TUJ1 expressing cells than both control and DnREST nucleofected cells (figure IV-5b and IV-5d). As shown in figure IV-6a, TUJ1 as well as

the REST-regulated neuronal proteins Snap25 and Synpasin1 were present in both rDnREST and rREST-VP16 infected cells after 5 days in culture. The expression of neuronal proteins was accompanied by a decrease in CNP, a protein expressed by oligodendrocytes. Therefore, REST repression appears to be required to prevent the expression of neuronal proteins in differentiating oligodendrocytes.

Consistent with changes in protein expression during oligodendrocyte differentiation, REST LOF led to multiple changes in gene expression (figure IV-3d, IV-6b, and IV-6c). Transcript levels were measured by qRT-PCR after 3 days in oligodendrocyte media and the fold difference in gene expression of rDnREST infected cells was measured relative to control rGFP infected cells. Genes known to be highly regulated by REST such as *Celsr3*, *SCG10*, and *Snap25* increased 5-10-fold with REST LOF (figure IV-6b) whereas other genes (both with and without identified RE1s) increased more modestly but significantly. Among these was Hes5 which contains an expanded RE1 (Otto et al., 2007) (figure IV-6c). As described previously, genes encoding myelin-specific genes declined (figure IV-3d). Notably, several genes involved in the regulation of oligodendrocyte differentiation, including *Id2*, *Id4*, *olig2*, *Nkx2.2*, and *YY1*, did not change (figure IV-6d). This data likely under-represents the magnitude of any changes in gene transcription since the infection efficiency was between 60-80%. These data suggest that REST regulates the expression of several neural-specific genes in differentiating oligodendrocytes as well as some genes involved in oligodendrocyte development, such as the differentiation inhibitor *Hes5*.

OPCs are highly plastic cells and their development in vitro is responsive to different media conditions (Raff et al., 1983). It is possible that factors found in the oligodendrocyte media contributed to the effects of REST LOF on OPC fate. To rule this out, I examined the effects of REST LOF on OPCs grown under other conditions, first in a defined media, also referred to as stem cell media (SCM), which is permissive to both glial and neuronal development (Liu et al., 2007a) and then in a type II astrocyte differentiation media containing 10% FBS.

OPCs infected with control rGFP or rDnREST were grown in proliferation media for 5 days, plated onto coverslips for 2 days, and switched to SCM. The expression of NG2, MBP, and TUJ1 was examined by immunofluorescence staining at

several time points following the media change (figures IV-7 - IV-10). The percent of infected cells positive for each marker antigen was counted under control and DnREST expressing conditions. Figure IV-9b shows that in control cultures, the percent of total NG2-positive cells decreased while the MBP-positive population increased over time. A small number of control cells (2.1%±0.2) expressed TUJ1 antigen and this did not change during the course of the experiment. . The percent of cells categorized as "other" (i.e., not identifiable as NG2, MBP, or TUJ1 positive cells) also increased by day 6. These are most likely O4-positive pre-oligodendrocytes that had not fully matured into MBP-positive cells. This pattern of development was altered by REST LOF. As the percentage of cells expressing NG2 decreased, a population of the rDnREST infected cells became TUJ1-positive (22.1±4.4%) whereas only a few cells expressed MBP by day 6 (2.4±0.6%). Therefore, REST LOF resulted in the formation of TUJ1-positive cells rather than MBP-positive cells when grown in stem cell media.

O4, A2B5, and GFAP-positive cells were also analyzed in the retroviral infected cultures after several time points in stem cell media. As was the case for cells grown in oligodendrocyte media, the appearance of the O4 antigen was delayed by about 2 days with REST LOF and the percent of cells expressing O4 remained significantly lower than in control cultures even after 6 days in stem cell media (figure IV-10). The percentage of cells expressing either GFAP or A2B5 was not affected by REST LOF (figure IV-7). In both control and rDnREST infected cells, the number of GFAP-positive cells remained low while the number cells expressing A2B5 decreased over time. Therefore, when OPCs were grown in stem cell media REST LOF prevented oligodendrocyte induction but did not promote the formation of GFAP/A2B5-positive 2As. Also, the O4 and MBP-negative cells in the DnREST cultures did not remain as NG2 or A2B5-positive OPCs. Rather, a large percentage of rDnREST infected cells expressed the neuronal maker TUJ1.

Western blot analysis confirmed TUJ1 protein expression in the DnREST cultures, as well as detected the neuronal proteins Synapsin1 and Snap25 after 5 days in stem cell media. At the RNA level, expression of *Tubb3* (Beta III tubulin), *Snap25*, and *Synapsin1*, increased with REST LOF (figure IV-11a and IV-11b) as well as other genes associated with neurons such as *DCX*. Also, consistent with the immunofluorescent

staining, *MBP* gene expression decreased with REST LOF (figure IV-11c). Therefore, the data obtained from the experiments using stem cell differentiation media suggest that REST LOF pushes OPCs to have a neuronal phenotype when grown in media permissive to both glial and neuronal development.

As previously stated, OPCs are highly plastic cells and their development in vitro is responsive to different media conditions. Next, the effects of REST LOF were examined on 2A development to determine whether REST also has a role in the differentiation of OPCs into 2A's. Similar to the above experiments, purified OPCs were grown for 2 days in proliferation media, infected with control rGFP or rDnREST and grown for an additional 5 days in proliferation media. Infected cells were plated onto coverslips for 2 days in proliferation media, switched to 2A differentiation media for 5 days, and cells were stained with antibodies against GFAP or TUJ1. The percent of infected cells expressing GFAP was higher in control cells (71.9±2.8%) compared to rDnREST infected cells (54.2±7.2%, figures IV-12a and IV-12b). This decrease in the percent of GFAP-positive cells with REST LOF was accompanied by an increase in percent of TUJ1-positive cells (DnREST = 39.1±8.9, control = 2.2±1.2, figures IV-13 and IV-14a) The fold difference in *GFAP* and *TUJ1* expression in rGFP and rDnREST cells after 3 days 2A media was measured relative to control infected OPCs at the time of media change. As shown in figure IV-12c, *GFAP* expression increased 41.3±4.5 fold in control rGFP cells compared to only 16.9±4.2 fold in rDnREST cells whereas *TUJ1* expression decreased in rGFP cells (-1.57±0.06 fold) and increased in rDnREST cells (1.91±0.35 fold, figure IV-14b). OPCs infected with either DnREST or REST-VP16 expressing adenoviruses and grown in 2A differentiation media for 5 days also had a higher percentage of TUJ1-positive infected cells than cells infected with a control GFP adenovirus (figure IV-14c). In addition to an increase in TUJ1 with REST LOF, Synapsin1, Neurofilament, SCG10, and Snap25 protein were detected in rDnREST and REST-VP16 retroviral infected cells after 5 days in 2A media (figures IV-15 and IV-16a).

In addition to the changes in *GFAP* and *TUJ1* transcript levels already discussed, the expression levels of multiple other genes changed with REST LOF after 3 days in 2A media. Several RE1 containing genes increased 4-14 fold (SCG10, CelsR3, and Snap25)

in rDnREST cultures relative to control cells (figure IV-16b). Similar to cells grown in oligodendrocyte media, myelin specific genes decreased with REST LOF while no change was detected in several genes involved in regulating oligodendrocyte development including *Id2*, *Id4*, and *YY1* (figure IV-4d). The table in Appendix-1 compares the changes in gene expression with REST LOF in OPCs after 3 days in oligodendrocyte or 2A media. These results support the hypothesis that REST-mediated gene repression is required for the differentiation of oligodendrocyte progenitor cells into either oligodendrocytes or 2As and inhibiting REST function induces neuronal protein and mRNA expression in OPCs under a variety of media conditions.

Effects of REST LOF: Clonal Analysis

One question that arises in the experiments described above is whether all of the cells or only a sub-population of the OPCs are responding to REST LOF. To address this, a detailed clonal analysis was performed. A description of the clonal analysis is shown in figure IV-17. Purified OPCs were grown in culture for 2 days, infected with control rGFP or rDnREST, and grown in proliferation media for 5 days. The cells were plated onto coverslips at clonal density (1000 cells/18mm round coverslip) and grown in proliferation media for 2 days to form clones. Some clones remained in proliferation media for additional time to examine REST LOF in OPC clones whereas other clones were switched to oligodendrocyte or stem cell media for 3 or 5 days or 2A media for 5 days. Under all conditions, GFP-expressing clones were identified and cell number and phenotype determined.

First, I analyzed clone size. In all media conditions, the REST LOF clones appeared to be smaller compared to control (figures IV-18 – IV-20). Although clonal size varied according to the media conditions, the mean clonal size of the DnREST infected cells was always smaller than that of control cells (figures IV-21a and IV-21b). As expected, the average control clone size was largest in OPC proliferation media after 5 days at clonal density (about 30 cells per clone, figure IV-21a). This was followed by stem cell media (about 17 cells per clone) and then oligodendrocyte media (about 10 cells per clone, figure IV-21a). At day 7, 2A control clones were the smallest compared to both oligodendrocyte and stem cell control clones (figure IV-21b). REST LOF clones

followed the same exact trend as control in all of the conditions and time points; however their overall mean clone size was much smaller. The decrease in mean clonal size was due to an increased number of small sized clones and a reduced number of large clones (figures IV-22 and IV-23).

To determine whether a decrease in cell proliferation was responsible for the smaller clone sizes with REST LOF, cells in non-clonal cultures were pulse-labeled with BrdU as described in Materials and Methods (figures IV-24 and IV-25). In control cultures, BrdU incorporation was highest when OPCs were grown in proliferation media (23.8±1.9%) followed by stem cell media (18.4±1.0%), oligodendrocyte media (13.4±1.3), and lowest in 2A media (3.6±1.8) (figures IV-24 and IV-25a). This is consistent with clonal size data in that OPC clones were the largest and 2A clones were the smallest. Looking at BrdU incorporation in DnREST cultures as a percent of control, figure IV-25b shows that there was a small but significant reduction in the rDnREST infected cells. This suggests that one function of REST in glial progenitor cells is to maintain cell proliferation, although we cannot rule out a contribution of increased cell death to the smaller clone size.

Clonal composition

The composition of individual clones was analyzed using antibodies against cell specific markers for each condition as described in figure IV-17. Because of the large amount of data obtained with the clonal analysis, the data is represented three different ways. First, I analyzed the phenotypes of the cells by scoring individual clones for the presence of at least one cell expressing either NG2, O4, MBP, GFAP, or TUJ1. As shown in figure IV-26, regardless of the media, 50% or more of the rDnREST infected clones contained TUJ1-positive cells. This rules out the possibility that the cultures contained a small population of neural stem-like cells that are driven to a neuronal phenotype by REST LOF. In addition, there were more TUJ1 expressing clones in oligodendrocyte, stem cell, and 2A media as compared to OPC media, suggesting that REST LOF has a greater effect on OPCs grown under differentiation conditions than on OPCs growing in proliferation media with added growth factors.

To look closer at the effects of REST LOF, I analyzed the cellular composition of each individual clone separately for each antigen in each media and represented it two ways. The 3D scatter plots shown compare the clone size, percent of cells positive for the denoted cell-type specific marker antigen, and the number of clones of that exact composition. The bar graphs are histograms displaying the percent of total clones containing different percent ranges of antigen positive cells. These data are organized by media and raw data tables for each individual clone can be found in Appendix-2.

Proliferation media (figures IV-27 – IV-29):

Both control and DnREST cultures contained a large number of 100% NG2-positive clones. However, this number was much lower in DnREST cultures (69.6%) compared to control (89.7%) (figure IV-29). The clones containing NG2-negative cells tended to be small and most contained less than 10 cells. In contrast with NG2 expression, the percent of TUJ1-positive cells was higher in DnREST clones compared to control (figure IV-29). The smaller the clone, the more likely they contained some or all TUJ1-positive cells (figure IV-28). All but one of the 100% TUJ1-positive DnREST clones contained 6 or less cells despite an average clone size of 20 cells. Therefore, DnREST expression resulted in an increase in clones containing NG2 negative and TUJ1 positive cells compared to control, and this effect was primarily seen in small clones. This suggests that, even in the presence of growth factors, some REST LOF exited the cell cycle and formed small, TUJ1-positive clones rather than remaining as NG2-positive, proliferating OPCs.

Oligodendrocyte media (figures IV-30 – IV-36):

When grown in oligodendrocyte media, most clones were heterogeneous in both size and composition. The number of total O4 expressing clones decreased from 84.5% to 61.7% with REST LOF (figure IV-36). This was primarily due to a large number of small clones (<5 cells) that were completely O4 negative within the DnREST group (44/91 clones) (figure IV-31). Consistent with O4 expression, over a broad range of clone sizes the clones derived from rDnREST infected cells contained fewer MBP positive cells than control infected clones after 5 days in oligodendrocyte media (figure IV-33).

73.3% of total control clones contained at least one MBP-positive cell compared to only 48.9% of total DnREST clones (figure IV-36). The absence of MBP-positive cells was prominent among smaller clones. Only 36.3% of control clones containing less than 20 cells lacked MBP expression compared to 72.0% of experimental clones. In larger clones containing greater than 20 cells, the number of MBP-negative control and DnREST clones were similar (23.5% and 26.3% respectively) however, only 9.6% of the total DnREST cells were MBP-positive compared to 21.8% of total control cells.

The number of TUJ1 expressing clones was greater than 9Xs higher with REST LOF compared to control (IV-35 and IV-36). All DnREST single cell clones and 20.4% of total DnREST clones were 100% TUJ1-positive, a phenotype that was absent in control clones. Consistent with the reduced expression of MBP in the small sized clones, 65.8% of total clones containing 10 or fewer cells expressed TUJ1 after REST LOF as opposed to only 3.2% of the control infected cells. Among the larger control clones (>10 cells/clone), only 11.5% had 1 or more TUJ1-positive cells whereas 50.% of the DnREST infected clones contained on average 11% TUJ1 positive cells. This data suggests that regardless of the capacity of an individual OPC to proliferate, REST function is required for timely and complete oligodendrocyte differentiation.

Stem cell media (figures (IV-37 – IV-43):

When the control rGFP and rDnREST infected clones were switched to stem cell media containing no growth or differentiation factors, the cells continued to proliferate more than the clones switched to either oligodendrocyte or 2A media (figure IV-21c). Consistent with the cells being in a more proliferative state and the lack of differentiation factors such as thyroid hormones, oligodendrocyte differentiation was modest under control conditions. Although a large number of control clones began expressing the immature oligodendrocyte marker O4 after three days in stem cell media (75.0%), most cells failed to fully mature into MBP expressing oligodendrocytes (figure IV-43). After 5 days in stem cell media, 51.8% of total control clones contained at least one MBP-positive cell, but only 16.5% of the total cells within these clones were MBP-positive. The expression of both O4 and MBP was altered with REST LOF (figures IV-38 and IV-43). Only 44.8% of total DnREST clones contained O4-positive cells compared to 75.0%

of control clones after 3 days in SCM (figure IV-43). The clones that were negative for O4 expression tended to be smaller in size. 76.5% of the O4-negative DnREST clones contained 5 or less cells whereas only 48.7% control clones in this size range lacked O4 expression.

The number of MBP expressing clones also decreased with REST LOF (31.7%) compared to control (51.8%). In both control and experimental conditions, smaller clones tended to be MBP-negative (figure IV-45). However, this effect was more pronounced in DnREST clones where 92.5% of the clones that contained 10 or less cells were absent for MBP compared to 78.6% of control clones (figure IV-45). When larger clones were examined, I observed that 85.0% of control clones containing more than 15 members had at least 1 MBP positive cell compared to only 22.8% of the DnREST clones.

TUJ1 expression was minimal among the control clones. Only 6.7% of all clones contained either 1 or 2 TUJ1 positive cells and no clones contained more than 2 (figure IV-42). In contrast, 68% of the DnREST infected clones contained at least one TUJ1-positive cell, 26.4% of which were 100% positive. Of these DnREST clones that contained all TUJ1-positive cells, 22 out of 26 contained 10 cells or less whereas no control clones in this size range expressed even a single TUJ1-positive cell (figure IV-42). This data suggests that perturbing REST function affects a majority of the OPC cell population and, regardless of the media, REST repression is required for proper oligodendrocyte development.

2A media (figures IV-44 – IV-48):

The effects of REST LOF on the expression of TUJ1 were more pronounced when the cells were grown in media to promote 2A differentiation. 83.9% of total rDnREST infected clones contained TUJ1 positive cells as opposed to 10.5% of total control rGFP infected clones (figure IV-48). Most control clones contained only 1 or 2 TUJ1-positive cells so that the total number of TUJ1-positive cells was small (33 out of 1550 total cells or 2.1%). In contrast, 64.9% of total rDnREST infected cells (491 out of 756 or 64.9%) expressed TUJ1. There was a strong tendency for the smaller clones to be 100% TUJ1 positive (figure IV-47). Of the 122 DnREST clones that contained 3 or less cells, 68.8% were 100% TUJ1-positive compared to only 2 of 60 (3.3%) control clones

within this size range. These were the only 100% TUJ1-positive control clones out of all control clones scored (2 of 202). When this population of small sized clones was analyzed for GFAP expression, control cultures contained more 100% GFAP positive clones (55.2%) than DnREST cultures (31.5%) and less GFAP negative clones (10.3% and 51.4% respectively, figure IV-45). Therefore, REST repression may be required for an OPC to develop into a type II astrocyte and the absence of REST repression, the cells begin to express neuronal characteristics.

DISCUSSION

Experimental approach

In this chapter I demonstrated that REST is required for both proper oligodendrocyte and type II astrocyte differentiation and for preventing the expression of neuronal properties. I used a loss of function approach to study the function of REST in oligodendrocyte lineage cells. In the initial studies I nucleofected plasmids coding for DnREST or REST-VP16 into cultured OPCs. I also infected cells with adenoviruses expressing the same REST LOF proteins. However, there are several disadvantages to using these techniques. In addition to infecting dividing OPCs, adenoviruses could also infect non-dividing oligodendrocytes and astrocytes. Also, adenoviral genes do not incorporate in the genome and their expression decreases as cells divide. Similarly, protein expression declines after 3-5 days following nucleofection. To overcome these limitations, I constructed retroviruses to express REST LOF proteins. Retroviral expression of exogenous proteins had several advantages over the other techniques for my experiments. First, large numbers of OPCs could be infected with high efficiency for biochemical and molecular biological experiments. Additionally, retroviral genes are unable to integrate into the host genome of non-dividing cells and therefore mature oligodendrocytes were excluded from my experiments. I could also study the long-term effects of REST LOF across multiple generations of dividing OPCs and after long periods of time in differentiation media. Lastly, infected OPCs could remain in proliferation media to allow for the expression of REST LOF proteins prior to the initiation of differentiation rather than simultaneously. In nucleofection and adenoviral experiments, cells were immediately placed in differentiation media to avoid a decrease in REST LOF protein expression during cell division. Retroviruses allowed me to first perturb REST function then initiate differentiation. Although this was an advantage, it meant the cells had to remain in proliferation media for a longer period of time.

OPCs grown in proliferation media for an extended period of time prior to the initiation of differentiation developed slower than cells grown in proliferation media for shorter periods. When OPCs were differentiated into oligodendrocytes

or astrocytes after only a few days in growth factors, greater than 90% of the cells expressed O4 or GFAP respectively. Longer culture periods in proliferation media caused a lag in the appearance of O4, GFAP, and MBP proteins, and a slight decrease in their expression. Therefore, in the nucleofection and adenoviral experiments where the cells were exposed to growth factors for a minimal period of time, more cells differentiated than in the retroviral infected cultures. However, in all instances, there was a significant decrease in oligodendrocyte and 2A maturation with REST LOF. Additionally, because retroviral infection efficiency was only between 60-80%, not every cell expressed DnREST and therefore some of the PCR and immunoblot data may under represent the magnitude of any changes.

The effects of REST LOF on oligodendrocyte and 2A development

During the onset of oligodendrocyte maturation, REST protein and transcript expression increased. This was accompanied by a corresponding decrease in the expression of RE1 containing genes. Perturbing REST function in OPCs prevented and delayed oligodendrocyte development and activated the expression of neuronal genes and proteins. These included the RE1 containing genes that were repressed during the onset of differentiation. One of these genes, Hes5, is a known inhibitor of oligodendrocyte development. Repression of Hes5 is required for an OPC to develop into an oligodendrocyte (Liu et al., 2006). Hes5 expression was derepressed when I infected OPCs with rDnREST and grew them in oligodendrocyte differentiation media. However, when the cells were grown in 2A media instead, Hes5 expression was not affected by REST LOF. Thus, REST does not regulate Hes5 during 2A differentiation but does so during oligodendrocyte development. Hes5 contains a structurally variant non-canonical expanded RE1 site (Johnson et al., 2007, Otto et al., 2007). These non-canonical sites tend to have weaker interactions with REST and are generally associated with cell or tissue-specific genes (Bruce et al., 2009). Therefore, higher levels of REST may be required to repress non-canonical RE1 sites such as that within the

Hes5 gene. Further studies are necessary to test this hypothesis and to evaluate REST/Hes5 interactions during glial differentiation.

In addition to regulating oligodendrocyte differentiation, REST is also required for proper 2A development. Perturbing REST function during this transition increased the number of neuron-like cells at the expense of GFAP-positive astrocytes. REST LOF differentially affected gene expression when the same pool of OPCs were placed in oligodendrocyte, stem cell, or 2A media suggesting that REST function can be modified by external factors. Following expression of DnREST, a larger number of genes were affected by 2A media as compared to oligodendrocyte media. The normal 4-fold increase in REST expression during the onset of oligodendrocyte induction may compete successfully with DnREST for RE1 binding; hence a reduced effect on gene transcription. Consistent with this idea, a larger percent of rDnREST infected cells expressed the neuronal marker TUJ1 when switched to 2A media compared to any other media. Kondo and Raff et al., (2004) suggested that the 2As phenotype may represent an intermediate stage between an OPC and a neural stem-like cell and therefore 2As might be poised (Ballas et al., 2005) to more readily respond to a loss of REST repression. Interestingly, perturbing REST function during the OPC to 2A transition also up-regulates both Myt1L and Ascl1. Forced expression of Myt1L, Ascl1, and Brn2 in differentiated fibroblasts is sufficient to convert the cells into functional neurons (Vierbuchen et al., 2010). It would be interesting to examine Brn2 expression in differentiating 2As following REST LOF. If Brn2 expression is also up-regulated, these 3 genes may together be responsible for the dramatic increase in TUJ1 expressing cells and clones when OPCs are grown in 2A media.

REST and OPC proliferation

The percent of REST LOF cells that incorporated BrdU decreased significantly in all media compared to control. This decrease could reflect a direct effect of REST LOF on cell cycle kinetics, an increase in cell death, or the differentiation of the cells into non-mitotic neuronal-like cells. There is

considerable controversy over whether REST regulates the self-renewal/pluripotency markers Oct4, Sox2, and Nanog and whether REST is required for the self-renewal of ES cells (Singh et al., 2008, Buckley et al., 2009, Jorgensen et al., 2009b, Yamada et al., 2010). Over-expression of Oct4/Sox2/Nanog along with Klf4 or c-myc in differentiated fibroblasts reverts the cells to an embryonic stem cell state (Takahashi and Yamanaka, 2006, Wernig et al., 2007, Mikkelsen et al., 2008, Deng et al., 2009). Similarly, OPCs revert back to pluripotent neural stem-like cells in a Sox2 dependant manner (Kondo and Raff, 2004). However, I did not detect a change in Sox2 expression following REST LOF under any conditions. Rather, I found that in all media the cells expressed mature neuronal genes and proteins including Snap25, Synapsin, Neurofilament, and SCG10. Furthermore, and as discussed above, in 2A media the cells up-regulate Myt1L and Ascl1 transcription, two of three proteins sufficient to convert differentiated fibroblasts into functional neurons (Vierbuchen et al., 2010). In this media, a majority of the cells were TUJ1-positive by immunofluorescence staining and had morphologies similar to neural-like cells. Therefore, I suggest that DnREST caused a direct transition to a neuronal phenotype rather than a step-wise conversion of OPCs to neural-stem like cells and that the decrease in proliferation was the result of OPCs differentiating into non-dividing neuronal like cells

Clonal analysis

One question that arises in the experiments described above is whether all of the cells or only a sub-population of the OPCs are responding to REST LOF. Because of the heterogeneous nature of OPCs, it is possible that individual cells respond to REST LOF differently. For example, REST could have a different function in immature OPCs compared to OPCs that have already committed to a certain fate. If REST repression is only required for fate determination and the initiation of glial differentiation, then these pre-committed OPCs may not be affected by REST LOF. In this case, the cells would maintain the ability to differentiate and thus exit the cell cycle and form a large number of small clones that contain many or all MBP or GFAP-positive cells. In addition, it is

possible that the cultures contain a small number of undifferentiated neural stem-like cells and that these are the cells that respond to REST LOF by expressing neuronal marker antigens. To address these questions, a detailed clonal analysis was performed. If all or most OPCs can respond to REST LOF, then a large number of clones would be predicted to contain fewer differentiated oligodendrocytes and astrocytes and more TUJ1-positiev cells. If only a sub-population of stem-like cells responded, the number of total clones expressing TUJ1 would be small.

Although clonal size varied according to the media, the mean clonal size was always smaller in DnREST infected cells compared to control. This decrease in clonal size was likely caused by the decrease in proliferation with REST LOF rather than cell death. Infection of neuronal precursor cells with retroviruses expressing DnREST does not increase the rate of cell death (Kohyama et al., 2010). Similarly, I did not detect a noticeable difference in activated caspase-3 immunostaining (data not shown) in rDnREST infected cultures compared to control cells in any of the media. However, the experiment was not replicated enough times for accurate quantification and statistical analysis. Therefore, I cannot rule out a possible contribution of increased cell death to the decreased clone size in REST LOF cultures.

Regardless of the media, greater than 50% of the clones contained at least one TUJ1-positive cell. This rules out the possibility that the neuronal phenotype observed with REST LOF was due to a small population of neural stem-like cells in the cultures. In all media, DnREST cultures contained more single and double cell clones than control. These small DnREST clones had a tendency to be TUJ1-positive (figure IV-49). This suggests that some DnREST clones exited the cell cycle, remained small, and began to develop into TUJ1 expressing cells at the expense of NG2-positive, O4-positive, MBP-positive, or GFAP-positive cells. It can not be ruled out that some cells expressed both TUJ1 and one or more of the glial antigens. We were unable to stain for more than one cell marker at a time because of technical limitations and the need to stain the clonal cultures for GFP. Nevertheless, many of the TUJ1-positive cells had distinct morphologies and cells with similar morphologies did not express GFAP or MBP.

Despite the decrease in O4-positive clones and total number of O4-positive cells, the number of clones 100% positive for O4 expression did not decrease with REST LOF. A similar result was observed in 2A media in that the number of 100% GFAP positive clones only slightly decreased in DnREST expressing cultures. In all cases, however; the number of clones containing zero cells positive for O4 or GFAP dramatically increased: this suggests that a subpopulation of the infected OPCs were already committed to develop as glia and REST LOF was not sufficient to perturb this commitment and glial fate. On the other hand, the large numbers of small cell clones that were 100% TUJ1-positive show that many OPCs had not yet committed to the glial lineage and REST LOF was able to modify their fate. The percent of small clones expressing glial markers decreased with REST LOF, probably because of the dramatic increase in single and double TUJ1-positive clones. These findings suggest that REST repression may only be required for the initiation of differentiation rather than at late stages of oligodendrocyte maturation. This could explain the increase in REST expression during the onset of oligodendrocyte differentiation as well as the decrease in REST expression I observed following peak myelination time within the optic nerve.

Summary

Here, I demonstrated that REST has an important function in oligodendrocyte lineage cells. REST repression is required for the proper development of an OPC into an oligodendrocyte or a type II astrocyte and loss of REST function induces neuronal properties. In the following chapter, I investigate the effects of REST LOF on the physiological properties of the cells as well as ask whether the expression of neuronal properties is stable when rDnREST-infected OPCs are transplanted into developing animals.

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Figure IV-1. Temporal changes in REST expression during oligodendrogenesis. (A) Real-time RT-PCR of REST mRNA expression throughout oligodendrocyte and type II astrocyte differentiation. Purified OPCs were grown in culture for 2-3 days and switched to oligodendrocyte or 2A differentiation media at time 0. Total RNA was extracted at the indicated time points post-media change. *REST* transcript levels are shown for oligodendrocyte (black) and 2A (gray) differentiating cells, and *MBP* transcript levels are shown for cells grown in oligodendrocyte media (red). mRNA expression is normalized to *GAPDH* and fold difference at each time point is relative to time 0 (OPCs). *REST* gene expression increases during the onset of oligodendrogenesis and peaks at 36hrs before slowly returning to baseline as the cells fully mature (indicated by MBP mRNA expression). *REST* also slightly increases during the onset of 2A differentiation. Error bars represent the standard deviation from the averages of 3 independent experiments. **(B)** Immunoblot showing REST protein levels increase during the onset of oligodendrocyte differentiation. Nuclear protein was isolated from OPCs and OPCs grown in oligodendrocyte media for 45hrs. Histone H3 antibody is used as a loading control. Data is representative of 3 independent experiments. **(C, D)** Graphs show real-time RT-PCR data of transcript levels 72hrs after oligodendrocyte induction when REST expression is still significantly above OPC levels. **(C)** After 72hrs in oligodendrocyte media (red), myelin genes (*MBP*, *MAG*, *PLP*) are up regulated whereas OPC specific genes (*NG2*, *PDGFaR*) are down regulated relative to OPCs (black). **(D)** Consistent with an increase in REST expression, REST regulated neuronal genes are further repressed in differentiating oligodendrocytes 3 days post media change (red) compared to OPCs (black). All genes are normalized to *GAPDH* and fold change is shown relative to OPCs. Error bars represent the standard deviation from the averages of 4-6 PCR runs from at least 2 independent experiments. *P-value $\leq$0.01, Student's t-test.

FIGURE IV-1

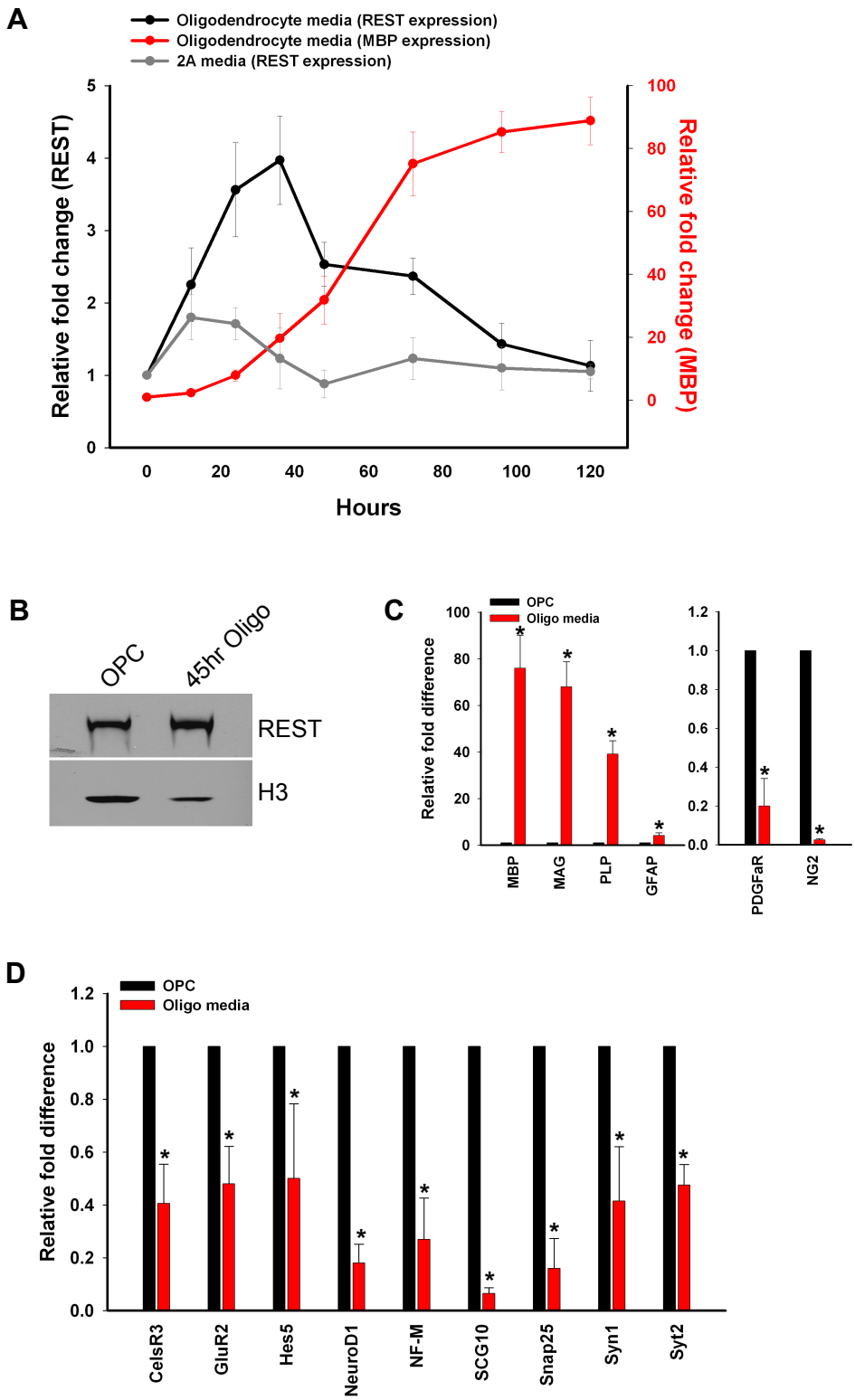

A
Oligodendrocyte media (REST expression)
Oligodendrocyte media (MBP expression)
2A media (REST expression)
Relative fold change (REST)
Relative fold change (MBP)
Hours
B
OPC
45hr Oligo
REST
H3
C
OPC
Oligo media
Relative fold difference
MBP
MAG
PLP
GFAP
PDGFaR
NG2
D
OPC
Oligo media
Relative fold difference
CelsR3
GluR2
Hes5
NeuroD1
NF-M
SCG10
Snap25
Syn1
Syt2

Figure IV-2 Perturbing REST function decreases the number of MBP expressing cells during oligodendrocyte differentiation. Purified OPCs were co-nucleofected with a plasmid expressing GFP and one expressing either DnREST, REST-VP16, or an empty vector, and grown in oligodendrocyte media for 5 days. **(A)** Immunofluorescence staining showing MBP expression (red) in nucleofected cells (green). DnREST and REST-VP16 decreased the number of MBP-positive cells compared to control but did not appear to inhibit MBP expression in non-nucleofected cells (arrows). **(B)** Lower magnification images showing MBP-positive cells in the nucleofected cultures. Cell nuclei were detected with DAPI (blue) to show the number of cells in each field. All scale bars indicate 50μm.

FIGURE IV-2

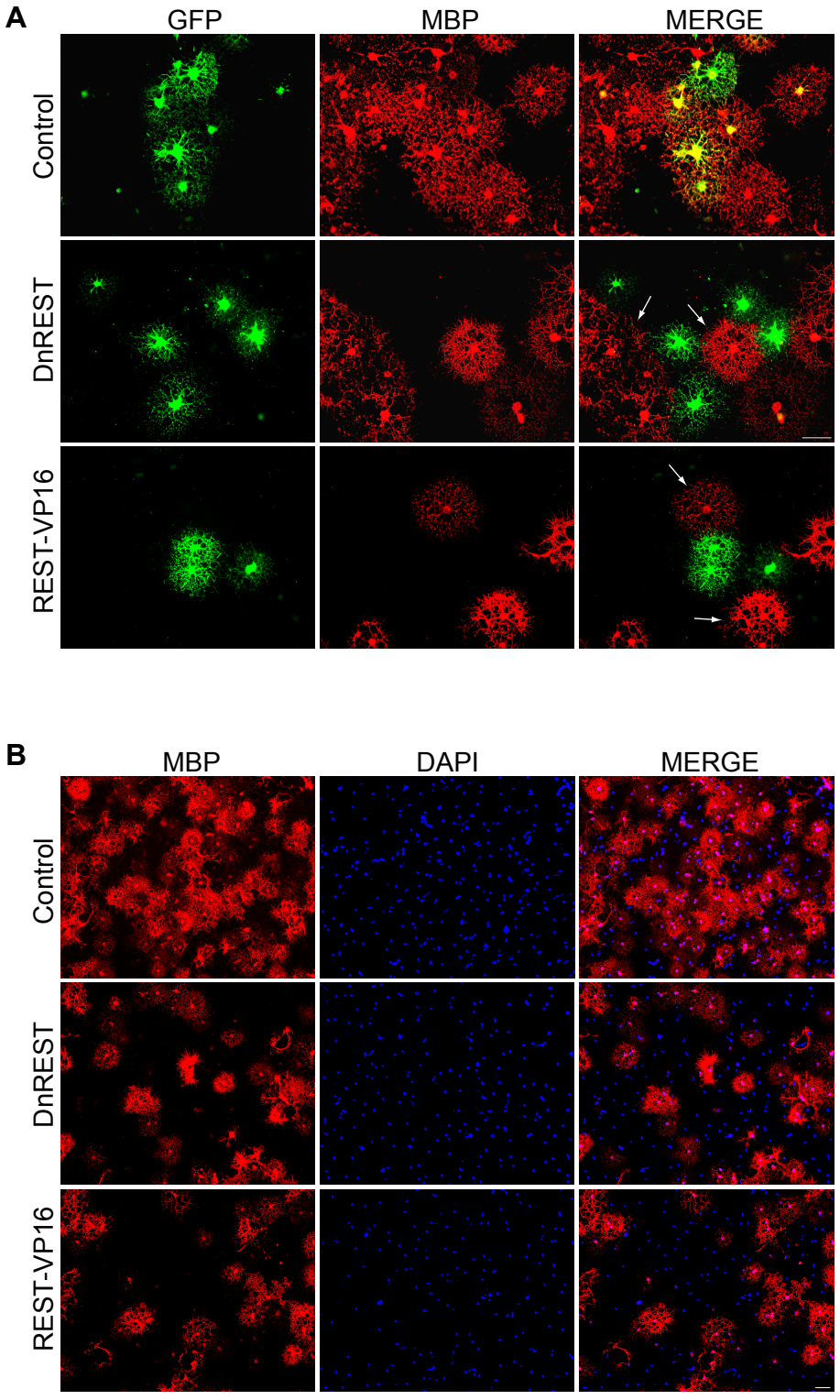

A
GFP
MBP
MERGE
Control
DnREST
REST-VP16
B
MBP
DAPI
MERGE
Control
DnREST
REST-VP16

Figure IV-3 REST LOF inhibits proper development of mature oligodendrocytes. (A) Quantitation of nucleofected cells from figure IV-2b. GFP-positive cells were scored for MBP expression at days 3 and 5 after oligodendrocyte induction in control, DnREST, and REST-VP16 nucleofected cultures. Data shown is percent of total GFP-positive cells. Error bars represent the standard deviation from the averages of three independent experiments. *P-value ≤0.02, **P-value ≤0.007, Student's t-test. **(B-D)** OPCs infected with control GFP or DnREST retroviruses were grown in OPC media for 5 days and switched to oligodendrocyte media. Cells were analyzed for MBP expression at the indicated time points subsequent to the initiation of differentiation. Both the percent of MBP-positive cells **(B)** and relative amounts of *MBP* mRNA **(C)** are decreased in differentiating oligodendrocytes after REST LOF. **(B)**. Error bars represent the standard deviation of the averages from at least three independent experiments. *P-value ≤0.001, Student's t-test. **(C)** Fold change is shown relative to OPCs at time of the initiation of oligodendrocyte differentiation (time 0) after normalization to *GAPDH*. Error bars represent the standard deviation from the averages of three independent experiments. *P-value ≤0.01, **P-value ≤0.005, Student's t-test. **(D)** Real-time RT-PCR of myelin gene expression 3 days after oligodendrocyte induction in control and DnREST retroviral infected cells. Fold difference in gene expression is shown relative to control infected cells after normalization to *GAPDH*. Error bars represent the standard deviation from 4-6 PCR runs from at least 2-3 independent experiments. *P-value ≤0.05, **P-value ≤0.005.

FIGURE IV-3

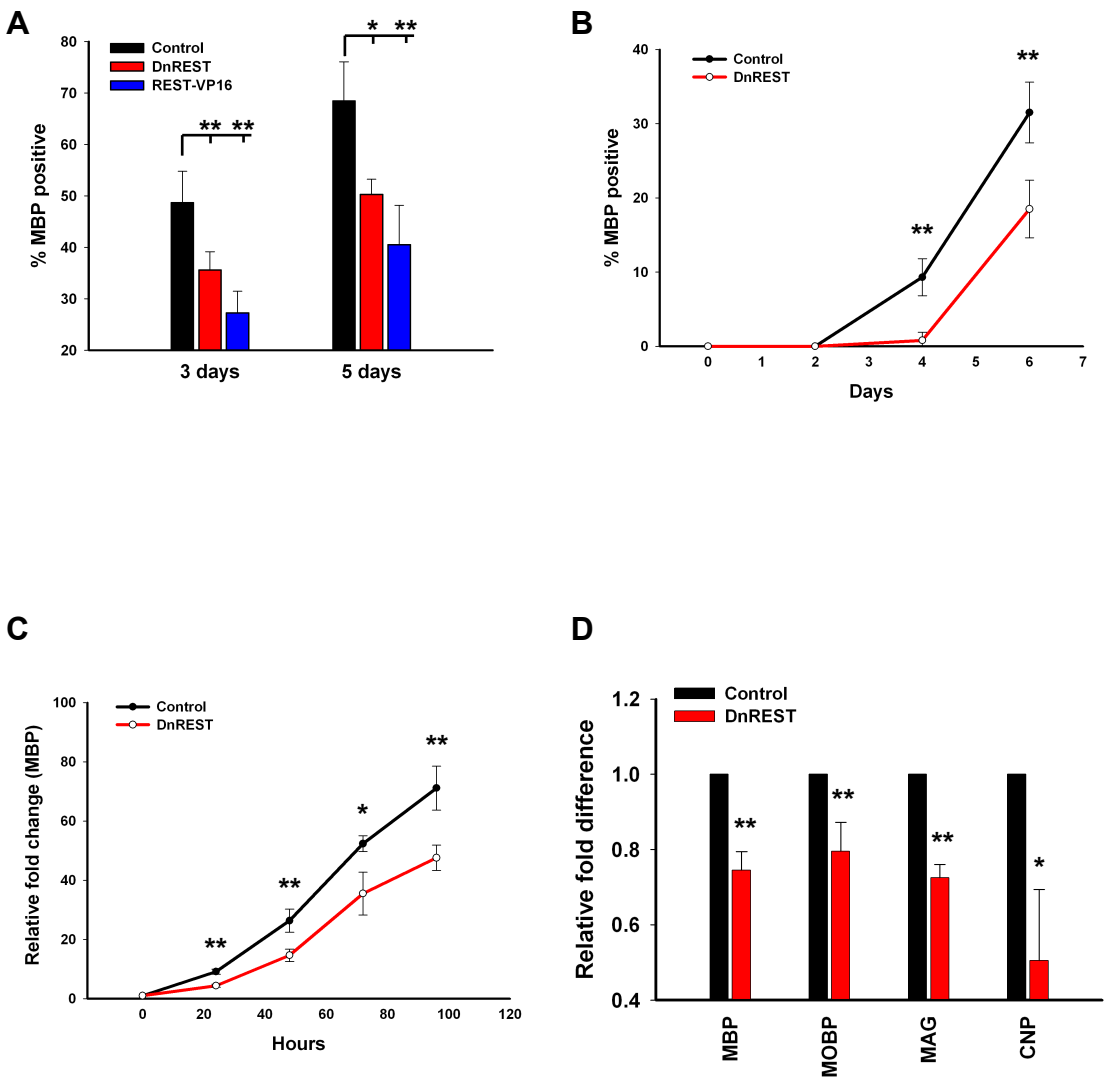

A
Control
DnREST
REST-VP16
% MBP positive
3 days
5 days
B
Control
DnREST
% MBP positive
Days
C
Control
DnREST
Relative fold change (MBP)
Hours
D
Control
DnREST
Relative fold difference
MBP
MOBP
MAG
CNP

Figure IV-4. REST LOF decreases the number of O4 expressing cells during oligodendrocyte differentiation. (A) Immunofluorescence staining showing O4 (red) expression in differentiating oligodendrocytes infected with control or DnREST retroviruses (green). Purified OPCs were infected and grown in proliferation media for 5 day followed by 3 days in oligodendrocyte differentiation media. The scale bar indicates 50μm. **(B)** Quantification of immunofluorescence staining showing the percent of retroviral infected cells expressing O4 at the indicated time points, time 0 being the time of media change and initiation of oligodendrocyte induction. There are less O4 expressing cells in DnREST infected cultures compared to control throughout differentiation. **(C)** Quantification of O4 expression in nucleofected OPCs shows a similar decrease in O4 expression with REST loss of function compared to control. Purified OPCs were co-nucleofected with plasmids expressing GFP and either DnREST, REST-VP16, or an empty vector and grown in oligodendrocyte differentiation media for 3 days. The number of O4 expressing cells is shown as a percent of the total nucleofected cells in each culture. All error bars represent the standard deviation from the averages of at least three independent experiments. *P-value $\leq$ 0.02, Student's t-test.

FIGURE IV-4

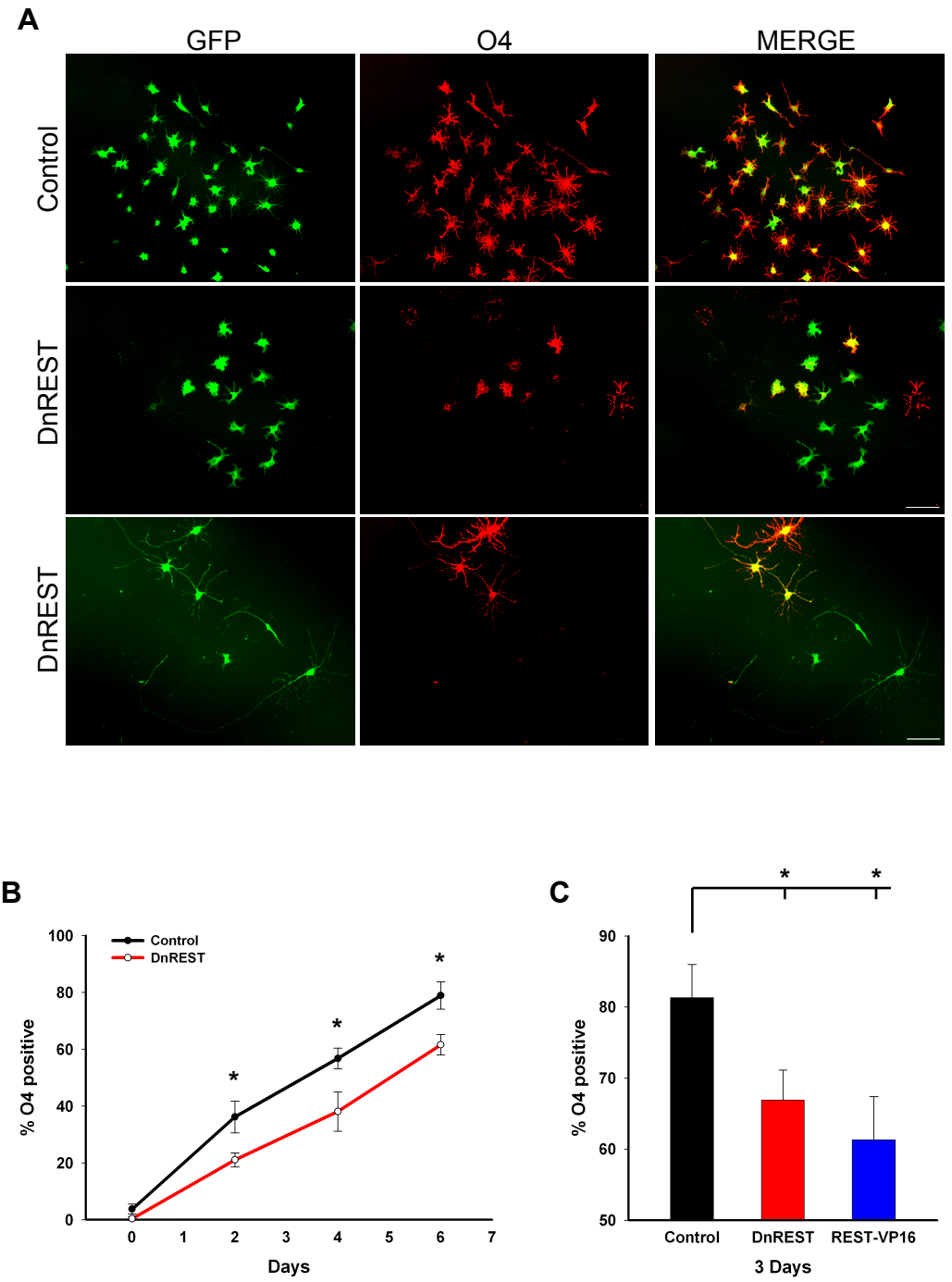

A
GFP
O4
MERGE
Control
DnREST
DnREST
B
Control
DnREST
% O4 positive
Days
C
% O4 positive
Control
DnREST
REST-VP16
3 Days

Figure IV-5. Loss of REST repression induces TUJ1 expression in differentiating oligodendrocytes. (A, C) Purified OPCs were infected with control GFP or DnREST expressing retroviruses, proliferated in culture for 5 days, and differentiated in oligodendrocyte media for 5 days. **(A)** Cultures were stained with an antibody against the neuronal marker TUJ1 (red) and infected cells (green) were analyzed. Many DnREST infected cells express TUJ1 whereas control infected cells expresses little to no TUJ1. **(C)** Quantification of immunofluorescence staining showing the percent of retroviral infected cells that express TUJ1 after 5 days in oligodendrocyte media. There is a significant increase in TUJ1 expression with REST loss of function compared to control. All error bars represent the standard deviation from the averages of four independent experiments. *P-value ≤0.0001, Student's t-test. **(B, D)** Purified OPCs were co-nucleofected with a plasmid expressing GFP and one expressing either DnREST, REST-VP16, or an empty vector, and grown in oligodendrocyte media for 5 days. **(B)** Immunofluorescence staining of nucleofected cells showing TUJ1 presenting cells when REST-VP16 is expressed. (**D)** Quantification of immunofluorescence staining showing the percent of nucleofected cells expressing TUJ1. There is a significant increase in TUJ1 expression with REST loss of function compared to control. All error bars represent the standard deviation from the averages of three independent experiments. *P-value ≤0.001, Student's t-test.

FIGURE IV-5

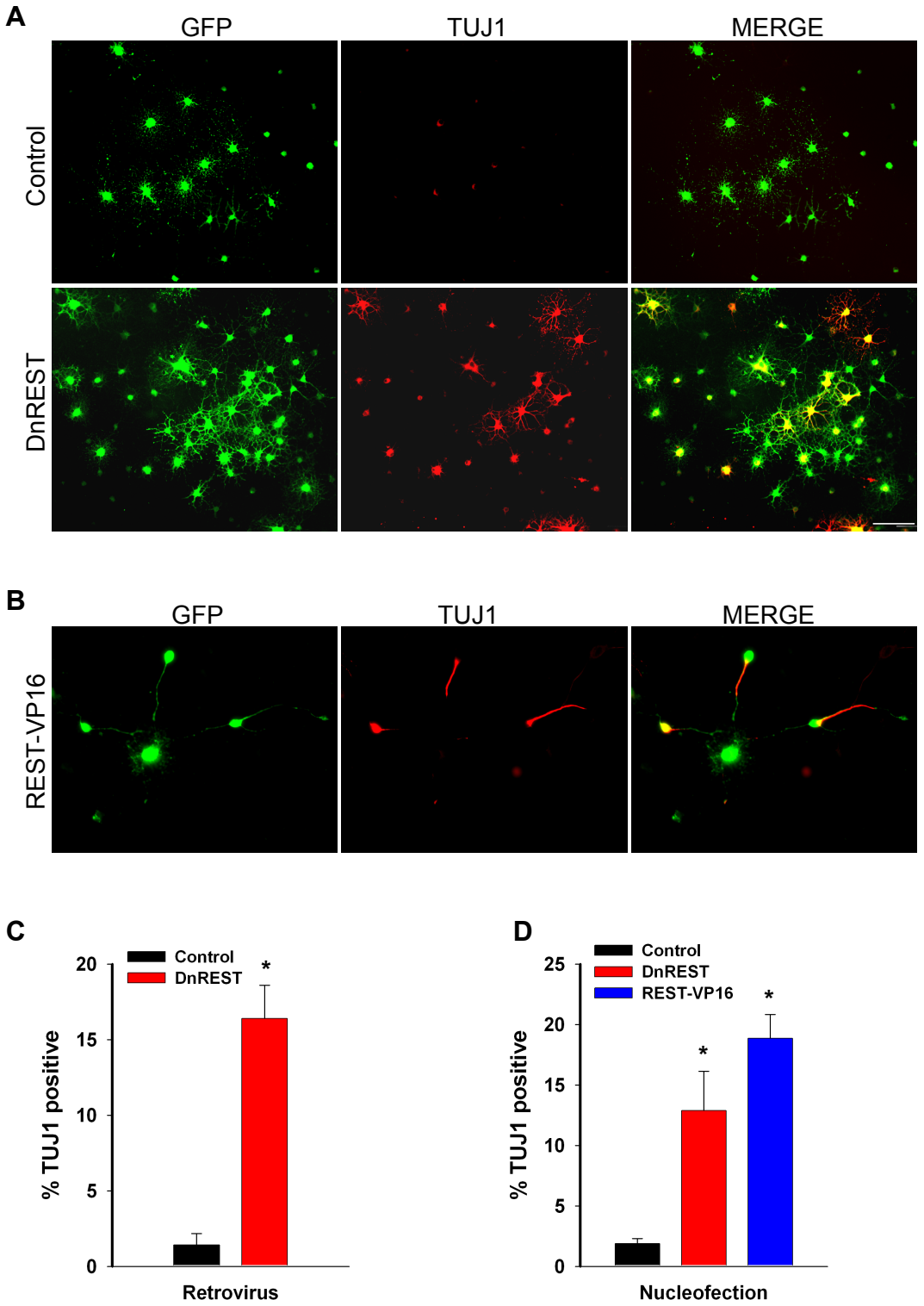

A
GFP
TUJ1
MERGE
Control
DnREST
B
GFP
TUJ1
MERGE
REST-VP16
C
Control
DnREST
*
% TUJ1 positive
20
15
10
5
0
Retrovirus
D
Control
DnREST
REST-VP16
*
*
% TUJ1 positive
25
20
15
10
5
0
Nucleofection

Figure IV-6. REST loss of function induces the expression of neuronal proteins and genes in OPCs after oligodendrocyte induction. Purified OPCs were grown in proliferation media for 5 days following infection with control GFP, DnREST, or REST-VP16 retroviruses. Cultures were switched to oligodendrocyte media for 5 days and protein expression was analyzed by immunoblot analysis **(A)**. CNP expression decreases and the neuronal proteins Beta III Tubulin (TUJ1), Synapsin1, and Snap25 are detected in DnREST and REST-VP16 infected cells but not in control cells. Beta-actin is used as a loading control. **(B-D)** After 5 days in proliferation media and 5 days in oligodendrocyte differentiation media, mRNA expression was analyzed by quantitative real-time PCR in control and DnREST infected cells. All graphs show the difference in gene expression with REST LOF relative to control cells after normalization to *GAPDH*. The genes are grouped by the amount of fold difference including large increases **(B)**, small increases **(C),** and little or no change in gene expression between DnREST and control cells **(D)**. All error bars represent the standard deviation from 4-6 total PCR runs from at least two independent experiments. *P-value $\leq$0.005, Student's t-test.

FIGURE IV-6

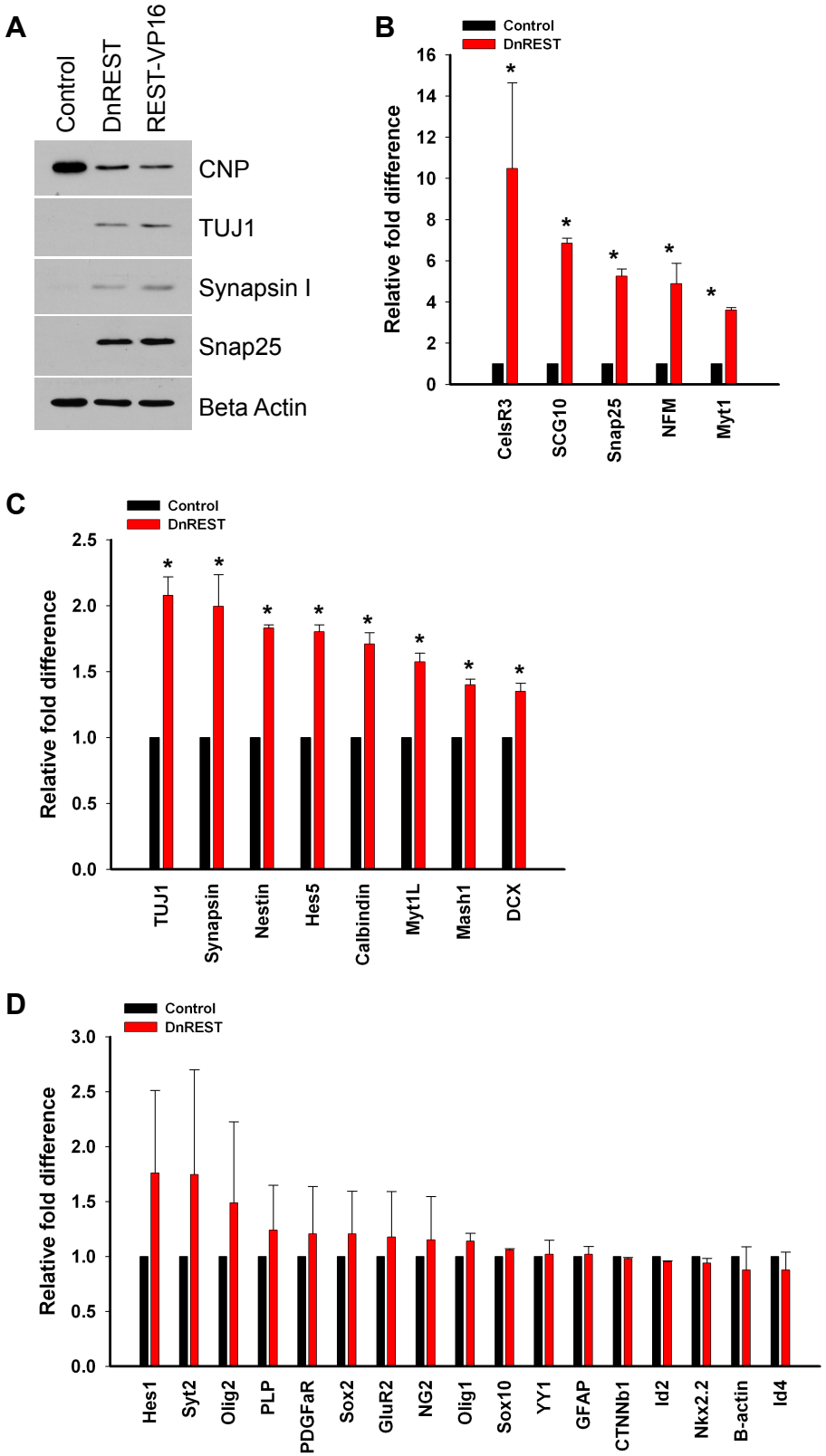

A
Control
DnREST
REST-VP16
CNP
TUJ1
Synapsin I
Snap25
Beta Actin
B
Control
DnREST
Relative fold difference
16
14
12
10
8
6
4
2
0
*
CelsR3
SCG10
Snap25
NFM
Myt1
C
Control
DnREST
Relative fold difference
2.5
2.0
1.5
1.0
0.5
0.0
TUJ1
Synapsin
Nestin
Hes5
Calbindin
Myt1L
Mash1
DCX
D
Control
DnREST
Relative fold difference
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Hes1
Syt2
Olig2
PLP
PDGFaR
Sox2
GluR2
NG2
Olig1
Sox10
YY1
GFAP
CTNNb1
Id2
Nkx2.2
B-actin
Id4

Figure IV-7. REST loss of function does not alter A2B5 or GFAP expression in OPCs after 6 days in stem cell media. Purified OPCs were infected with control GFP or DnREST retroviruses, grown in proliferation media for 5 days, and switched to stem cell media. **(A)** Immunofluorescence staining showing infected cells (green) that express A2B5 (red) after 4 days in stem cell media. **(B)** Quantification of immunofluorescence staining showing the percent of infected cells that express A2B5 at time 0 (OPCs at the time of media change), and after 4 and 6 days in stem cell media. A2B5 expression decreases at a similar rate in control and DnREST infected cells over time, and both conditions contain a similar number of A2B5 expressing cells at each time point**. (C)** Quantification of GFAP expression shows that a similar number of control and DnREST infected cells express GFAP at each time point in stem cell media. OPCs grown in stem cell media do not develop into GFAP expressing cells and the number of GFAP-positive cells in these cultures remains under 3% of the total population. All error bars represent the standard deviation of the averages of four independent experiments. Scale bar represents 50μm.

FIGURE IV-7

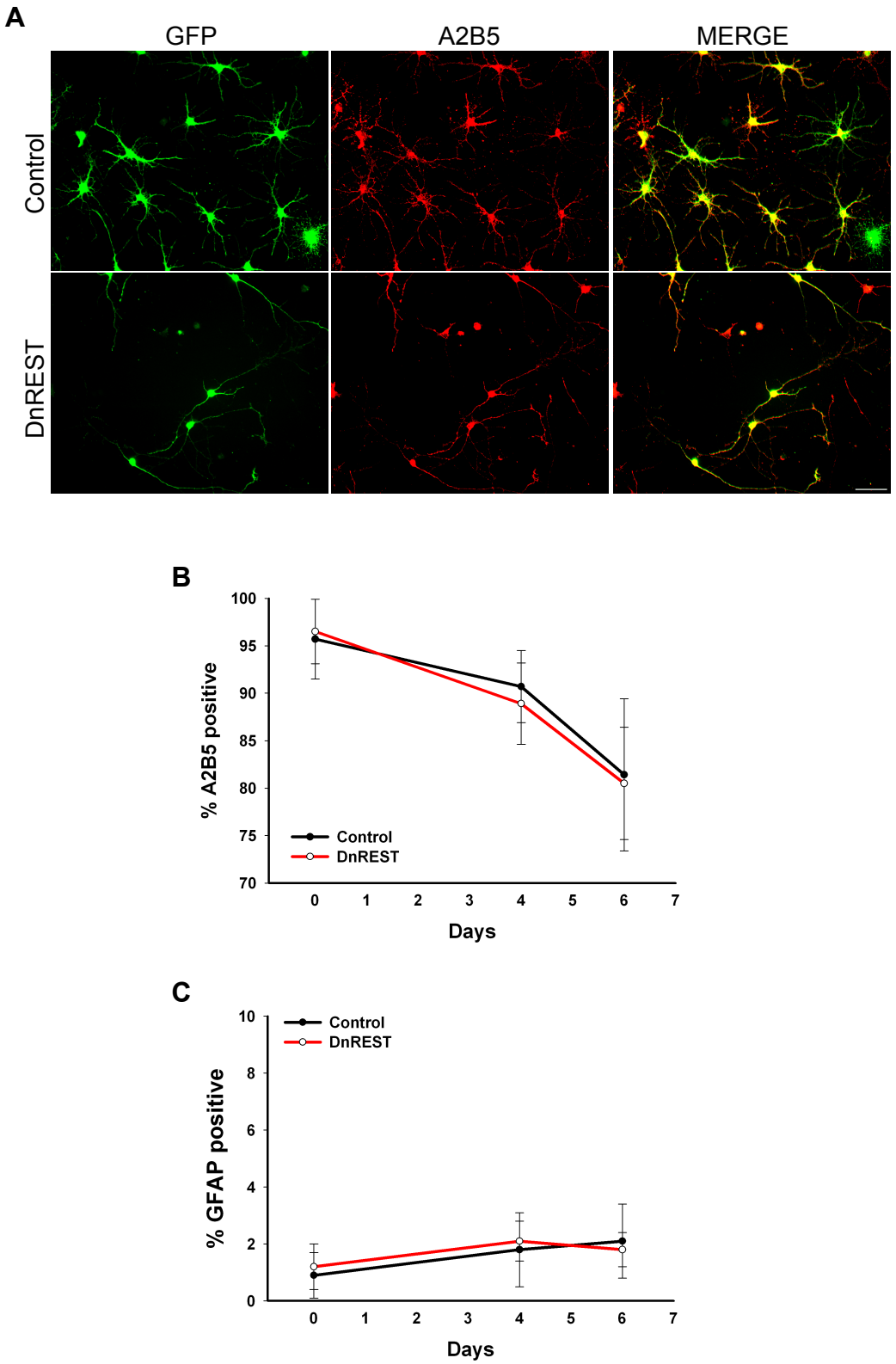

A
GFP
A2B5
MERGE
Control
DnREST
B
% A2B5 positive
100
95
90
85
80
75
70
Control
DnREST
0
1
2
3
4
5
6
7
Days
C
% GFAP positive
10
8
6
4
2
0
Control
DnREST
0
1
2
3
4
5
6
7
Days

Figure IV-8. Expression of MBP and TUJ1 in OPCs grown in stem cell media. Purified OPCs were infected with retroviruses expressing either DnREST or GFP alone as a control. Cells were grown in proliferation media for 5 days followed by 4 days in stem cell media. **(A**) Immunofluorescence staining showing the expression of the mature oligodendrocyte protein MBP (red) in infected cells (green). Some control cells begin to express MBP (arrows) after 4 days in stem cell media. DnREST expression decreases the number of MBP positive cells but does not inhibit MBP expression in non-infected cells within the same culture (arrowhead). **(B)** Unlike MBP, the neuronal marker TUJ1 (red) is expressed by a large number of DnREST infected cells but not by control cells. Higher magnification (bottom) shows TUJ1 positive cells in the DnREST cultures with differing morphologies including those of an oligodendrocyte lineage cell (arrow) and neuronal-like cells (arrowheads). NG2 expression was also analyzed, see figure IV-9 for images and quantification of immunofluorescence staining. All scale bars represent 50μm.

FIGURE IV-8

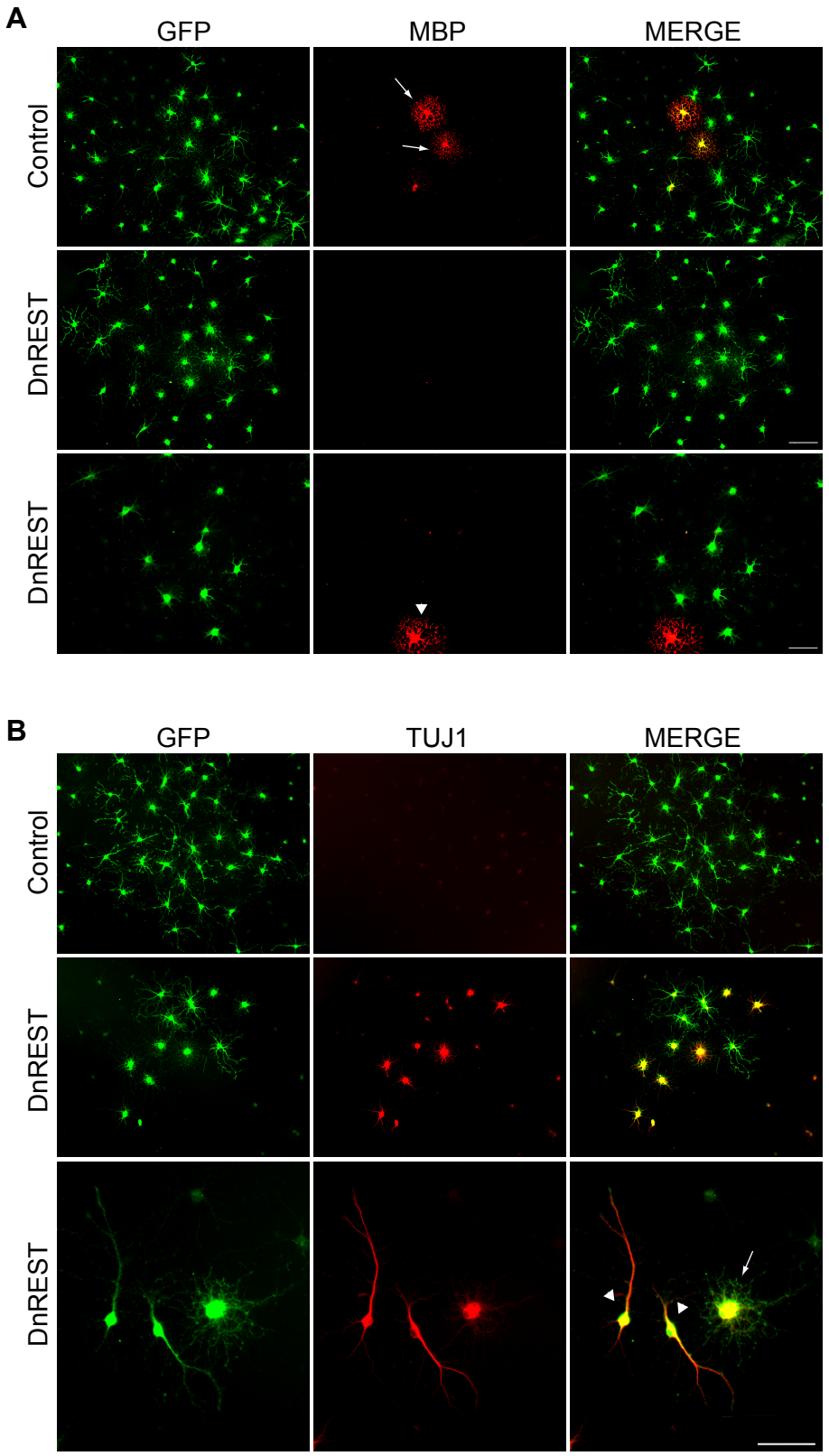

A
GFP
MBP
MERGE
Control
DnREST
DnREST
B
GFP
TUJ1
MERGE
Control
DnREST
DnREST

Figure IV-9. TUJ1 expression increases at the expense of MBP-positive cells in DnREST infected OPCs grown in stem cell media. This is a continuation of figure IV-8. **(A)** Immunofluorescence staining showing infected cells (green) that express NG2 (red) after 4 days in stem cell media. A large number of control and DnREST cells express NG2, although there is some variation in cell morphology between the cultures. Scale bars represent 50μm**. (B)** Stacked bar graph showing the quantification of immunofluorescence staining at the indicated time points subsequent from media change to SCM. The bars are set to 100% and broken down by the percent of cells expressing NG2 (black bars), MBP (blue bars), TUJ1 (purple bars), and the percent of cells negative for all of these markers termed Other (gray bars). Over time, the number MBP-positive cells increases in control cultures whereas TUJ1-positive cells increase in DnREST cultures. n=4.

FIGURE IV-9

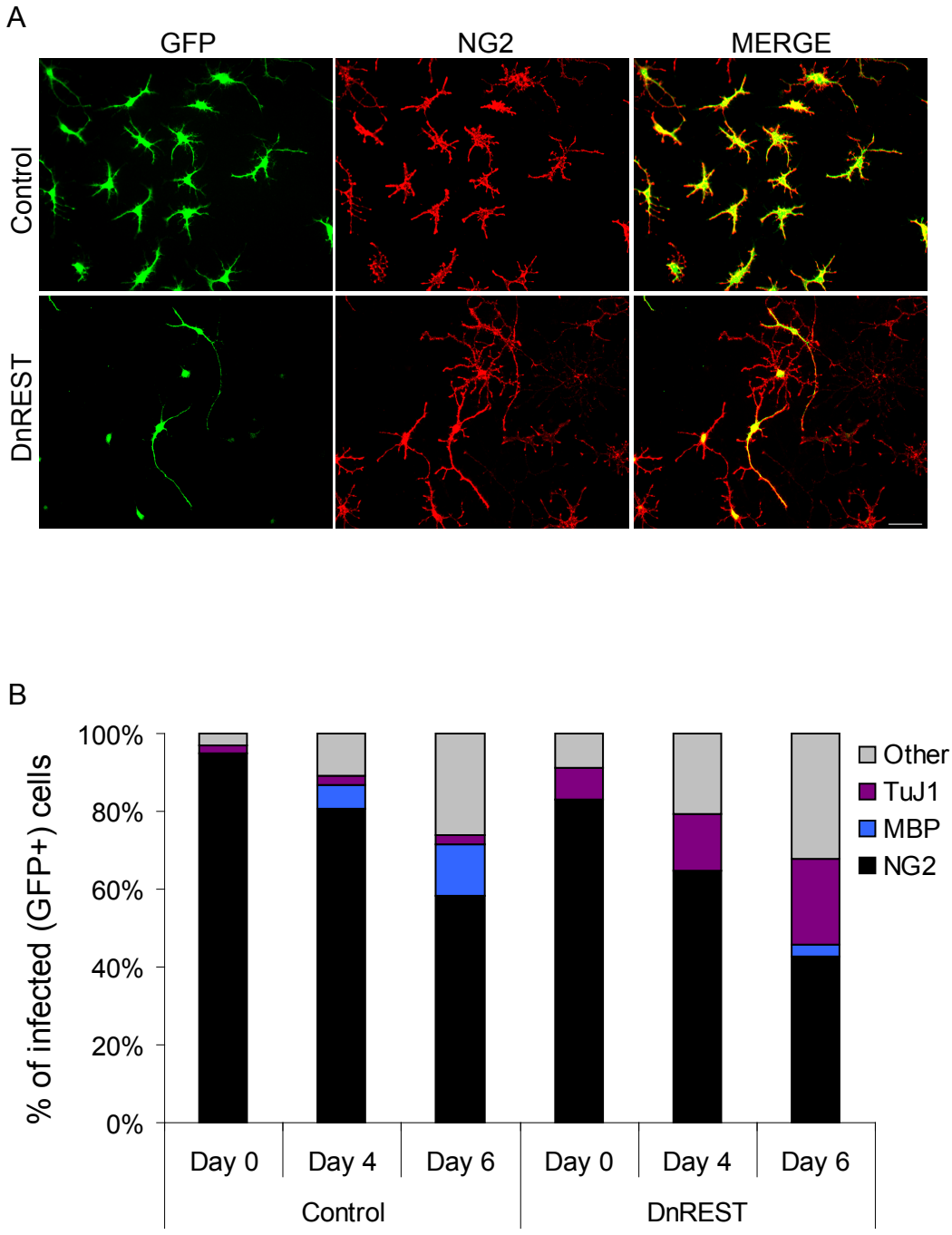

A
GFP
NG2
MERGE
Control
DnREST
B
% of infected (GFP+) cells
100%
80%
60%
40%
20%
0%
Day 0
Day 4
Day 6
Day 0
Day 4
Day 6
Control
DnREST
Other
TuJ1
MBP
NG2

Figure IV-10. REST loss of function decreases the number of O4 expressing cells when OPCs are grown in stem cell media. (A, B) Immunofluorescence staining showing O4 (red) expression in OPCs grown in stem cell media. Purified OPCs were infected with control GFP or DnREST expressing retroviruses and grown in proliferation media for 5 day followed by 4 **(A)** or 6 **(B)** days in stem cell media. The DnREST infected cell population contained fewer O4 positive cells than control cultures. The scale bar indicates 50μm. **(C)** Quantification of immunofluorescence staining showing the percent of infected cells expressing O4 at the indicated time points subsequent to media change. There are fewer O4 expressing cells in DnREST infected cultures (red line) compared to control (black line) throughout time. Error bars represent the standard deviation from the averages of four independent experiments. *P-value $\leq$0.05, Student's t-test.

FIGURE IV-10

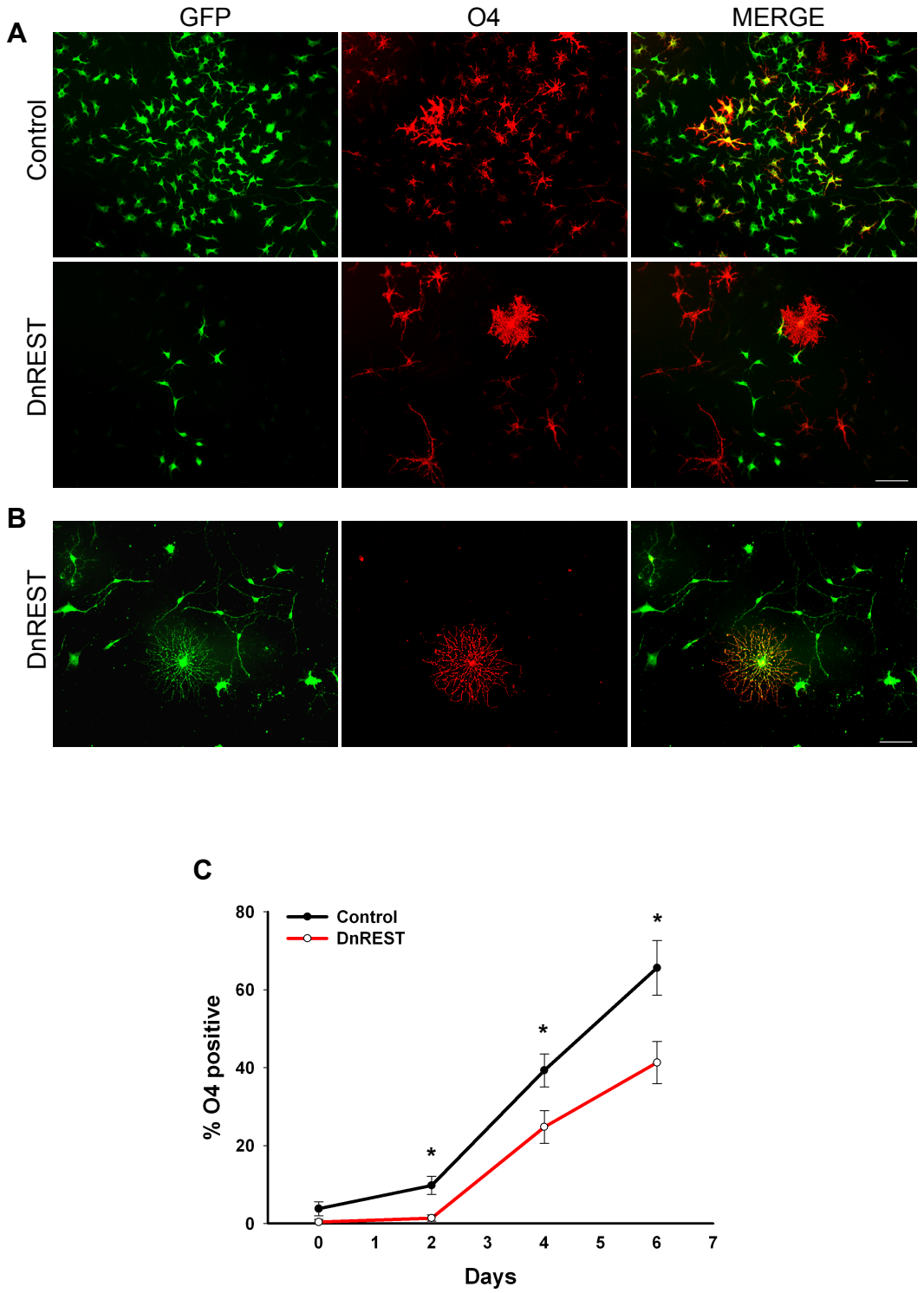

A
GFP
O4
MERGE
Control
DnREST
B
DnREST
C
Control
DnREST
% O4 positive
Days
0
20
40
60
80
0
1
2
3
4
5
6
7
*
*
*

Figure IV-11. REST LOF induces the expression of neuronal proteins and genes in OPCs grown in stem cell media. Purified OPCs were infected with GFP or DnREST retroviruses, grown in proliferation media for 5 days, and switched to stem cell media. **(A)** Immunoblot analysis showing detection of the neuronal proteins Snap25, Synapsin1, and Beta III tubulin (TUJ1) after 5 days in SCM. Beta-actin is used as a loading control. **(B-C)** After 5 days in proliferation media and 5 days in stem cell media, mRNA expression was analyzed by quantitative real-time PCR in control and DnREST infected cells. Both graphs show the difference in gene expression with REST LOF relative to control cells after normalization to *GAPDH*. The genes are grouped by the amount of fold difference. All error bars represent the standard deviation from 4-6 total PCR runs from at least two independent experiments. *P-value $\leq$0.05, **P-value $\leq$0.005 Student's t-test.

FIGURE IV-11

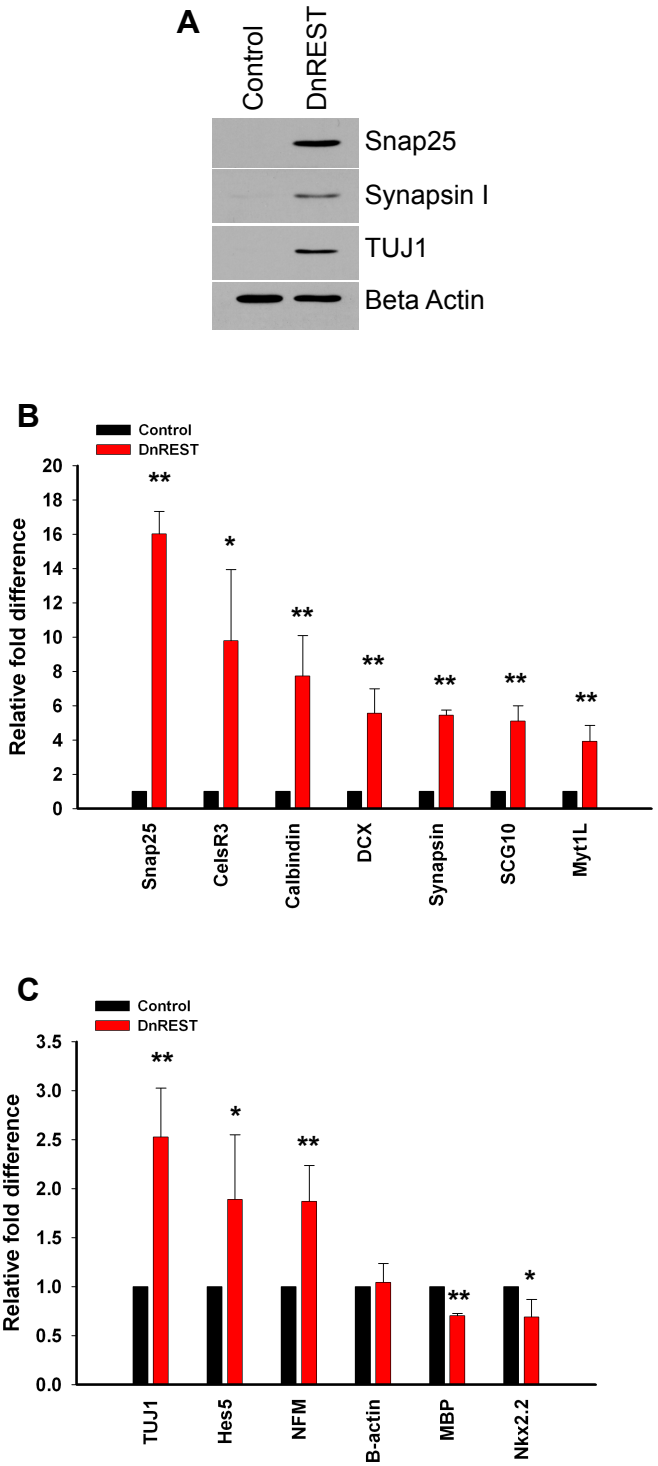

A
Control
DnREST
Snap25
Synapsin I
TUJ1
Beta Actin
B
Control
DnREST
Relative fold difference
Snap25
CelsR3
Calbindin
DCX
Synapsin
SCG10
Myt1L
C
Control
DnREST
Relative fold difference
TUJ1
Hes5
NFM
B-actin
MBP
Nkx2.2

Figure IV-12. REST loss of function decreases GFAP expression in differentiating type II astrocytes. Purified OPCs were infected with retroviruses expressing either DnREST or GFP alone as a control. After 5 days in proliferation media, the cells were placed in media containing 10% FBS to promote 2A differentiation for an additional 5 days. Cultures were stained with antibodies against GFP (green) and GFAP (red) **(A)**. DnREST expressing cells contain fewer GFAP positive cells than control cultures. Although both cultures are heterogeneous in cell morphology, cells infected with DnREST retroviruses tend to have longer processes and a slightly different overall morphology than control cells (arrows). The scale bar represents 50μm. **(B)** Quantification of immunofluorescence staining showing a decrease in the percent of infected cells that express GFAP with REST loss of function compared to control. Error bars represent the standard deviation from the averages of four independent experiments. *P-value ≤0.001, Student's t-test. **(C)** Consistent with GFAP protein, *GFAP* mRNA expression also decreases when REST function is perturbed. Quantitative RT-PCR shows an increase in *GFAP* expression in control cells after 3 days in 2A media. This increase is significantly less in DnREST expressing cells. Fold difference is shown relative to control infected OPCs at time 0 (time of media change to 2A differentiation media) after normalization to *GAPDH*. Error bars represent the standard deviation from the averages of three independent experiments. *P-value ≤0.001, Student's t-test.

FIGURE IV-12

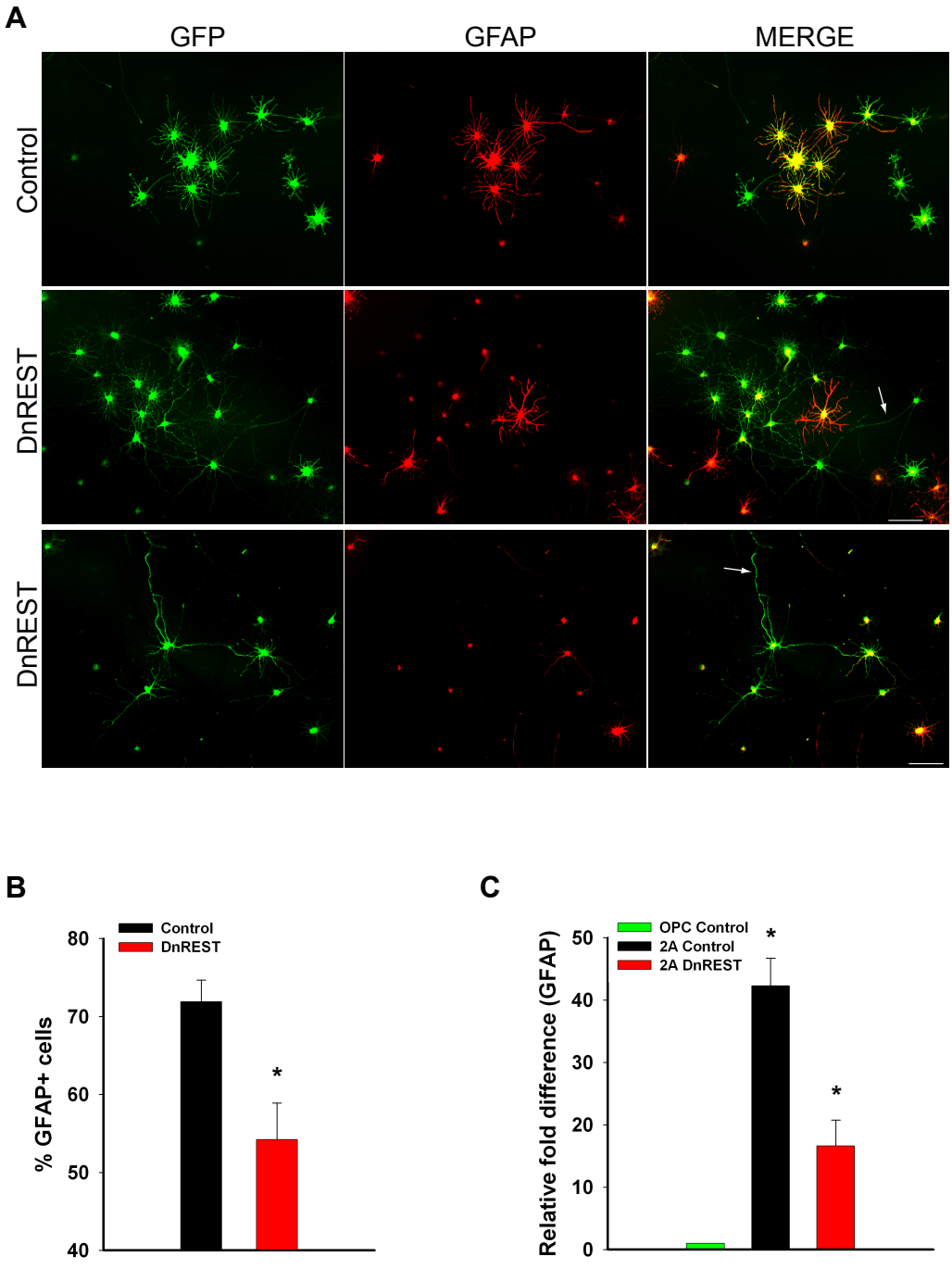

A
GFP
GFAP
MERGE
Control
DnREST
DnREST
B
Control
DnREST
% GFAP+ cells
80
70
60
50
40
*
C
OPC Control
2A Control
2A DnREST
Relative fold difference (GFAP)
50
40
30
20
10
0
*
*

Figure IV-13. OPCs grown in 2A differentiation media express the neuronal marker TUJ1 when REST function is perturbed. Purified OPCs were infected with retroviruses expressing either DnREST or GFP alone as a control. Cells were grown in proliferation media for 5 days followed by 5 days in 2A differentiation media. Immunofluorescence staining showing DnREST infected cells (green) that are positive for TUJ1 (red) expression. Control 2A cells do not express TUJ1. Note the difference in morphology between the DnREST and control cultures. For quantification of and TUJ1 expression, see figure IV-14. All scale bars indicate 50μm.

FIGURE IV-13

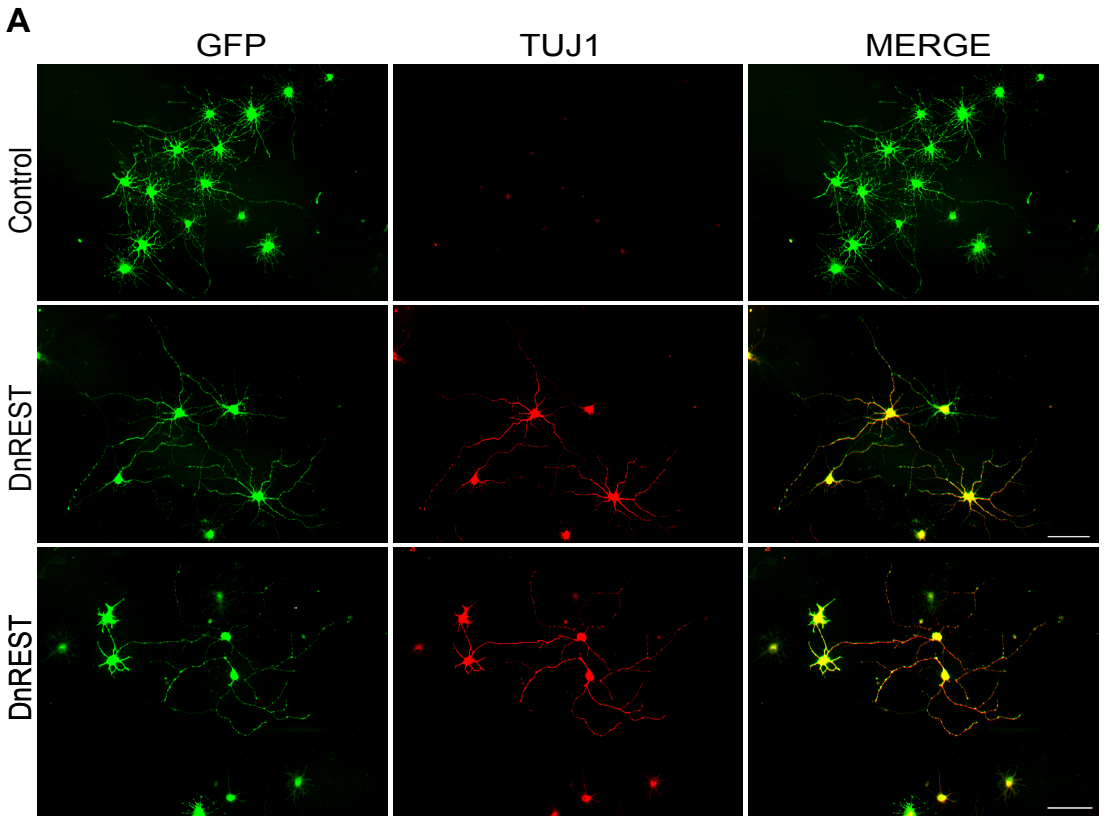


Figure IV-14. REST LOF results in an increase in the number of TUJ1 positive cells. (A and **B)** Purified OPCs were infected with retroviruses expressing either DnREST or GFP alone as a control. After 5 days in proliferation media, the cells were placed in media containing 10% FBS to promote 2A differentiation and TUJ1 expression analyzed 5 days later. **(A)** Quantification of immunofluorescence staining showing as the percent of infected cells that are positive for TUJ1. Very few control cells express TUJ1 as opposed to the DnREST cultures in which over one third of the infected cells are TUJ1-positive. Error bars represent the standard deviation from the averages of four independent experiments. *P-value ≤0.0001, Student's t-test. **(B)** Quantitative real-time PCR analysis of *Tubb3* (TUJ1) transcript expression in the retroviral infected cells after 5 days in proliferation media followed by 3 days in 2A media. The fold difference is shown relative to control OPCs at time 0 (the time of media change from proliferation to 2A media) after normalization to *GAPDH*. *Tubb3* mRNA levels decrease as OPCs develop in to 2A's. Conversely, *Tubb3* mRNA expression increases and with REST loss of function. Error bars represent the standard deviation from three independent experiments. *P-value ≤0.01, Student's t-test. **(C)** The increase in TUJ1 protein expression after infecting OPCs with DnREST, REST-VP16, or control GFP expressing adenoviruses. Following infection, cells were grown in 2A differentiation media for 4 days and stained with an antibody against TUJ1. TUJ1 expression is shown as a percent of total infected cells for each group. Error bars represent the standard deviation from three independent experiments. *P-value ≤0.01, Student's t-test.

FIGURE IV-14

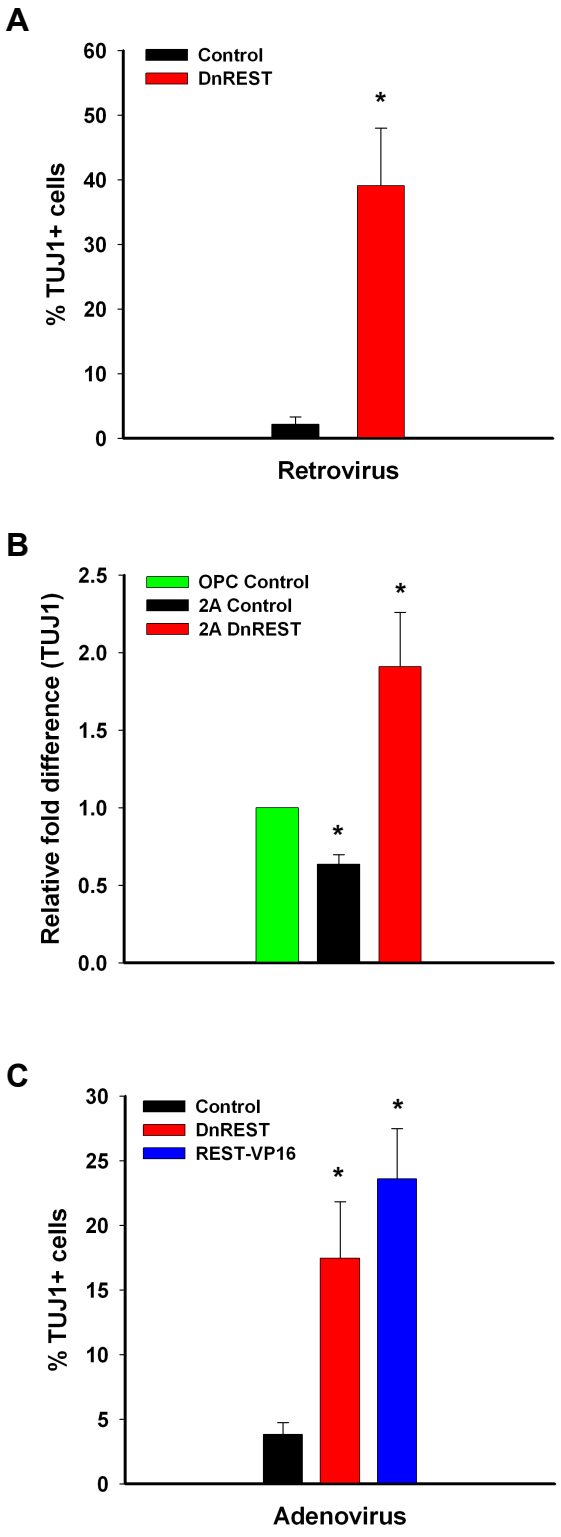

A
Control
DnREST
% TUJ1+ cells
60
50
40
30
20
10
0
*
Retrovirus
B
OPC Control
2A Control
2A DnREST
Relative fold difference (TUJ1)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
C
Control
DnREST
REST-VP16
% TUJ1+ cells
30
25
20
15
10
5
0
*
*
Adenovirus

Figure IV-15. Immunodetection of neuronal proteins in differentiating 2A's after adenovirus infection. Purified OPCs were infected with control GFP or REST-VP16 expressing adenoviruses and switched to 2A media for 4 days. Images show Synapsin I **(A)**, SCG10 **(B)**, and Neurofilament **(C)** protein expression in REST-VP16 adenoviral infected cells but not in control. Scale bars indicate 50μm.

FIGURE IV-15

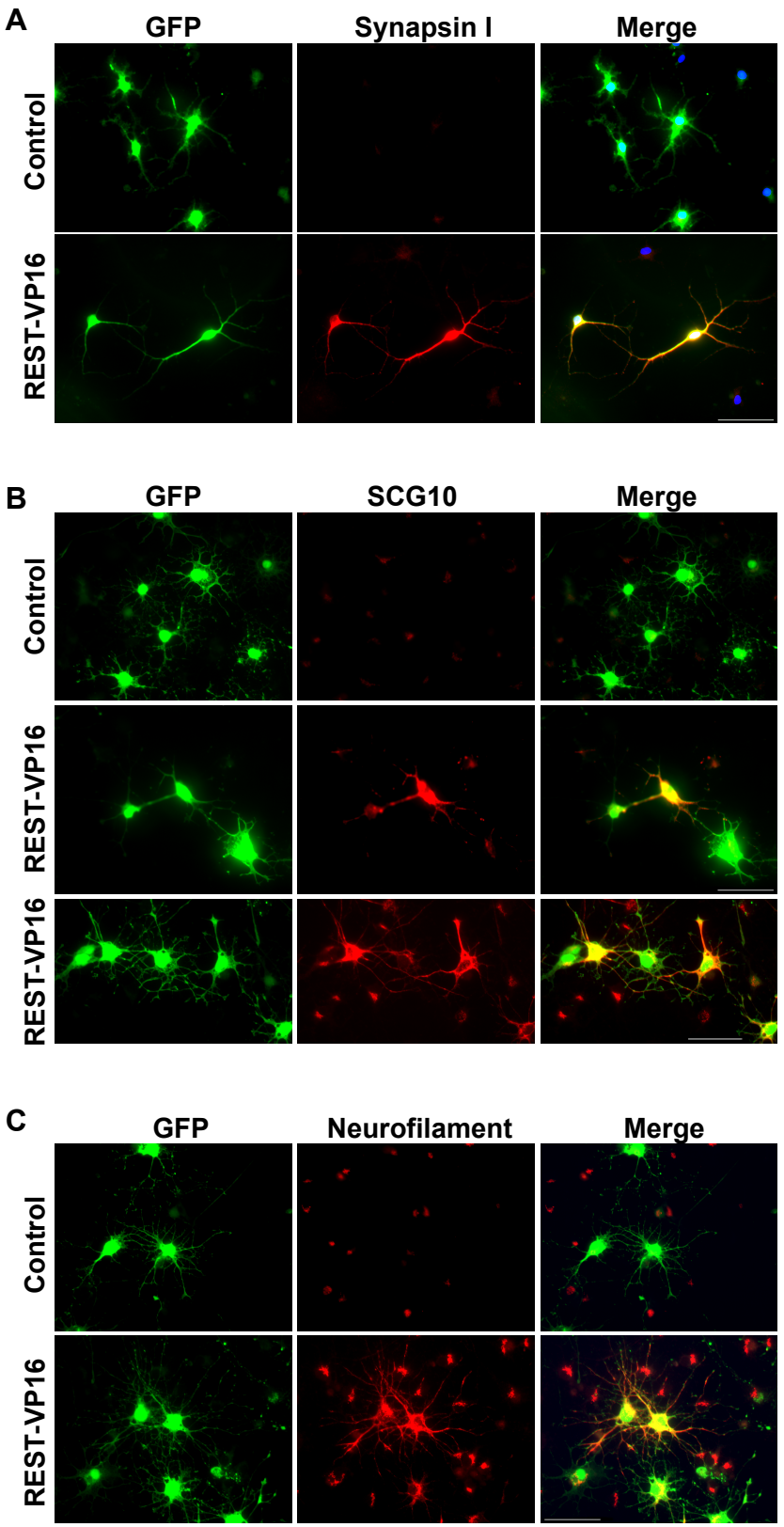

A
GFP
Synapsin I
Merge
Control
REST-VP16
B
GFP
SCG10
Merge
Control
REST-VP16
REST-VP16
C
GFP
Neurofilament
Merge
Control
REST-VP16

Figure IV-16. REST loss of function induces the expression of neuronal proteins and genes in OPCs after 2A induction. Purified OPCs were grown in proliferation media for 5 days following infection with control GFP, DnREST, or REST-VP16 retroviruses and switched to 2A media. **(A)** Immunoblot analysis showing neuronal protein expression in infected cells after 5 days in 2A media. TUJ1, Neurofilament, Synapsin1, and Snap25 proteins are present in DnREST and REST-VP16 expressing cells but not in control cells. Beta-actin is used as a loading control. **(B-D)** Quantitative real time PCR of infected cells after 3 days in 2A media showing gene expression changes with REST loss of function. The fold difference in gene expression in DnREST infected cells is shown relative to control infected cells after normalization to *GAPDH*. The genes are grouped by the amount of fold difference including large increases **(B)** moderate increases **(C),** and no change or decreases **(D)**. RE1 containing neuronal genes show the greatest fold difference with REST loss of function whereas genes involved in oligodendrocyte development, such as MAG, are further repressed compared to control cells. All error bars represent the standard deviation from 4-6 PCR runs from at least two independent experiments. *P-value $\leq$0.05, **P-value $\leq$0.005, Student's t-test.

FIGURE IV-16

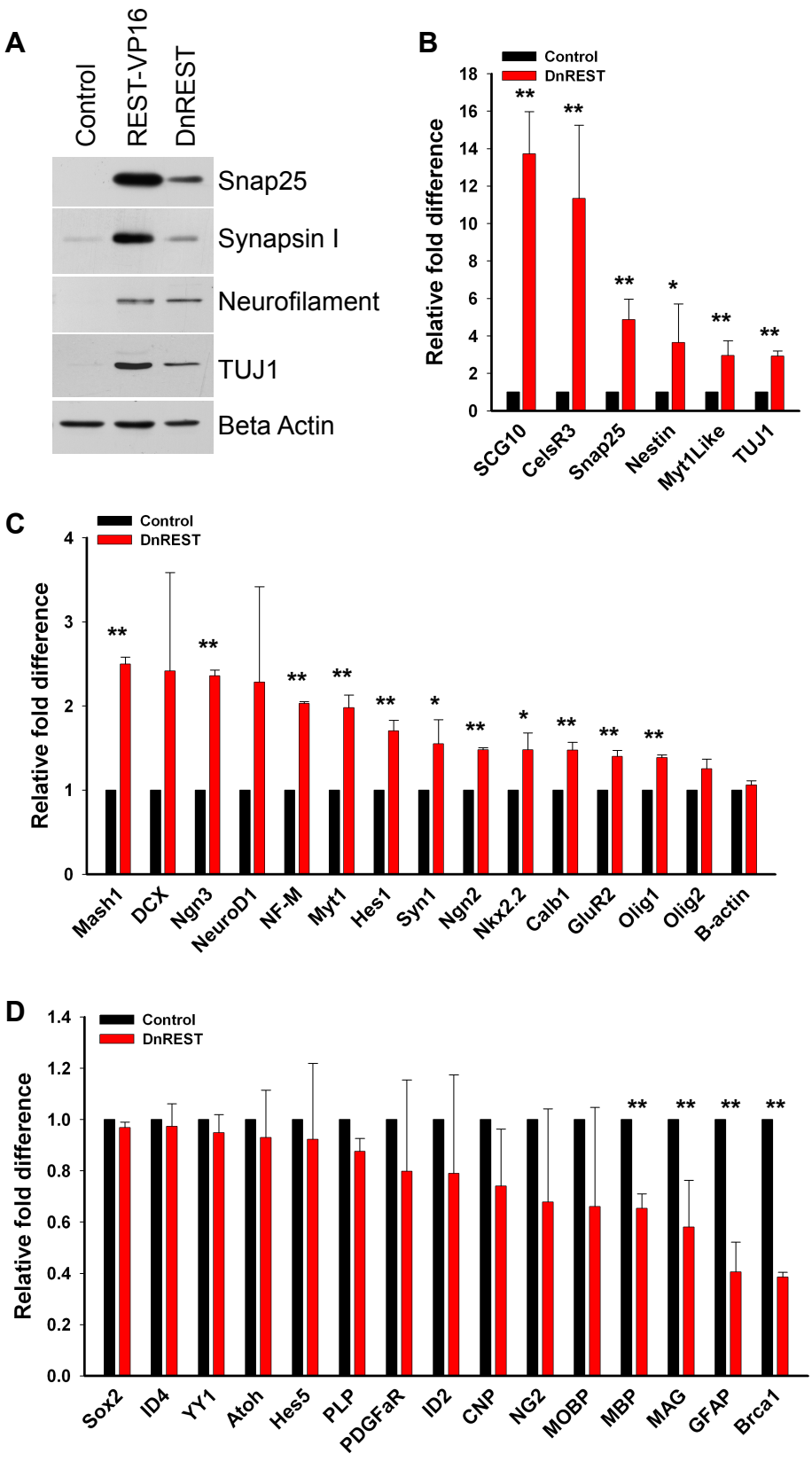

A
Control
REST-VP16
DnREST
Snap25
Synapsin I
Neurofilament
TUJ1
Beta Actin
B
Control
DnREST
Relative fold difference
SCG10
CelsR3
Snap25
Nestin
Myt1Like
TUJ1
C
Control
DnREST
Relative fold difference
Mash1
DCX
Ngn3
NeuroD1
NF-M
Myt1
Hes1
Syn1
Ngn2
Nkx2.2
Calb1
GluR2
Olig1
Olig2
B-actin
D
Control
DnREST
Relative fold difference
Sox2
ID4
YY1
Atoh
Hes5
PLP
PDGFaR
ID2
CNP
NG2
MOBP
MBP
MAG
GFAP
Brca1

Figure IV-17. Overview of clonal analysis. Purified OPCs were plated in 100mm tissue culture dishes in proliferation media for 1-2 days. Cells were infected with retroviruses expressing either DnREST or GFP alone as a control. OPCs were grown for 5 days in proliferation media post-infection. One dish of control or DnREST infected cells were passed onto a large number of 18mm glass coverslips at clonal density so that all infected cells in each condition originated from the same population in each experiment. Clones were grown for 2 days in proliferation media followed by additional time (2, 3, or 5 days) in proliferation media **(A)** or oligodendrocyte **(B)**, stem cell **(C)**, or 2A **(D)** differentiation media. Clones were stained with cell specific antibodies as indicated and clonal size and composition were analyzed in the different conditions. All cells were immunostained with an anti-GFP antibody to enhance GFP fluorescence. GFP expression was used to identify infected clones.

FIGURE IV-17

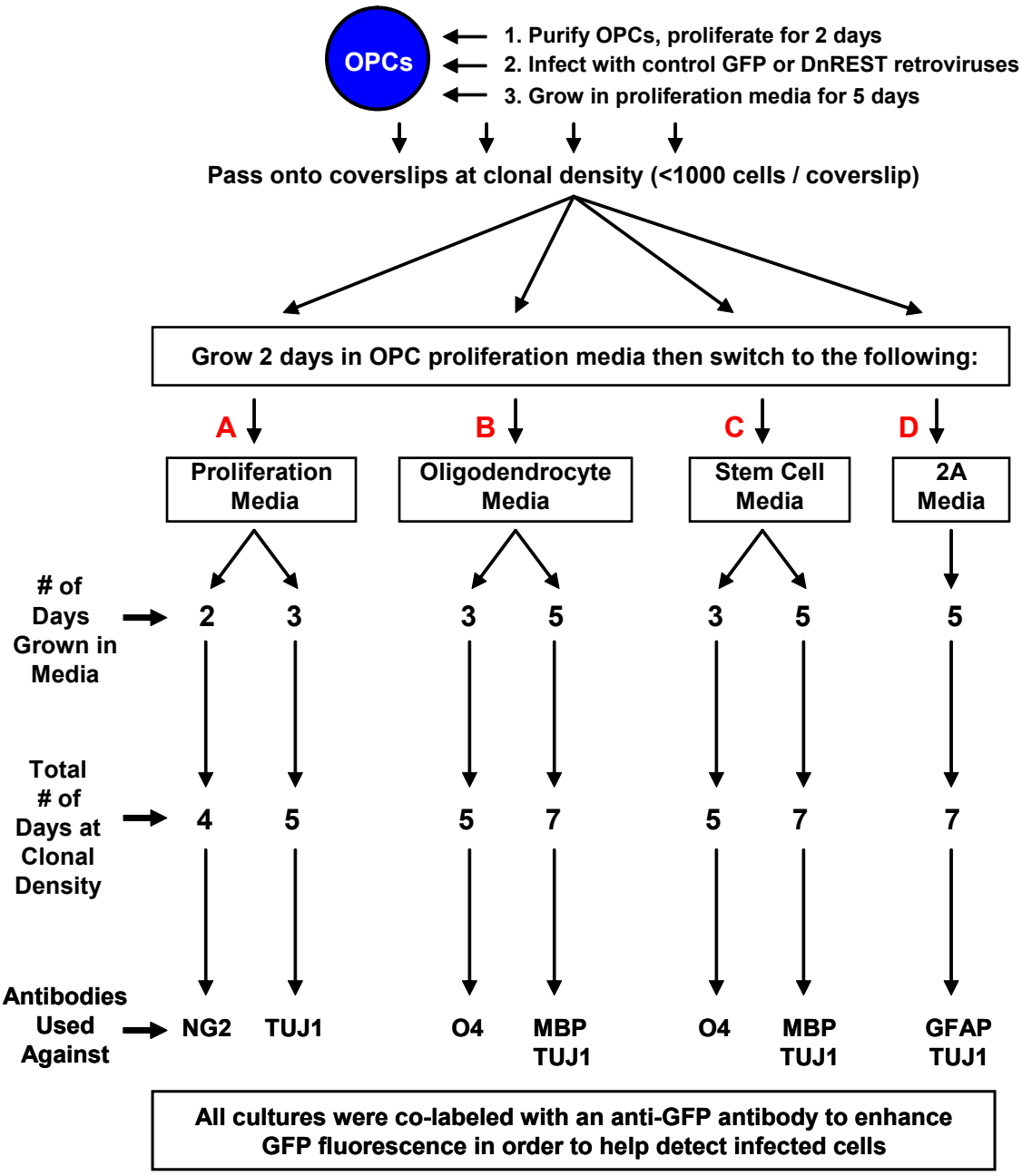

OPCs
1. Purify OPCs, proliferate for 2 days
2. Infect with control GFP or DnREST retroviruses
3. Grow in proliferation media for 5 days
Pass onto coverslips at clonal density (<1000 cells / coverslip)
Grow 2 days in OPC proliferation media then switch to the following:
A
B
C
D
Proliferation Media
Oligodendrocyte Media
Stem Cell Media
2A Media
# of Days Grown in Media
2
3
3
5
3
5
5
Total # of Days at Clonal Density
4
5
5
7
5
7
7
Antibodies Used Against
NG2
TUJ1
O4
MBP TUJ1
O4
MBP TUJ1
GFAP TUJ1
All cultures were co-labeled with an anti-GFP antibody to enhance GFP fluorescence in order to help detect infected cells

Figure IV-18. Appearance of clones in oligodendrocyte differentiation media. Cells were grown as described in figure IV-17b. Immunofluorescence images show infected clones from control **(A)** and experimental **(B)** cultures at days 5 (left) and 7 (right). Scale bars indicate 50μm.

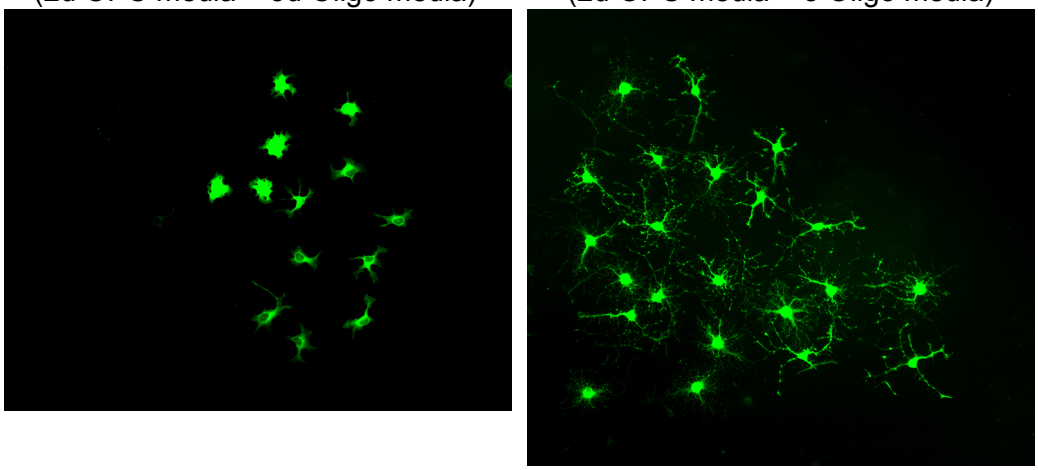
FIGURE IV-18

A

CONTROL

5 day clones
(2d OPC media + 3d Oligo media)

7 day clones
(2d OPC media + 5 Oligo media)



B

DnREST

5 day clones
(2d OPC media + 3d Oligo media)

7 day clones
(2d OPC media + 5 Oligo media)

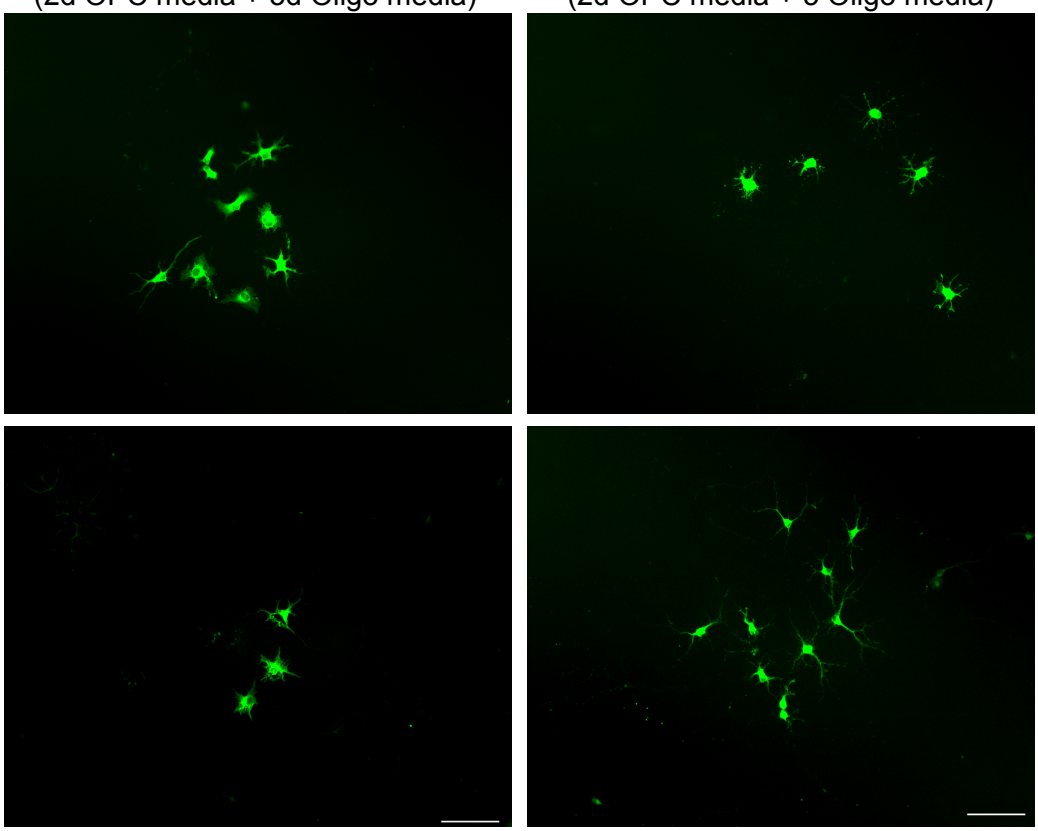
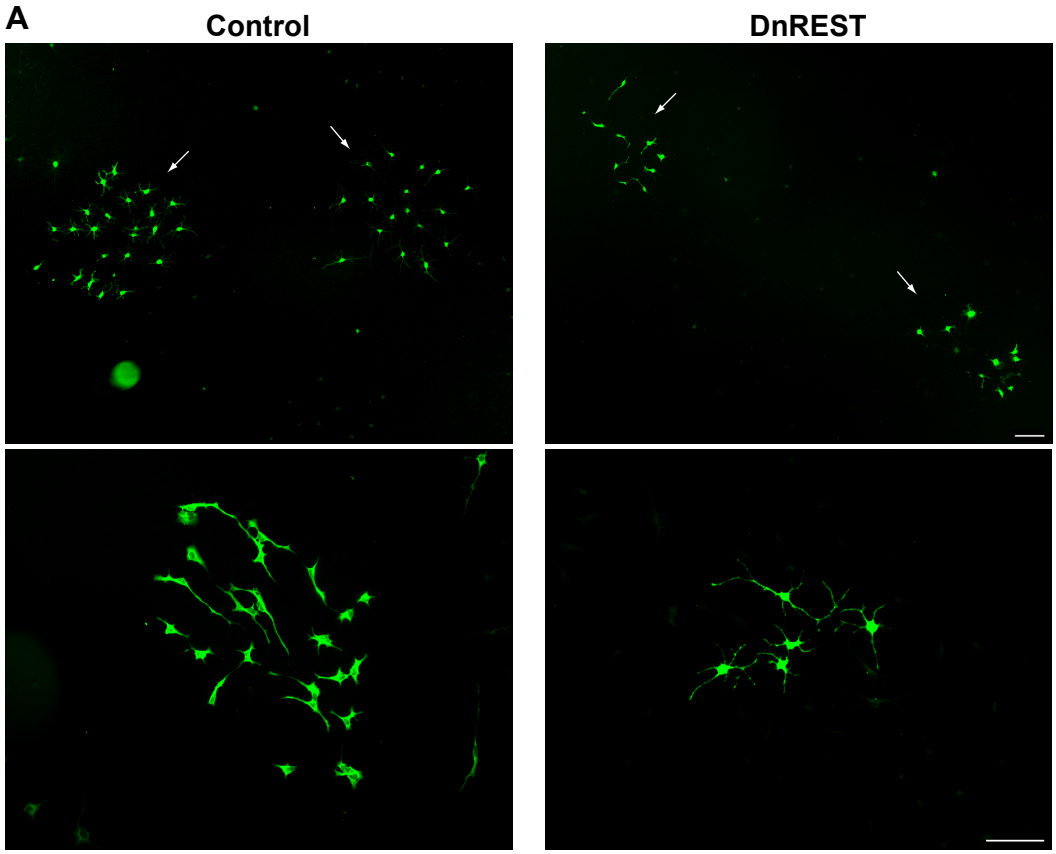


Figure IV-19. Appearance of clones in stem cell differentiation media. Cells were grown as described in figure IV-17c. **(A)** (Top) Immunofluorescence staining showing low power images of infected clones from control (left) and experimental (right) cultures at day 5. There are two separate clones in each condition as indicated by the arrows. Higher magnification images show single infected clones at day 5 (bottom, **A)** and day 7 **(B)**. Scale bars indicate 50μm.

FIGURE IV-19

5 DAY CLONES
(2d OPC media + 3d SCM media)



7 DAY CLONES
(2d OPC media + 5d SCM media)

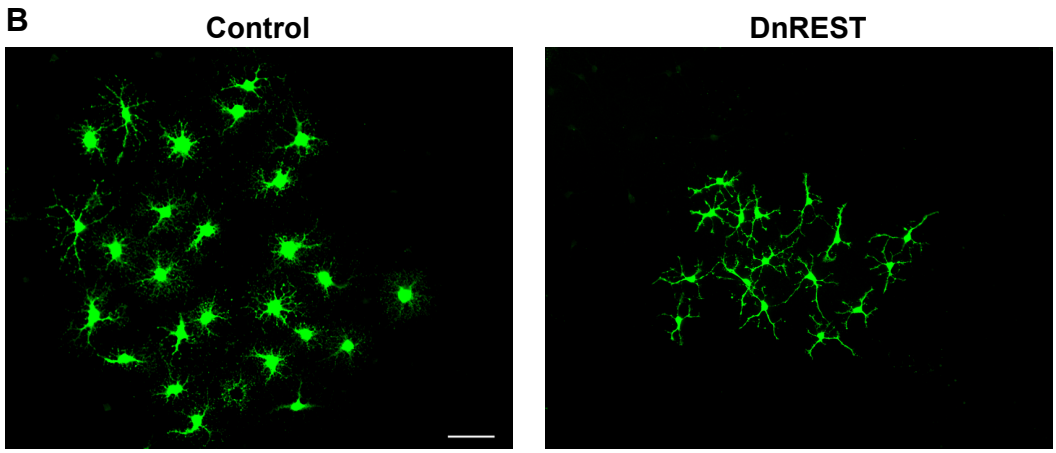


Figure IV-20. Appearance of clones in type II astrocyte differentiation media. Cells were grown as described in figure IV-17d. Immunofluorescence images show infected clones from control (left) and experimental (right) cultures at day 7. Scale bars indicate 50μm.

FIGURE IV-20

7 DAY CLONES
(2d OPC media + 5d 2A media)

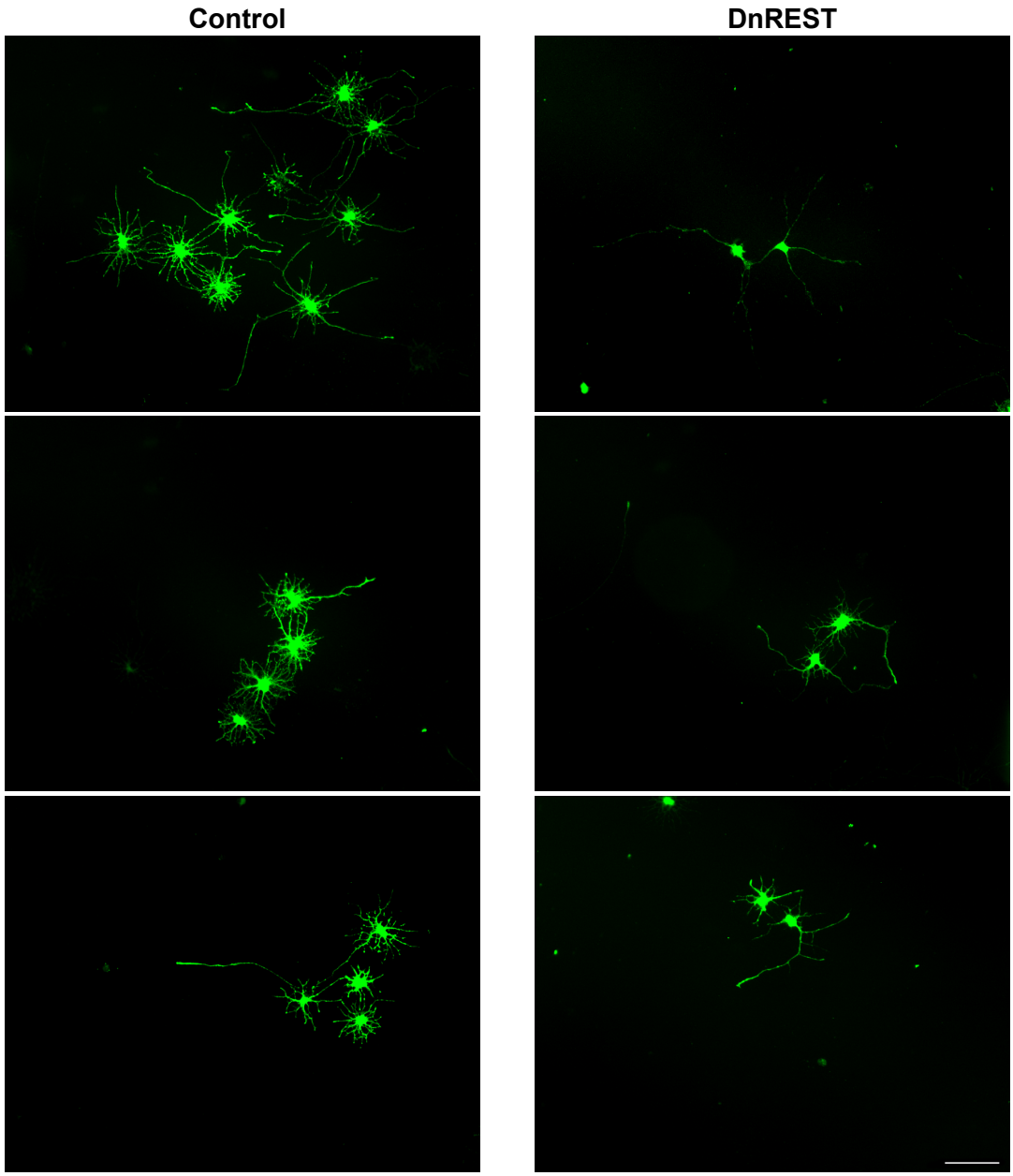


Figure IV-21 Loss of REST repression results in a smaller average clone size. The bar graphs compare the mean clonal size of control and DnREST clones at different time points grown under different conditions. Cells were grown as described in figure IV-17, time points and media conditions are indicated. **(A)** Mean clone size after 5 days at clonal density. **(B)** Mean clone size after 7 days at clonal density. In all cases, the mean size of DnREST expressing clones is smaller than that of control clones. All error bars represent the standard deviation from at least 4 separate experiments. *P-value ≤0.01, **P-value ≤0.005, Student's t-test.

FIGURE IV-21

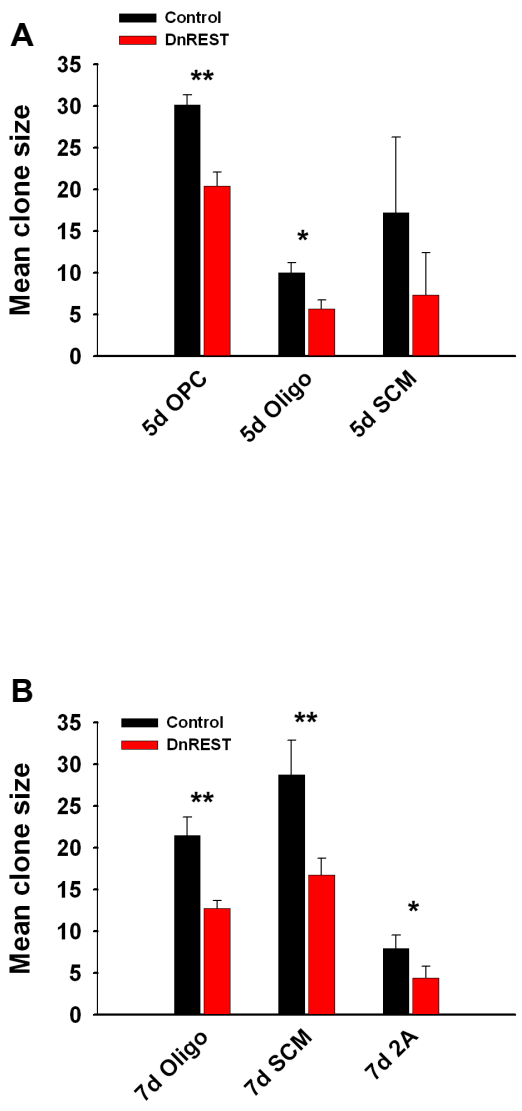

A
Control
DnREST
Mean clone size
35
30
25
20
15
10
5
0
**
*
5d OPC
5d Oligo
5d SCM
B
Control
DnREST
Mean clone size
35
30
25
20
15
10
5
0
**
**
*
7d Oligo
7d SCM
7d 2A

Figure IV-22. Variations in clone size with REST LOF in proliferation media and oligodendrocyte differentiation media. Cells were grown in the indicated media as described in figure IV-17 and the number of cells per clone counted. Both control and experimental clones continue to expand in OPC media **(A)** and in oligodendrocyte media **(B)**. A higher percent of DnREST clones in both OPC and oligodendrocyte media fall into the smaller sized ranges than do control clones. These data represent total clones and averages are not shown; total clone number **(A)** 4d control n = 58, 5d control n = 74, 5d DnREST n = 69, 7d DnREST n = 75, **(B)** 5d control n = 167, 7d control n = 103, 5d DnREST n = 160, 7d DnREST n=99.

FIGURE IV-22

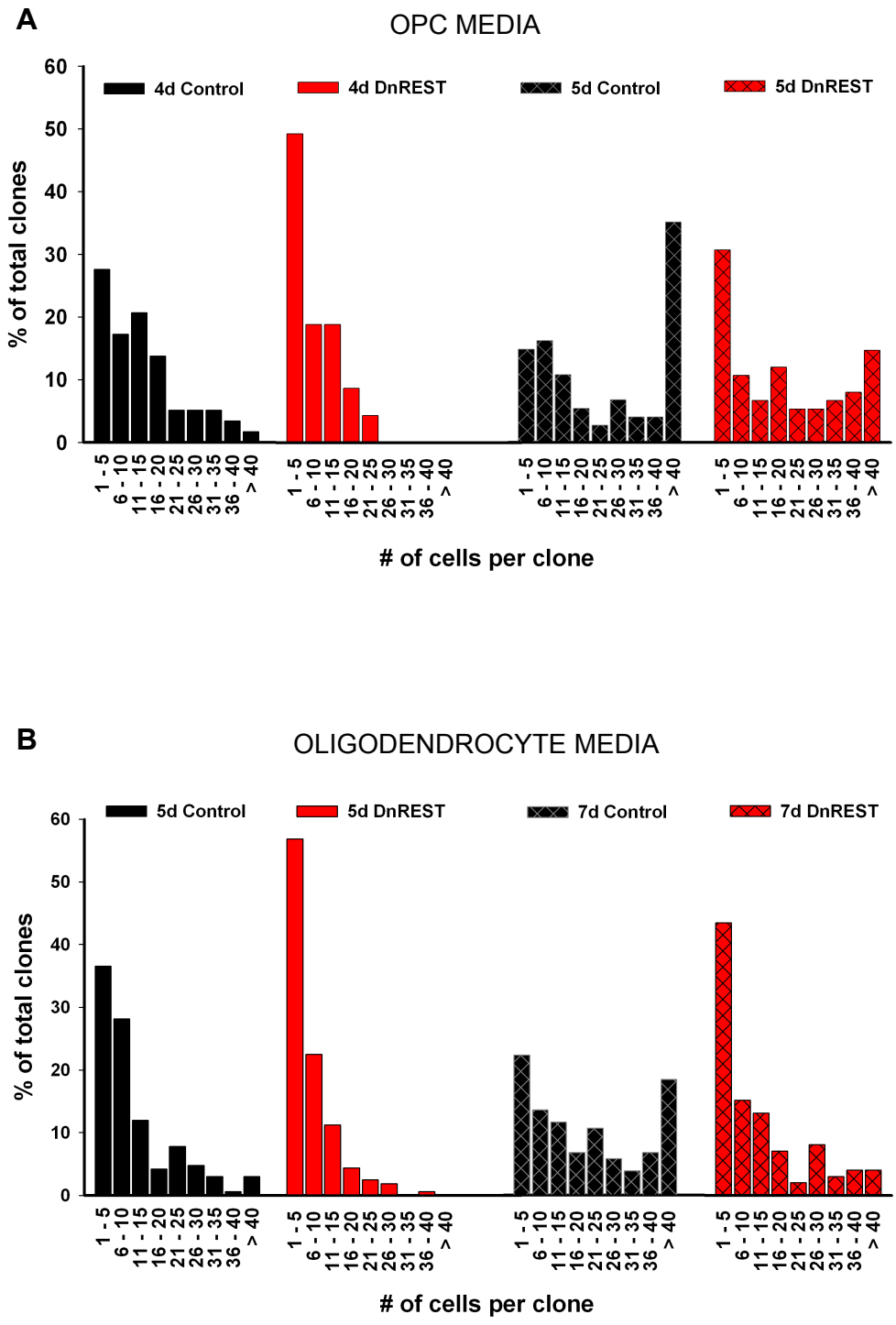
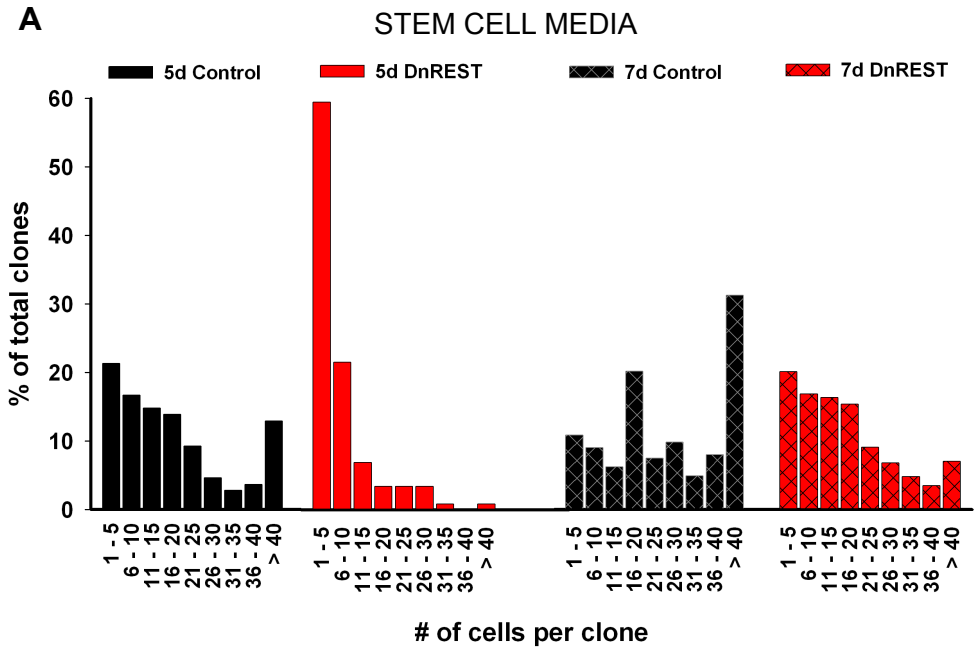

A
OPC MEDIA
4d Control
4d DnREST
5d Control
5d DnREST
% of total clones
# of cells per clone
B
OLIGODENDROCYTE MEDIA
5d Control
5d DnREST
7d Control
7d DnREST
% of total clones
# of cells per clone

Figure IV-23. Variations in clone size with REST LOF in stem cell differentiation media and type II astrocyte differentiation media. Cells were grown as described in figure IV-17. The number of cells per clone was counted and sorted. **(A)** Both control and experimental clones continue to grow between days 5 and 7 in stem cell media. However, a higher percent of DnREST clones fall into the smaller sized ranges than do control clones. This is also true for clones grown in type II astrocytes media **(B)**. These data represent total clones and averages are not shown; total clone number **(A)** 5d control n = 108, 7d control n = 387, 5d DnREST n = 116, 7d DnREST n = 397, **(B)** 7d control n = 475, 7d DnREST n=473.

FIGURE IV-23


A
STEM CELL MEDIA
5d Control
5d DnREST
7d Control
7d DnREST
% of total clones
0
10
20
30
40
50
60
1 - 5
6 - 10
11 - 15
16 - 20
21 - 25
26 - 30
31 - 35
36 - 40
> 40
# of cells per clone

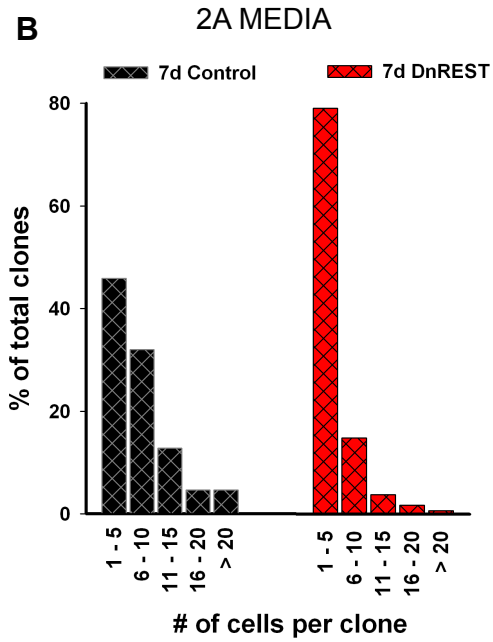

B
2A MEDIA
7d Control
7d DnREST
% of total clones
0
20
40
60
80
1 - 5
6 - 10
11 - 15
16 - 20
> 20
# of cells per clone

Figure IV-24. BrdU labeling of differentiating OPCs. Purified OPCs were infected with control GFP or DnREST expressing retroviruses, and grown in proliferation media for 4 days followed by a media switch to oligodendrocyte media **(A)**, stem cell media **(B)**, or 2A media **(C)**. After 44 hours cells were labeled with BrdU and immunostained as described under Experimental Procedures. Infected cells (green) that are labeled with BrdU (red) are indicated by arrows. In all media, fewer cells are labeled with BrdU with REST LOF compared to control. Scale bars indicate 50μm.

FIGURE IV-24

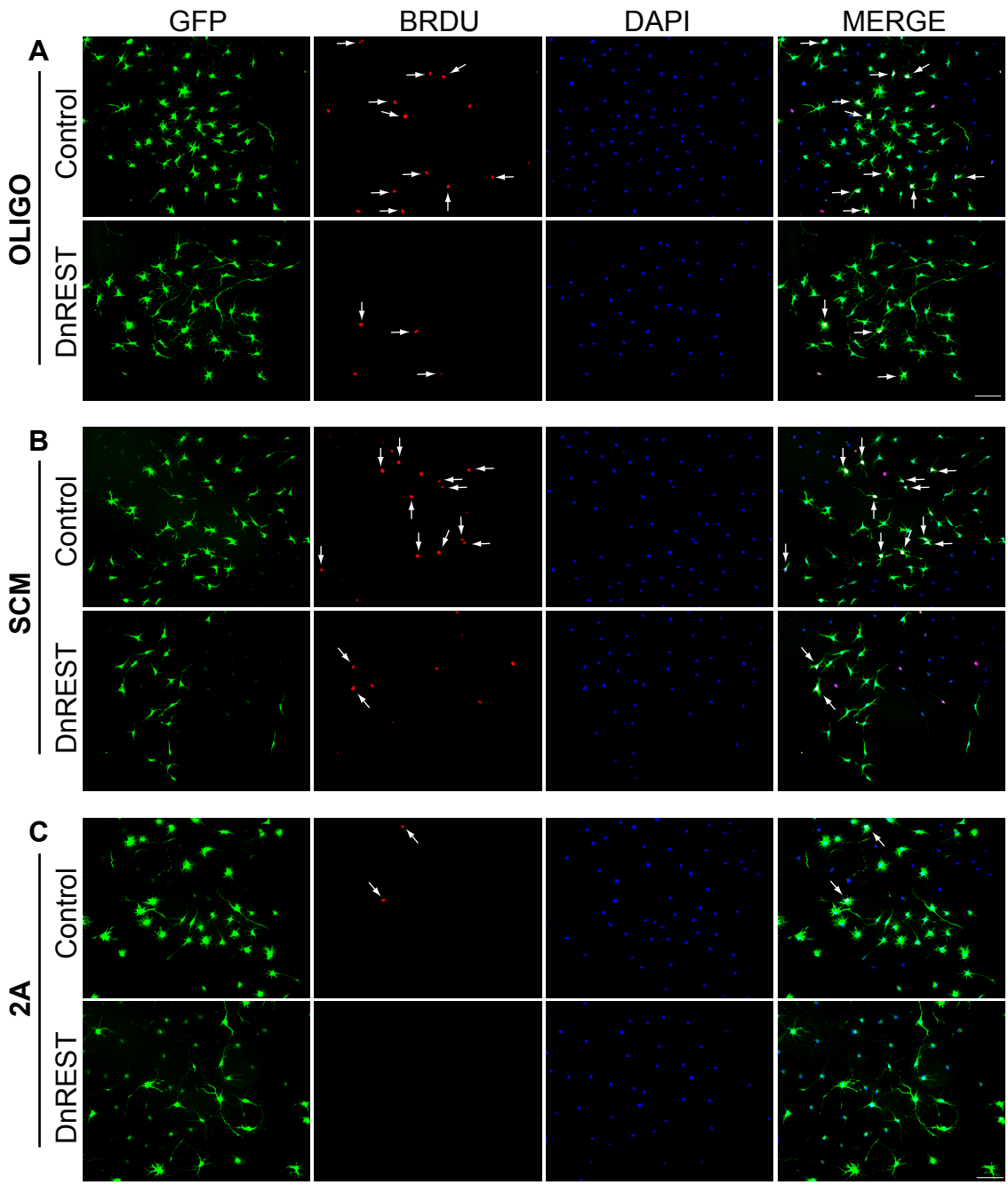

GFP
BRDU
DAPI
MERGE
A
OLIGO
Control
DnREST
B
SCM
Control
DnREST
C
2A
Control
DnREST

Figure IV-25. REST LOF decreases proliferation rate. Purified OPCs were infected with control GFP or DnREST expressing retroviruses and grown in proliferation media for 4 days followed by a media switch to the indicated media. Cells were labeled with BrdU after 44 hours and immunostained as described under Experimental Procedures. **(A)** Quantification of immunofluorescence staining showing the percent of BrdU incorporation in total control infected cells. Proliferation rate decreased when cells were switched from OPC media and growth factors were removed. Error bars represent the standard deviation from 3 separate experiments. *P-value ≤0.05, **P-value ≤0.005, Student's t-test. **(B)** REST LOF decreases proliferation rate in all media. BrdU incorporation is shown as a percent of control. Error bars represent the standard deviation from 3 independent experiments. *P-value ≤0.005, Student's t-test.

FIGURE IV-25

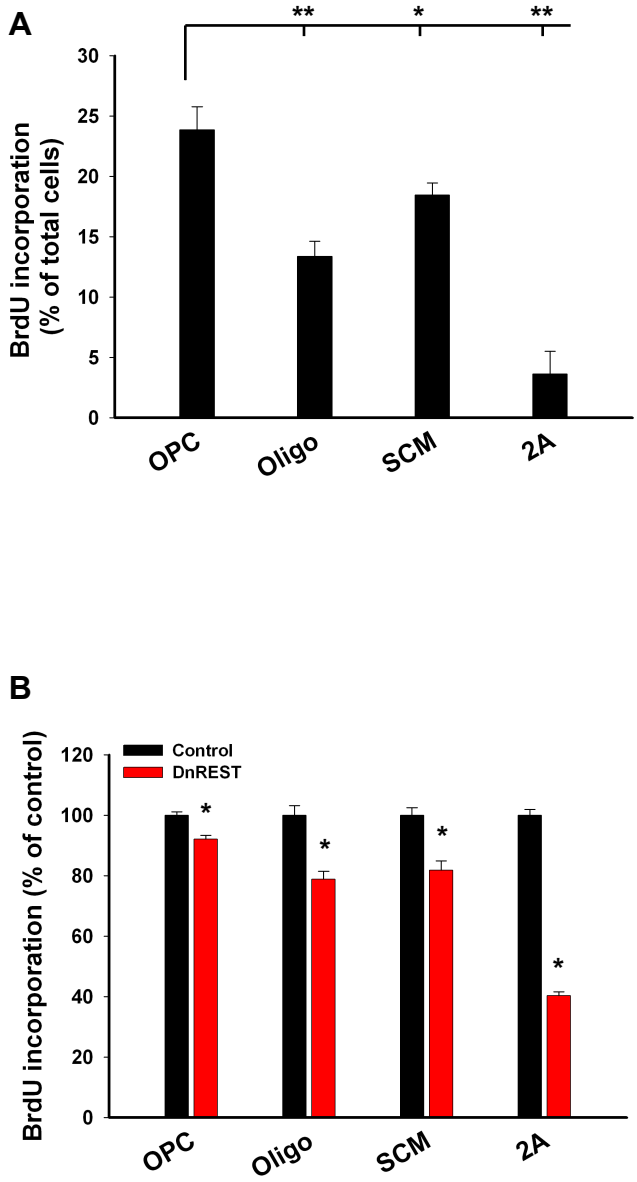

A
**
*
**
BrdU incorporation (% of total cells)
30
25
20
15
10
5
0
OPC
Oligo
SCM
2A
B
Control
DnREST
BrdU incorporation (% of control)
120
100
80
60
40
20
0
*
*
*
*
OPC
Oligo
SCM
2A

Figure IV-26. Clonal composition: Percent of clones containing antigen positive cells. Clonal analysis was performed as described in figure IV-17. The percent of clones containing at least one cell positive for the indicated marker antigen is shown for each media. Error bars represent the standard deviation from at least 3-4 separate experiments.

FIGURE IV-26

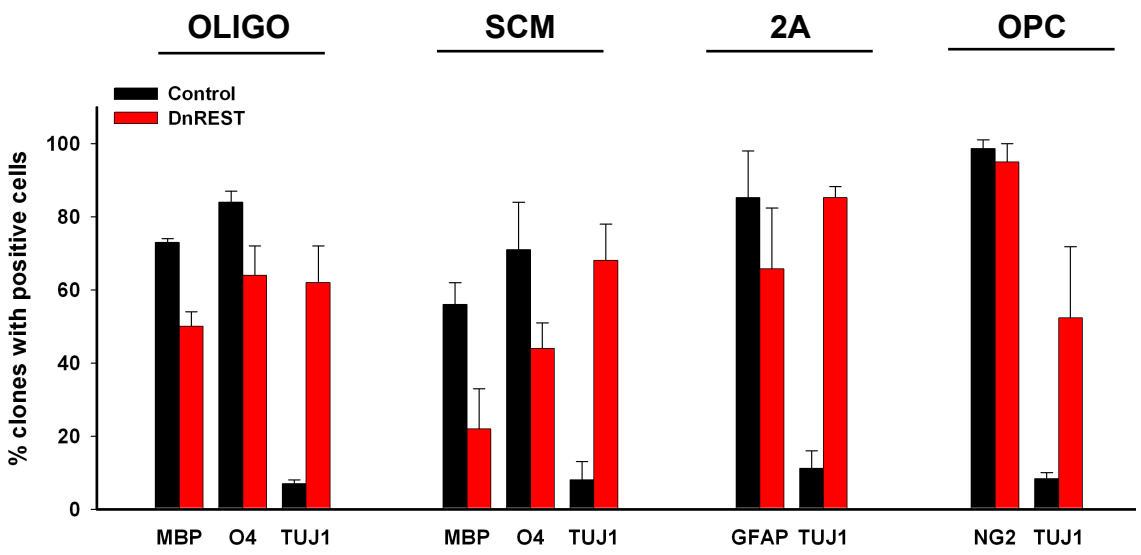

OLIGO
SCM
2A
OPC
Control
DnREST
% clones with positive cells
100
80
60
40
20
0
MBP
O4
TUJ1
MBP
O4
TUJ1
GFAP
TUJ1
NG2
TUJ1

Figure IV-27. OPC media: NG2 expression in individual clones. Clonal analysis was performed as described in figure IV-17a for NG2 expression. The size and the percent of NG2-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More DnREST clones contain NG2-negative cells than control clones. REST LOF appears to primarily affect smaller clones. Total number of clones: control n=58, DnREST n=69

FIGURE IV-27

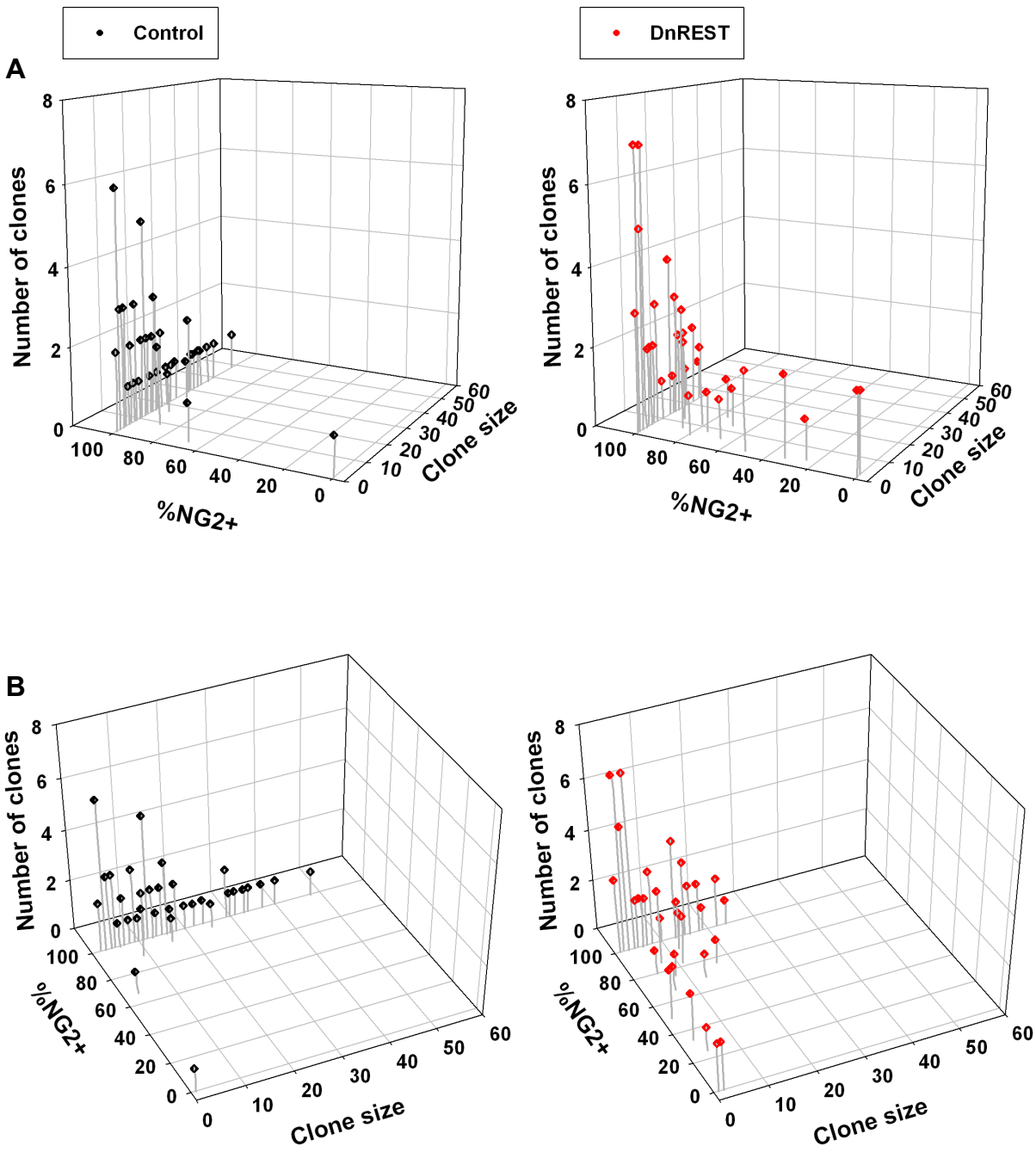

A
Control
DnREST
Number of clones
%NG2+
Clone size
B
Number of clones
%NG2+
Clone size

Figure IV-28. OPC media: TUJ1 expression in individual clones. Clonal analysis was performed as described in figure IV-17a for TUJ1 expression. The size and the percent of TUJ1-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More DnREST clones contain TUJ1-positive cells than control clones. REST LOF appears to have the largest effect on TUJ1 expression in small clones. Total number of clones: control n=74, DnREST n=75.

FIGURE IV-28

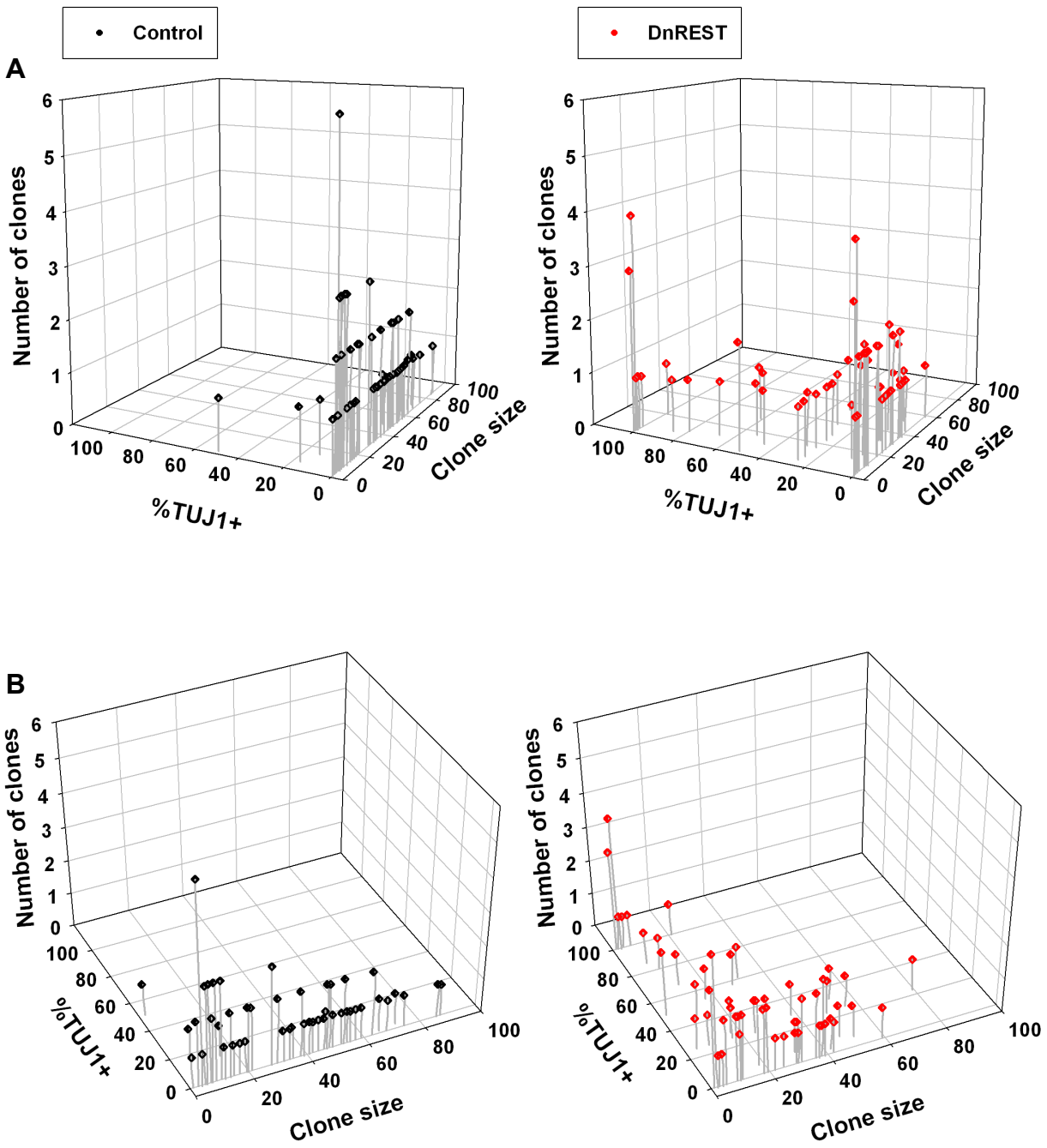

A
Control
DnREST
Number of clones
%TUJ1+
Clone size
B
Number of clones
%TUJ1+
Clone size

Figure IV-29. Clonal composition of OPCs grown in proliferation media. Clonal analysis was performed as described in IV-17a. The histogram shows a summary of the percent of total clones containing the indicated percentage of NG2 or TUJ1-positive cells. The number of clones expressing NG2 decreases slightly while the number of clones expressing TUJ1 increases with REST LOF. Total number of clones: NG2 control n=58, DnREST n=69, TUJ1 control n=74, DnREST n=75.

FIGURE IV-29

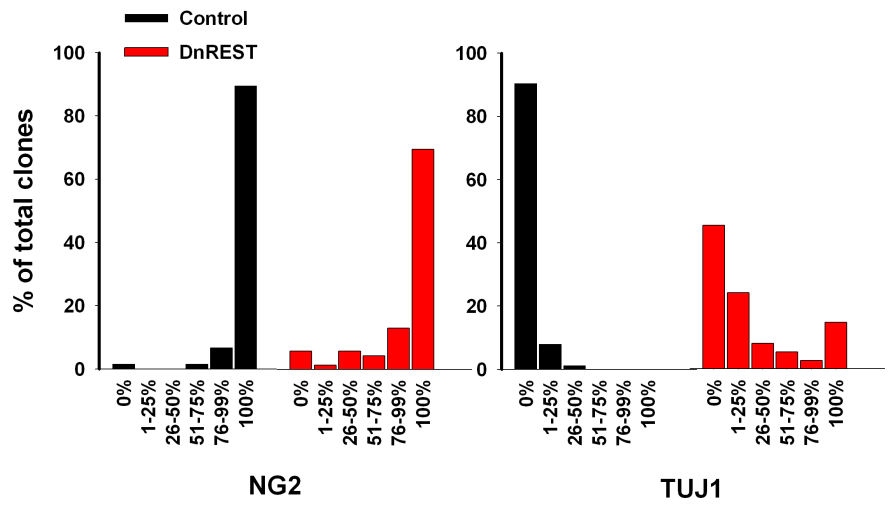

Control
DnREST
% of total clones
100
80
60
40
20
0
0%
1-25%
26-50%
51-75%
76-99%
100%
NG2
TUJ1

Figure IV-30. OPC clones in oligodendrocyte differentiation media: The number of O4 expressing clones decrease with REST LOF. Clonal analysis was performed as described in figure IV-17b. After 3 days in oligodendrocyte media clones were immunostained with an antibody against O4 (red) and infected clones (greens) were identified and analyzed. The images show a single control clone expressing O4 (left) and two DnREST clones (right). Although O4 expression decreased with REST LOF, some infected cells were O4-positive (far right). Scale bars indicate 50μM.

FIGURE IV-30

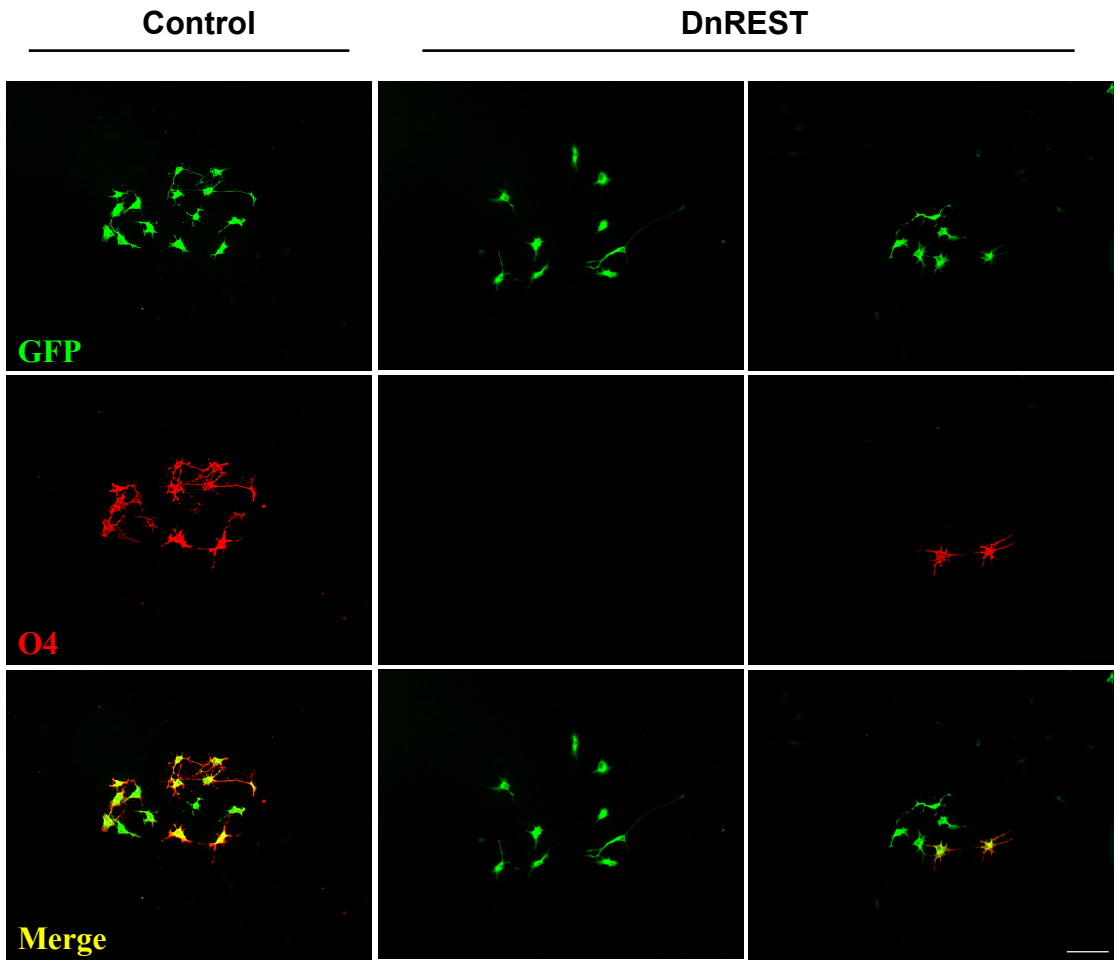


Figure IV-31. Oligodendrocyte media: O4 expression in individual clones. Clonal analysis was performed as described in figure IV-17b for O4 expression. The size and the percent of O4-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More DnREST clones contain fewer or no O4-positive cells than control clones. Total number of clones: control n=167, DnREST n=160.

FIGURE IV-31

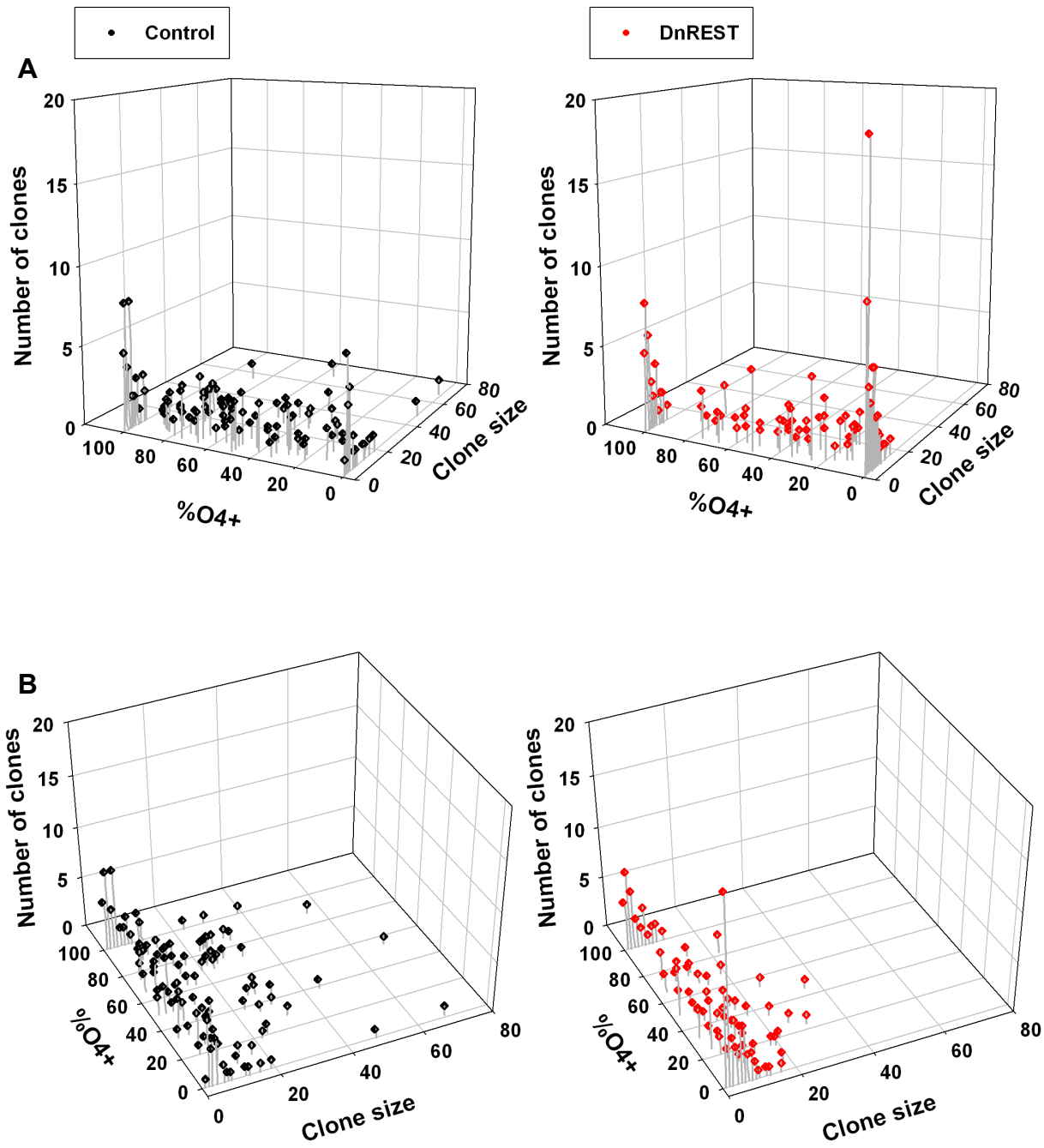

A
Control
DnREST
Number of clones
%O4+
Clone size
B
Number of clones
%O4+
Clone size

Figure IV-32. OPC clones in oligodendrocyte differentiation media: The number of MBP expressing clones decreases with REST LOF. Clonal analysis was performed as described in figure IV-17b. After 5 days in oligodendrocyte media clones were immunostained with an antibody against MBP (red) and infected clones (greens) were identified and analyzed. The images show a two-cell control clone containing an MBP-positive cell (left) and an MBP-negative DnREST clone (right, arrow). The scale bar indicates 50μm.

FIGURE IV-32

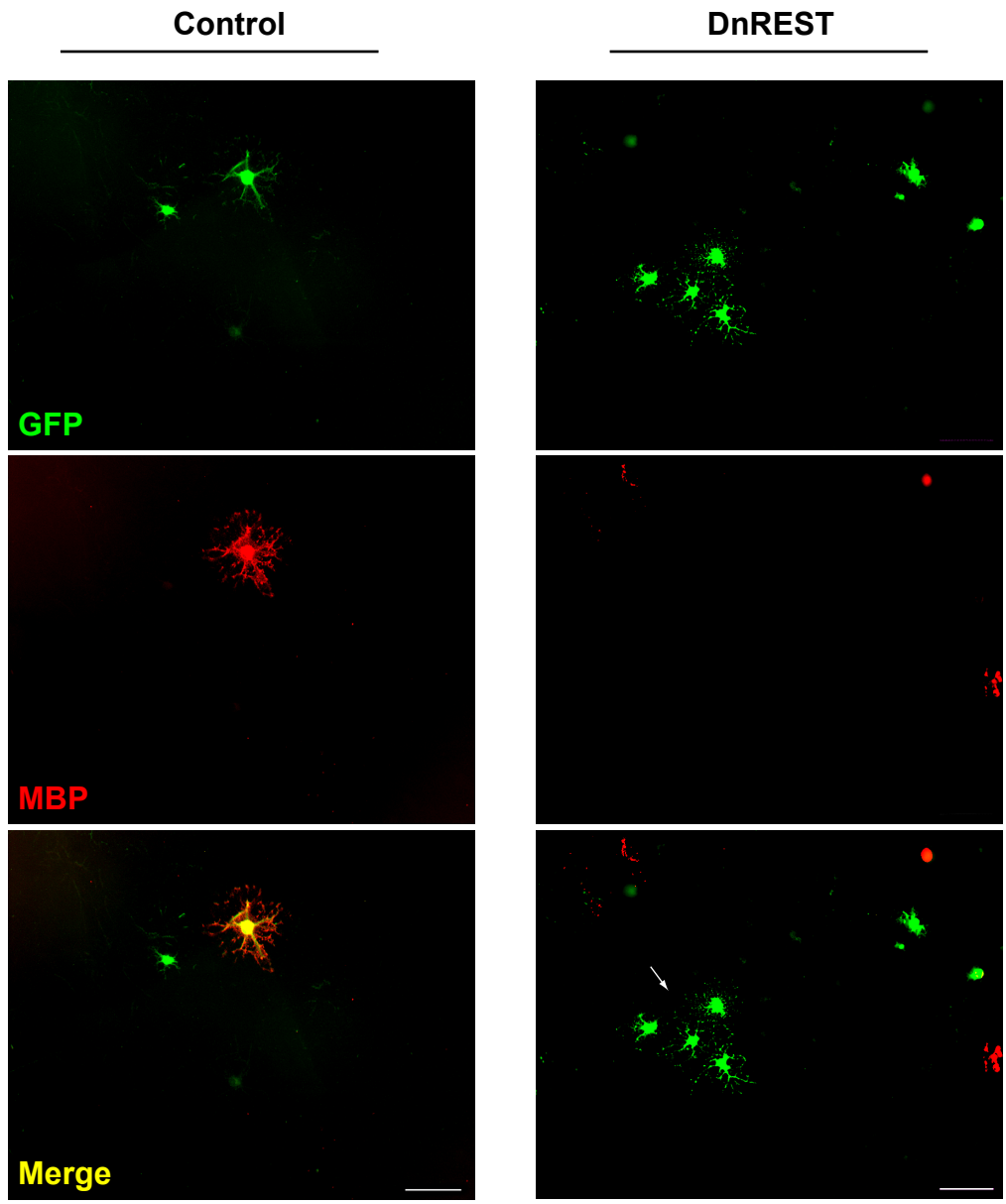


Figure IV-33. Oligodendrocyte media: MBP expression in individual clones. Clonal analysis was performed as described in figure IV-17b for MBP expression. The size and the percent of MBP-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More control clones contain a higher percentage of MBP-positive cells than DnREST clones. Total number of clones: control n=45, DnREST n=45.

FIGURE IV-33

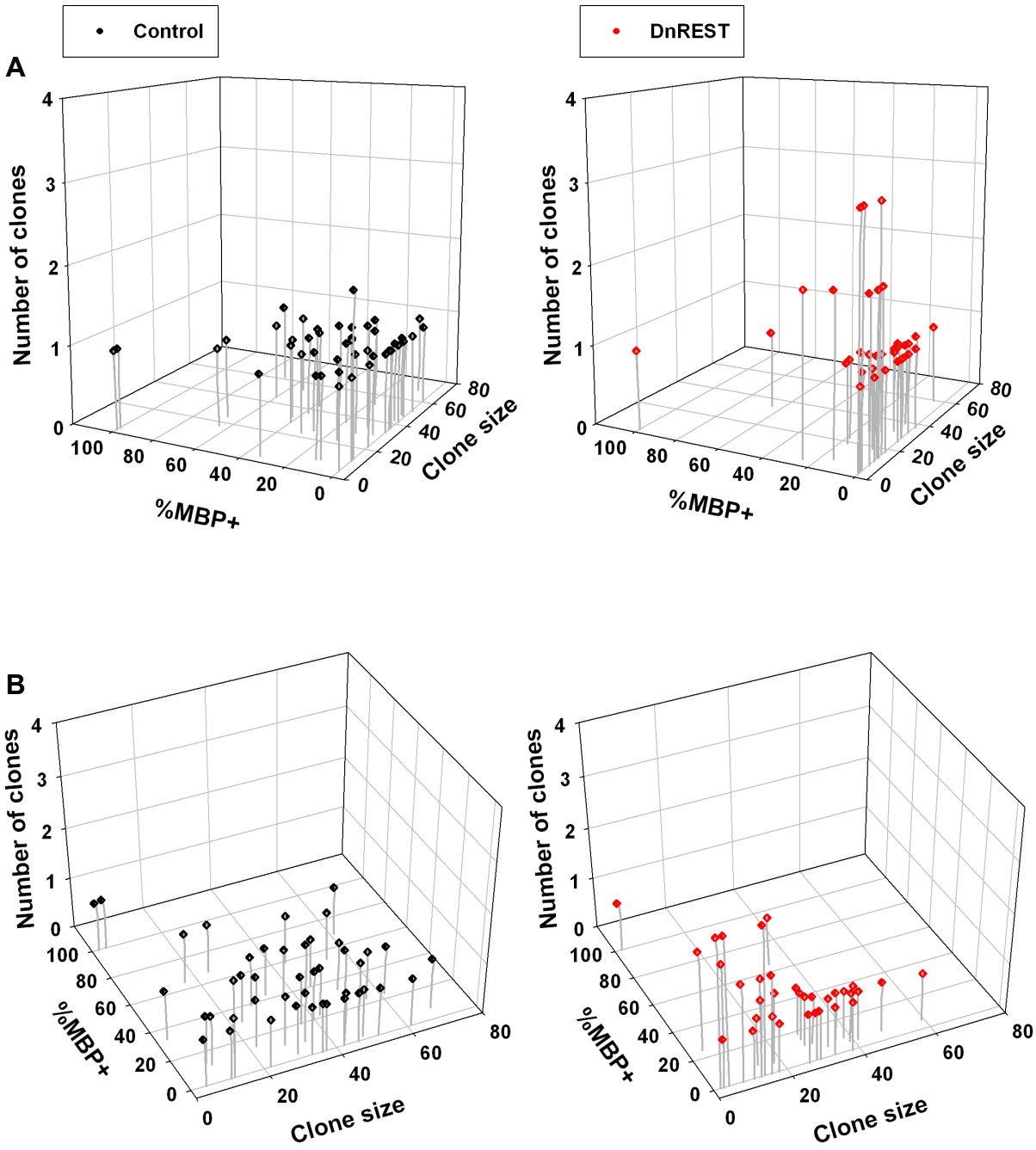

A
Control
DnREST
Number of clones
%MBP+
Clone size
B
Number of clones
%MBP+
Clone size

Figure IV-34. OPC clones in oligodendrocyte differentiation media: The number of TUJ1 expressing clones increases with REST LOF. Clonal analysis was performed as described in figure IV-17b. After 5 days in oligodendrocyte media clones were immunostained with an antibody against TUJ1 (red) and infected clones (greens) were identified and analyzed. The images show a single TUJ1-negative control clone (left) and a TUJ1 expressing DnREST clone (right).

FIGURE IV-34

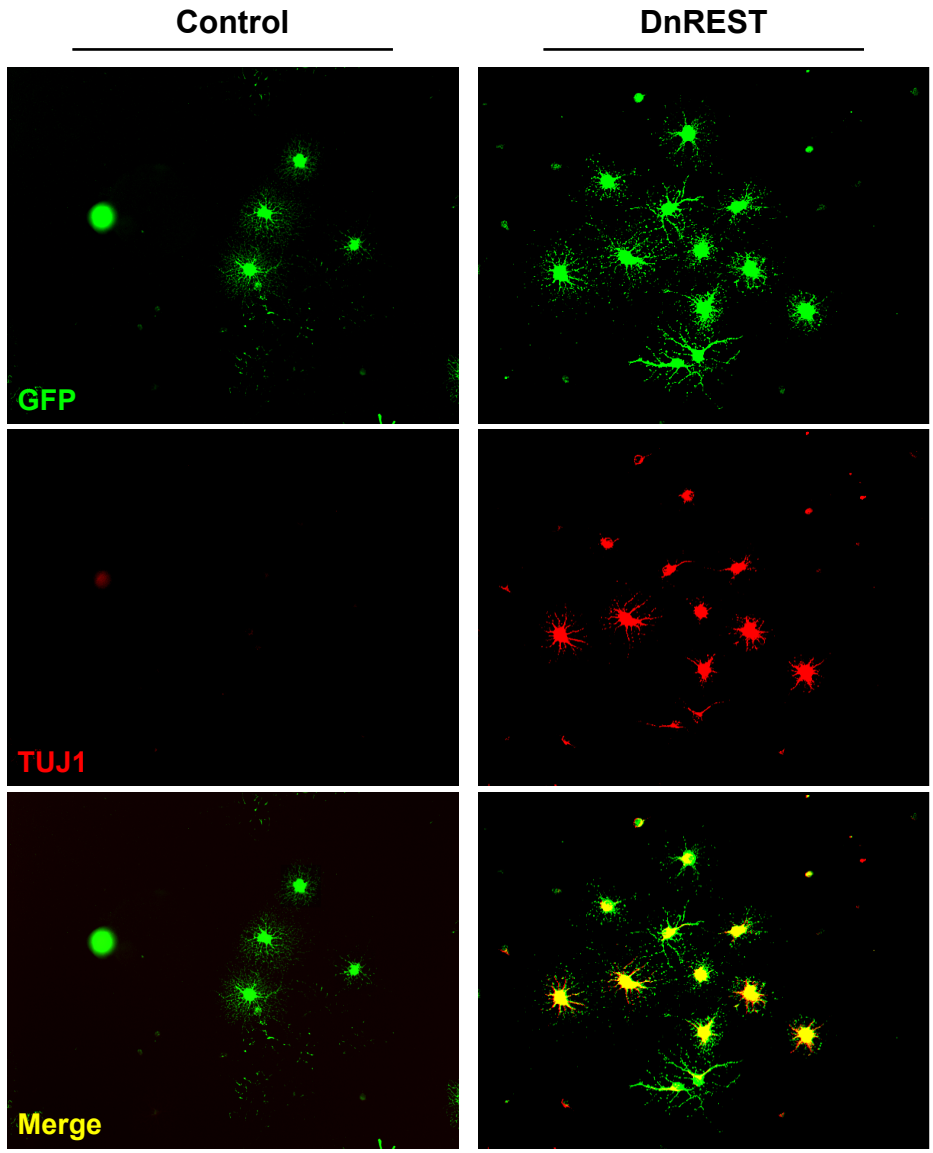


Figure IV-35. Oligodendrocyte media: TUJ1 expression in individual clones. Clonal analysis was performed as described in figure IV-17b for TUJ1 expression. The size and the percent of TUJ1-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. Unlike DnREST clones, most control clones do not contain TUJ1-positive cells. Total number of clones: control n=58, DnREST n=54.

FIGURE IV-35

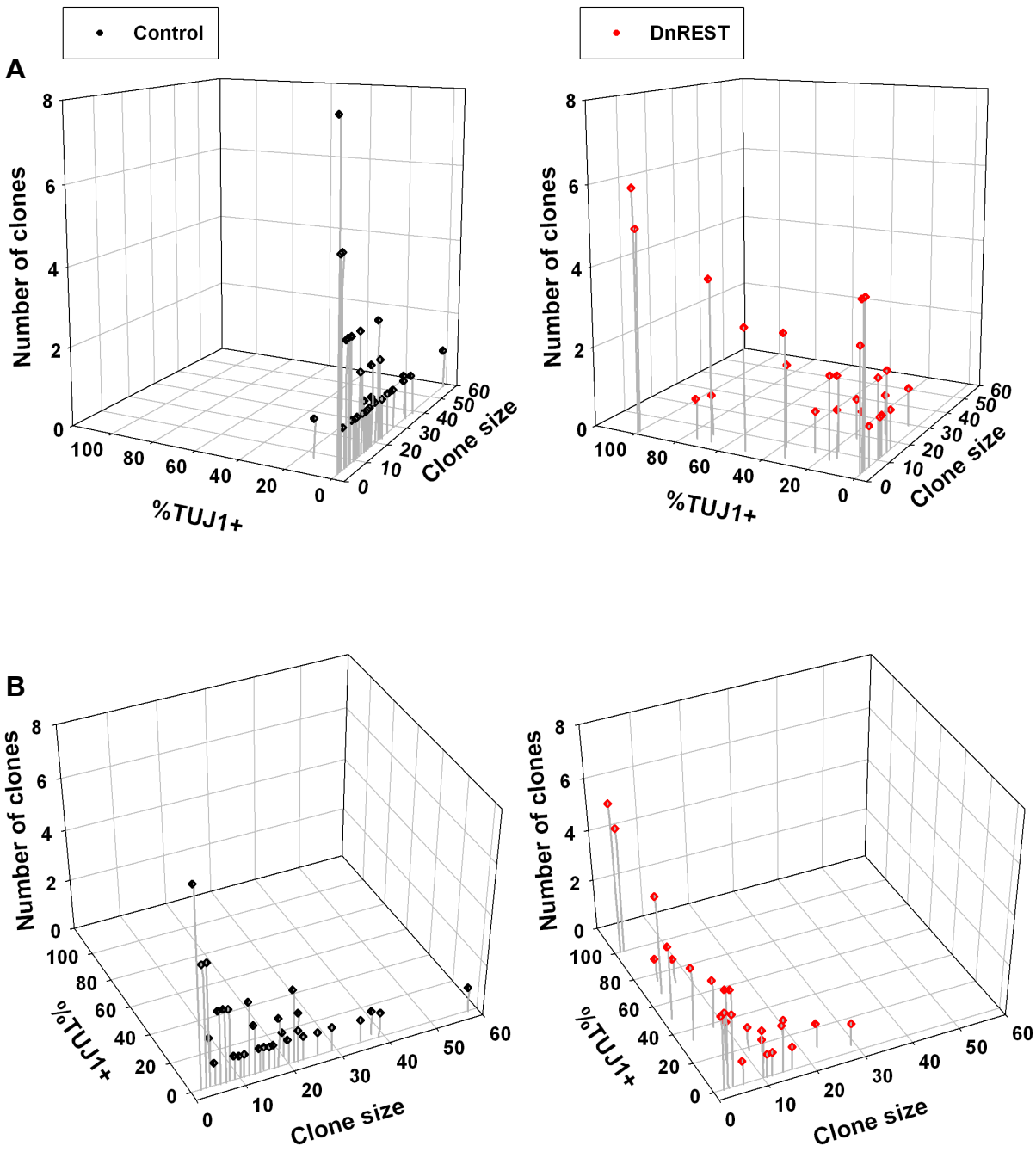

A
Control
DnREST
Number of clones
%TUJ1+
Clone size
B
Number of clones
%TUJ1+
Clone size

Figure IV-36. Clonal composition of OPCs grown in oligodendrocyte differentiation media. Clonal analysis was performed as described in IV-17b. The histogram shows a summary of the percent of total clones containing the indicated percentage of MBP, O4, or TUJ1-positive cells. The number of clones expressing MBP and O4 decrease while the number of TUJ1 expressing clones increases with REST LOF. Total number of clones: O4 control n=167, DnREST n=160, MBP control n=45, DnREST n=45, TUJ1 control n=58, DnREST n=54.

FIGURE IV-36

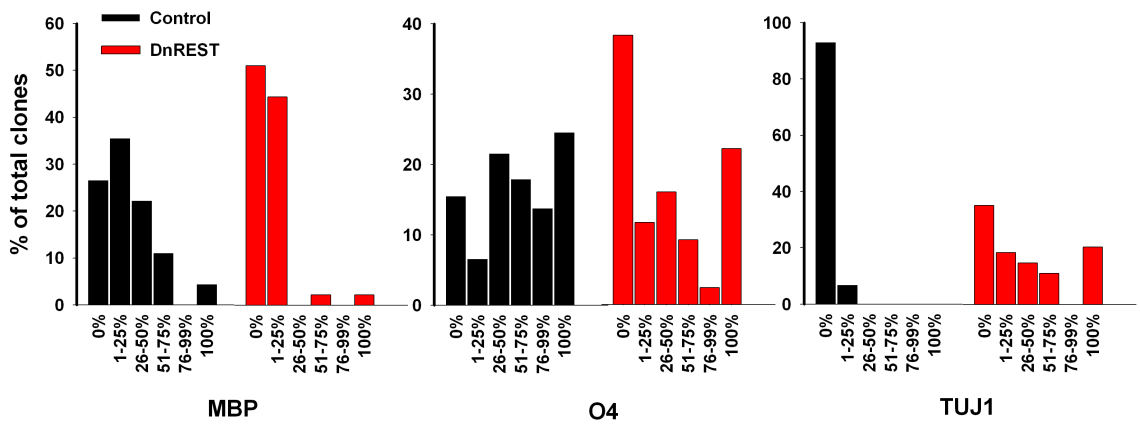

Control
DnREST
% of total clones
60
50
40
30
20
10
0
0%
1-25%
26-50%
51-75%
76-99%
100%
MBP
40
30
20
10
0
O4
100
80
60
40
20
0
TUJ1

Figure IV-37. OPC clones in stem cell media: O4 expression decreases with REST LOF. Clonal analysis was performed as described in figure IV-17c. After 3 days in oligodendrocyte media clones were immunostained with an antibody against O4 (red) and infected clones (greens) were identified and analyzed. **(A)** The images show a single control clone expressing O4 (left) and two DnREST clones (right). REST LOF decreased the number of O4 expressing clones but did not affect the uninfected clones in the same cultures (arrows, **A** and **C**). **(B)** High power image of an O4-negative DnREST clone with unusually long processes (arrowhead). Scale bars indicate 50μm.

FIGURE IV-37

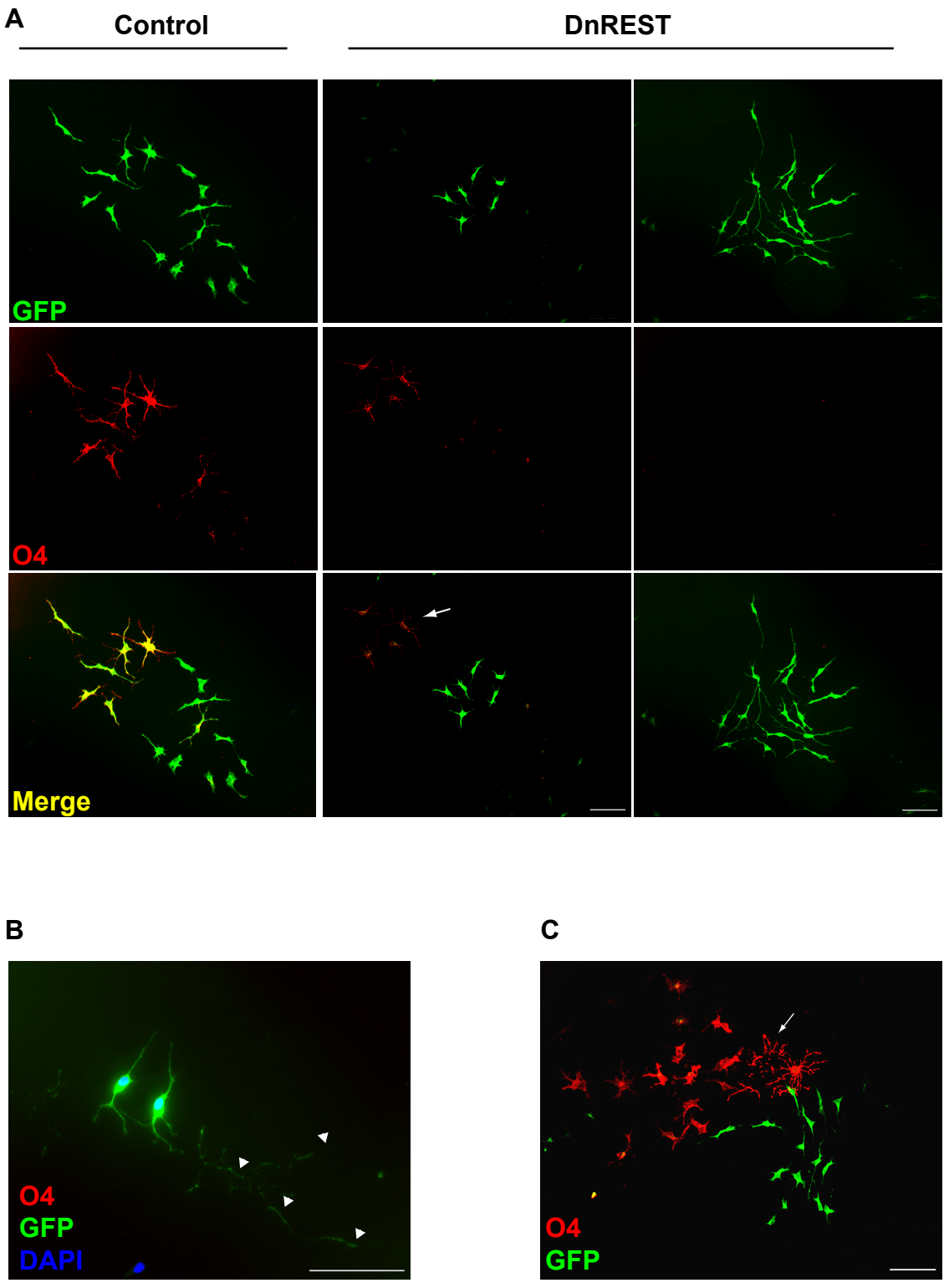


Figure IV-38. Stem cell media: O4 expression in individual clones. Clonal analysis was performed as described in figure IV-17c for O4 expression. The size and the percent of O4-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More DnREST clones contain fewer or no O4-positive cells than control clones. Total number of clones: control n=108, DnREST n=116.

FIGURE IV-38

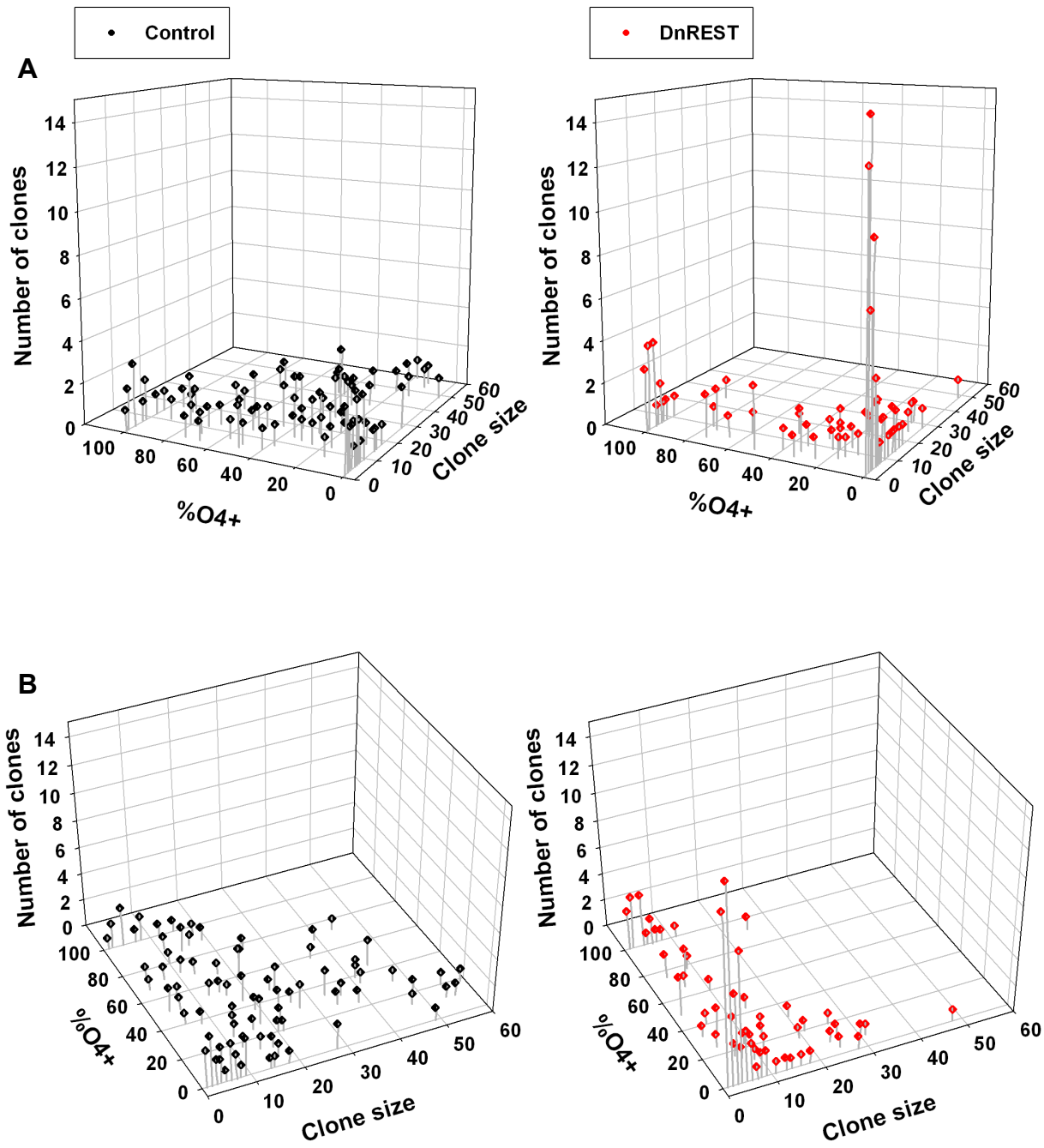

A
Control
DnREST
Number of clones
%O4+
Clone size
B
Number of clones
%O4+
Clone size

Figure IV-39. OPC clones in stem cell media: The number of MBP expressing clones decrease with REST LOF. Clonal analysis was performed as described in figure IV-17c. After 5 days in stem cell media clones were immunostained with an antibody against MBP (red) and infected clones (greens) were identified and analyzed. The images show a single control clone containing MBP-positive cells (left) and an MBP-negative DnREST clone (right). Scale bars indicate 50μm.

FIGURE IV-39

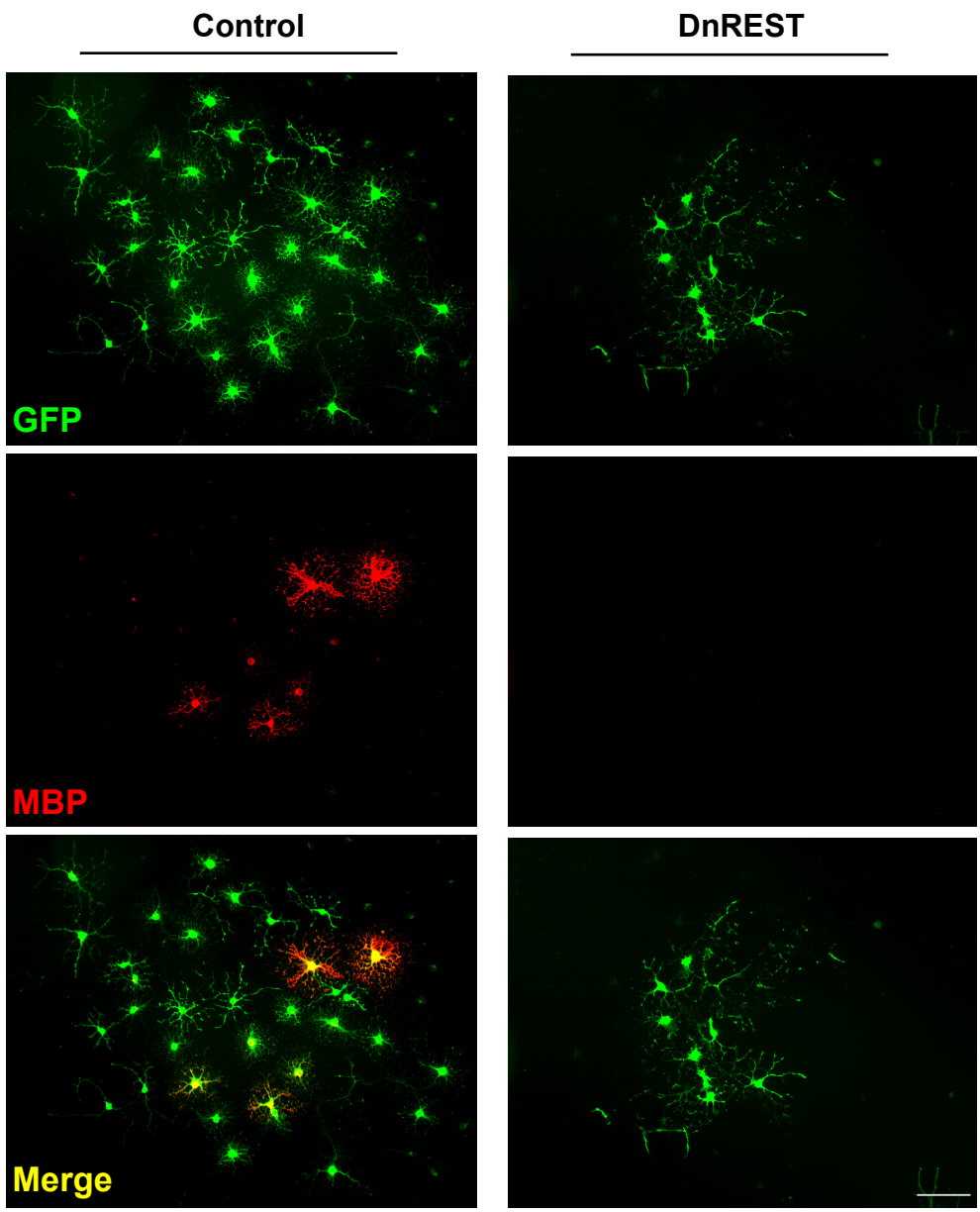


Figure IV-40. Stem cell media: MBP expression in individual clones. Clonal analysis was performed as described in figure IV-17c for MBP expression. The size and the percent of MBP-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More DnREST clones contain some or all MBP-negative cells than control clones. Total number of clones: control n=251, DnREST n=251.

FIGURE IV-40

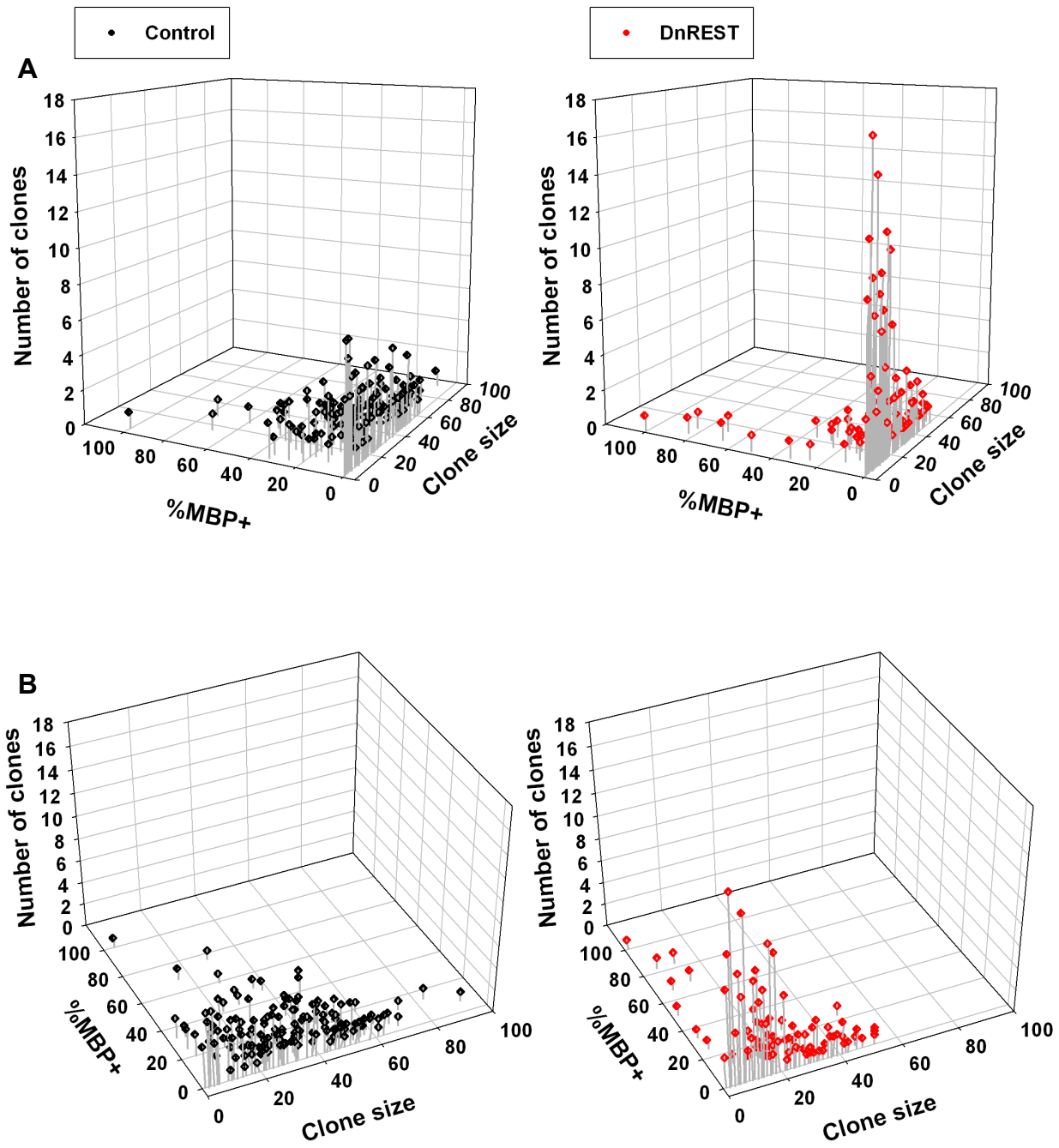

A
Control
DnREST
Number of clones
%MBP+
Clone size
B
Number of clones
%MBP+
Clone size

Figure IV-41. OPC clones in stem cell media: The number of TUJ1 expressing clones increases with REST LOF. Clonal analysis was performed as described in figure IV-17c. After 5 days in oligodendrocyte media clones were immunostained with an antibody against TUJ1 (red) and infected clones (greens) were identified and analyzed. The images show a single TUJ1-negative control clone (left) and a TUJ1 expressing DnREST clone (right). Scale bars indicate 50μm.

FIGURE IV-41

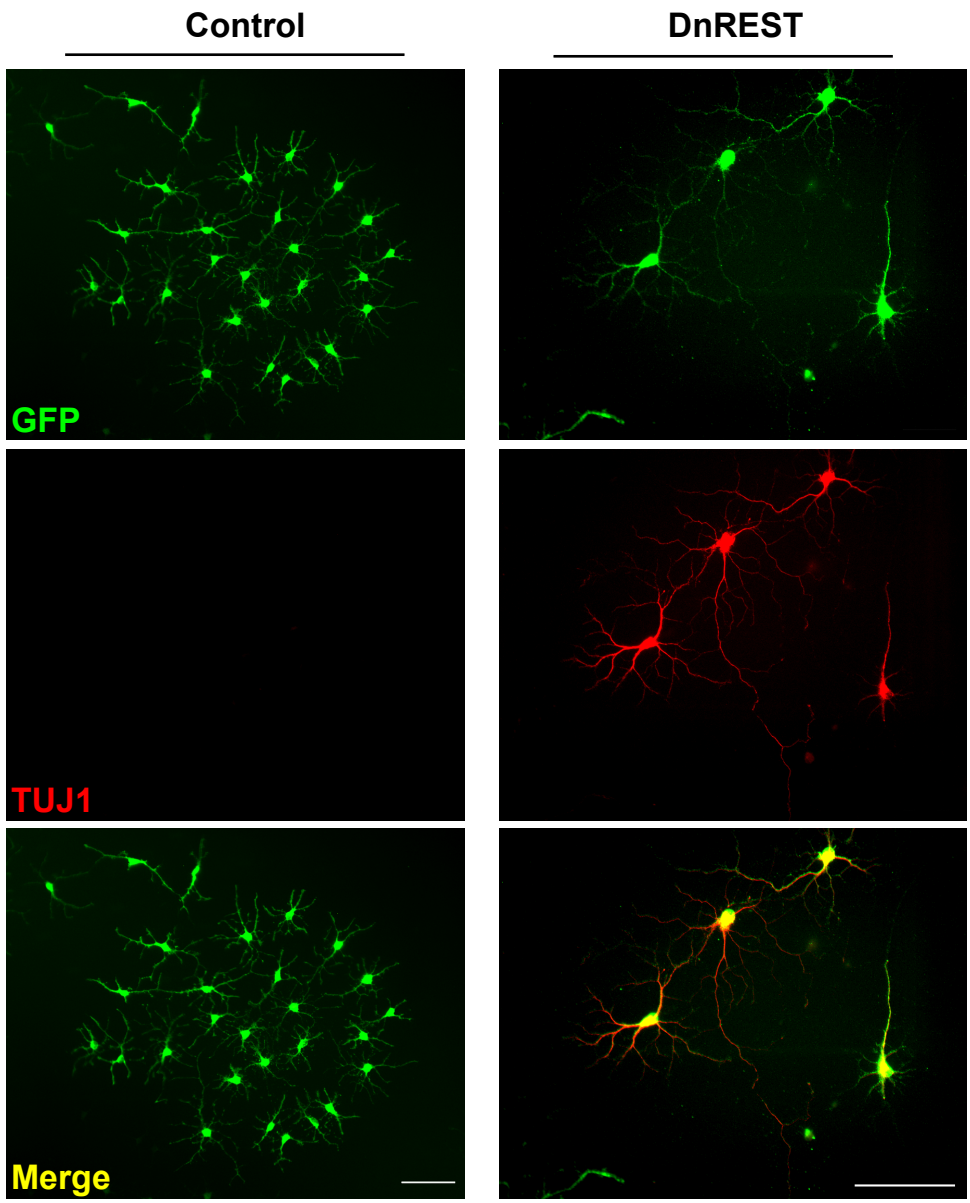


Figure IV-42. Stem cell media: TUJ1 expression in individual clones. Clonal analysis was performed as described in figure IV-17c for TUJ1 expression. The size and the percent of TUJ1-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. Many DnREST clones contain at least one TUJ1-positive cell whereas most control clones do not express any TUJ1. Total number of clones: control n=136, DnREST n=146.

FIGURE IV-42

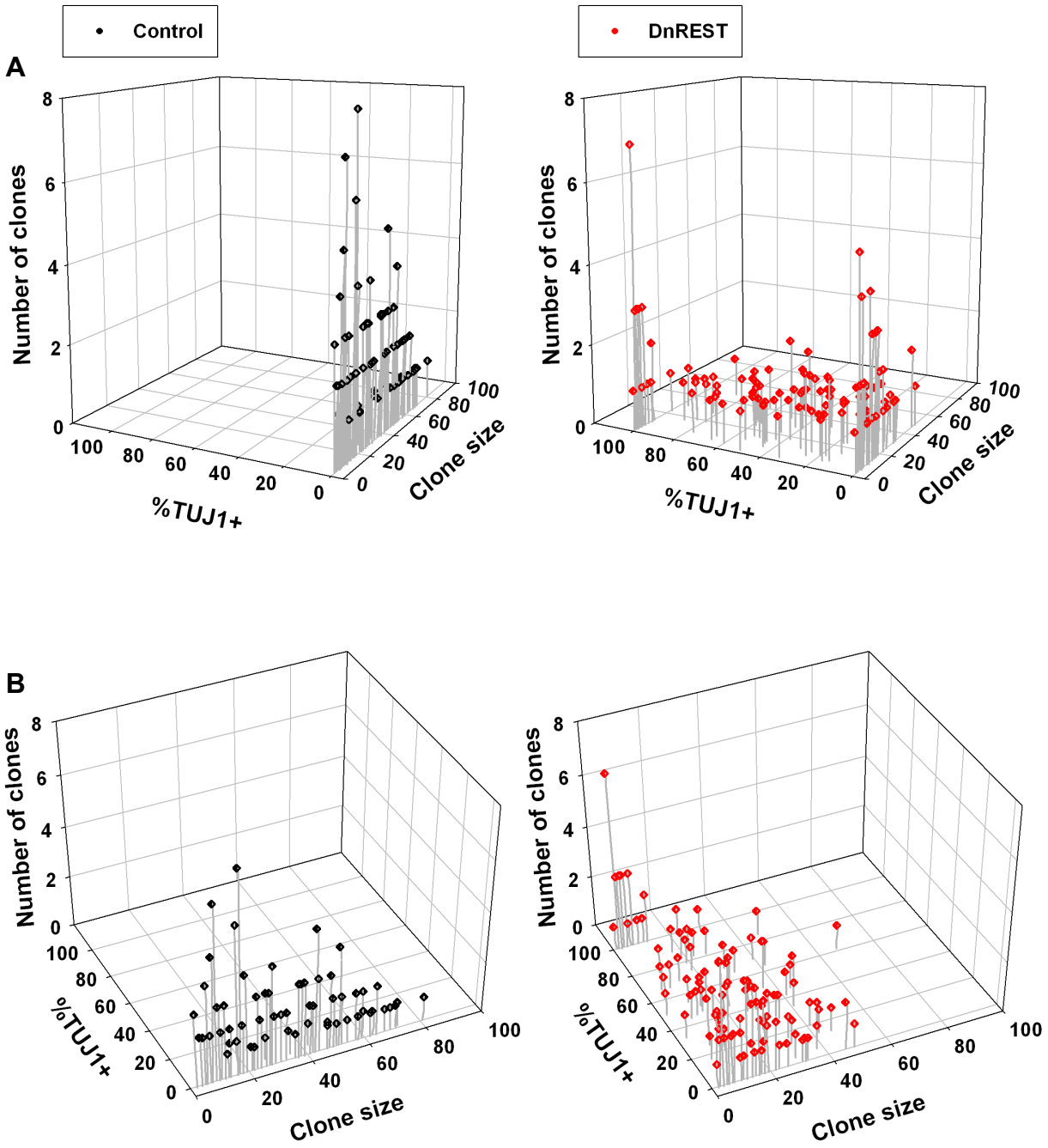

A
Control
DnREST
Number of clones
%TUJ1+
Clone size
B
Number of clones
%TUJ1+
Clone size

Figure IV-43. Clonal composition of OPCs grown in stem cell media. Clonal analysis was performed as described in IV-17c. The histogram shows a summary of the percent of total clones containing the indicated percentage of MBP, O4, or TUJ1-positive cells. The number of clones expressing MBP and O4 decrease while the number of TUJ1 expressing clones increases with REST LOF. Total number of clones: O4 control n=108, DnREST n=116, MBP control n=251, DnREST n=251, TUJ1 control n=136, DnREST n=146.

FIGURE IV-43

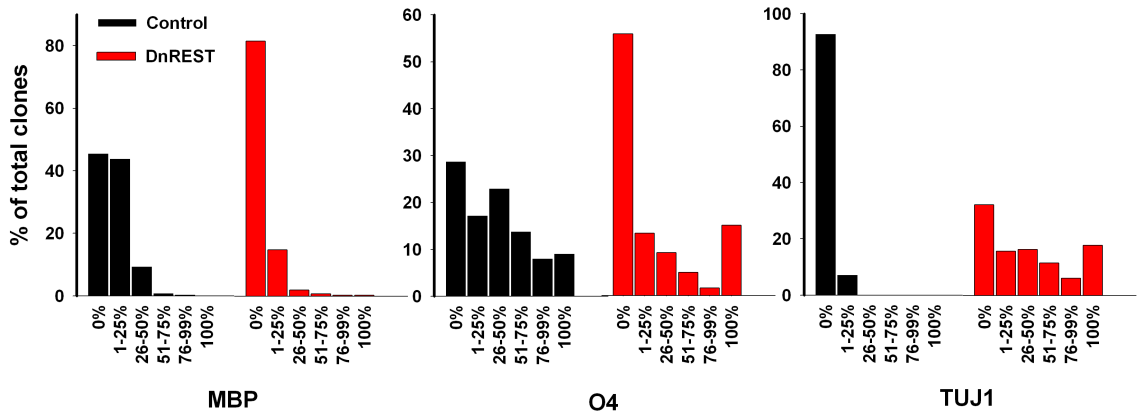

Control
DnREST
% of total clones
0
20
40
60
80
0%
1-25%
26-50%
51-75%
76-99%
100%
MBP
0
10
20
30
40
50
60
O4
0
20
40
60
80
100
TUJ1

Figure IV-44. OPC clones in 2A differentiation media: The number of GFAP expressing clones increases with REST LOF. Clonal analysis was performed as described in figure IV-17d. After 5 days in oligodendrocyte media clones were immunostained with an antibody against GFAP (red) and infected clones (greens) were identified and analyzed. **(A)** The images show a single GFAP-positive control clone (left) and a GFAP-negative DnREST clone (right). **(B)** Image shows a single GFAP-positive clone infected with the DnREST expressing retrovirus. Although the number of GFAP expressing clones decreases with REST LOF, many clones are still GFAP-positive. All scale bars indicate 50μm.

FIGURE IV-44

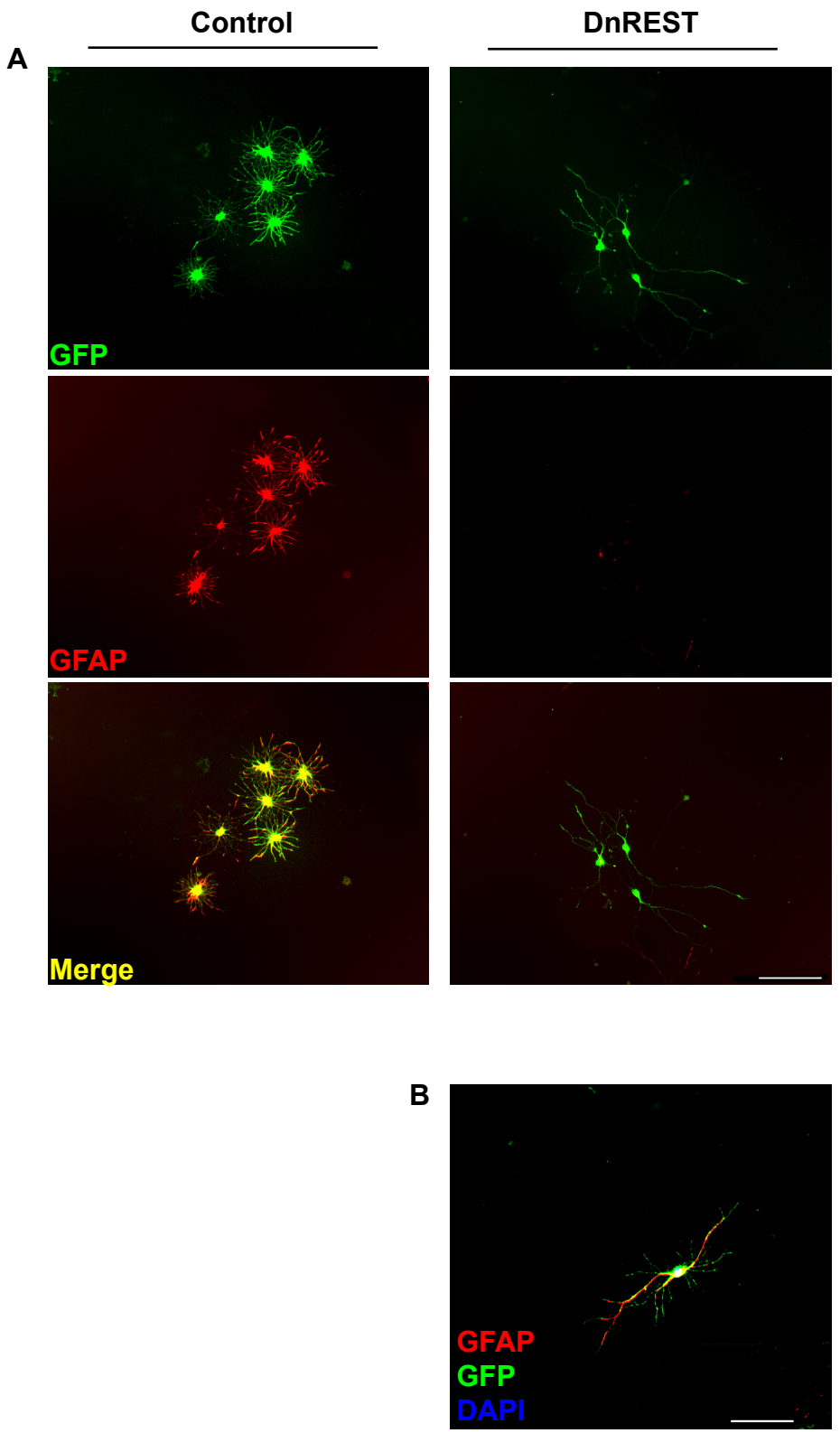


Figure IV-45. Type II Astrocyte media: GFAP expression in individual clones. Clonal analysis was performed as described in figure IV-17d for GFAP expression. The size and the percent of GFAP-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. More DnREST clones contain fewer or no GFAP-positive cells than control clones. Total number of clones: control n=273, DnREST n=279.

FIGURE IV-45

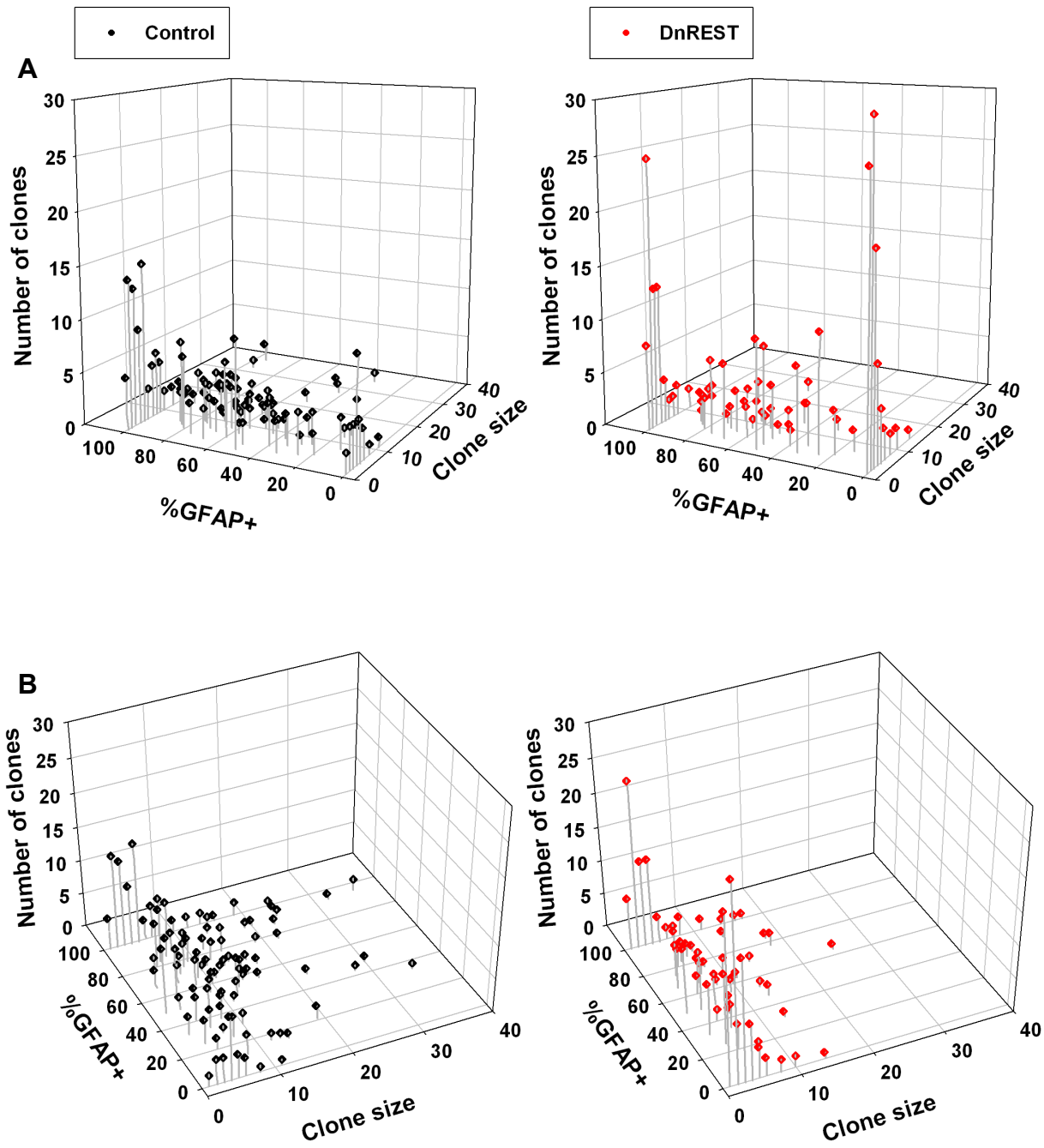

A
Control
DnREST
Number of clones
%GFAP+
Clone size
B
Number of clones
%GFAP+
Clone size

Figure IV-46. OPC clones in 2A differentiation media: The number of TUJ1 expressing clones increases with REST LOF. Clonal analysis was performed as described in figure IV-17d. After 5 days in oligodendrocyte media clones were immunostained with an antibody against TUJ1 (red) and infected clones (green) were identified and analyzed. The images show a single TUJ1-negative control clone (left) and two TUJ1 expressing DnREST clones (right). Note the difference in morphology between control and DnREST cells. Scale bars indicate 50μm.

FIGURE IV-46

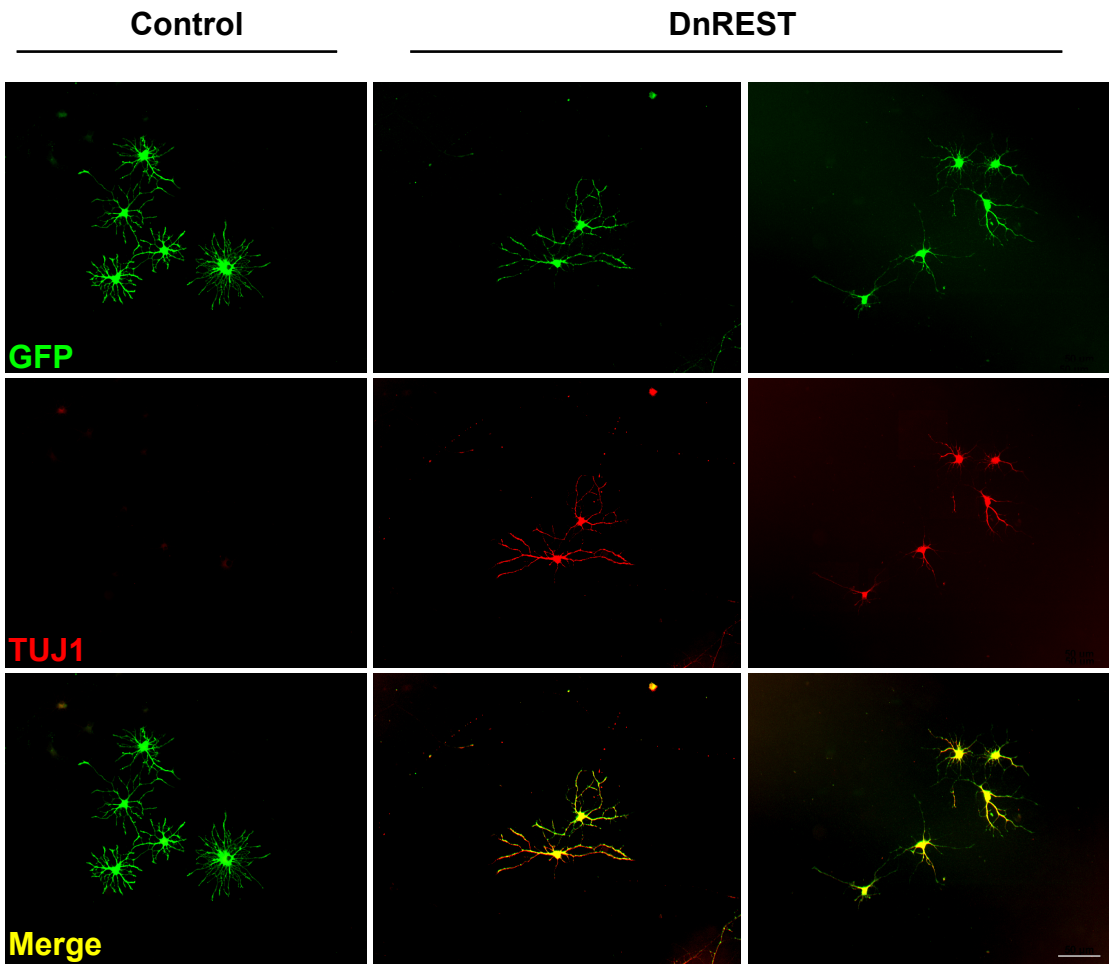


Figure IV-47. Type II Astrocyte media: TUJ1 expression in individual clones. Clonal analysis was performed as described in figure IV-17d for TUJ1 expression. The size and the percent of TUJ1-positive cells are shown for each individual clone in control (black) and DnREST (red) cultures. The scatter plots are shown at 2 angles for ease of data display **(A and B)**. Most DnREST clones contain at least one TUJ1-positive cell whereas a majority of the control clones do not express any TUJ1. Total number of clones: control n=202, DnREST n=194.

FIGURE IV-47

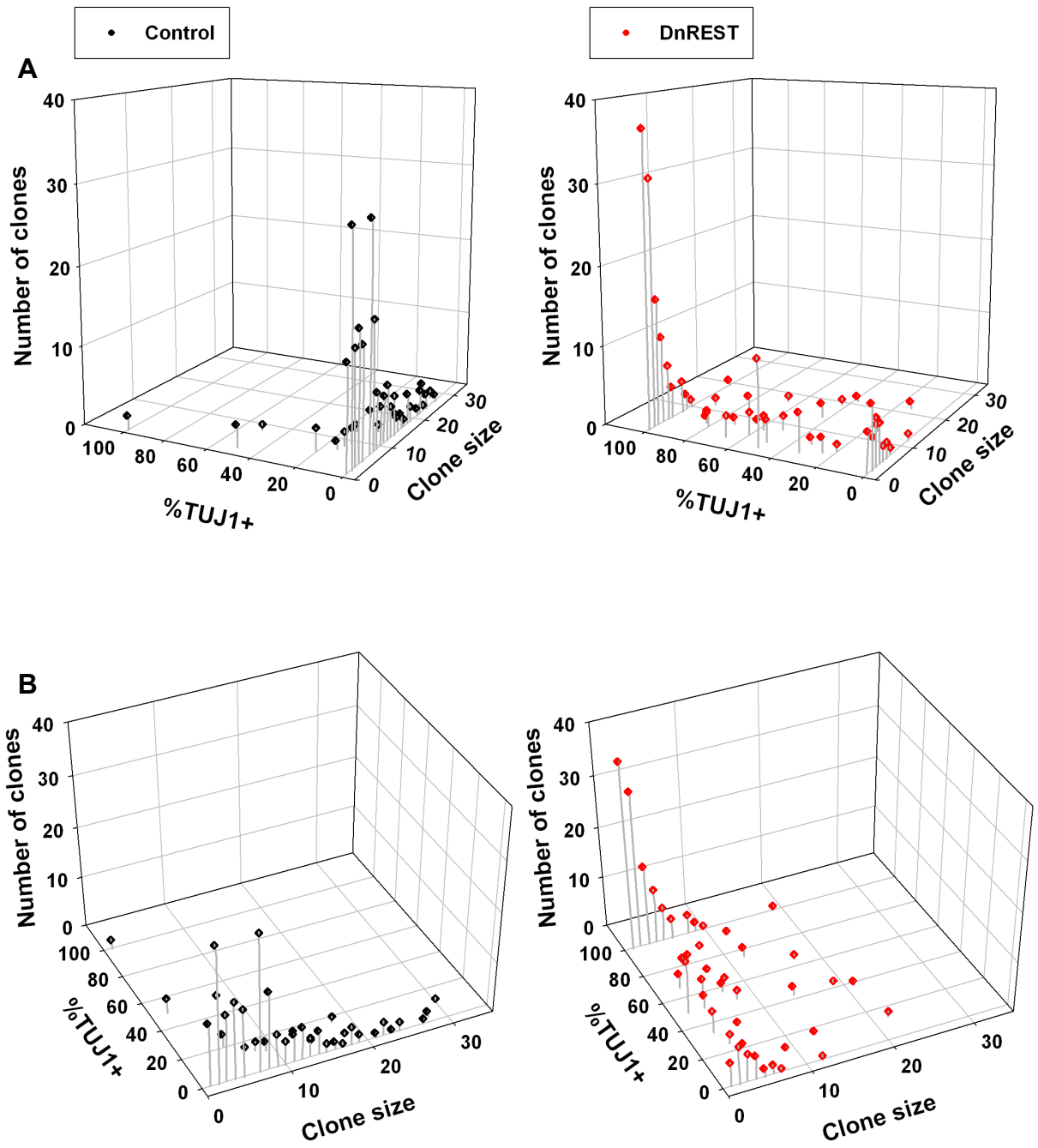

A
Control
DnREST
Number of clones
%TUJ1+
Clone size
B
Number of clones
%TUJ1+
Clone size

Figure IV-48. Clonal composition of OPCs grown in 2A differentiation media. Clonal analysis was performed as described in IV-17d. The histogram shows a summary of the percent of total clones containing the indicated percentage of GFAP or TUJ1-positive cells. The number of clones expressing GFAP decreases while the number of clones expressing TUJ1 increases with REST LOF. Total number of clones: GFAP control n=273, DnREST n=279, TUJ1 control n=202, DnREST n=194.

FIGURE IV-48

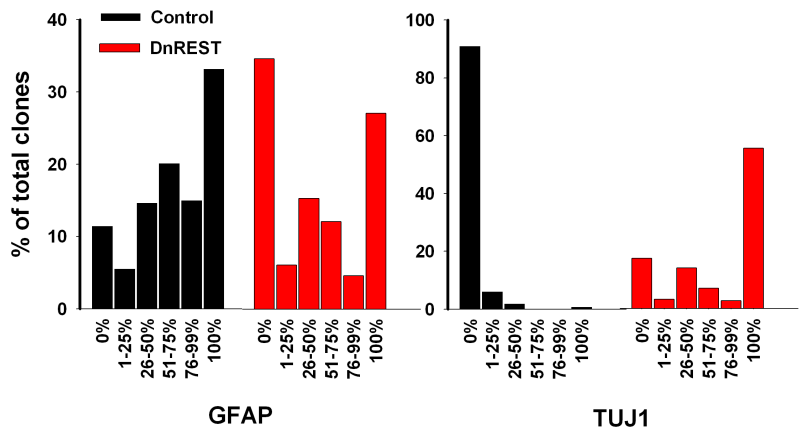
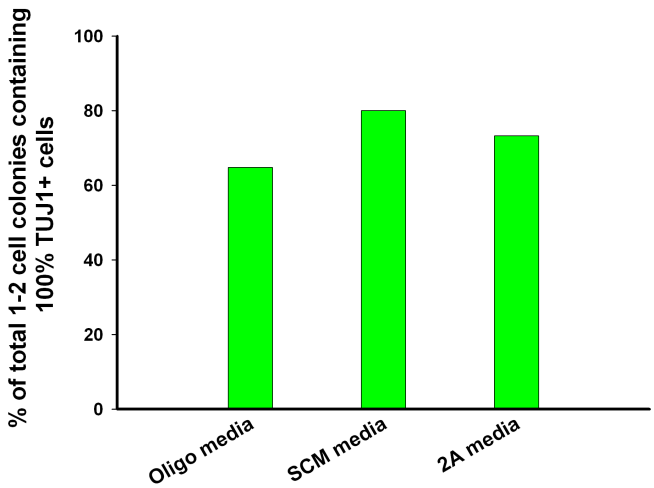

Control
DnREST
% of total clones
40
30
20
10
0
100
80
60
40
20
0
0%
1-25%
26-50%
51-75%
76-99%
100%
GFAP
TUJ1

Figure IV-49. Small clones express TUJ1 with REST LOF. Clonal analysis was performed as described in figure IV-17 for TUJ1 expression. The graph shows the percent of total 1 and 2 cell DnREST infected clones that are 100% TUJ1-positive after 5 days in the indicated media.

FIGURE IV-49


% of total 1-2 cell colonies containing 100% TUJ1+ cells
100
80
60
40
20
0
Oligo media
SCM media
2A media

CHAPTER V
MISCELLANEOUS ODDS AND ENDS

INTRODUCTION

This chapter contains the results from two incomplete experiments that I find interesting and important to discuss. The data obtained from these experiments is preliminary but opens the door to future studies that should be investigated in this particular subject matter.

The first set of experiments was performed in collaboration with Jessica Helm from Dr. Lonnie Wollmuth's laboratory. The purpose of this study was to assay the electrophysiological properties of OPCs following REST LOF. In Chapter's III and IV, I demonstrated that the transcriptional repressor REST is an important regulator of OPC cell fate determination and oligodendrocyte differentiation. REST LOF promotes the expression of numerous neuronal genes and proteins. Although these cells have a neuronal phenotype, they cannot be classified as neurons without studying their electrophysiological properties. REST functions as a transcriptional repressor through the recruitment of HDACs and other epigenetic regulators to the DNA (Andres et al., 1999, Huang et al., 1999, Naruse et al., 1999, Grimes et al., 2000, Roopra et al., 2000, Humphrey et al., 2001, You et al., 2001, Roopra et al., 2004, Ooi and Wood, 2007). Blocking HDACs in OPCs promotes lineage plasticity and induces the formation of electrically excitable TUJ1-positive neurons (Liu et al., 2007a). Therefore, I wanted to determine if the TUJ1-positive neuronal-like cells obtained with REST LOF are functional neurons.

The second set of experiments was a group effort performed by Dr. Joel Levine, Victoria Latella, and myself. We wanted to study the effect of REST LOF on OPCs in vivo. OPCs behave differently depending on the external environment (Raff et al., 1983, Kondo and Raff, 2000). In Chapters III and IV, I demonstrated that OPCs develop a neuronal phenotype when REST function is perturbed. Here, I wanted to determine whether the neuronal phenotype I observed with REST

LOF in vitro was stable in the LOF cells when transplanted back into a normal oligodendrogenic environment. To do this, I transplanted infected OPCs into p2 rat corpus callosum and after sacrificing the animals, Dr. Levine and Victoria analyzed the behavior and phenotype of the transplanted cells.

The corpus callosum is a white matter region that contains the myelinated commissural nerve fibers that connect the two cerebral hemispheres. The corpus callosum is just beneath the cerebral cortex which is a grey matter area containing nerve cell bodies. Myelin sheaths are first seen in the corpus callosum of rodents about 11-15 days after birth (Sturrock, 1980, Nunez et al., 2000), making it the ideal location to study oligodendrocyte differentiation during this period. OPCs transplanted into p2 rats typically generate oligodendrocytes in the corpus callosum with minimal astrocyte or neuronal development after one week (Liu et al., 2007a). However, when HDACs are inhibited, the number of transplanted OPCs expressing astrocytic or neuronal markers dramatically increases at the expense of cells expressing oligodendrocyte lineage markers (Liu et al., 2007a). I wanted to determine if perturbing REST function would have a similar effect on transplanted OPCs considering we see a similar effect in vitro.

RESULTS

Electrophysiological studies

For these initial studies, I used a combination of REST LOF methods and grew the cells in several types of media to study their electrophysiological properties. I infected purified OPCs with adenoviruses expressing DnREST, REST-VP16, or GFP alone as a control, or with control GFP or DnREST expressing retroviruses. After infection, I grew the cells in 2A media or SCM with or without bFGF for 4 to 6 days. Infected cells were identified on the basis of GFP expression. 7 out of 12 LOF cells fired repetitive action potentials following current injection (figure V-1b). All control cells studied fired either one or no action potentials and no control cells fired repetitive action potentials (figure V-1a). 3-fold greater current injections were required to elicit these responses. In addition to firing repetitive action potentials, experimental cells also had higher Na+ current densities (92±22 pA/pF, n=11, figure V-1d) compared to control cells (44±13 pA/pF, n=13, figure V-1c). These inward currents were blocked by the sodium channel blocker tetrodotoxin (TTX). The higher current densities in REST LOF cells could be a result of more channels per surface area or higher conductance. These preliminary studies suggest that REST LOF promotes not only the expression of neuronal proteins such as TUJ1 but also the development of electrical excitability, a hallmark of neurons. Due to the small number of cells analyzed, further studies are needed to confirm this conclusion.

Transplantation studies

I purified, infected, and transplanted control rGFP or rDnREST infected OPCs into the corpus callosum of p2 rat pups as described in Experimental Procedures. The injection site is indicated in red in figure V-2. Two days post transplantation, many of the cells remained at the injection site (figure V-3a). By day 9, the control cells migrated extensively within the white matter (figure V-3b). DnREST expressing cells also migrated extensively from the injection site within the white matter (figure V-3), but some of the cells exited the white matter

and colonized the cortex adjacent to the injection site (figure V-3d). Unlike the unipolar and bipolar control cells (figure V-4a), several of the DnREST cells had multiple processes and more complex morphologies (figure V-4b) and expressed the neuronal-specific antigen NeuN (figure V-4c). Although further studies are needed to confirm these findings, these preliminary data suggest that REST LOF promotes OPCs to give rise to cells having a neuronal phenotype in an in vivo environment.

DISCUSSION

Electrophysiological studies

In this chapter I demonstrated that in addition to expressing neuronal proteins and genes following REST LOF, some OPCs also develop electrophysiological properties normally associated with neurons. Since this was a pilot study, the cells were grown in various external environments. Therefore, the 12 experimental cells studied were a combination of cells grown in different media and expressing either DnREST or REST-VP16. We did not find a noticeable difference in the number of cells that fired multiple action potential between media or DnREST versus REST-VP16 expression suggesting that the effects were due to REST LOF, not media conditions. However, we did not analyze enough cells to substantiate this conclusion. Unfortunately, we were not able to continue this project. These preliminary results suggest that REST is directly involved in restricting the neuronal fate choice of an OPC and loss of REST repression may be sufficient to promote the development of electrically excitable neurons.

Transplantation studies

Following transplantation of control OPCs into p2 rat corpus callosum, we found that the cells migrated within the white matter and remained NeuN-negative. The transplanted cells were unipolar and bipolar, characteristic of actively migrating glial progenitor cells (de Castro and Bribian, 2005). This result is similar to work from Dr. Patrizia Casaccia-Bonnefil's laboratory. They also found that over 80% of OPCs transplanted into p2 rats migrated within the corpus callosum and expressed the oligodendrocyte marker CC1. Less than 4% of these cells expressed the neuronal antigen NeuN after one week (Liu et al., 2007a). While many of the DnREST infected OPCs behaved similarly, some of the cells migrated out of the white matter and colonized the cortex and these cortical cells expressed the neuronal marker NeuN.

The data discussed in this chapter, although incomplete, suggests that REST functions to inhibit the neurogenic potential of OPCs. Perturbing REST function may be sufficient to induce OPCs to differentiate into functional neurons. Lastly, the neuronal phenotype observed in OPCs following REST LOF was stable when transplanted back into a normal oligodendrogenic environment. This suggests that, in addition to OPCs grown in culture, REST may also have a functional role in OPC cell fate determination and oligodendrocyte differentiation in vivo. It would be interesting to further explore these possibilities.

Figure V-1. OPCs fire repetitive action potentials after REST LOF. Purified OPCs were infected with control GFP, DnREST, or REST-VP16 expressing adenoviruses and grown for 5 days in 2A media. **(A)** Control infected cells fired either 1 or no action potential even with 3-fold greater current injection than REST LOF cells. **(B)** The REST LOF cells fired repetitive action potentials after current injection (7 of 12). REST LOF cells also displayed higher current densities **(D)** as compared to control cells **(C)**.

FIGURE V-1

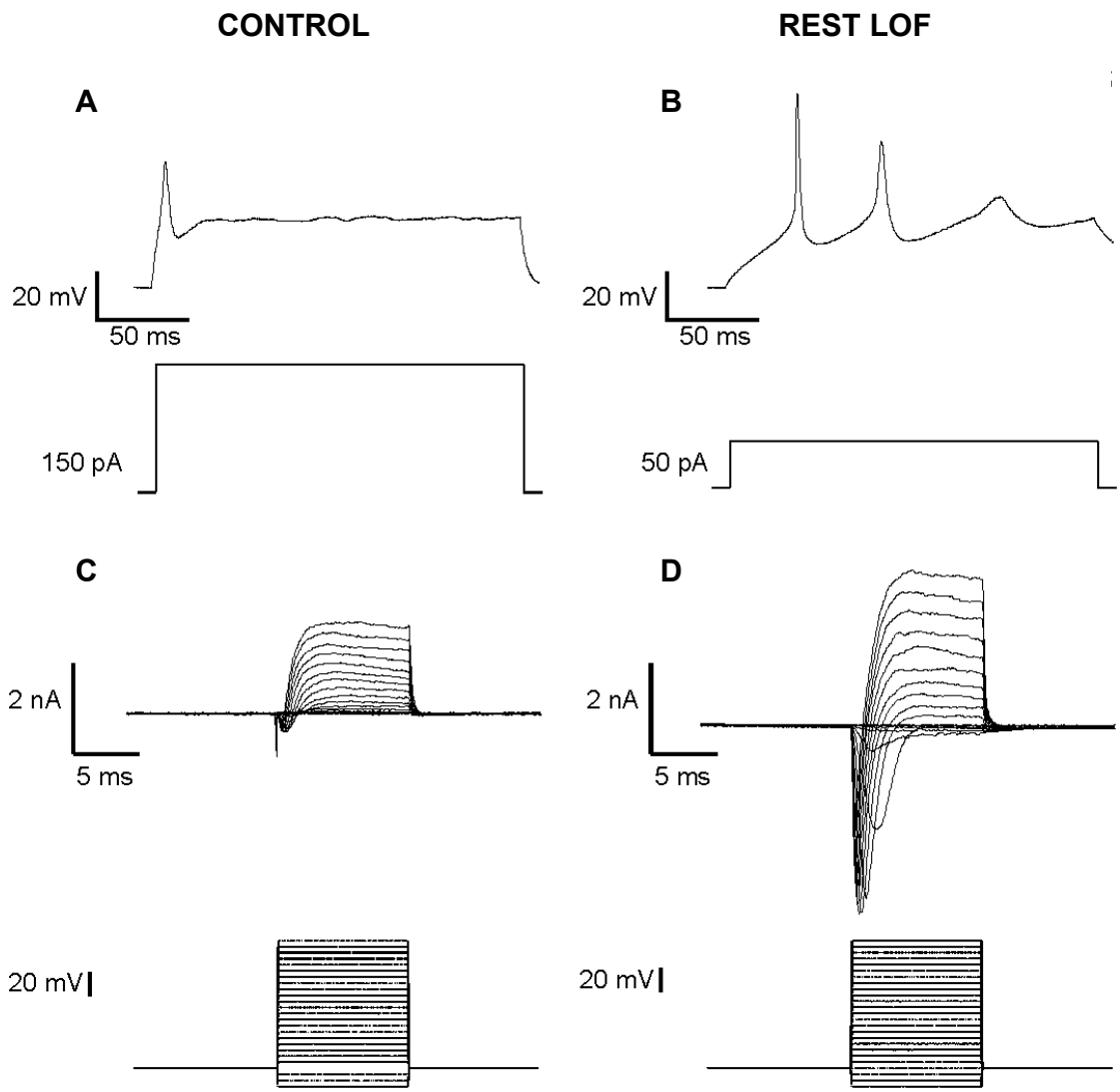

CONTROL
REST LOF
A
B
20 mV
50 ms
150 pA
20 mV
50 ms
50 pA
C
D
2 nA
5 ms
20 mV
2 nA
5 ms
20 mV

Figure V-2. Transplanted control cells migrate within the white matter. Control cells transplanted into the corpus callosum of p2 rats. **(A)** Image of the injection site 2 days post-injection. Most of the transplanted cells remain at the injection site at this time point. **(B)** By day 9, many cells migrate extensively (arrows) but remain within the white matter which is typical of cells adapting an oligodendrocyte fate. All scale bars indicate 100μm

FIGURE V-2

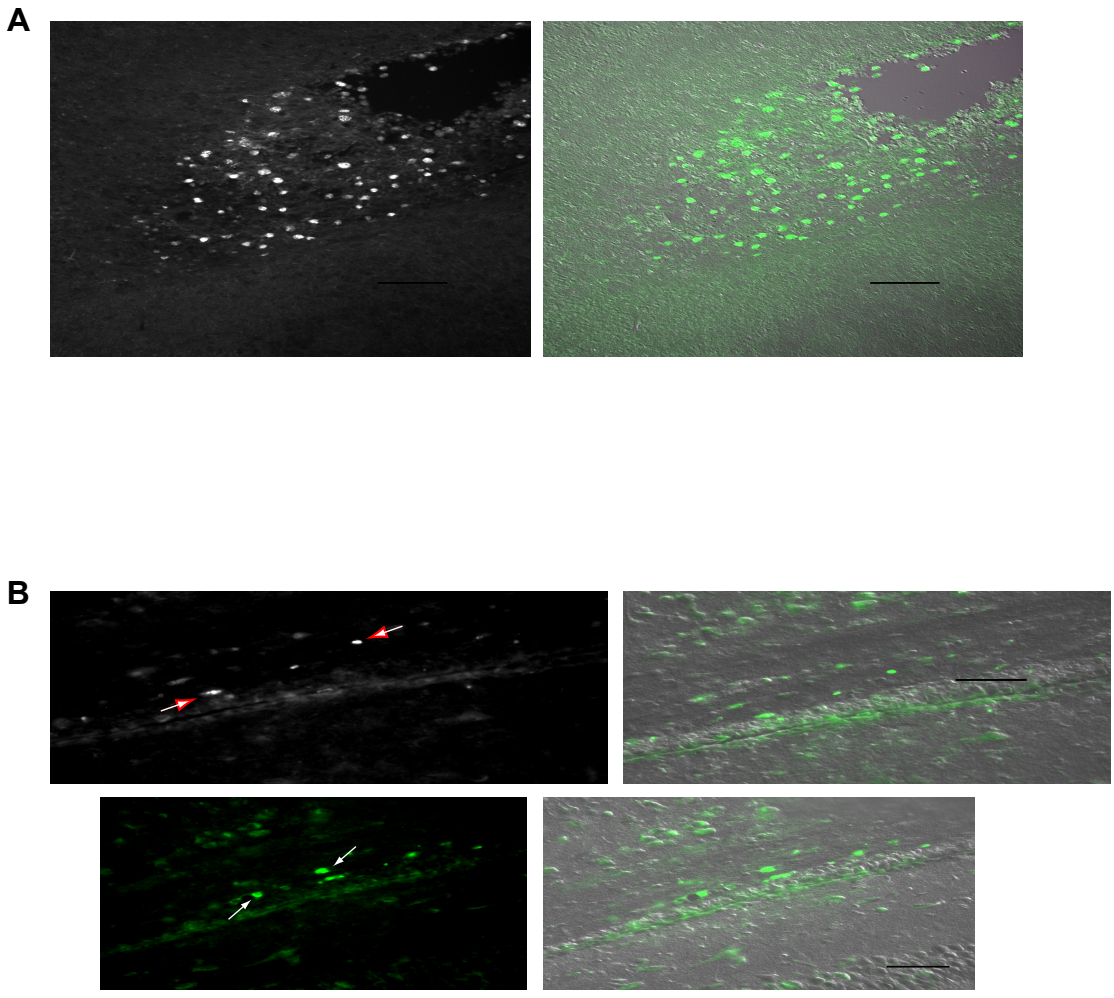


Figure V-3. Transplanted OPCs expressing DnREST migrate within and outside of the white matter. DnREST infected OPCs 9 days post-transplantation into the corpus callosum of p2 rats. As was the case with control cells, DnREST cells (green) migrate extensively from the injection site. The injection site is indicated with an arrow **(A)**. Within the white matter, the migrating cells disperse both contralateral **(B)** and ipsilateral **(C)** of the injection site. **(D)** Migrating OPCs also exit the white matter when REST function is perturbed (red) and colonize the cortex. Scale bars indicate **(A, B** and **D)** 100μM, **(C)** 20μm.

FIGURE V-3

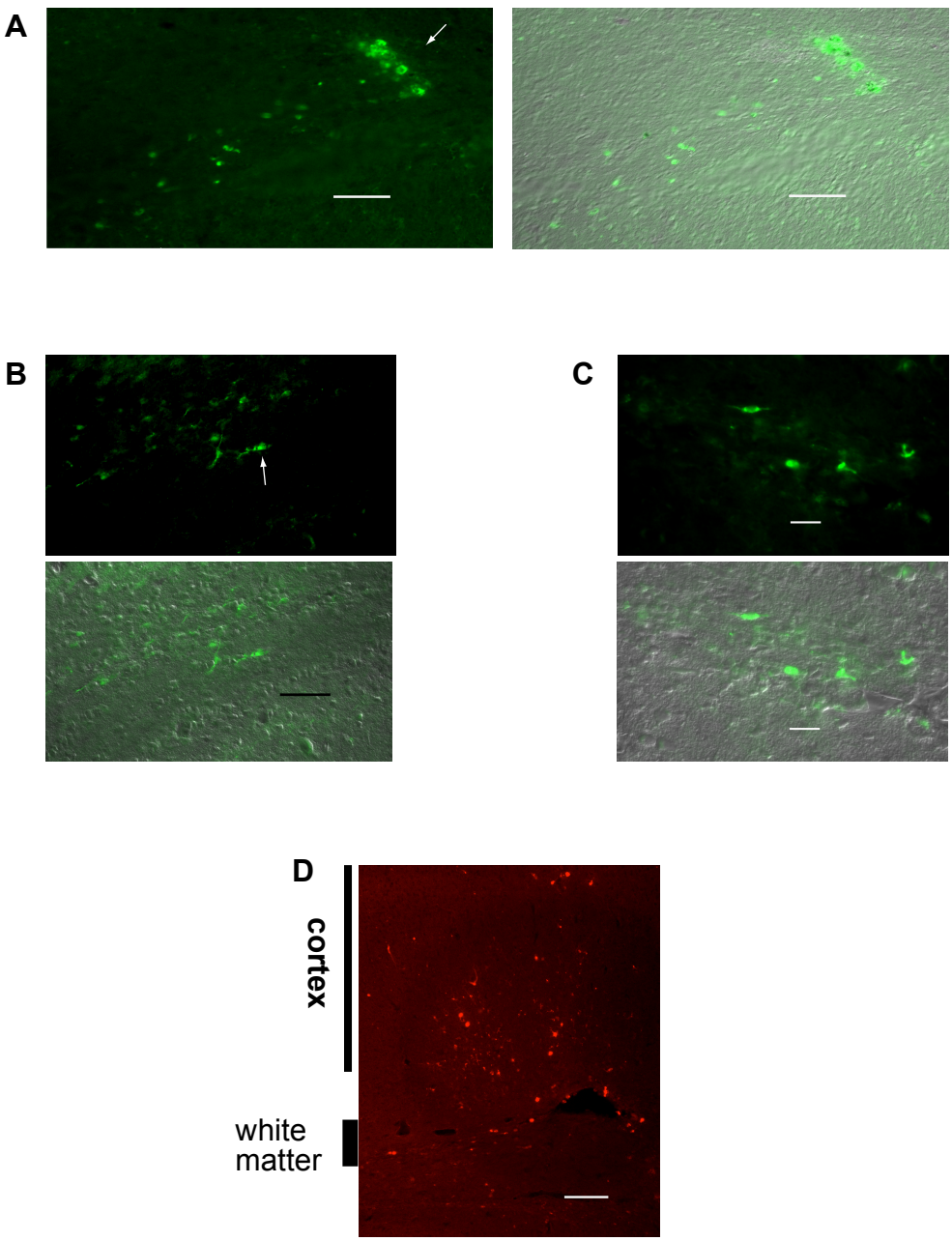
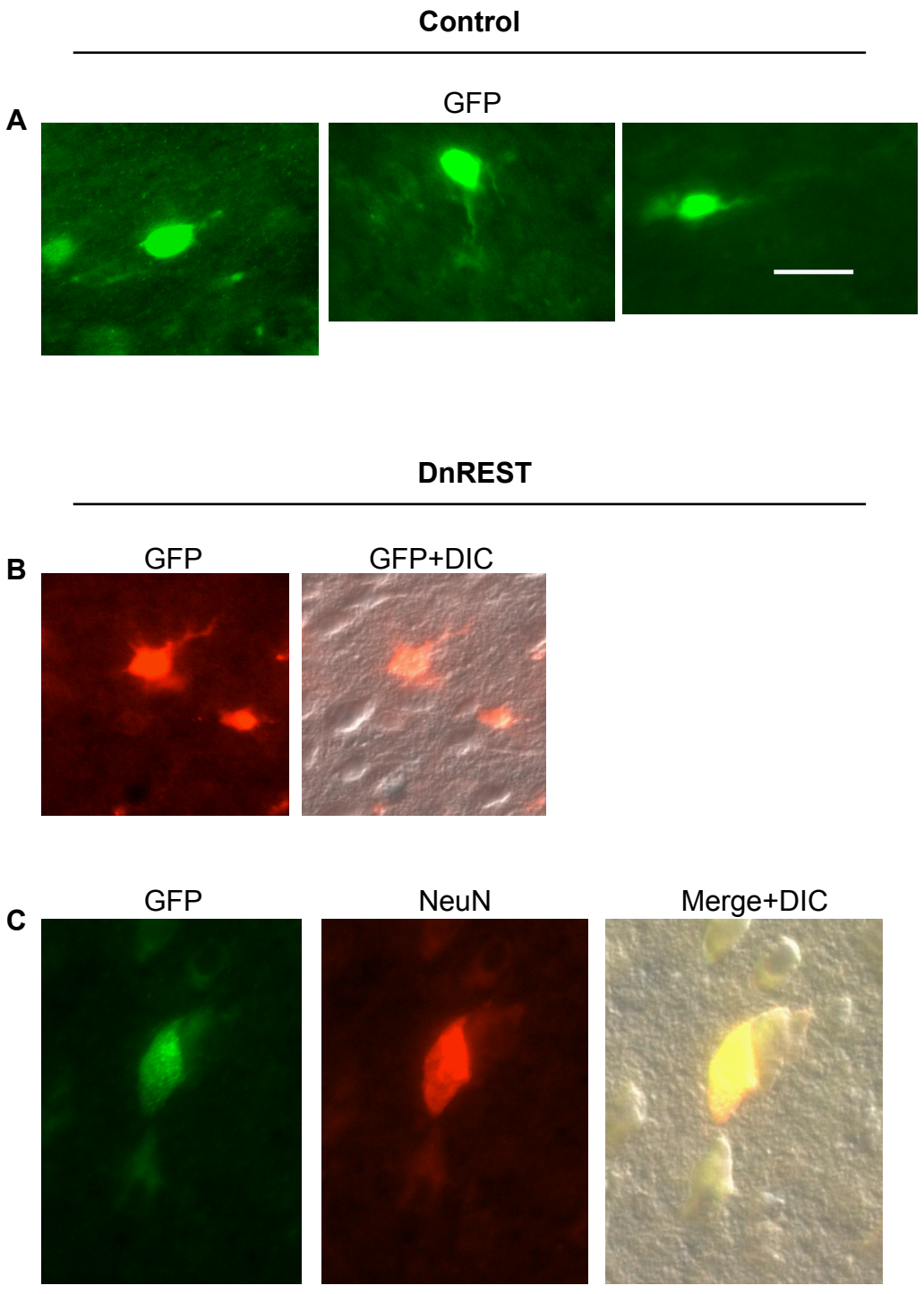


Figure V-4. Transplanted OPCs express the immature neuronal antigen NeuN following REST LOF. (A) Control infected OPCs 9 days post transplantation into the corpus callosum of p2 rats. Immunofluorescence images of individual cells show that they are unipolar or bipolar. This is characteristic of actively migrating glial progenitors. **(B)** Unlike control cells, many DnREST infected cells have multiple processes**. (C)** Immunofluorescence image showing a DnREST infected OPC that is positive for NeuN, a maker antigen for immature neurons. Sale bar indicates 20μm.

FIGURE V-4



CHAPTER VI
GENERAL DISCUSSION

REST and glial development

The timing of oligodendrocyte differentiation is dictated by the activation of pro-oligodendrocyte genes and the repression of differentiation inhibitor genes. Blocking HDAC-mediated gene repression in OPCs prevents oligodendrocyte development and promotes the expression of neuronal properties (Liu et al., 2007a, Lyssiotis et al., 2007). REST regulates the expression of many neuronal genes in non-neuronal cells by recruiting HDACs and other repressors to the DNA. In this dissertation, I demonstrated that REST is an important regulator in oligodendrocyte development. REST is required for the timely and complete differentiation of oligodendrocytes and prevents OPCs from developing neuronal properties.

Following the completion of my experiments, two other groups reported a potential role for REST in glial development. In the first study, Abrajano et al., (2009) performed a genome-wide binding experiment using ChIP on chip assays to identify REST and CoREST binding sites during several stages of oligodendrocyte maturation (Abrajano et al., 2009). They found that REST physically interacts with a set of genes throughout all stages of oligodendrocyte development. They also identified unique sites that were bound by REST at only one stage of differentiation. The number of these unique sites progressively increased as the cells developed into mature oligodendrocytes. Therefore, in addition to differentially regulating gene expression in different cell types, REST may temporally regulate different genes as a single cell type develops. Abrajano et al's., (2009) findings suggest that REST has multiple roles during the process of oligodendrogenesis. The work presented here is the first functional analyses of REST during oligodendrogenesis.

When I examined REST expression during oligodendrocyte development, I found that REST expression increases during the onset of differentiation. This increase may enable REST to bind to different targets as oligodendrocytes mature.

Abrajano et al., (2009) also examined the expression levels of genes that were not bound by REST in premyelinating oligodendrocytes but were bound in myelinating oligodendrocytes. Among these genes, 197 were up-regulated whereas 83 were downregulated. Since REST may also function as an activator of gene transcription (Bessis et al., 1997, Kallunki et al., 1997, Armisen et al., 2002, Kuwabara et al., 2004), it is possible that the high levels of REST in differentiating OPCs may also lead to the activation of some genes.

The second group examined the role of REST during BMP-induced astrocyte differentiation of embryonic day 14 neural progenitor cells (Kohyama et al., 2010). They demonstrated that REST is up-regulated during astrocyte development, similar to what I observed following the onset of oligodendrocyte differentiation. Kohyamea et al., (2010) identified a Smad-binding element in the regulatory region of REST. Smad is a downstream transcription factor of BMP2. During BMP-induced astrocyte differentiation, Smad associated with the regulatory region of REST and induced its transcription. This suggests that the spike in REST expression during the onset of oligodendrocyte differentiation may also be the result of an external cue.

In my studies, I used thyroid hormones (THs) to promote oligodendrocyte differentiation. THs not only promote oligodendrocyte development in culture (Almazan et al., 1985, Koper et al., 1986), they also regulate the timing of oligodendrocyte development in vivo (Billon et al., 2001). For example, myelination is accelerated in hyperthyroid animals and delayed in hypothyroid animals (Walters and Morell, 1981, Dussault and Ruel, 1987, Rodriguez-Pena et al., 1993, Marta et al., 1998). The next section discusses a potential role for thyroid hormones in mediating REST upregulation during oligodendrogenesis.

A role for thyroid hormones in REST regulation

Thyroid hormones alter gene expression by binding to nuclear receptors. Nuclear receptors are a large family of ligand-regulated transcription factors that respond to endocrine hormones (Lee and Privalsky, 2005). Nuclear receptors typically bind as protein dimers to specific sites in the DNA and recruit

corepressors or coactivators to repress or activate gene expression respectively (Chen and Li, 1998, Jenster, 1998, Koenig, 1998, Xu et al., 1999, Ito and Roeder, 2001, Lee et al., 2001, Ordentlich et al., 2001, Rachez and Freedman, 2001, McKenna and O'Malley, 2002, Privalsky, 2004, Lee and Privalsky, 2005). Included in the nuclear receptor family are the receptors for thyroid hormones (TRs) and retinoic acid (RARs). TRs and RARs have the ability to heterodimerize and interact with retinoic acid response elements (RARE) (Lee and Privalsky, 2005). In the absence of hormone, RAR/TR heterodimers typically recruit corepressors whereas in the presence of the thyroid hormone they recruit coactivators (Lee and Privalsky, 2005).

Ballas et al., (2005) demonstrated that REST contains a retinoic response element upstream of its transcriptional start site. In post-mitotic cortical neurons, REST is transcriptionally repressed by the recruitment of a complex containing retinoic acid receptor (RAR) to this response element. Therefore, it is possible that in the presence of thyroid hormone RAR/TR heterodimers bind to the RARE site in the REST promoter and recruit coactivators to activate transcription. This would account for the spike in REST expression when OPCs are placed in oligodendrocyte differentiation media containing thyroid hormones. Thyroid hormone mediated REST upregulation may be a required pathway in the timing of oligodendrocyte differentiation in normal development. Regulation of REST transcription during oligodendrocyte induction would implicate THs in a previously unrecognized role.

REST and the initiation of differentiation

Following oligodendrocyte induction, the increase in protein expression is maintained for at least 45 hours. This sustained increase in protein level is in contrast to the down-regulation of REST protein that occurs during retinoic-acid induced differentiation of ES cells into neurons (Ballas et al., 2005, Westbrook et al., 2008). The rapid proteasomal degradation of REST is necessary for neuronal differentiation. Similarly, REST LOF induced neuronal properties in differentiating OPCs at the expense of cells expressing O4 and MBP. However,

REST LOF did not inhibit all OPCs from developing into oligodendrocytes. In my clonal analysis, the number of clones that contained 100% O4-positive cells did not change with REST LOF despite an overall decrease in O4 expression. This suggests that a subpopulation of OPCs were already committed to develop into oligodendrocytes and REST LOF was not sufficient to inhibit their development. Therefore, it's possible that REST may only be required for the initiation of oligodendrocyte differentiation. This would explain the need for increased REST levels during the onset of oligodendrocyte maturation. It is important to note that REST has a second function in glial development in that it also represses neuronal genes (see below). Because of this, the OPCs that developed into O4 and MBP-positive cells following REST LOF likely maintained abnormal expression of some REST regulated genes.

A proposed model for REST in oligodendrocyte development

Among the potential targets for REST regulation is Hes5. Hes5 is a downstream target of the Notch pathway and is a known inhibitor of oligodendrocyte maturation. Hes5 recruits HDACs to repress myelin genes and binds to activators of oligodendrocyte differentiation inhibiting their function (Liu et al., 2006). In addition to Hes5, several other proteins are known to function as inhibitors of oligodendrocyte differentiation including Id2, Id4, and Tcf4. While Hes5 is downstream of the Notch pathway, the transcription factor Tcf4 is a downstream effector of the canonical Wnt signaling pathway. Tcf4 activates Id2 and Id4 expression while repressing genes necessary for oligodendrocyte development (Ye et al., 2009, Liu and Casaccia, 2010). The Id proteins inhibit differentiation by forming heterodimers with pro-oligodendrocyte transcription factors (Samanta and Kessler, 2004, Li et al., 2009). When bound to Id proteins, these transcription factors cannot activate genes required for oligodendrocyte development.

In order for oligodendrocytes to mature, inhibitors of differentiation need to be repressed. The transcription factor YY1 represses Tcf4 and Id4 during oligodendrocyte development (He et al., 2007). YY1 expression increases during

differentiation and is necessary but not sufficient for OPCs to terminally differentiate into oligodendrocytes (He et al., 2007). Similarly, overexpression of REST in neural progenitor cells inhibits their ability to differentiate into neurons but has no effect on MBP expression (Kohyama et al., 2010). When I overexpressed REST in OPCs, I did not detect a noticeable difference in O4 or MBP expression by immunofluorescence staining after 3 or 5 days in stem cell media (data not shown). This suggests that, like YY1, REST is required but not sufficient for oligodendrocyte maturation.

Although YY1 has no effect on Hes5 transcription, my results show that Hes5 can be regulated by REST. Hes5 is transcriptionally repressed during the onset of differentiation in parallel with increased REST expression. Hes5 contains an expanded RE1 and was derepressed in differentiating oligodendrocytes following REST LOF. Perturbing REST function had no effect on YY1, Id2, or Id4 during oligodendrocyte differentiation. It seems possible that there is a division of labor during oligodendrocyte differentiation such that factors like YY1 are responsible for repressing Wnt mediated inhibition while Notch inhibition is overcome by REST.

I propose a model in which REST has two roles in OPC differentiation (VI-1). First, REST is required for the timely and complete differentiation of oligodendrocytes by working in parallel with YY1 to repress inhibitors of differentiation. During the initiation of differentiation, factors such as thyroid hormones upregulate REST expression. This increase enables REST to bind to the expanded RE1 in Hes5 and repress its expression while YY1 represses Wnt mediated inhibition. The second function of REST during OPC development is to inhibit genes associated with the functional properties of neurons such as SCG10, Nav1.2, and Synapsin. Because OPCs can develop into neurons, neuronal genes likely need to be tightly regulated for OPCs to have an alternate fate choice.

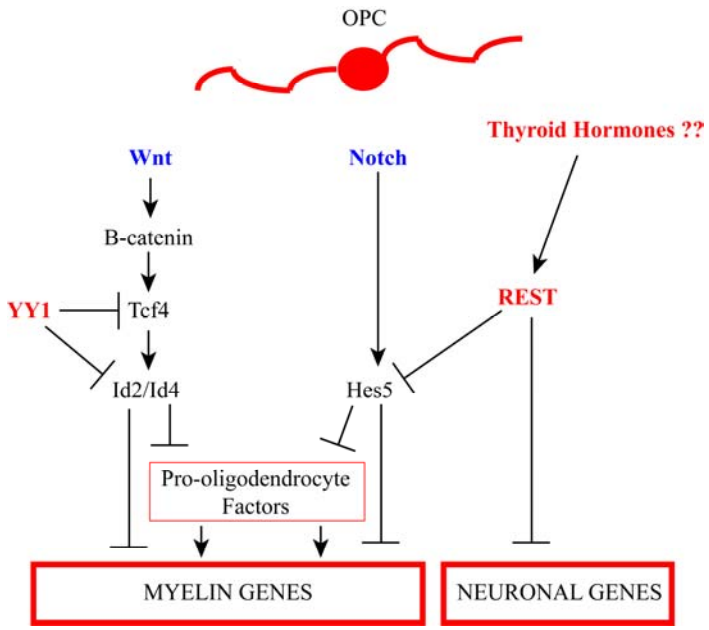


Figure VI-1. Proposed model for REST during oligodendrocyte differentiation

A role for REST in demyelinating diseases

The finding that REST repression is required for proper oligodendrocyte development suggests that REST repression may also be necessary for remyelination in diseases such as multiple sclerosis. In MS, the autoimmune reaction results in the loss of myelin and the formation of demyelinated lesions. If axons remain unmyelinated they become vulnerable to atrophy. This axonal loss leads to the deterioration associated with the later stages of MS. The efficiency of remyelination decreases with age despite the recruitment of OPCs to demyelinated MS lesions (Shields et al., 1999, Sim et al., 2002, Woodruff et al., 2004, Fancy et al., 2010). Impaired OPC differentiation in these lesions is a consequence of both intrinsic changes (Shen and Casaccia-Bonnefil, 2008, Shen et al., 2008b) and extracellular cues such as increased levels of inhibitory signals (John et al., 2002) or a lack of differentiation signals (Mastronardi et al., 2003). Administration of thyroid hormone to rats with experimental autoimmune encephalomyelitis (an animal model used for studying demyelinating diseases including MS) decreases the number of proliferating cells and increases the

number of cells expressing the oligodendrocyte marker MBP (Calza et al., 2002). This is interesting because thyroid hormones may increase REST expression which is required for proper oligodendrocyte development.

One idea why myelination fails is that, during the time when OPCs migrate and proliferate to fill demyelinated lesions, inhibitory regulators of differentiation are activated to prevent premature differentiation before enough cells are generated (Casaccia-Bonnefil and Liu, 2003, Fancy et al., 2010). These inhibitory pathways must be deactivated at the precise time for OPCs to respond to pro-differentiation signals and mature into myelinating cells. Therefore, it has been suggested that the failure to remyelinate is a result of a disturbance in the timing of this process rather than a single pathway (Fancy et al., 2010).

Following demyelination in young animals, the inhibitors of differentiation Hes5, Id2, and Id4 can be repressed in OPCs through the recruitment of HDACs to their promoters (Shen et al., 2008b). However, this is not the case in aged animals. There is a decrease in HDAC activity in OPCs and inhibitors of oligodendrocyte differentiation including Hes5, Id2, and Id4 continue to be expressed (Shen et al., 2008b), contributing to remyelination failure. My studies demonstrated that REST expression may also decrease with age. In addition to a decrease in HDAC expression, lower REST levels could account for the lack of HDAC recruitment to the Hes5 promoter. This is especially true if the burst in REST expression I observed during the onset of oligodendrocyte differentiation is required for Hes5 repression in vivo. Low levels of REST in adult OPCs could prevent HDAC recruitment to Hes5. This could lead to Hes5 expression and contribute to inefficient oligodendrocyte differentiation and remyelination.

Future directions

Many experiments could be performed in order to further understand the role of REST in oligodendrocyte lineage cells. The first would be to complete the studies I discussed in Chapter V. Additional electrophysiological experiments are necessary to determine if REST LOF in OPCs is sufficient to induce neuronal

differentiation. My studies demonstrated that perturbing REST function increases neuronal gene and protein expression. Although 7 out of 12 REST LOF cells fired multiple action potentials, additional experiments are needed to confirm this finding. Similarly, further studies are necessary to understand the role of REST in OPC specification and differentiation in an in vivo environment. This could be achieved by additional transplant studies, direct injection of REST LOF viruses, and the generation of conditional knockout mice.

It would also be of interest to examine the components of the REST complex in OPCs. In stem cells, REST recruits CoREST, mSin3, HDACs, and MeCP2 to RE1 sites (Ballas et al., 2005). In contrast to differentiated fibroblasts, REST does not interact with the histone methyltransferase G9a in stem cells and the DNA within and surrounding RE1 regions remains unmethylated (Ballas et al., 2005). Because OPCs have the ability to develop into neurons, it would be interesting to determine if OPCs are more like stem cells with REST regulated genes poised for expression. This could be done by performing ChIP assays using antibodies against G9a or H3K9 dimethylated histones, di- and trimethylated H3K4, and by examining the DNA surrounding RE1s to determine the DNA methylation status.

Additionally, it would be interesting to study how REST itself is regulated during the OPC to neuron transition. During embryonic neuronal differentiation, REST undergoes proteasomal degradation and is transcriptionally repressed (Ballas et al., 2005). During the differentiation of adult neural stem cells, a small double stranded non-coding RNA may convert REST from a repressor to an activator (Kuwabara et al., 2004). REST has also been shown to be negatively regulated by the brain specific isoform of REST, REST4 (Tabuchi et al., 2002), and by cytoplasmic sequestering (Shimojo, 2008). In would be interesting to determine if REST is regulated in a similar fashion during the OPC to neuron transition as it is in embryonic or adult neuronal differentiation of neural stem cells.

Future studies should also be performed to examine how the increase in REST expression is regulated during the onset of oligodendrocyte differentiation.

BMP2 (Kohyama et al., 2010), Wnt signaling (Nishihara et al., 2003), and Oct4/Sox2/nanog (Boyer et al., 2005) have been shown to activate REST transcription. Thyroid hormones may also have the ability to activate REST transcription through the RARE site in the regulatory region of REST. A luciferase reporter assay could be performed to determine if the RARE site in REST is sufficient to activate transcription in OPCs following exposure to thyroid hormones. ChiP analysis could also be performed to determine if TR/RAR heterodimers bind to the retinoic response element in REST.

Lastly, REST has been linked to many diseases including cancer (see introduction for details). REST can function as a tumor suppressor or an oncogene. Because a role for REST has been implicated in numerous types of cancer and it is an important regulator of glial development, it would be interesting to examine REST in glial tumors. Although several groups have studied REST in gliomas, there is a general lack of knowledge in this particular area of research (Blom et al., 2006, Zhang et al., 2009a). Considering the complexity of REST regulation and its involvement in glial specification and differentiation, it would be interesting to further investigate REST in gliomas as well as other types of neurodegenerative and demyelinating diseases including multiple sclerosis.

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APPENDIX I
GENE CHANGES DURING DIFFERENTIATION

Oligodendrocyte Media		Type II Astrocyte Media	
Gene Name	**Relative Fold Difference ± SD**	**Gene Name**	**Relative Fold Difference ± SD**
CelsR3	10.48 ± 4.16 **	SCG10	13.73 ± 2.24 **
SCG10	6.85 ± 0.25 **	CelsR3	11.33 ± 3.92 **
Snap25	5.25 ± 0.35 **	Snap25	4.88 ± 1.09 **
NFM	4.89 ± 0.98 **	Nestin	3.65 ± 2.05 *
Myt1	3.61 ± 0.12 **	Myt1L	2.95 ± 0.78 **
BIIITub	2.08 ± 0.28 **	BIIITub	2.93 ± 0.27 **
Syn1	1.96 ± 0.24 **	ATOH	2.50 ± 0.08 **
Nestin	1.83 ± 0.03 **	DCX	2.42 ± 1.17
Hes5	1.82 ± 0.05 **	NGN3	2.36 ± 0.07 **
Hes1	1.76 ± 0.75	NeuroD1	2.29 ± 1.13
Syt2	1.75 ± 0.95	NF-M	2.03 ± 0.02 **
Calb1	1.71 ± 0.08 **	Myt1	1.98 ± 0.15 **
Myt1L	1.58 ± 0.07 **	Hes1	1.71 ± 0.13 **
Olig2	1.49 ± 0.74	Syn1	1.55 ± 0.29 *
Mash1	1.40 ± 0.04 **	Nkx2.2	1.48 ± 0.20 **
DCX	1.35 ± 0.06 **	NGN2	1.48 ± 0.025 **
PLP	NC	Calb1	1.47 ± 0.09 **
Sox2	NC	GluR2	1.40 ± 0.07 **
PDGFaR	NC	Olig1	1.39 ± 0.03 **
GluR2	NC	Olig2	1.25 ± 0.11 *
NG2	NC	B-actin	NC
Olig1	NC	Sox2	NC
Sox10	NC	ID4	NC
YY1	NC	YY1	NC
GFAP	NC	Mash1	NC
CTNNb1	NC	Hes5	NC
Id2	NC	PLP	NC
Nkx2.2	NC	PDGFaR	NC
B-actin	NC	ID2	NC
Id4	NC	CNP	0.74 ± 0.22
MOBP	0.79 ± 0.07 **	NG2	0.68 ± 0.36
MBP	0.74 ± 0.05 **	MOBP	0.66 ± 0.38
MAG	0.72 ± 0.03 **	MBP	0.65 ± 0.05 **
CNP	0.50 ± 0.31 *	MAG	0.58 ± 0.18 **
		GFAP	0.41 ± 0.11 **
		Brca1	0.39 ± 0.02 **

* P-value < 0.05
** P-value < 0.005
NC = No Change

APPENDIX II
RAW CLONAL DATA

Control clones
NG2 expression
OPC media

Clone size	# of NG2– cells	# of NG2+ cells	%NG2+	Number of clones
1	1	0	0.00	1
2	0	2	100.00	2
3	1	2	66.67	1
3	0	3	100.00	6
4	0	4	100.00	3
5	0	5	100.00	3
6	0	6	100.00	1
7	0	7	100.00	2
8	0	8	100.00	1
9	1	8	88.89	2
9	0	9	100.00	3
10	0	10	100.00	1
11	0	11	100.00	2
12	0	12	100.00	5
13	0	13	100.00	2
14	0	14	100.00	1
15	0	15	100.00	2
16	1	15	93.75	1
16	0	16	100.00	3
17	0	17	100.00	1
18	0	18	100.00	2
20	0	20	100.00	1
22	0	22	100.00	1
24	0	24	100.00	1
25	1	24	96.00	1
29	0	29	100.00	2
30	0	30	100.00	1
31	0	31	100.00	1
33	0	33	100.00	1
34	0	34	100.00	1
37	0	37	100.00	1
40	0	40	100.00	1
48	0	48	100.00	1
Total number of clones counted:				58

DnREST clones
NG2 expression
OPC media

Clone size	# of NG2– cells	# of NG2+ cells	%NG2+	Number of clones
1	0	1	100.00	3
1	1	0	0.00	2
2	2	0	0.00	2
2	1	1	50.00	2
2	0	2	100.00	7
3	2	1	33.33	2
3	0	3	100.00	5
4	3	1	25.00	1
4	0	4	100.00	7
5	1	4	80.00	1
5	0	5	100.00	2
6	2	4	66.67	1
6	0	6	100.00	2
7	1	6	85.71	3
7	0	7	100.00	2
8	2	6	75.00	1
8	0	8	100.00	3
10	0	10	100.00	1
11	2	9	81.82	2
12	1	11	91.67	2
13	4	9	69.23	1
13	0	13	100.00	4
14	0	14	100.00	1
15	0	15	100.00	3
16	0	16	100.00	2
17	4	13	76.47	1
18	0	18	100.00	2
19	0	19	100.00	1
22	0	22	100.00	2
24	0	24	100.00	1
Total number of clones counted:				69

Control clones
TUJ1 expression
OPC media

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
1	1	0	0.00	1
2	1	1	50.00	1
3	3	0	0.00	2
4	4	0	0.00	6
5	5	0	0.00	1
6	6	0	0.00	3
6	5	1	16.67	1
7	7	0	0.00	3
8	8	0	0.00	2
9	9	0	0.00	3
11	11	0	0.00	3
12	12	0	0.00	1
14	14	0	0.00	2
15	15	0	0.00	1
15	13	2	13.30	1
17	17	0	0.00	1
19	19	0	0.00	1
20	20	0	0.00	2
21	21	0	0.00	2
28	28	0	0.00	3
30	30	0	0.00	2
32	32	0	0.00	1
34	34	0	0.00	1
35	35	0	0.00	1
38	38	0	0.00	2
39	39	0	0.00	1
41	41	0	0.00	1
42	42	0	0.00	1
44	44	0	0.00	1
46	46	0	0.00	1
47	47	0	0.00	2
48	48	0	0.00	2
48	46	2	4.17	1
49	49	0	0.00	1
52	52	0	0.00	1
53	53	0	0.00	2
54	54	0	0.00	1
55	55	0	0.00	1
57	57	0	0.00	1
59	59	0	0.00	1
63	63	0	0.00	2
66	65	1	1.52	1
68	68	0	0.00	1
72	70	2	2.78	1
74	74	0	0.00	1
86	86	0	0.00	1
87	87	0	0.00	1
93	93	0	0.00	1
Total number of clones counted:				74

DnREST clones
TUJ1 expression
OPC media (page 1)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
1	1	0	0.00	3
1	0	1	100.00	3
2	2	0	0.00	4
2	1	1	50.00	2
2	0	2	100.00	4
3	3	0	0.00	1
3	0	3	100.00	1
4	4	0	0.00	1
4	3	1	25.00	1
4	0	4	100.00	1
5	5	0	0.00	2
6	0	6	100.00	1
7	1	6	85.71	1
8	6	2	25.00	1
9	9	0	0.00	2
10	2	8	80.00	1
10	10	0	0.00	2
11	11	0	0.00	2
11	6	5	45.45	1
12	4	8	66.67	1
13	12	1	7.69	1
16	12	4	25.00	1
17	12	5	29.41	1
17	8	9	52.94	1
18	18	0	0.00	2
18	17	1	5.56	2
19	19	0	0.00	2
21	0	21	100.00	1
22	22	0	0.00	1
24	18	6	25.00	1
25	25	0	0.00	1
28	21	7	25.00	1
28	12	16	57.14	1
29	29	0	0.00	1
30	30	0	0.00	1
31	31	0	0.00	2
31	29	2	6.45	1
31	12	19	61.29	1
32	30	2	6.25	1
36	36	0	0.00	2
37	37	0	0.00	1
38	38	0	0.00	1
38	27	11	28.95	1
39	39	0	0.00	1
41	38	3	7.32	2
42	42	0	0.00	1
42	41	1	2.38	1
47	43	4	8.51	1

DnREST clones
TUJ1 expression
OPC media (page 2)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
49	37	12	24.49	1
51	48	3	5.88	1
53	36	17	32.08	1
56	42	14	25.00	1
59	59	0	0.00	1
79	61	18	22.78	1
Total number of clones counted:				75

Control clones
O4 expression
Oligodendrocyte media (page 1)

Clone size	# of O4– cells	# of O4+ cells	%O4+	Number of clones
1	1	0	0.00	1
1	0	1	100.00	5
2	2	0	0.00	7
2	1	1	50.00	2
2	0	2	100.00	8
3	3	0	0.00	4
3	2	1	33.33	1
3	1	2	66.67	2
3	0	3	100.00	4
4	4	0	0.00	5
4	2	2	50.00	3
4	1	3	75.00	3
4	0	4	100.00	8
5	4	1	20.00	1
5	3	2	40.00	3
5	2	3	60.00	1
5	1	4	80.00	1
5	0	5	100.00	2
6	6	0	0.00	2
6	4	2	33.33	1
6	3	3	50.00	1
6	2	4	66.67	3
6	1	5	83.33	2
6	0	6	100.00	2
7	7	0	0.00	1
7	5	2	28.57	3
7	4	3	42.86	2
7	3	4	57.14	1
7	2	5	71.43	1
7	1	6	85.71	2
7	0	7	100.00	3
8	8	0	0.00	1
8	7	1	12.50	2
8	6	2	25.00	1
8	4	4	50.00	2
8	2	6	75.00	1
8	1	7	87.50	1
8	0	8	100.00	1
9	7	2	22.22	1
9	4	5	55.56	2
9	3	6	66.67	3
10	7	3	30.00	2
10	6	4	40.00	1
10	4	6	60.00	1
10	1	9	90.00	1
10	0	10	100.00	3
11	8	3	27.27	1
11	2	9	81.82	1
11	0	11	100.00	2
12	12	0	0.00	1

Control clones
O4 expression
Oligodendrocyte media (page 2)

Clone size	O4 negative	# of O4+ cells	%O4+	Number of Clones
12	11	1	8.33	1
12	8	4	33.33	3
12	7	5	41.67	1
12	6	6	50.00	1
13	13	0	0.00	1
13	8	5	38.46	1
13	2	11	84.62	1
13	1	12	92.31	1
14	12	2	14.29	1
14	5	9	64.29	1
14	4	10	71.43	1
14	3	11	78.57	1
14	2	12	85.71	1
16	16	0	0.00	1
16	6	10	62.50	1
16	2	14	87.50	1
17	15	2	11.76	1
17	4	13	76.47	1
19	19	0	0.00	1
20	6	14	70.00	1
22	18	4	18.18	1
22	6	16	72.73	1
23	14	9	39.13	1
23	7	16	69.57	1
23	4	19	82.61	1
23	0	23	100.00	1
24	19	5	20.83	1
24	6	18	75.00	1
24	5	19	79.17	2
24	4	20	83.33	1
25	7	18	72.00	1
25	4	21	84.00	1
26	17	9	34.62	1
26	14	12	46.15	1
27	7	20	74.07	1
27	4	23	85.19	1
28	15	13	46.43	1
29	14	15	51.72	1
29	0	29	100.00	1
30	19	11	36.67	1
31	4	27	87.10	1
32	23	9	28.13	1
32	18	14	43.75	1
32	9	23	71.88	1
32	5	27	84.38	1
39	0	39	100.00	1
44	27	17	38.64	1
48	48	0	0.00	1
56	7	49	87.50	1
68	67	1	1.47	1
68	31	37	54.41	1
Total number of clones counted:				167

DnREST clones
O4 expression
Oligodendrocyte media (page 1)

Clone size	# of O4– cells	# of O4+ cells	%O4+	Number of clones
1	1	0	0.00	10
1	0	1	100.00	5
2	2	0	0.00	19
2	1	1	50.00	5
2	0	2	100.00	8
3	3	0	0.00	5
3	2	1	33.33	3
3	1	2	66.67	2
3	0	3	100.00	6
4	4	0	0.00	4
4	3	1	25.00	5
4	1	3	75.00	3
4	0	4	100.00	3
5	5	0	0.00	6
5	4	1	20.00	2
5	3	2	40.00	2
5	2	3	60.00	1
5	0	5	100.00	2
6	6	0	0.00	6
6	5	1	16.67	1
6	4	2	33.33	1
6	3	3	50.00	1
6	0	6	100.00	4
7	7	0	0.00	3
7	5	2	28.57	1
7	4	3	42.86	1
7	3	4	57.14	1
7	2	5	71.43	1
7	0	7	100.00	1
8	8	0	0.00	3
8	7	1	12.50	1
8	0	8	100.00	2
9	9	0	0.00	2
9	8	1	11.11	2
9	2	7	77.78	1
9	0	9	100.00	2
10	10	0	0.00	1
10	6	4	40.00	1
11	9	2	18.18	2
11	8	3	27.27	2
11	7	4	36.36	1
11	5	6	54.55	1
11	3	8	72.73	1
11	0	11	100.00	1
12	12	0	0.00	1
12	8	4	33.33	1
12	7	5	41.67	1
12	4	8	66.67	1
12	3	9	75.00	1
13	13	0	0.00	1

DnREST clones
O4 expression
Oligodendrocyte media (page 2)

Clone size	O4 negative	# of O4+ cells	%O4+	Number of Clones
13	11	2	15.38	1
14	5	9	64.29	1
14	2	12	85.71	1
15	8	7	46.67	1
16	16	0	0.00	1
16	9	7	43.75	1
18	17	1	5.56	1
18	15	3	16.67	1
18	11	7	38.89	1
19	16	3	15.79	1
19	6	13	68.42	1
21	17	4	19.05	1
22	4	18	81.82	2
23	15	8	34.78	1
26	19	7	26.92	1
26	12	14	53.85	1
30	23	7	23.33	1
36	20	16	44.44	1
Total number of clones counted:				160

Control clones
MBP expression
Oligodendrocyte media

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
2	0	2	100.00	1
3	2	1	33.33	1
4	4	0	0.00	1
4	0	4	100.00	1
8	7	1	12.50	1
9	8	1	11.11	1
11	11	0	0.00	1
12	12	0	0.00	2
14	13	1	7.14	1
17	6	11	64.71	1
22	22	0	0.00	1
22	19	3	13.64	1
23	16	7	30.43	1
24	8	16	66.67	1
26	19	7	26.92	1
28	17	11	39.29	1
29	26	3	10.34	1
30	29	1	3.33	1
33	33	0	0.00	1
33	19	14	42.42	1
34	31	3	8.82	1
36	36	0	0.00	1
36	29	7	19.44	1
37	37	0	0.00	1
37	23	14	37.84	1
40	32	8	20.00	1
42	42	0	0.00	1
42	33	9	21.43	1
43	42	1	2.33	1
43	27	16	37.21	1
44	18	26	59.09	1
45	27	18	40.00	1
46	46	0	0.00	1
48	47	1	2.08	1
51	37	14	27.45	1
51	34	17	33.33	1
52	52	0	0.00	1
52	43	9	17.31	1
54	25	29	53.70	1
56	43	13	23.21	1
61	61	0	0.00	1
61	47	14	22.95	1
61	19	42	68.85	1
69	64	5	7.25	1
Total number of clones counted:				45

DnREST clones
MBP expression
Oligodendrocyte media

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
2	2	0	0.00	3
2	0	2	100.00	1
3	3	0	0.00	1
4	4	0	0.00	3
4	3	1	25.00	2
7	6	1	14.29	2
8	8	0	0.00	2
11	11	0	0.00	1
13	13	0	0.00	2
14	14	0	0.00	3
14	13	1	7.14	1
16	16	0	0.00	2
18	18	0	0.00	1
18	17	1	5.56	1
18	15	3	16.67	1
22	18	4	18.18	1
26	26	0	0.00	1
28	28	0	0.00	1
28	25	3	10.71	1
28	24	4	14.29	1
28	23	5	17.86	1
29	29	0	0.00	1
30	27	3	10.00	1
33	33	0	0.00	1
33	31	2	6.06	1
34	12	22	64.71	1
36	33	3	8.33	1
38	38	0	0.00	1
38	35	3	7.89	1
39	37	2	5.13	1
41	39	2	4.88	1
41	37	4	9.76	1
48	45	3	6.25	1
59	56	3	5.08	1
Total number of clones counted:				45

Control clones
TUJ1 expression
Oligodendrocyte media

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
2	2	0	0.00	8
3	3	0	0.00	5
4	4	0	0.00	5
5	5	0	0.00	1
6	6	0	0.00	3
7	7	0	0.00	3
7	6	1	14.29	1
8	8	0	0.00	3
9	9	0	0.00	1
10	10	0	0.00	1
11	11	0	0.00	1
12	12	0	0.00	3
13	13	0	0.00	2
14	14	0	0.00	1
15	15	0	0.00	1
16	16	0	0.00	1
17	17	0	0.00	1
18	18	0	0.00	2
20	20	0	0.00	1
20	19	1	5.00	1
21	21	0	0.00	3
22	22	0	0.00	2
23	23	0	0.00	1
23	22	1	4.35	1
26	26	0	0.00	1
29	29	0	0.00	1
35	35	0	0.00	1
38	37	1	2.63	1
39	39	0	0.00	1
58	58	0	0.00	1
Total number of clones counted:				58

DnREST clones
TUJ1 expression
Oligodendrocyte media

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
1	0	1	100.00	6
2	2	0	0.00	3
2	1	1	50.00	3
2	0	2	100.00	5
3	3	0	0.00	4
3	2	1	33.33	3
3	1	2	66.67	4
4	4	0	0.00	4
4	1	3	75.00	1
6	6	0	0.00	1
6	5	1	16.67	2
7	6	1	14.29	2
7	2	5	71.43	1
8	6	2	25.00	1
8	5	3	37.50	2
10	10	0	0.00	2
11	11	0	0.00	1
11	9	2	18.18	1
12	12	0	0.00	1
12	11	1	8.33	1
14	14	0	0.00	2
16	16	0	0.00	1
18	15	3	16.67	1
23	21	2	8.70	1
29	28	1	3.45	1
Total number of clones counted:				54

Control clones
O4 expression
Stem cell media (page 1)

Clone size	# of O4– cells	# of O4+ cells	%O4+	Number of clones
1	1	0	0.00	3
1	0	1	100.00	1
2	2	0	0.00	4
2	0	2	100.00	2
3	3	0	0.00	2
3	1	2	66.67	1
4	4	0	0.00	2
4	2	2	50.00	2
4	1	3	75.00	1
4	0	4	100.00	3
5	5	0	0.00	1
5	3	2	40.00	1
6	6	0	0.00	3
6	3	3	50.00	1
7	7	0	0.00	2
7	6	1	14.29	1
7	3	4	57.14	1
7	2	5	71.43	1
7	0	7	100.00	1
8	8	0	0.00	1
8	5	3	37.50	1
8	0	8	100.00	2
9	9	0	0.00	3
10	2	8	80.00	1
11	3	8	72.73	1
11	1	10	90.91	1
12	10	2	16.67	1
12	9	3	25.00	1
12	0	12	100.00	1
13	9	4	30.77	1
13	6	7	53.85	1
13	4	9	69.23	1
14	14	0	0.00	1
14	9	5	35.71	1
14	2	12	85.71	2
15	15	0	0.00	1
15	13	2	13.33	1
15	7	8	53.33	1
15	0	15	100.00	1
16	12	4	25.00	1
16	8	8	50.00	1
16	2	14	87.50	1
17	16	1	5.88	1
17	15	2	11.76	1
17	6	11	64.71	1
18	18	0	0.00	1
18	13	5	27.78	2
18	10	8	44.44	2
18	1	17	94.44	1
19	12	7	36.84	1

Control clones
O4 expression
Stem cell media (page 2)

Clone size	O4 negative	# of O4+ cells	%O4+	Number of Clones
19	2	17	89.47	1
20	16	4	20.00	1
21	17	4	19.05	1
21	8	13	61.90	2
22	16	6	27.27	1
23	7	16	69.57	1
24	15	9	37.50	1
24	13	11	45.83	1
25	14	11	44.00	2
25	6	19	76.00	1
26	17	9	34.62	1
27	19	8	29.63	2
28	28	0	0.00	2
33	22	11	33.33	2
34	25	9	26.47	1
36	25	11	30.56	1
36	15	21	58.33	1
38	29	9	23.68	1
39	12	27	69.23	1
41	27	14	34.15	1
41	25	16	39.02	1
42	24	18	42.86	1
44	12	32	72.73	1
45	25	20	44.44	2
47	41	6	12.77	1
47	33	14	29.79	1
49	49	0	0.00	1
49	39	10	20.41	1
54	48	6	11.11	1
55	44	11	20.00	1
56	50	6	10.71	1
59	48	11	18.64	1
Total number of clones counted:				108

DnREST clones
O4 expression
Stem cell media (page 1)

Clone size	# of O4– cells	# of O4+ cells	%O4+	Number of clones
1	1	0	0.00	13
1	0	1	100.00	3
2	2	0	0.00	15
2	1	1	50.00	3
2	0	2	100.00	4
3	3	0	0.00	7
3	2	1	33.33	1
4	4	0	0.00	10
4	3	1	25.00	1
4	1	3	75.00	2
4	0	4	100.00	4
5	5	0	0.00	4
5	3	2	40.00	1
5	0	5	100.00	1
6	6	0	0.00	3
6	5	1	16.67	1
6	4	2	33.33	2
6	2	4	66.67	1
6	0	6	100.00	2
7	7	0	0.00	1
7	6	1	14.29	1
7	0	7	100.00	1
8	8	0	0.00	2
8	2	6	75.00	2
8	0	8	100.00	1
9	9	0	0.00	2
9	8	1	11.11	1
9	7	2	22.22	1
9	6	3	33.33	1
9	2	7	77.78	1
10	8	2	20.00	1
10	4	6	60.00	1
11	11	0	0.00	1
11	0	11	100.00	1
12	10	2	16.67	1
13	13	0	0.00	1
13	10	3	23.08	1
14	14	0	0.00	1
14	10	4	28.57	1
14	8	6	42.86	1
16	16	0	0.00	1
18	18	0	0.00	1
19	16	3	15.79	1
20	14	6	30.00	1
21	17	4	19.05	1
24	22	2	8.33	1
25	24	1	4.00	1
25	2	23	92.00	1
26	23	3	11.54	1
26	21	5	19.23	1

DnREST clones
O4 expression
Stem cell media (page 2)

Clone size	O4 negative	# of O4+ cells	%O4+	Number of Clones
28	28	0	0.00	1
30	28	2	6.67	1
31	29	2	6.45	1
48	48	0	0.00	1
Total number of clones counted:				116

Control clones
MBP expression
Stem cell media (page 1)

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
1	1	0	0.00	4
2	2	0	0.00	7
3	3	0	0.00	6
3	2	1	33.33	2
4	4	0	0.00	7
4	3	1	25.00	2
4	0	4	100.00	1
5	5	0	0.00	4
5	4	1	20.00	2
6	6	0	0.00	5
6	4	2	33.33	1
9	9	0	0.00	5
9	8	1	11.11	1
10	10	0	0.00	1
10	8	2	20.00	1
11	11	0	0.00	4
11	9	2	18.18	1
12	12	0	0.00	2
12	9	3	25.00	1
14	14	0	0.00	4
14	10	4	28.57	1
15	15	0	0.00	1
15	13	2	13.33	1
15	12	3	20.00	1
16	13	3	18.75	1
16	5	11	68.75	1
17	17	0	0.00	2
17	16	1	5.88	1
18	18	0	0.00	5
18	17	1	5.56	2
18	16	2	11.11	1
18	15	3	16.67	3
18	14	4	22.22	2
18	12	6	33.33	1
19	19	0	0.00	1
19	18	1	5.26	2
19	16	3	15.79	2
19	12	7	36.84	2
21	21	0	0.00	2
21	19	2	9.52	1
22	22	0	0.00	2
22	21	1	4.55	1
22	19	3	13.64	2
22	13	9	40.91	1
23	23	0	0.00	3
23	21	2	8.70	1
23	19	4	17.39	1
23	17	6	26.09	1
24	24	0	0.00	5
24	23	1	4.17	1

Control clones
MBP expression
Stem cell media (page 2)

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
24	22	2	8.33	1
26	26	0	0.00	3
26	24	2	7.69	1
26	23	3	11.54	1
26	22	4	15.38	1
27	27	0	0.00	2
27	11	16	59.26	1
28	28	0	0.00	2
28	27	1	3.57	2
28	19	9	32.14	2
28	16	12	42.86	1
28	15	13	46.43	1
29	29	0	0.00	2
29	26	3	10.34	2
29	23	6	20.69	1
29	7	22	75.86	1
30	26	4	13.33	1
30	23	7	23.33	1
32	32	0	0.00	2
32	28	4	12.50	1
32	24	8	25.00	1
33	33	0	0.00	3
33	32	1	3.03	2
33	31	2	6.06	1
33	28	5	15.15	1
34	33	1	2.94	1
35	27	8	22.86	1
35	25	10	28.57	1
36	36	0	0.00	4
36	33	3	8.33	1
36	32	4	11.11	1
36	18	18	50.00	1
38	38	0	0.00	5
38	34	4	10.53	1
38	32	6	15.79	1
38	31	7	18.42	2
38	20	18	47.37	1
39	39	0	0.00	2
39	38	1	2.56	1
39	28	11	28.21	1
41	38	3	7.32	1
41	34	7	17.07	1
41	31	10	24.39	1
41	28	13	31.71	1
41	27	14	34.15	1
42	42	0	0.00	3
42	39	3	7.14	3
42	34	8	19.05	1
42	29	13	30.95	1
43	43	0	0.00	2

Control clones
MBP expression
Stem cell media (page 3)

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
43	42	1	2.33	2
43	36	7	16.28	1
44	44	0	0.00	1
44	31	13	29.55	1
45	31	14	31.11	1
46	46	0	0.00	2
46	45	1	2.17	1
46	42	4	8.70	1
46	38	8	17.39	2
47	47	0	0.00	2
47	41	6	12.77	1
47	30	17	36.17	2
48	48	0	0.00	2
48	47	1	2.08	1
48	45	3	6.25	1
48	40	8	16.67	1
49	49	0	0.00	1
49	45	4	8.16	2
50	47	3	6.00	1
50	37	13	26.00	1
51	51	0	0.00	4
51	49	2	3.92	1
51	47	4	7.84	1
51	45	6	11.76	1
51	43	8	15.69	1
51	40	11	21.57	1
51	27	24	47.06	1
52	43	9	17.31	2
53	53	0	0.00	2
53	52	1	1.89	1
53	49	4	7.55	1
53	46	7	13.21	2
54	54	0	0.00	2
56	54	2	3.57	1
57	55	2	3.51	1
58	58	0	0.00	2
59	55	4	6.78	1
60	49	11	18.33	1
61	61	0	0.00	1
62	57	5	8.06	1
63	63	0	0.00	2
63	60	3	4.76	1
67	63	4	5.97	1
69	67	2	2.90	1
73	64	9	12.33	1
83	71	12	14.46	1
93	87	6	6.45	1
Total number of clones counted:				251

DnREST clones
MBP expression
Stem cell media (page 1)

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
1	1	0	0.00	3
1	0	1	100.00	1
2	2	0	0.00	9
2	1	1	50.00	1
3	3	0	0.00	12
3	2	1	33.33	1
4	4	0	0.00	17
4	3	1	25.00	1
5	5	0	0.00	5
6	6	0	0.00	10
6	2	4	66.67	1
6	1	5	83.00	1
7	7	0	0.00	8
8	8	0	0.00	15
8	7	1	12.50	1
9	9	0	0.00	3
10	10	0	0.00	4
11	11	0	0.00	9
12	12	0	0.00	10
12	11	1	8.33	1
12	2	10	83.33	1
13	13	0	0.00	7
13	4	9	69.23	1
14	14	0	0.00	8
15	15	0	0.00	5
16	16	0	0.00	12
16	15	1	6.25	4
16	14	2	12.50	1
17	17	0	0.00	2
17	16	1	5.88	1
17	15	2	11.76	1
17	13	4	23.53	1
18	18	0	0.00	11
18	17	1	5.56	1
18	16	2	11.11	1
18	15	3	16.67	1
19	19	0	0.00	3
19	18	1	5.26	2
20	18	2	10.00	1
21	21	0	0.00	7
21	19	2	9.52	2
21	18	3	14.29	1
21	17	4	19.05	2
22	22	0	0.00	1
22	18	4	18.18	1
22	16	6	27.27	1
23	23	0	0.00	3
23	15	8	34.78	1
24	24	0	0.00	4
24	23	1	4.17	1

DnREST clones
MBP expression
Stem cell media (page 2)

Clone size	# of MBP– cells	# of MBP+ cells	%MBP+	Number of Clones
25	25	0	0.00	2
26	26	0	0.00	3
26	25	1	3.85	1
26	23	3	11.54	1
26	19	7	26.92	1
28	28	0	0.00	3
28	25	3	10.71	1
29	29	0	0.00	1
29	28	1	3.45	1
29	22	7	24.14	1
30	30	0	0.00	1
31	31	0	0.00	1
31	30	1	3.23	1
32	32	0	0.00	4
33	33	0	0.00	1
34	34	0	0.00	1
34	31	3	8.82	2
36	36	0	0.00	3
36	35	1	2.78	1
37	37	0	0.00	2
38	38	0	0.00	2
39	39	0	0.00	2
41	41	0	0.00	3
42	42	0	0.00	1
43	43	0	0.00	1
43	41	2	4.65	1
46	46	0	0.00	2
46	45	1	2.17	1
48	48	0	0.00	1
48	37	11	22.92	1
52	52	0	0.00	1
53	52	1	1.89	1
54	52	2	3.70	1
Total number of clones counted:				251

Control clones
TUJ1 expression
Stem cell media (page 1)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
2	2	0	0.00	3
4	4	0	0.00	2
5	5	0	0.00	2
6	6	0	0.00	4
7	7	0	0.00	2
8	8	0	0.00	5
9	9	0	0.00	7
10	10	0	0.00	3
11	11	0	0.00	2
12	12	0	0.00	3
13	13	0	0.00	1
14	14	0	0.00	2
16	16	0	0.00	6
16	15	1	6.25	1
17	17	0	0.00	8
18	18	0	0.00	2
18	17	1	5.56	1
19	19	0	0.00	4
21	21	0	0.00	1
22	22	0	0.00	1
23	23	0	0.00	3
24	24	0	0.00	2
26	26	0	0.00	3
27	27	0	0.00	3
27	26	1	3.70	1
28	28	0	0.00	4
29	29	0	0.00	2
31	31	0	0.00	2
33	33	0	0.00	2
35	34	1	2.86	1
36	36	0	0.00	1
37	37	0	0.00	3
38	38	0	0.00	3
39	39	0	0.00	3
41	41	0	0.00	2
42	42	0	0.00	2
42	40	2	4.76	1
43	43	0	0.00	5
44	44	0	0.00	3
47	47	0	0.00	1
48	48	0	0.00	3
48	47	1	2.08	1
49	49	0	0.00	2
50	50	0	0.00	1
51	51	0	0.00	4
52	52	0	0.00	2
54	54	0	0.00	1
57	57	0	0.00	2
58	58	0	0.00	1
59	59	0	0.00	2

Control clones
TUJ1 expression
Stem cell media (page 2)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
60	59	1	1.67	1
62	62	0	0.00	1
63	63	0	0.00	1
64	64	0	0.00	2
67	67	0	0.00	1
69	69	0	0.00	1
71	71	0	0.00	1
72	71	1	1.39	1
81	81	0	0.00	1
Total number of clones counted:				136

DnREST clones
TUJ1 expression
Stem cell media (page 1)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
1	0	1	100.00	1
2	2	0	0.00	1
2	1	1	50.00	1
2	0	2	100.00	7
3	3	0	0.00	2
3	2	1	33.33	1
3	0	3	100.00	3
4	4	0	0.00	5
4	0	4	100.00	3
5	5	0	0.00	2
5	2	3	60.00	1
5	0	5	100.00	3
6	6	0	0.00	4
6	5	1	16.67	1
6	2	4	66.67	1
6	0	6	100.00	1
7	7	0	0.00	2
7	6	1	14.29	2
7	0	7	100.00	3
8	8	0	0.00	2
8	6	2	25.00	2
9	5	4	44.44	1
9	3	6	66.67	1
9	2	7	77.78	1
9	0	9	100.00	1
10	10	0	0.00	1
10	8	2	20.00	1
11	11	0	0.00	1
11	9	2	18.18	1
11	6	5	45.45	1
11	5	6	54.55	1
11	0	11	100.00	1
12	12	0	0.00	4
12	9	3	25.00	1
12	0	12	100.00	2
13	9	4	30.77	1
13	7	6	46.15	1
13	4	9	69.23	1
14	14	0	0.00	3
14	10	4	28.57	1
14	7	7	50.00	1
15	15	0	0.00	1
15	13	2	13.33	1
15	7	8	53.33	1
16	16	0	0.00	3
16	15	1	6.25	1
16	9	7	43.75	1
17	17	0	0.00	1
17	2	15	88.24	1
18	18	0	0.00	3

DnREST clones
TUJ1 expression
Stem cell media (page 2)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
18	14	4	22.22	1
18	11	7	38.89	1
18	8	10	55.56	1
19	19	0	0.00	2
19	5	14	73.68	1
19	4	15	78.95	1
19	3	16	84.21	1
20	19	1	5.00	1
22	22	0	0.00	2
22	12	10	45.45	1
22	3	19	86.36	1
23	23	0	0.00	1
23	19	4	17.39	1
23	4	19	82.61	1
23	0	23	100.00	1
24	20	4	16.67	1
24	10	14	58.33	1
26	26	0	0.00	1
26	21	5	19.23	1
26	16	10	38.46	1
26	11	15	57.69	1
27	20	7	25.93	1
27	5	22	81.48	1
28	17	11	39.29	1
28	16	12	42.86	1
28	11	17	60.71	1
29	18	11	37.93	1
29	9	20	68.97	1
30	29	1	3.33	1
30	1	29	96.67	1
31	31	0	0.00	1
31	21	10	32.26	1
31	11	20	64.52	1
32	32	0	0.00	1
32	28	4	12.50	1
32	27	5	15.63	1
33	33	0	0.00	1
33	23	10	30.30	1
34	24	10	29.41	1
36	18	18	50.00	2
38	36	2	5.26	1
39	12	27	69.23	1
41	33	8	19.51	1
41	27	14	34.15	1
41	24	17	41.46	1
42	36	6	14.29	1
42	15	27	64.29	1
43	35	8	18.60	1
44	24	20	45.45	1
46	46	0	0.00	2

DnREST clones
TUJ1 expression
Stem cell media (page 3)

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
46	40	6	13.04	1
47	23	24	51.06	1
47	7	40	85.11	1
49	49	0	0.00	1
67	26	41	61.19	1
Total number of clones counted:				146

Control clones
GFAP expression
2A media (page 1)

Clone size	# of GFAP– cells	# of GFAP+ cells	%GFAP+	Number of clones
1	1	0	0.00	2
1	0	1	100.00	5
2	2	0	0.00	4
2	1	1	50.00	10
2	0	2	100.00	14
3	3	0	0.00	4
3	2	1	33.33	3
3	1	2	66.67	3
3	0	3	100.00	13
4	4	0	0.00	10
4	3	1	25.00	4
4	2	2	50.00	2
4	1	3	75.00	3
4	0	4	100.00	9
5	5	0	0.00	4
5	4	1	20.00	2
5	3	2	40.00	4
5	2	3	60.00	7
5	1	4	80.00	7
5	0	5	100.00	15
6	6	0	0.00	3
6	4	2	33.33	3
6	2	4	66.67	3
6	1	5	83.33	8
6	0	6	100.00	3
7	5	2	28.57	1
7	4	3	42.86	4
7	3	4	57.14	1
7	2	5	71.43	3
7	1	6	85.71	3
7	0	7	100.00	5
8	8	0	0.00	1
8	7	1	12.50	3
8	6	2	25.00	3
8	5	3	37.50	2
8	3	5	62.50	4
8	2	6	75.00	4
8	1	7	87.50	3
8	0	8	100.00	6
9	5	4	44.44	2
9	4	5	55.56	2
9	3	6	66.67	4
9	2	7	77.78	4
9	0	9	100.00	5
10	7	3	30.00	2
10	5	5	50.00	1
10	4	6	60.00	2
10	3	7	70.00	1
10	1	9	90.00	2
10	0	10	100.00	2

Control clones
GFAP expression
2A media (page 2)

Clone size	# of GFAP– cells	# of GFAP+ cells	%O4+	Number of clones
11	11	0	0.00	1
11	6	5	45.45	1
11	4	7	63.64	1
11	3	8	72.73	1
12	10	2	16.67	1
12	6	6	50.00	2
12	4	8	66.67	1
12	3	9	75.00	3
12	2	10	83.33	2
12	0	12	100.00	2
13	11	2	15.38	1
13	6	7	53.85	3
13	4	9	69.23	1
14	12	2	14.29	1
14	6	8	57.14	1
14	5	9	64.29	2
14	4	10	71.43	1
14	3	11	78.57	2
14	1	13	92.86	1
14	0	14	100.00	2
15	6	9	60.00	1
15	5	10	66.67	1
15	2	13	86.67	2
15	0	15	100.00	1
16	7	9	56.25	1
16	5	11	68.75	1
16	0	16	100.00	1
17	6	11	64.71	1
19	15	4	21.05	2
19	4	15	78.95	1
19	2	17	89.47	1
19	0	19	100.00	2
20	2	18	90.00	1
22	11	11	50.00	1
22	5	17	77.27	1
23	3	20	86.96	1
24	2	22	91.67	1
24	1	23	95.83	1
24	0	24	100.00	1
28	16	12	42.86	1
30	16	14	46.67	1
32	2	30	93.75	1
35	23	12	34.29	1
36	2	34	94.44	2
Total number of clones counted:				273

DnREST clones
GFAP expression
2A media (page 1)

Clone size	# of GFAP– cells	# of GFAP+ cells	%GFAP+	Number of clones
1	1	0	0.00	26
1	0	1	100.00	8
2	2	0	0.00	30
2	1	1	50.00	10
2	0	2	100.00	25
3	3	0	0.00	19
3	2	1	33.33	8
3	1	2	66.67	7
3	0	3	100.00	13
4	4	0	0.00	9
4	3	1	25.00	11
4	2	2	50.00	9
4	1	3	75.00	7
4	0	4	100.00	13
5	5	0	0.00	5
5	4	1	20.00	4
5	3	2	40.00	2
5	2	3	60.00	3
5	1	4	80.00	3
5	0	5	100.00	4
6	6	0	0.00	3
6	4	2	33.33	4
6	3	3	50.00	5
6	2	4	66.67	4
6	1	5	83.33	3
6	0	6	100.00	2
7	6	1	14.29	2
7	4	3	42.86	1
7	2	5	71.43	2
7	1	6	85.71	2
7	0	7	100.00	2
8	8	0	0.00	2
8	4	4	50.00	1
8	3	5	62.50	1
8	2	6	75.00	1
8	1	7	87.50	1
8	0	8	100.00	3
9	4	5	55.56	2
10	10	0	0.00	2
10	4	6	60.00	1
10	3	7	70.00	2
11	4	7	63.64	1
11	0	11	100.00	2
13	9	4	30.77	1
13	7	6	46.15	2
13	6	7	53.85	1
13	1	12	92.31	1
14	14	0	0.00	1
14	4	10	71.43	1
14	0	14	100.00	1

DnREST clones
GFAP expression
2A media (page 2)

Clone size	# of GFAP– cells	# of GFAP+ cells	%O4+	Number of clones
16	0	16	100.00	1
17	0	17	100.00	1
18	4	14	77.78	2
18	3	15	83.33	1
25	9	16	64.00	1
Total number of clones counted:				279

Control clones
TUJ1 expression
2A media

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
1	1	0	0.00	13
1	0	1	100.00	2
2	2	0	0.00	28
2	1	1	50.00	3
3	3	0	0.00	14
4	4	0	0.00	16
5	5	0	0.00	14
5	4	1	20.00	3
7	7	0	0.00	28
7	6	1	14.29	1
8	8	0	0.00	16
8	7	1	12.50	2
8	4	4	50.00	1
9	9	0	0.00	7
9	8	1	11.11	2
10	10	0	0.00	5
11	11	0	0.00	6
12	12	0	0.00	7
13	13	0	0.00	4
13	11	2	15.38	1
14	14	0	0.00	5
14	13	1	7.14	1
15	15	0	0.00	2
16	16	0	0.00	2
17	17	0	0.00	1
18	18	0	0.00	4
18	17	1	5.56	1
18	15	3	16.67	1
19	19	0	0.00	2
21	21	0	0.00	1
22	22	0	0.00	3
23	23	0	0.00	1
24	24	0	0.00	2
27	27	0	0.00	1
28	27	1	3.57	1
30	27	3	10.00	1
Total number of clones counted:				202

DnREST clones
TUJ1 expression
2A media

Clone size	# of TUJ1– cells	# of TUJ1+ cells	%TUJ1+	Number of Clones
1	1	0	0.00	5
1	0	1	100.00	37
2	2	0	0.00	8
2	1	1	50.00	11
2	0	2	100.00	31
3	3	0	0.00	6
3	2	1	33.33	5
3	1	2	66.67	3
3	0	3	100.00	16
4	4	0	0.00	5
4	3	1	25.00	2
4	2	2	50.00	3
4	0	4	100.00	11
5	5	0	0.00	2
5	4	1	20.00	1
5	2	3	60.00	3
5	1	4	80.00	2
5	0	5	100.00	7
6	6	0	0.00	2
6	4	2	33.33	1
6	1	5	83.33	1
6	0	6	100.00	4
7	7	0	0.00	1
7	3	4	57.14	2
7	2	5	71.43	1
8	4	4	50.00	2
8	3	5	62.50	1
8	1	7	87.50	1
8	0	8	100.00	4
9	8	1	11.11	1
9	0	9	100.00	2
10	0	10	100.00	1
12	12	0	0.00	1
12	3	9	75.00	2
12	1	11	91.67	1
13	11	2	15.38	1
14	8	6	42.86	2
17	6	11	64.71	1
19	11	8	42.11	1
19	0	19	100.00	1
21	13	8	38.10	1
22	19	3	13.64	1
Total number of clones counted:				194