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**14. ABSTRACT**

The goals of our work are to develop means of identifying cells, and individuals, that present with a more basal oxidized redox state and to identify molecular mechanisms that functionally integrate such an oxidized state with observations that the multiple environmental insults suggested to be involved in autism pathogenesis also occur in many children that do not develop ASD. This suggests there is an underlying vulnerability phenotype that makes some children more vulnerable to such stressors. The regulatory pathway that we discovered to be central to redox-based modulation of cell division, differentiation and survival affects multiple cellular functions that appear to be related to the pathological changes seen in the CNS of children with autism. Moreover, the integration of these findings with the Nrf2 pathway provides mechanisms that may provide better markers of a more oxidative state and have the added value of explaining why the more oxidized cells of an individual with ASD do not reset themselves to create a normal redox balance. These discoveries provide new targets for resetting these metabolic problems, and continuation of this work looks likely to identify agents that could be used to address the oxidative abnormalities that look increasingly likely to be relevant to the understanding of ASD pathogenesis.

**15. SUBJECT TERMS**
Redox; intracellular redox state; glutathione; brain; lymphocytes; c-Met; dendritic complexity; hippocampus; Fyn; c-Cbl; redox/Fyn/c-Cbl pathway; receptor tyrosine kinase; Nrf2; Keap 1; oligodendrocyte progenitor cell

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## 1. Introduction

This is the final summary project report for Project 2 of grant W81XWH-08-1-0702.

This report both summarizes and integrates the work enabled by our DOD funding within a much larger realm of research that was inspired by this funding and that I think has placed us in a much firmer position to investigate and understand metabolic contributions to the pathogenesis of autism spectrum disorders (ASDs) and also to other childhood developmental disorders. The support provided by the DOD enabled studies to be conducted on a specific set of questions that we proposed, and the reviewers agreed, would shed new light on ASD pathogenesis and risk factors. Moreover, the DOD funding greatly enhanced the success of parallel efforts and opened up new ways of approaching multiple important diseases. Indeed, analysis of the problem of disease-related vulnerabilities to physiological stressors has set the stage disease-related insights of potentially very broad relevance (as discussed in Reportable Outcomes).

As will be seen, this report provides a detailed synthesis of our work that details the research findings, their possible relevance to the understanding of ASD pathogenesis and the implications of our discoveries for future studies on this important problem. Thus, we report studies carried out in regards to the specific aims of the DOD-supported research, and also have taken care to indicate where synergy with other efforts helped us to better achieve the goals of the DOD-funded research.

The initial goals of this project were to develop means of identifying cells, and individuals, that present with a more basal oxidized redox state and to identify molecular mechanisms that functionally integrate such an oxidized state with observations that the multiple environmental insults suggested to be involved in autism pathogenesis also occur in many children that do not develop ASD. This suggests there is an underlying vulnerability phenotype that makes some children more vulnerable to such stressors. The regulatory pathway that we discovered to be central to redox-based modulation of cell division, differentiation and survival affects multiple cellular functions that appear to be related to the pathological changes seen in the CNS of children with autism. Moreover, the integration of these findings with analysis of the Nrf2 pathway provides mechanisms that may provide better markers of a more oxidative state and has the added value of explaining why the more oxidized cells of an individual with ASD do not reset themselves to create a normal redox balance.

As this work progressed, and parallel work inspired by our findings progressed, it became critical to understand what roles these redox-related pathways were playing in normal development in order to understand how redox abnormalities might lead to developmental abnormalities. This was necessary because there is an enormous difference between the implications of creating a pathogenic process that shifts the set points of normal development in ways that are inappropriate but does not invoke novel mechanisms and pathogenic processes that are founded in true disruptions of normal development in which pathology occurs due to the introduction of regulatory misinformation of a type that does not normally exist (such as occurs in severe mutation-caused diseases of development).

Our work was successful in identifying points of intersection with development that resulted from alterations in the normal physiological process of redox regulation. It was critical in this work to understand whether our analysis of oxidative regulation of cell function represented a general

paradigm for studying the problem of metabolic contributions to normal and pathological development. What would be the next example of such a regulator? As will be discussed, a metabolic intervention examined in parallel with our own experiments led to surprising insights into the possible importance of micronutrient deficiencies as contributory factors to CNS changes that occur in the brains of children with autism. Moreover, the interplay between this micronutrient deficiency (which is iron deficiency in the absence of anemia) and oxidizing insults generated by exposure to environmental toxicants is opening up rich new areas for study.

In brief, our work demonstrated that cells can indeed have different basal redox set points, we discovered a molecular mechanism that modulates such differences, found that these differences modulate responsiveness to inducers of differentiation and further discovered molecular mechanisms that translate changes in oxidative status into cell cycle exit and differentiation of oligodendrocyte progenitor cells (OPCs).

These discoveries offer specific predictions about development in children that may have an abnormally oxidized metabolic status, which is that they would show increased myelination early and a reduction in age-appropriate myelination. Our findings further predict that children with an increased oxidative status will show decreased dendritic complexity.

We further provide data that predictions derived by knowledge of the molecular mechanism that regulates differentiation in OPCs also successfully predicted in vivo reductions in levels of a key regulator of dendritic complexity in the hippocampus, and further successfully predicts reductions in dendritic complexity in mice that are more oxidized.

Thus, this research revealed multiple means by which increased oxidative status (which is typical of children with ASDs) can alter normal development in the CNS. Moreover, through interaction with colleagues working on other potential insults to the developing CNS, it was possible to encourage their interest in working on ASD-related questions. This interaction, which was also an important outcome of our DOD funding, has led to new insights into how iron deficiency in the absence of anemia during early gestation might be an important risk factor for pathogenesis of ASDs (see Section 4.7 for a brief overview of these parallel findings).

Application of these findings to the understanding of ASD pathogenesis predicts that abnormalities in oxidation occurring during gestation and early development can cause developmental abnormalities such as occur in ASDs, and that detection and treatment of such abnormalities early may provide a means of reducing the frequency of these disorders. Moreover, our results suggest earlier initiation of such treatments would be predicted to have greater benefit by enabling subsequent development to occur in an appropriate redox environment.

## 2. BODY

The original aims of this project, and the Sections in which they were addressed, are as follows:

***Project 2,*** *Aim 1: Analysis of the correlation between redox abnormalities in cells of the peripheral blood and cells of the developing brain*

- a) DoD regulatory review and approval of our UAMS IACUC-approved protocol (months

1-4)

- b) Optimization of methodologies for studying other metabolic aspects of redox balance (months 1-4; while we are awaiting DoD approval we will use established cell lines (that do not require regulatory approval) to optimize all analytic parameters relevant to the remaining components of Task 1)
- c) Analysis of the redox status (by dihydrocalcein fluorescence) of peripheral blood cells in multiple mouse strains at two different ages (Months 4-8)
- d) Analysis of the redox status of multiple CNS populations in multiple mouse strains (Months 3-12)
- e) Analysis of additional metabolic aspects of redox balance in cells of blood and developing brain (Months 3-12)
- f) Analysis of proteins that contribute to redox balance in the above cell populations (Months 12-18)
- g) Statistical Analysis and manuscript writing (Years 2 and 3)
- *Aim 2: Analysis of the biological consequences of strain-dependent redox differences present in oligodendrocyte progenitor cells*
- a) Analysis of the relationship between redox state of oligodendrocyte progenitor cells isolated from different mouse strains and their ability to undergo division and differentiation in vitro (Months 6-18)
- b) Analysis of the correlates of redox status with the time course of myelination in vivo (Months 12-24)
- c) Analysis of the consequences of strain-associated differences in redox status for responsiveness of progenitor cells to thyroid hormone as an inducer of oligodendrocyte generation (Months 12-30)
- d) Analysis of the consequences of strain-associated differences in redox status for vulnerability of progenitor cells to physiological stressors of putative relevance to ASD pathogenesis (Months 12-30)
- e) Analysis of the role of activation of the redox/Fyn/c-Cbl pathway in 2a-d (Months 18-36)
- f) Statistical Analysis and manuscript writing: (Years 2 and 3)

**2.1 Overview of results**

Successful science needs to open up new doorways, especially in response to puzzling results. That has been the nature of this portion of our research as we have attempted to integrate some of the most sophisticated aspects of precursor cell function with redox status, one of the most fundamental of all regulators of biological function.

I will first present a brief summary of the results that to be discussed, so as to make the path of this research clearer.

The first steps in this research proceeded as predicted, with findings of redox-based control of vulnerability and findings of the correlations between the redox status of cells of the hematopoietic system with cells of the CNS.

We next integrated these results with analyses of normal developmental mechanisms because it was not possible to tell how our redox studies were integrated with the regulation of normal

development. This portion of the work had two components, one related to cell-intrinsic contributions to redox status and normal development and the second related to the role of cell-extrinsic signaling molecules in modulating precursor cell function via changes in redox state.

Our studies on cell-intrinsic control of redox status during normal development led to identification of molecular mechanisms involved in this control and integrated a regulatory pathway critical in response to environmental toxicants (the Nrf2-regulated anti-oxidant response element pathway that controls expression of phase 2 detoxifying enzymes) with normal developmental control of redox state. This work also inspired parallel studies (conducted with other funding) on another disease with redox abnormalities, a disease called ataxia telangiectasia (A-T). Relevant results from our A-T research will be discussed at the end of the discussion of the DOD-sponsored research due to their potential relevance. Although they were not funded by this grant, questions asked in the A-T research were both informed by the research conducted under this grant and helped inform us about new questions that needed to be asked.

Our studies on cell-extrinsic control of redox status in regulation of development integrated the pathway being studied in the control of environmental toxicants with such critical signaling molecules as thyroid hormone (TH) and bone morphogenetic protein (BMP). This led us to the discovery that what we had originally identified as a potential pathogenic pathway that was initiated by exposure to environmental toxicants represents a critical central regulator of the balance between division and differentiation in dividing progenitor cells of the developing central nervous system. This critical finding enables us to think more productively about the relatively mild pathological features (at the cellular level) of ASDs.

This linkage between oxidative changes caused by environmental toxicants and normal signaling processes also was important in enabling us to begin understanding what the mechanistic interface might be between environmental insults and normal developmental processes.

With this new knowledge, we then returned to analysis of strains of animals in which we previously had defined differences in redox status, and found that these differences were no longer detectable. The supplier had not changed, and our vivarium staff could not suggest any changes that they had made that might be relevant to these differences. Attempting to understand these differences has not reached a satisfactory conclusion, and it raises great concerns about comparing strain studies from different laboratories if such fundamental properties can be changed for unknown reasons. This was an unexpected problem in this work, as other aspects of our studies are extremely robust. This problem is discussed in the detailed section of this report.

One of the reasons why we are confident that the changes in redox state analyzed between different strains is not due to any changes in our own protocols is that our studies on developmental differences in redox regulation in different CNS regions have not changed at all during this same time frame. This enabled us to better understand the molecular basis of redox differences and the consequences of these differences, which was part of the core goals of this research program. Thus, we were able to extend our understanding of redox control of development in the CNS in important ways despite the problems with variability in mouse strain performance.

In addition, we introduced at this time a new metabolic regulator into our overall program on developmental insults (which extends beyond this DOD funding) in order to determine the specificity of the changes associated with redox status. The laboratory of our colleague, Dr. Margot

Mayer-Proschel, investigates the effects of gestational iron deficiency on development. This was of particular interest to us as differences in iron levels and metabolism can have profound effects on redox state and on normal development. Moreover, it has been suggested that iron deficiency and autism may have some interesting areas of overlap at the levels of cellular and behavioral pathologies. Again, this is an area that was not pursued with grant funding but was an area that helped inform some of our thinking, and in which the research pursued with DOD funding had a very significant effect on the way in which that research progressed. The key lessons learned thus far in this work are briefly considered after discussing the DOD-funded research.

The information provided by our colleagues also was of great interest in the context of ASDs due to findings that taking pre-conception vitamin supplementation can reduce the risk of having an autistic child by half. While these studies (from UC-Davis) were interpreted in terms of folate deficiency, pre-conception vitamin supplements are rich in iron and iron deficiency is very common in pregnancy.

The comparisons between effects of redox changes and effects of sub-clinical iron deficiency have been greatly informative and surprising in two respects. First, there are areas of convergent outcomes between these effects that suggest where a convergence between a redox insult and iron deficiency could function as collaborating risk factors. In fact, it appears that the co-ordinate insults of subclinical iron deficiency and exposure to environmental toxicants may be very relevant to understanding abnormalities in childhood neurological development.

As indicated, an unexpected benefit of our comparisons with iron deficiency (which were not funded by the DOD funding, but which would not have happened otherwise as the parallel efforts were what inspired critical questions to be asked) was the discovery of a remarkable congruence between effects of early gestational iron deficiency and CNS changes seen in autism. Thus, even though no funding from the DOD project was used to fund the iron studies, the comparison of information obtained in the two lines of research appears to have identified an entirely new area for exploring a metabolic insult as a risk factor for development of ASDs.

Thus, the funding from the DOD enabled important new advances related to understanding the role of redox changes in control of normal CNS development. Moreover, this funding enabled us to interact productively with a laboratory pursuing an entirely different problem and influenced them to look at autism-related questions that they otherwise would not have pursued. This unexpected benefit of the DOD funding looks like it may be quite profound in its importance, as will be discussed in the detailed section of this report.

Finally, the research carried out in this effort profoundly altered our approach to the analysis of multiple other clinical problems, leading to critical breakthroughs related to other diseases (as discussed in Reportable Outcomes).

**2.2 Specific results related to Aims of the Project. (NB: Information is presented in a sequence that is easiest to follow scientifically, and the Aims and subaims to which particular experiments are relevant are indicated for each results section.)**

*2.2.1 Redox differences due to the development are stable, those that are due to strain differences are not. (Aim 1, b-e)*

We first addressed the problem that strain differences may themselves provide differences between cell populations that may or may not be relevant to redox status. To this end, we focused on goals 1e and 1f in order to first broaden the parameters that would be applied to the populations of goals 1b and 1c. By focusing on differences in basal redox state between oligodendrocyte progenitor cells (OPCs) of the developing corpus callosum and cortex (Power et al, 2002), we were able to identify multiple physiological and protein expression parameters that are related to differences in redox status without concerns about whether differences were simply due to other effects of strain differences. This is an ideal situation in which to define redox-related differences in metabolite and protein expression without concern about differences that might be due to strain differences but unrelated to redox status. This work led to the identification of a wide range of further potential differences (Figures 1-3), including differences in levels of glutathione, in the ratio of ATP:ADP and the ratio of reduced:oxidized pyrimidine nucleotides, and differences in levels of bcl-2 and superoxide dismutase-1 and in the levels of γ-glutamyl-cysteinyl-synthase heavy chain (γ-GCS), the rate-limiting enzyme in glutathione biosynthesis (Deneke & Fanburg, 1989).

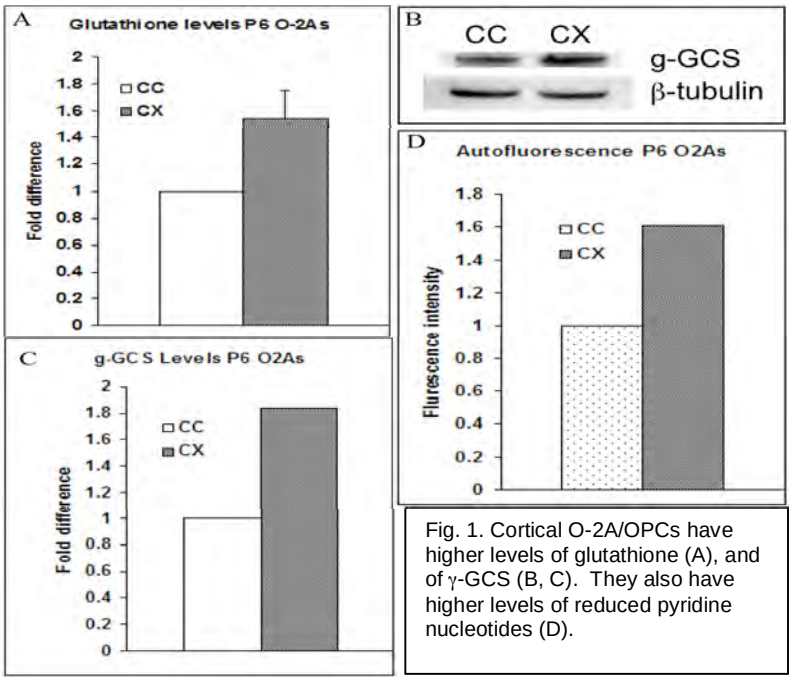



Fig. 1. Cortical O-2A/OPCs have higher levels of glutathione (A), and of γ-GCS (B, C). They also have higher levels of reduced pyridine nucleotides (D).

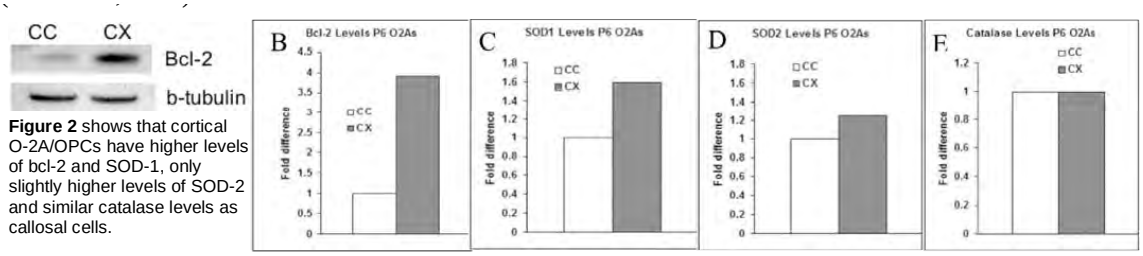



**Figure 2** shows that cortical O-2A/OPCs have higher levels of bcl-2 and SOD-1, only slightly higher levels of SOD-2 and similar catalase levels as callosal cells.

We next applied our analyses to the lymphoid system in two strains of mice (CBA and SJL) in which we had found very different basal redox states in the CNS. The primary goal in this part of Project 2 was to determine whether analysis of lymphoid and myeloid populations, or any other readily obtainable cell populations, would provide information on redox state that mirrored the redox state found in the progenitor cells of the developing CNS. As it is not possible to obtain CNS cells from children with autism, it is critical to determine whether other cell

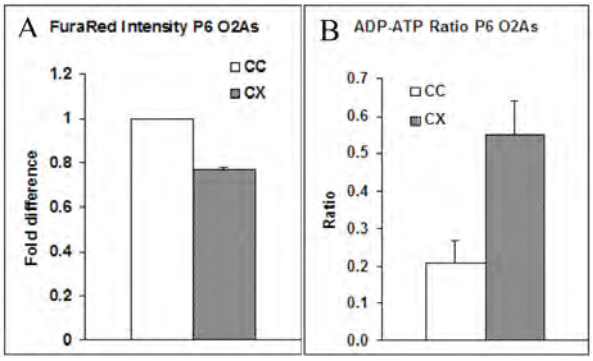



**Figure 3** demonstrates that cortical O-2A/OPCs have a higher level of free calcium, as indicated by FuraRed, and also have a higher ratio of ADP:ATP.

populations can be used to obtain information on basal redox status.

Analysis of multiple cell populations from the bloodstream revealed that both B lymphocyte and T lymphocyte populations showed the same differences in redox status as we previously detected in oligodendrocyte progenitor cells (**Figure 4, Table 1**). In contrast, these differences are not as reliably detected in myeloid populations, such as macrophages and neutrophils (**Figure 5**).

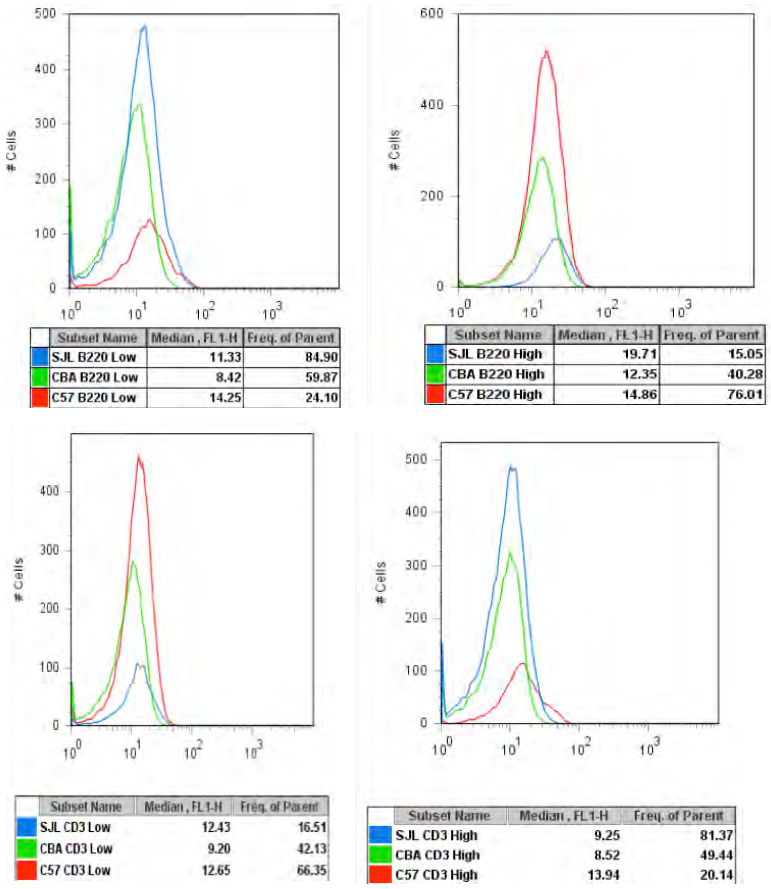
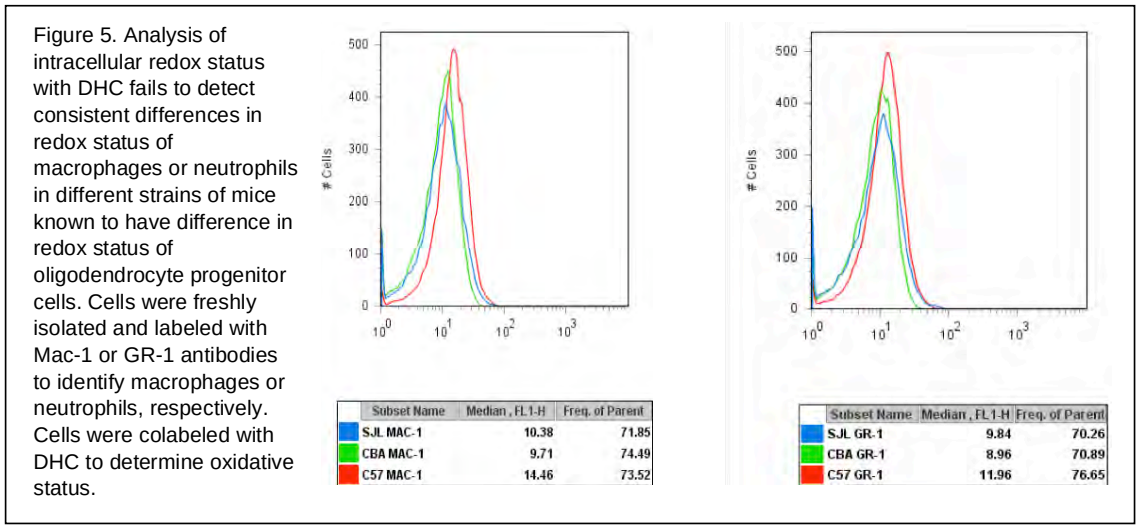



**Figure 4.** Analysis of intracellular redox status with DHC reveals consistent differences in redox status of B cells and T cells in different strains of mice known to have difference in redox status of oligodendrocyte progenitor cells. Cells were freshly isolated and labeled with B220 or CD3 antibodies to identify B-cells or T-cells, respectively. Cells were colabeled with DHC to determine oxidative status. As B220+ cells and CD3+ cells both contained two distinct populations of cells with high or low levels of antigen expression, these were examined separately. As seen for T cells, analysis of the CD3-low population revealed a greater degree of difference for SJL vs CBA cells than seen for the CD3- high populations.

| ***Redox*** | Low | | | High | | |
|---|---|---|---|---|---|---|
| | CBA | C57 | SJL | CBA | C57 | SJL |
| B cells | 8.42 | 14.25 | 11.33 | 12.35 | 14.86 | 19.71 |
| T cells | 9.20 | 12.65 | 12.43 | 8.52 | 13.94 | 9.25 |

**Table 1**. Summary of analysis of B cell and T cell populations with DHC. The numbers in red indicate the most oxidized populations. As shown, with the exception of CD3-high cells from SJL mice, the populations derived from SJL or C57BL/6 mice are from 20%-69% more brightly labeled (i.e, more oxidized) than comparable antigenically defined populations isolated from CBA mice.




We found that the redox status of T cells and B cells correlated well with our previous observations on the redox status of oligodendrocyte progenitor cells, regardless of the intensity of staining with either CD3 or B220 antibody. CD3+ T cells derived from either SJL or CBA mice were consistently more oxidized than those derived from CBA mice.

The results obtained in this part of the work were very encouraging in that they appeared to provide a means of assessing basal metabolic status without requiring examination directly of cells of the CNS. To test this hypothesis we examined the redox state of OPCs freshly isolated from the corpus callosum of 7 day postnatal (P7) CBA and SJL mice, using DHS fluorescence, and found that the cells derived from SJL mice were indeed more oxidized (**Figure 6**).

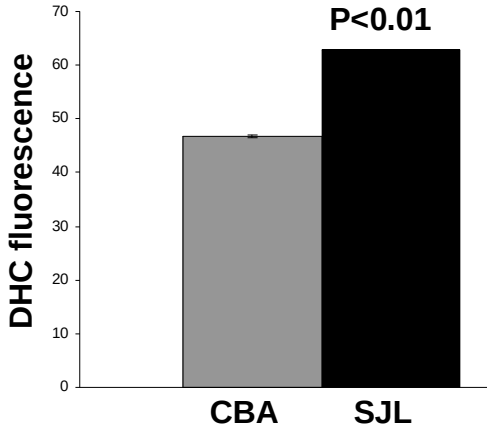



**Figure 6** Examination of the redox state of freshly isolated OPCs from SJL and CBA mice shows that cells derived from SJL mice are intrinsically more oxidized as determined by DHC fluorescence.

*2.2.2 Changes in dendritic arborization represent a potential convergence point between redox effects and neuronal development*

One of the critical effects of oxidizing cells is to activate the redox/Fyn/c-Cbl pathway, in which increases in oxidation cause sequential activation of Fyn kinase and the c-Cbl ubiquitin ligase, leading to degradation of a group of receptor tyrosine kinases (RTKs) that are vital for cell division and differentiation. The importance of oxidation-based activation of the redox/Fyn/c-Cbl pathway for understanding differentiation is presented in Section 2.2.9.2-5. Before discussing this portion of the research, however, there is a final set of results related to strain differences in redox state of potential interest in respect to understanding possible contributions of increased oxidative status to pathogenesis of ASDs.

One of the target receptors of the redox/Fyn/c-Cbl pathway is the c-Met receptor, a protein that plays an important role in many biological processes but is of particular interest in the context of autism. The connection between the c-Met receptor and autism is twofold. First, a polymorphism in the promoter region of the gene encoding this receptor has been observed at high frequency in autism. Moreover, examination of the brains of individuals carrying this polymorphism

demonstrates a reduced level of the c-Met protein in their CNS (Campbell et al, 2007; Campbell et al, 2006). As activation of the redox/Fyn/c-Cbl pathway also leads to reductions in levels of the c-Met protein, such activation represents another means of achieving the lower protein levels that are of interest in regards to autism. The second reason for being particularly interested in either mutations or regulatory pathways that cause a reduction in the level of the c-Met protein has to do with the function of this protein in modulating dendrite generation in neurons of the hippocampus. As the hippocampus is central to learning, and alterations in learning ability are of considerable concern in a large number of children diagnosed with autism spectrum disorders, we were particularly interested in this problem. Moreover, it has been previously reported that there is a reduced complexity of dendritic structure in the hippocampus of children with autism (Raymond et al, 1996).

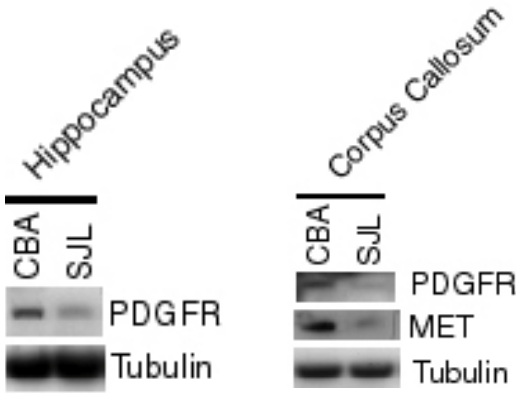



**Figure 7.** Examination of the redox state freshly isolated OPCs from SJL and CBA mice shows that cells derived from SJL mice are intrinsically more oxidized as determined by DHC fluorescence.

When we analyzed the levels of the c-Met protein in the hippocampus of SJL mice, we found that these protein levels were much reduced compared with that found in the hippocampus of CBA mice (**Figure 7**). This is precisely as predicted by the redox/Fyn/c-Cbl hypothesis, as the SJL mice we examined were intrinsically more oxidized than CBA mice.

If the current understanding of the role of the c-Met protein is correct then a reduction in levels of this protein in the developing hippocampus should lead to a reduction in dendrite formation. Such an observation would provide a potentially direct linkage between oxidative abnormalities and a disruption in normal neuronal development.

We therefore analyzed dendritic complexity in the hippocampus of SJL and CBA mice using other funding. We focused our attention on animals that were 15 days old, as development during this period corresponds with that seen in the human CNS during the first 6 to 12 months of life. This analysis was conducted by using Golgi staining to reveal neuronal architecture **(Figure 8**). These analyses revealed several striking differences between hippocampus dendritogenesis in CBA vs SJL mice. Dendritic complexity in SJL mice was markedly reduced as compared with CBA mice of the same age, with the quantity of dendrites being markedly lower in the hippocampi of SJL mice (**Figure 9, Table 2**). This was reflected in a grouping of the dendritic numbers towards the lower number of values, as compared with the broader distribution of values for CBA mice. Similar outcomes were obtained for analysis of dendritic nodes, which were more numerous in the

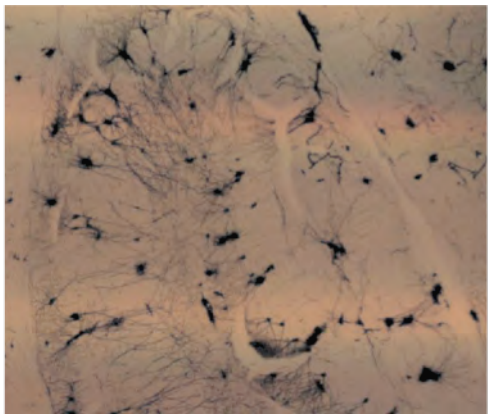


**Figure 8.** Golgi staining of the hippocampus of a P15 mouse reveals detailed neuronal architecture. Detailed quantitative analysis of images such as these provides the data presented in Figure 9 and Table 2.

hippocampus of CBA mice than in SJL mice. As predicted from such outcomes, the number of dendritic ends was also lower in SJL mice.

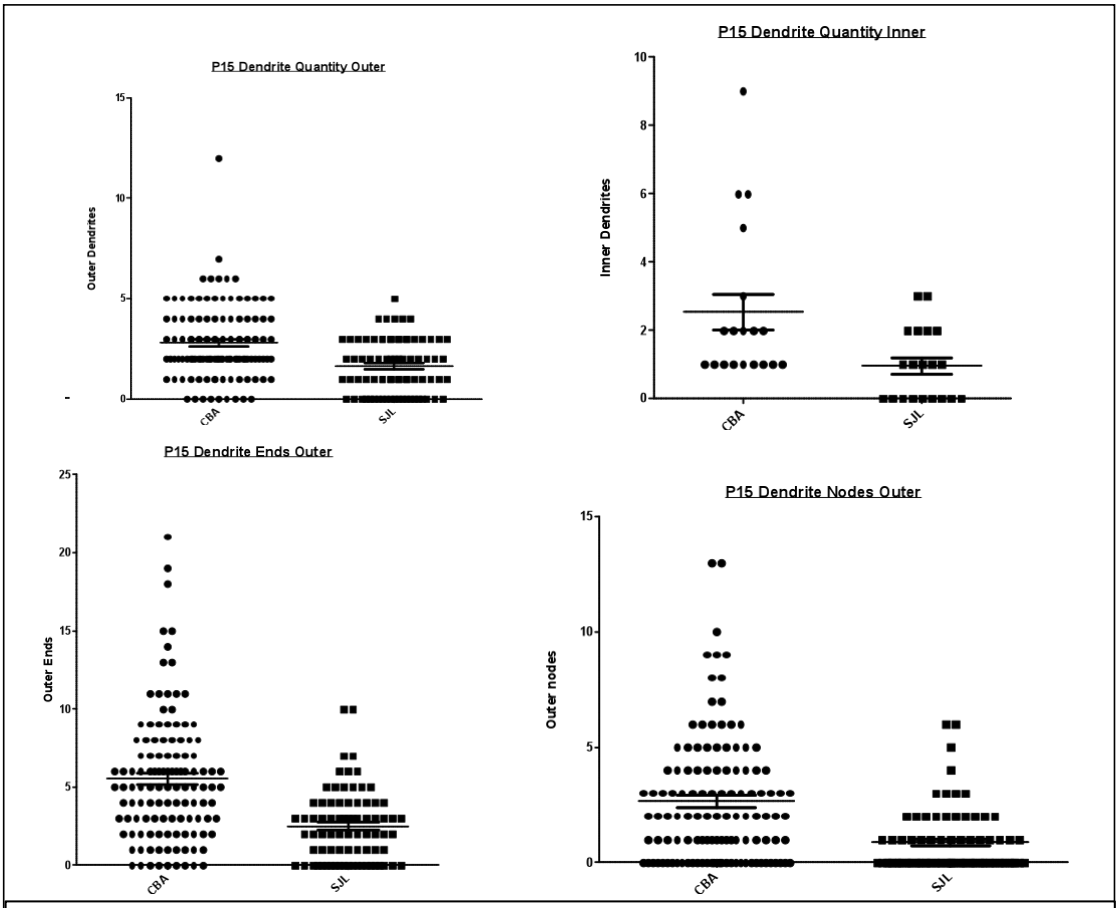



**Figure 9.** Analysis of dendritic complexity in SJL and CBA mice reveals reduced complexity in the hippocampus of P15 SJL mice as compared with that seen in CBA mice. Neurons were separately analyzed according to their location in the hippocampus. As see, the number of dendrites and the number of nodes clusters at lower values for neurons in the hippocampus of SJL mice. Most striking is the low number of SJL neurons with more than 4 outer ends or 3 outer nodes.

| Neuronal parameter analyzed | CBA *n* | CBA mean | SJL *n* | SJL mean | Preliminary *p* values |
|---|---|---|---|---|---|
| **Dendrite nodes outer** | 119 | 2.65 | 78 | 0.87 | <.0001 |
| **Dendrite nodes inner** | 19 | 1.21 | 20 | 0.25 | 0.0368 |
| **Dendrite ends outer** | 119 | 5.52 | 78 | 2.49 | <.0001 |
| **Dendrite ends inner** | 19 | 3.79 | 20 | 1.2 | 0.0081 |
| **Dendrite quantity outer** | 119 | 2.8 | 78 | 1.63 | <.0001 |
| **Dendrite quantity inner** | 19 | 2.53 | 20 | 0.95 | 0.0084 |

**Table 2**. Summary of findings to date on analysis of dendritic complexity in the hippocampus of P15 SJL and CBA mice. The value for *n* is the number of neurons analyzed in their entirety. For each strain, neurons from a minimum of 3 different mice were examined. Standard deviations are not provided for this analysis as it is ongoing, and p values should be considered as preliminary. Nonetheless, it is clear that the predicted outcome of less dendritic complexity in the developing hippocampus of the more oxidized SJL strain of mice, which have lower c-Met levels, looks likely to be correct.

Thus, these studies revealed a significant decrease in the number of intersections in apical and basal dendrites and in the total length of apical and basal dendrites along specific branch orders. This phenotype of impaired dendritic complexity is similar to what has been described in ASDs (i.e (Penzes et al, 2011)).

*2.2.3 An unexpected change in inter-strain redox status*

The problem with our analyses on the lymphoid system emerged after we completed our analysis of the molecular basis for developmentally-regulated differences in redox status and tried to apply these findings to the analysis of strain differences. We purchased new mice from our original suppliers (Charles River and Jackson Laboratories) and found that the previously identified redox differences no longer could be found. At the same time, the developmentally regulated redox differences observed in different regions of the CNS were stable and precisely as seen in the past.

We worked on the changes in redox differences between different mouse strains for several months and found it impossible to reconstitute what were easily detectable differences in the past. Due to other aspects of our work we were well aware of the potential for such events to occur, as we were aware of transgenic strains in which mutations had been lost due to the animal care staff selecting for dams with larger litter sizes (which was likely due to not having the mutation). As we had seen that litters in oxidized (SJL) mice had fewer pups than litters of more reduced (CBA) mice, it may have been that selection for healthier animals unintentionally selected for mice that were not as oxidized. We were also aware (in work carried out in other laboratories) of changes in response to traumatic injury to the CNS due to changes in the diet put into place without consultation with the scientists running the experiments. In the end, though, it became clear that continuing to work to solve this problem was causing resources to be expended without advancing our understanding of the cellular and molecular aspects of cell-intrinsic differences in redox biology.

We are very concerned about the above problems in respect to papers claiming different outcomes on particular strains when putatively identical experiments are conducted in different laboratories.

It is important to note, however, that the prediction that an increased basal redox state does predict earlier myelination, in that the more oxidized corpus callosum initiates and completes myelination earlier than the more oxidized cortex (Yakovlev & Lecours, 1967). Thus, despite the problems with changes in basal redox status in particular strains of mice, this prediction of our studies appears to be correct.

*2.2.4 Cell-intrinsic redox differences confer differences in vulnerability to physiological stressors and also are inherited from dividing OPCs to non-dividing oligodendrocytes*

While the problem of variability in mice of the same strain purchased at different times is a problem that merits attention, devoting extensive resources to solving this problem was not going to further our investigation of the fundamental questions underlying this grant, or more generally to the understanding of problems related to ASDs. We therefore focused our attention on applying other strategies for testing the key hypotheses of our proposal. Specifically, we focused on the robust

differences in redox state observed in OPCs isolated from different regions of the developing CNS. These experiments provided the opportunity to address identical questions within a single strain of animals.

We next focused our attention to the question of whether cell-intrinsic differences in redox status alter cellular vulnerability to physiological stressors of potential relevance in ASDs, one of the key hypotheses of our proposal.

We first asked the question of whether vulnerability to tumor necrosis factor-α (TNF-α), an inflammatory cytokine that is thought to be elevated in children with ASDs, shows a random or non-random distribution in its ability to kill oligodendrocytes derived from different OPC clones. We and others had previously shown that oligodendrocytes themselves are killed by exposure to TNF-α but these studies also had the surprising outcome that cell killing reached a plateau of vulnerability beyond which increases in TNF-α concentration did not increase the proportion of cells killed (Mayer & Noble, 1994). For example, exposure to TNF-α of oligodendrocytes, derived from O-2A/OPCs isolated from optic nerve or corpus callosum of 7-day old (P7) rat pups (Ibarrola et al, 1996; Noble & Murray, 1984; Raff et al, 1983), revealed relatively small differences in the extent of cell death induced by 10, 50 or 100 ng/ml of this cytokine. Our hypothesis was that this plateau predicted a non-random distribution of sensitivity.

Clonal analysis of vulnerability revealed that the probability of a clone containing TNF-α–resistant oligodendrocytes was not randomly distributed, such that large clones rarely showed high levels of oligodendrocyte death (**Fig. 10**). To determine if TNF-α resistance was randomly dispersed in a population, purified OPCs were grown at clonal density for 5 days, induced with thyroid hormone (TH) to differentiate into oligodendrocytes and then exposed to 20 ng/ml TNF-α for 48 hrs. The percentage of living versus dead oligodendrocytes in randomly chosen clones was determined using MTT analysis combined with microscopy (Mayer et al, 1994; Mayer & Noble, 1994). Clones in which ≥50% of oligodendrocytes were killed by 20 ng/ml TNF-α all contained <40 total cells, while in 14/17 clones with >20 cells the majority of oligodendrocytes were not killed by TNF-α.

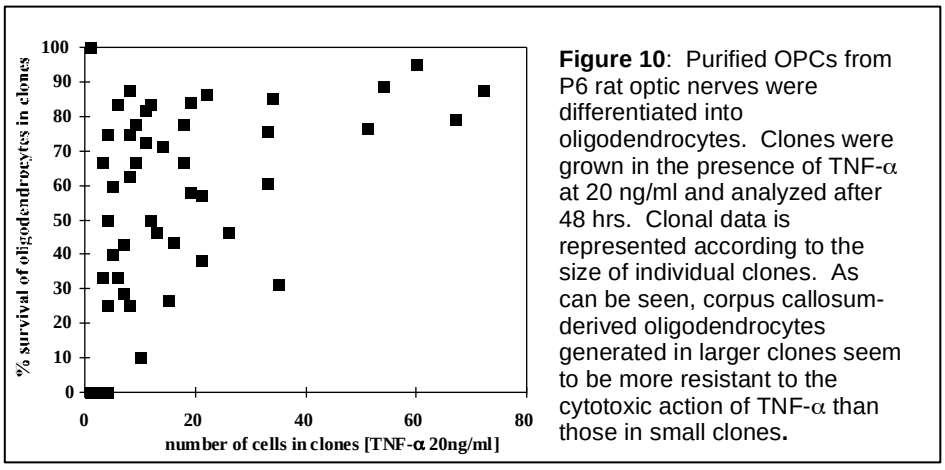



**Figure 10**: Purified OPCs from P6 rat optic nerves were differentiated into oligodendrocytes. Clones were grown in the presence of TNF-α at 20 ng/ml and analyzed after 48 hrs. Clonal data is represented according to the size of individual clones. As can be seen, corpus callosum-derived oligodendrocytes generated in larger clones seem to be more resistant to the cytotoxic action of TNF-α than those in small clones.

*2.2.5 Vulnerability of oligodendrocytes to TNF-α correlates with the intracellular redox state of their ancestral progenitor cell*

The discovery that vulnerability to TNF-α correlates with clonal size raised the possibility that these characteristics are physiologically related. Our previous studies on the balance between self-

renewal and differentiation in dividing O-2A/OPCs demonstrated a correlation between the intracellular redox state of a progenitor and the extent of division occurring in the clone derived from that cell (Smith et al, 2000). This correlation raises the question of whether progenitor cell redox state also correlates with the vulnerability of the oligodendrocytes they generate. To test this possibility, OPCs isolated from P7 corpus callosum were purified into relatively more reduced or oxidized populations as previously (Smith et al, 2000), induced to differentiate into oligodendrocytes by exposure to TH (Barres et al, 1994; Ibarrola et al, 1996), and then exposed to TNF-α for 3 days.

As shown in **Figure 11**, OPCs with different intracellular redox states gave rise to oligodendrocytes with different degrees of vulnerability to TNF-α. DHCM-Rosamine$^{low}$ progenitors (i.e., those that were more reduced at the time of isolation) gave rise to oligodendrocytes resistant to killing by TNF-α. Treatment with 1, 10 or 20 ng/ml of TNF-α had no effect on these cells, in which viability remained at 20% above control values, further indicating that these oligodendrocytes also were resistant to the background cell death that normally occurs in culture. In striking contrast, DHCM-Rosamine$^{high}$ progenitors (i.e, those that were more oxidized) generated oligodendrocytes vulnerable to TNF-α, with exposure to 10ng/ml TNF-α reducing cell number by 40% and 20ng/ml reducing viability by 60%.

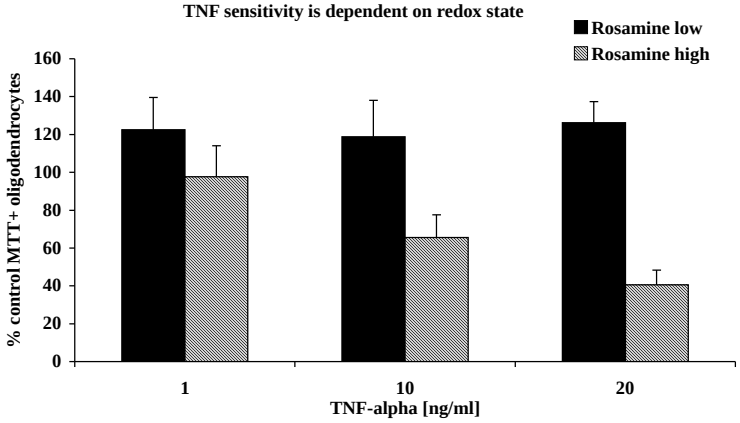



**Figure 11**: Corpus callosum-derived O-2A/OPCs were separated into DHCM-Rosamine$^{high}$ and DHCM-Rosamine$^{low}$ by FACS-mediated purification. Oligodendrocytes generated from these two populations were treated for 5 days with TNF-α at various doses and the % of live cells was determine by MTT assay and DAPI staining. Oligodendrocytes derived from the DHCM-Rosamine$^{low}$ (= more reduced) progenitor cells were at concentrations above 10ng/ml significantly less (*= p<0.01, *=p<0.001) sensitive to TNF-alpha than cells derived from DHCM-Rosamine$^{high}$ progenitor populations.

*2.2.6 Progenitor cell redox state predicts the vulnerability and redox state of oligodendrocytes generated in vivo*

In experiments presented so far, we used OPCs isolated from a defined tissue, and separated different redox populations from the heterogeneous population of cells present in that tissue. We previously discovered, however, that the redox status of cells is not only different among cells of the same tissue but may be even more different between progenitors isolated from different tissues (Power et al, 2002). For example, OPCs isolated from cortex are on average more reduced than those isolated from corpus callosum or optic nerve. This difference in redox status correlates with the capacity of cortical progenitor cells to undergo extensive self-renewal in the absence of differentiation in vitro (Power et al, 2002). The observation that the redox status of progenitors seems to be preserved in the resulting oligodendrocytes (as in **Figure 3**) prompted us to determine whether the vulnerability of oligodendrocytes generated in vivo in different CNS regions was predictable from the redox status of the progenitor cells found in that tissue. We isolated oligodendrocytes directly from P7 cortex (from which progenitors are relatively more reduced) and from corpus callosum (from which progenitors are relatively more oxidized) and compared the

vulnerability of these freshly isolated oligodendrocytes to TNF-α, as well as determining their relative redox status.

We found that oligodendrocytes isolated from cortex were more reduced and more resistant to the effects of TNF-α than callosum-derived oligodendrocytes. Cortex-derived oligodendrocytes showed no response to 20 ng/ml of TNF-α, while viability of corpus callosum oligodendrocytes was reduced by >60%. In agreement with the earlier noted correspondence between the redox status of progenitors and the oligodendrocytes they generate, oligodendrocytes generated in vivo in the cortex, where O-2A/OPCs are intrinsically more reduced than those in the corpus callosum, were themselves more reduced than oligodendrocytes isolated from corpus callosum.

Thus, in sum the prediction that being more oxidized is a successful predictor of vulnerability to physiological stressors thought to be of relevance to the pathogenesis of ASD.

*2.2.7 Transient modulation of O-2A/OPC redox state alters vulnerability of subsequently generated oligodendrocytes*

We next focused on two questions of potential relevance to developing novel approaches to ASD treatment. Understanding the physiological basis by which progenitors from different regions of the CNS generate oligodendrocytes of different redox states and vulnerabilities to TNF-α could theoretically enable protection from the effects of this inflammatory cytokine. Of even greater interest is the question of whether such knowledge would enable progenitor cells to be "re-programmed" so as to, e.g., generate oligodendrocytes resistant to TNF-α-induced death. Thus, the goal in these experiments was to determine whether a state of increased vulnerability could be altered by interventions of potential therapeutic relevance.

As one of the major regulators of intracellular redox state is glutathione, and we previously found

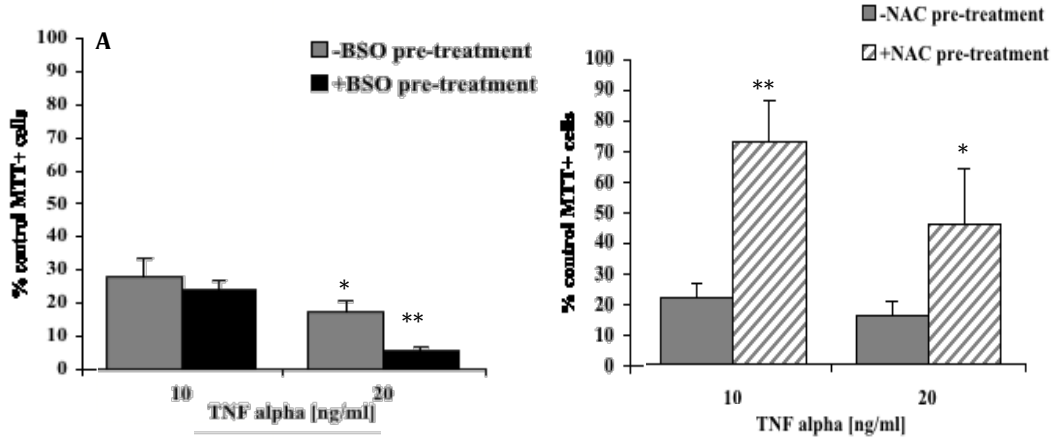



**Figure 12**: Optic nerve-derived progenitor cells were purified, plated onto coverslips and treated for 2 days with BSO **(A)** or NAC **(B).** An aliquot of cells was then stained with DHCM-Rosamine to confirm that NAC-treated cells contained more reduced and BSO-treated cells contained more oxidized cells than untreated control cultures. Cultures were then washed and treated with PDGF (1ng/ml) and thyroid hormone to induced oligodendrocyte differentiation. When cultures contained >90% oligodendrocytes (3-5 days), cells were exposed for additional 5 days to TNF-α at the indicated concentration. The percentage of live cells was determined using DAPI staining. Oligodendrocytes derived from oxidized progenitor cells were significantly more vulnerable to 10 and 20ng/ml TNF-α, while oligodendrocytes derived from pre-reduced progenitor cells were significantly less vulnerable to TNF-α at all concentrations. (*=p ≤ 0.01, **= p < 0.001)

that modification of glutathione content can rescue oligodendrocytes from TNF-α-induced death (Mayer & Noble, 1994), we next examined the glutathione content of OPCs and oligodendrocytes isolated from cortex and corpus callosum. We found that both cortex-derived populations had 2-fold higher levels of glutathione than their callosal counterparts (as detected with monochlorobimane (Fernandez-Checa & Kaplowitz, 1990; Rice et al, 1986)).

We next found that transient exposure of progenitors to pharmacological modifiers of glutathione content was sufficient to re-program the vulnerability of oligodendrocytes generated from such cells. To alter glutathione content we exposed progenitors for 48 hrs to BSO (to decrease glutathione content) or NAC (to increase glutathione content), switched cells to fresh medium (lacking BSO or NAC) and induced oligodendrocyte generation, followed by exposure to TNF-α. As shown in **Figure 12A**, oligodendrocytes generated from progenitors transiently exposed to 1μM BSO were more vulnerable to TNF-α than control cells. In contrast, if OPCs were transiently exposed to NAC prior to oligodendrocyte generation, there was a dramatic increase in the proportion of oligodendrocytes that survived exposure to TNF-α (**Figure 12B**).

As glutamate is also released as a consequence of inflammation in the CNS, we also determined whether treatment of OPCs with NAC prior to the generation of oligodendrocytes rendered these cells resistant to glutamate-induced cell death. As shown in **Figure 13**, this was indeed the case, and pre-treatment of progenitor cells with NAC was sufficient to provide protection to the oligodendrocytes generated from these cells.

Thus, these experiments showed that vulnerability to physiological stressors caused by basal differences in redox status could be altered by pharmacological intervention.

*2.2.8 Cell-intrinsic differences in redox state appear to be established by cell-intrinsic differences in Nrf2-mediated activation of the antioxidant response element (the work of the no-cost extension)*

The above problems are precisely the problems that are at the core of the goal of this grant, which is to improve our understanding of oxidative abnormalities in ASD. Our work has revealed a mechanism that ties increased oxidative status together with problems ranging from increased vulnerability to physiological stressors (including environmental toxicants) to reduced levels of a receptor (c-Met) mutated in ASDs to reduced dendritic complexity in the hippocampus.

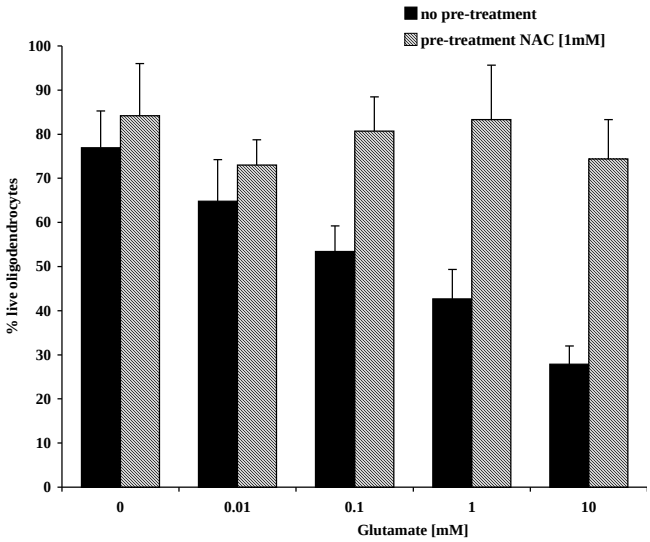



**Figure 13**: Optic nerve derived progenitor cells were purified, plated onto coverslips and treated for 2 days with NAC. An aliquot of cells was then stained with Rosamine to confirm that NAC treated cells were more reduced than control cells. Cultures were then washed and treated with PDGF (1ng/ml) and TH to induce oligodendrocyte differentiation. When cultures contained >90% GalC cells (3-5 days), cells were exposed for additional 3 days to glutamate at the indicated concentration. The percentage of live cells was determined using DAPI staining. Oligodendrocytes derived from pre-reduced progenitor cells were significantly less vulnerable to glutamate at concentrations higher than 0.1mM (**=p<0.001).

In our no-cost extension we proposed to carefully use the remaining resources in this research program to investigate how cells control their redox set points, with a focus of attention on Nrf2-mediated regulation of the antioxidant response element pathway.

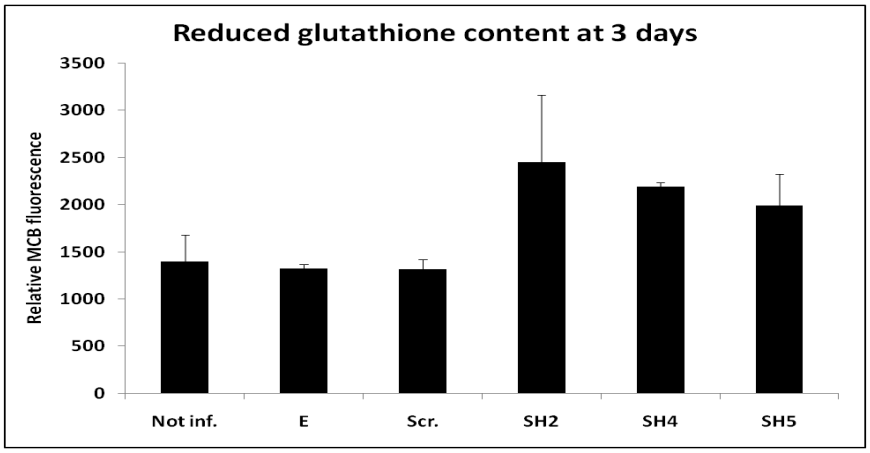



**Figure 14.** *Keap-1 knockdown increases the glutathione content of O-2A/OPCs.* CC O-2A/OPCs were infected with lentiviruses coding for an empty pLKO.1 vector (E), scrambled shRNA sequence (Scr.), or Keap1 shRNA (3 constructs, SH2, SH4, SH5) or left uninfected. After selection cells were grown in serum-free chemically-defined medium, supplemented with PDGFaa for 3 days and then stained with MCB and analyzed on a plate reader. Values are expressed as relative MCB fluorescence normalized for background signal and cell number.

Based on the findings presented thus far, and the goals of understanding the control of redox differences at a mechanistic and functional level, we next focused attention on the developmentally regulated differences between OPCs from from the corpus callosum and the cortex. As discussed in Section 2.2.1, when compared to corpus callosum (CC) derived, cortical (CX) OPCs were found to be more reduced, self-renewed extensively and were very resistant to inducers of differentiation.

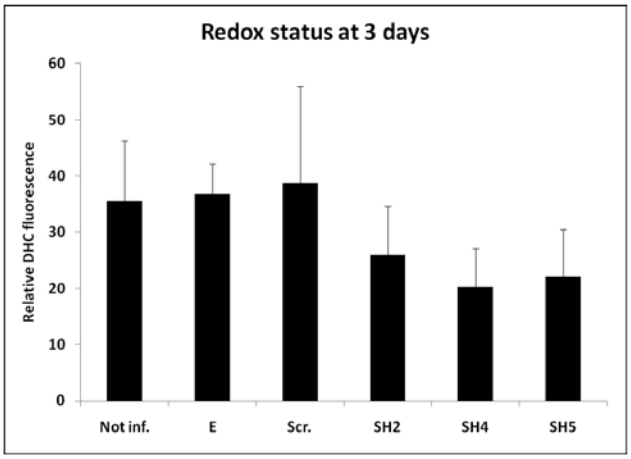



**Figure 15.** *Keap-1 knockdown makes O-2A/OPCs more reduced.* CC O-2A/OPCs were infected with lentiviruses coding for an empty pLKO.1 vector (E), scrambled shRNA sequence (Scr.), or Keap1 shRNA (3 constructs, SH2, SH4, SH5) or left uninfected. After selection cells were grown in serum-free chemically-defined medium supplemented with PDGFaa for 3 days and then stained with DHC and analyzed on plate reader. Values are expressed as relative DHC fluorescence normalized for background signal and cell number.

The difference between cortical and callosal OPCs enabled us to examine the basis for cell intrinsic redox modulation. The data presented in Section 2.2.1 suggested to us a potential role of the Nrf2 pathway in epigenetic control of redox state. The cortical OPCs were found to have higher levels of catalase, superoxide dismutases (SOD) and γ-glutamyl cysteine synthetase (GCS), more reduced glutathione (GSH). These outcomes converge on Nrf2 regulation of the antioxidant response element (ARE). If this were correct then enhancing Nrf2 activity in CC OPCs should induce them to exhibit the characteristics of CX OPCs and conversely, inhibiting Nrf2 in CX OPCs should induce them to exhibit the characteristics of CC OPCs.

We first tested the hypothesis that activating Nrf2 signaling by knocking down the inhibitor Keap1 increases corpus callosum (CC) OPC reduced glutathione content and makes them more reduced. As shown in Figure 14, this prediction was correct as infection with various shRNA constructs used to reduce levels of Keap1 all were associated with 30-60% increases in reduced glutathione content. We further found (**Figure 15**) that decreasing the levels of Keap1 with shRNA also was associated

with OPCs becoming generally more reduced (a broader measurement of physiological status than focus on reduced glutathione, although these two parameters generally are correlated).

We next tested the hypothesis that altering levels of Nrf2 activity by reducing levels of Keap1 suppresses differentiation induced by exposure to thyroid hormone (TH). We previously have found that induction of differentiation by TH requires making cells more oxidized , and that keeping cells reduced prevents the induction of oligodendrocyte generation by TH exposure. As shown in **Figure 16**, knockdown of Keap1 with shRNA inhibits TH-induced differentiation over a prolonged time period.

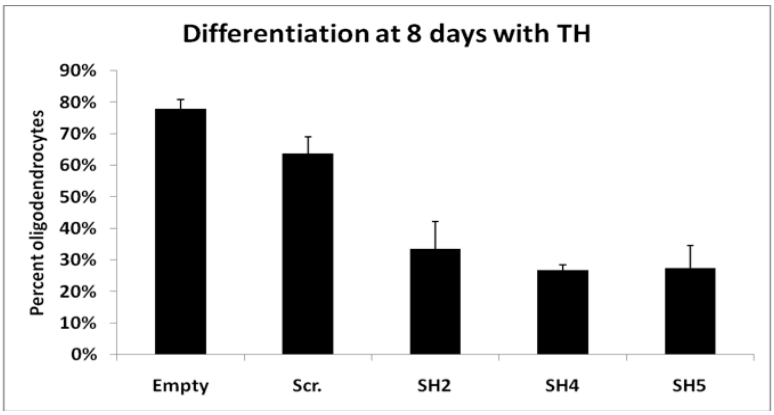



**Figure 16.** *Decreasing the levels of Keap-1 inhibits TH-induced differentiation of O-2A/OPCs into oligodendrocytes.* CC O-2A/OPCs were infected with lentiviruses coding for an empty pLKO.1 vector (E), scrambled shRNA sequence (Scr.), or Keap1 shRNA (3 constructs, SH2, SH4, SH5). After selection cells were grown in DMEM SIT-, supplemented with PDGFaa and TH for 8 days and then stained with GalC and DAPI and imaged using the Cyntellect cell scanner. Cells were then counted manually on a computer and a proportion of GalC positive cells from total DAPI positive cells plotted.

If our overriding hypothesis is correct, then reducing Nrf2 levels should have the opposite effect from reducing levels of Keap-1. As shown in **Figures 17-19**, this is indeed the case and decreasing the levels of Nrf2 leads to decreases in glutathione content, generation of a more oxidized state and an enhanced response to TH. In this case, our starting population was O-2A/OPCs isolated from the cortex (CX), which are intrinsically more reduced than those isolated from the CC.

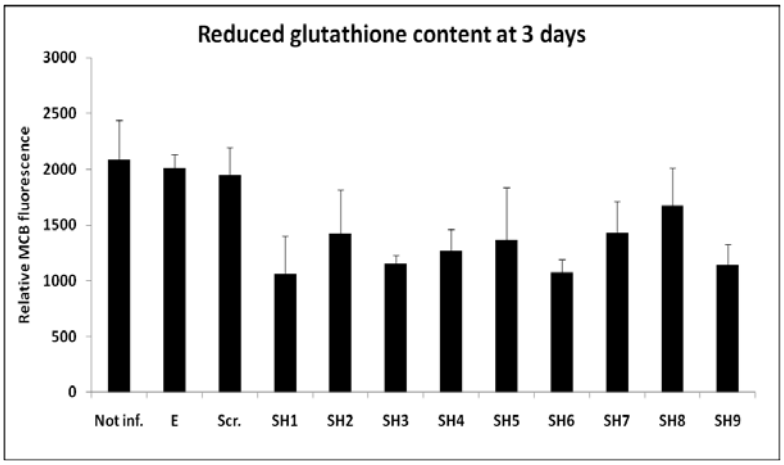



**Figure 17.** *Decreasing levels of Nrf2 is associated with decreases in levels of reduced glutathione in O-2A/OPCs.* CX O-2A/OPCs were infected with lentiviruses coding for an empty pLKO.1 vector (E), scrambled shRNA sequence (Scr.), or Nrf2 shRNA (9 constructs, SH1 through SH9) or left uninfected. After selection cells were grown in DMEM SIT-, supplemented with PDGFaa for 3 days and then stained with MCB and analyzed on plate reader. Values are expressed as relative MCB fluorescence normalized for background signal and cell number.

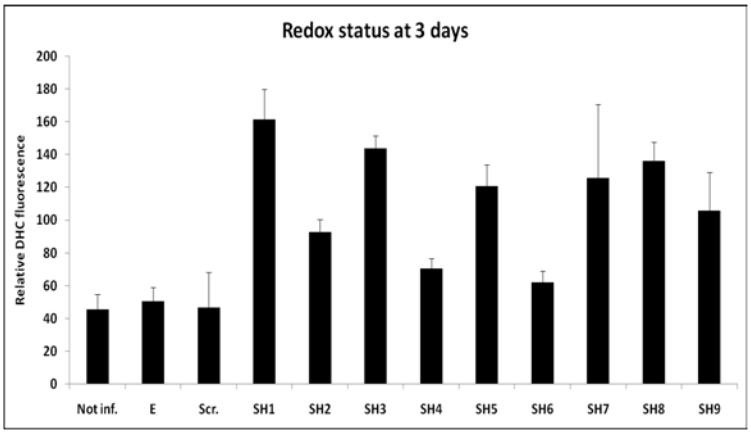



**Figure 18.** *Decreasing levels of Nrf2 is associated with making cortical O-2A/OPCs more oxidized.* CX O-2A/OPCs were infected with lentiviruses coding for an empty pLKO.1 vector (E), scrambled shRNA sequence (Scr.), or Nrf2 shRNA (9 constructs, SH1 through SH9) or left uninfected. After selection cells were grown in DMEM SIT-, supplemented with PDGFaa for 3 days and then stained with DHC and analyzed on plate reader. Values are expressed as relative DHC fluorescence normalized for background signal and cell number.

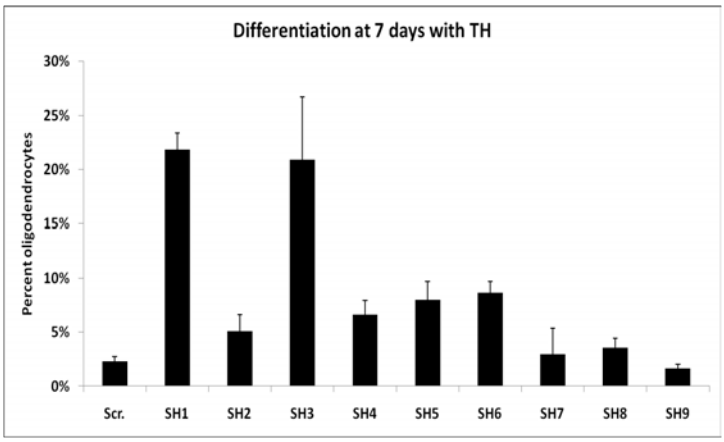



**Figure 19.** *Decreasing levels of Nrf2 is associated with increased differentiation of cortical O-2A/OPCs exposed to TH.* CX O-2A/OPCs were infected with lentiviruses coding for an empty pLKO.1 vector (E), scrambled shRNA sequence (Scr.), or Nrf2 shRNA (9 constructs, SH1 through SH9). After selection cells were grown in DMEM SIT-, supplemented with PDGFaa and TH for 7 days and then stained with GalC and DAPI and imaged using the Cyntellect cell scanner. Cells were then counted manually on a computer and a proportion of GalC positive cells from total DAPI positive cells plotted.

We also have tested the hypothesis that the biological regulation of the redox state in different populations of OPCs is modulated by Nrf2 activity. As shown in **Figure 20**, we found that cortical O-2A/OPCs had higher levels of Nrf2 than those isolated from the corpus callosum. Gamma-glutamyl-cysteinyl-synthase (GCLC) was also elevated in the CX cells, as would be predicted from increased Nrf2 levels. In contrast, the levels of Keap-1, catalase (CAT) and superoxide dismutase-1 (SOD1) were very similar in these two populations.

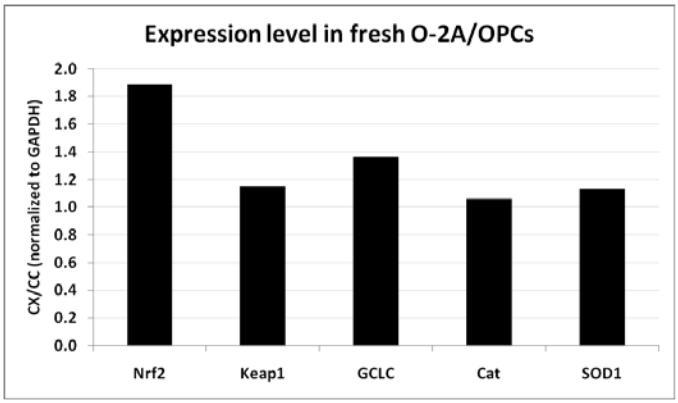



**Figure 20.** *Comparison of levels of redox-state regulators in cortical (CX) and corpus callosal (CC) O-2A/OPCs indicates that Nrf2 levels are specifically elevated in the more reduced CX cells.* O-2A/OPCs were isolated and sorted as usual and their RNA isolated for cDNA synthesis and Q-PCR analysis. Two replicates so no error bars yet. GCLC also looks like it is elevated by about 30%. Data was analyzed using the delta Ct method of Pfaffl, M.W. (2001) and values expressed as a ratio of CX to CC.

Thus, these experiments successfully tested the hypothesis that altering the basal redox set point could alter the responsiveness of OPCs to inducers of differentiation into oligodendrocytes.

*2.2.9 Mechanistic analysis of how redox regulation controls precursor cell function:*

In order to derive a mechanistic analysis that helps to understand the control of the generation of myelin-forming cells (one of the goals of this project) it is necessary to go beyond genetic manipulation to also include analysis of the cell-extrinsic signaling molecules that provide the environmental cues that control precursor cell function.

Through this work, which is directed to both project aims, we have been able to define a molecular mechanism that integrates intracellular redox state, multiple cell signaling pathways, regulation of mitochondrial release of reactive oxidative species (ROS), control of receptor tyrosine kinase (RTK) degradation and also multiple components of cell cycle regulation into a single pathway that appears to lie at the heart of regulating the balance between self-renewal and differentiation in dividing OPCs. These studies thus offer a broad-ranging integration of the ways in which increases in oxidative status can alter the function of dividing progenitor cells so as to cause premature generation of oligodendrocytes (as a possible explanation for the early supra-normal levels of while matter seen in children with ASDs (Ben Bashat et al, 2007; Courchesne, 2004; Courchesne et al, 2001; Hardan et al, 2000; Herbert et al, 2003; Herbert et al, 2004; Mostofsky et al, 2007)).

In our second report in 2011 we noted that in order to understand how it is that changes in redox status are translated into changes in self-renewal probability in oligodendrocyte progenitor cells, we have been examining the effects of such alterations on the regulation of the redox/Fyn/c-Cbl pathway (RFC). This pathway converts small increases in oxidative status into accelerated degradations of receptor tyrosine kinases that are targets of the c-Cbl ubiqutin ligase. In the context of the previous section, however, Nrf2 knockdown, which causes a more oxidized redox status, also causes increases in c-Cbl activation. This leads to loss of receptors, such as the platelet-derived growth factor receptor-$\alpha$, that are required for continued division of these progenitor cells.

We have extended these results in such a manner as to develop an entirely new synthesis as to how redox state controls OPC function (as summarized in the Conclusions section of this report). With this work we have a much more profound understanding of the problems on which this grant was focused, and can offer a highly predictive mechanistic framework for future analysis of this problem. Moreover, it has turned out that this analysis also provides a critical control point in the biology of glioblastomas and breast cancers (and indeed has led to a series of new discoveries related to novel therapeutic approaches that appear to be applicable to a variety of different kinds of cancers).

*2.2.9.1 TH-induced differentiation requires opening of the membrane permeability transition pore*

Before discussing this next component of our studies, it is necessary to provide additional background information. Basal division of OPCs is promoted by platelet-derived growth factor (PDGF (Ibarrola et al, 1996; Noble et al, 1988)), and progenitors derived from such CNS regions as the postnatal optic nerve growing in the presence of PDGF will generate non-dividing oligodendrocytes with a probability of ~0.5 (Yakovlev et al, 1998). If cells are grown in the presence of thyroid hormone (TH, one of the most extensively studied inducers of oligodendrocyte generation (for reviews see, e.g., (Bernal, 2005; Mayer-Proschel et al, 2001; Raff et al, 1998; Rodriguez-Pena, 1999)), then the progenitors become more oxidized and are more likely to generate oligodendrocytes (Smith et al, 2000; Yakovlev et al, 1998). If these pro-oxidative changes are prevented, however, then the effects of TH on oligodendrocyte generation also are prevented (Smith et al, 2000). In contrast, co-exposure of dividing OPCs to neurotrophin-3 (NT-3) makes cells more reduced and enhances self-renewal. Inhibition of synthesis of glutathione (a tripeptide that is one of the central regulators of cellular redox state) prevents the effects of NT-3 on redox state and also prevents NT-3-mediated enhancement of self-renewal. Thus, despite the well-studied effects of these signaling molecules on gene transcription and signaling cascades, antagonizing the redox changes they produce prevents their effects on self-renewal and differentiation, thus underscoring the importance of intracellular redox status as a regulator of this balance.

As a clear example of a physiological signal that increases intracellular oxidative status and can alter the timing of oligodendrocyte generation, we initially focused our attention on TH. The first question we examined was whether analysis of TH and of mitochondrial function would enable us to integrate the following observations: (i) Although most studies on TH-induced oligodendrocyte differentiation have focused on the role of this hormone in regulation of gene expression by its receptors (e.g., (Bernal, 2005; Dugas et al, 2012; Nygård et al, 2003; Pombo et al, 1999; Tokumoto et al, 1999)), studies on mice lacking all TH receptor (THR) alpha and beta isoforms does not reveal any detectable changes in the timing of myelination (Baas et al, 2002). Such results raise the possibility that TH may regulate the timing of myelination by effects independent of the role of these receptors in nuclear transcriptional regulation. (ii) Our studies suggest that one potential such regulator is redox state. We found that the ability of TH to make OPCs more oxidized is essential to the induction of oligodendrocyte generation by TH, that co-exposure to the anti-oxidant N-acetyl-L-cysteine (NAC) blocks the effects of TH on cessation of division and initiation of oligodendrocyte generation and that treatment with a chemical pro-oxidant was as effective as TH at inducing oligodendrocyte generation from dividing progenitor cells(Smith et al, 2000). (iii) Through a pathway separate from the effects controlled by nuclear THRs, TH can stimulate mitochondrial respiration, thus enhancing production of reactive oxidative species (ROS) and also induces opening of the mitochondrial membrane permeability transition pore (MPTP) (Kalderon et al, 1995; Wrutniak-Cabello et al, 2001; Yehuda-Shnaidman et al, 2010; Yehuda-Shnaidman et al, 2005).

In other words, these experiments tested the hypothesis that it is the pro-oxidant effects of TH that are critical in inducing oligodendrocyte generation and asked how these pro-oxidative effects originate. It is important to point out that alterations in mitochondrial function, of a nature that would be expected to make cells more oxidized, are of interest as a possible contributory factor in generation of ASDs (e.g., (Frye et al, 2013a; Frye et al, 2013b; James et al, 2009)).

To test the hypothesis that TH-induced MPTP opening functionally links promotion of oligodendrocyte generation with mitochondrial-induced increases in oxidative status, we first exposed O-2A/OPCs dividing in the presence of PDGF to TH in the presence or absence of cyclosporine A (CysA), a pharmacological inhibitor of MPTP opening (Broekemeier et al, 1989; Crompton et al, 1988). This prevented TH-induced increases in oxidative status (**Figure 21A**) and suppressed oligodendrocyte generation**.** Detailed clonal analysis of differentiation demonstrated that by the third day of TH exposure most clones contained at least one (43.4%), two (20.8%), three (7.5%) and as many as four (3.8%) oligodendrocytes (**Figure 21C**). In contrast, less than a third (29%) of the clones in the TH/CysA double treatment contained any oligodendrocytes at all at this time point (**Figure 21D**). At day 6 (data not shown) most (68%) of the TH-treated clones contained oligodendrocytes, and 23% consisted entirely of oligodendrocytes. In contrast, in TH/CysA double-treated cultures, only 11.5% of clones contained only oligodendrocytes while 59.8% had no oligodendrocytes at all and contained only progenitor cells. The decreases in oligodendrocyte generation were not due to induction of cell death, as CysA did not alter cell viability (TH = 7.9% ± 0.7%) vs. TH+CysA 3.7% ± 1%).

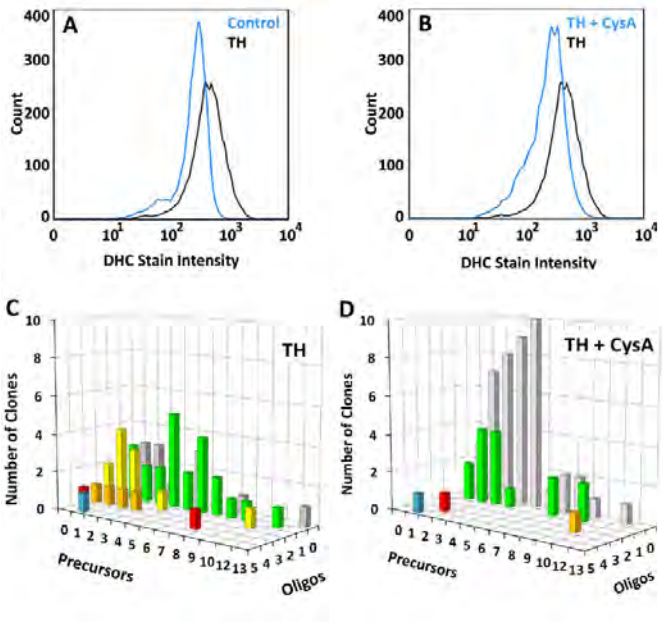



*Figure 21. Mitochondrial permeability transition pore (MPTP) inhibitor Cyclosporin A prevents TH-induced O-2A/OPC oxidation and differentiation.* (A) Flow cytometric analysis shows that a 3-day TH treatment makes O-2A/OPCs more oxidized. (B) Pharmacological MPTP inhibitor cyclosporin A (CysA) prevents intracellular oxidation by TH. (C) Clonal analysis shows that a 3-day TH treatment induces robust oligodendrocyte generation. (D) Clones treated with MPTP inhibitor CysA and TH show very little differentiation and most contain only precursors.

Genetic inhibition of MPTP opening by Bcl-2 overexpression (e.g., (Marzo et al, 1998; Reed et al, 1998)) also prevented TH-induced increases in oxidative status, and was even more potent than CysA at preventing TH-induced differentiation (**Figure 22**). In cultures infected with a control lentivirus, 30% of the clones contained only progenitor cells on Day 3 and 70% of total clones had 2 or less progenitor cells. In contrast, 44% of clones arising from cells overexpressing Bcl-2 consisted wholly of progenitor cells after 3 days of TH exposure, and only 31% of total clones contained 2 or fewer progenitor cells. On day 6 (data not shown), only 2% of the clones in cultures infected with empty virus consisted wholly of progenitor cells, and 48% had 2 or less progenitor cells in them. When Bcl-2 overexpressing cells were grown in identical conditions, 42% of the clones consisted only of progenitor cells and only 20% of clones had 2 or less progenitors in them.

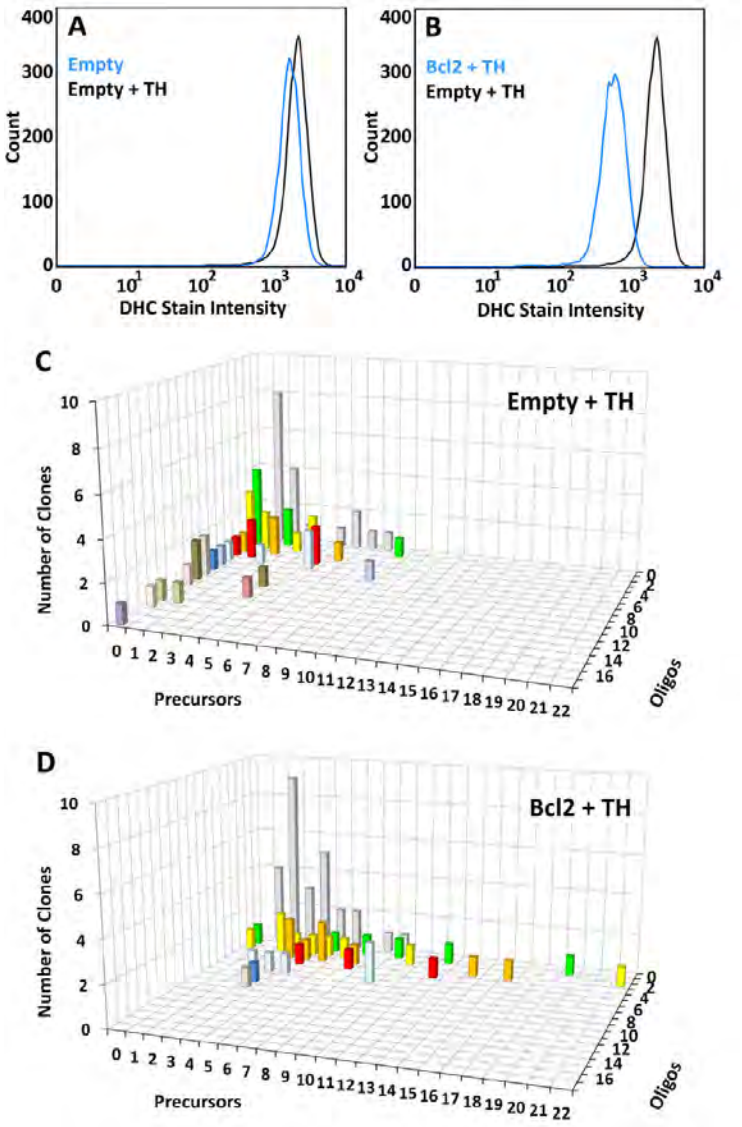



*Figure 22. Mitochondrial permeability transition pore (MPTP) inhibition by Bcl2 prevents TH-induced O-2A/OPC oxidation and differentiation.* (A,B) Expression of Bcl2, and endogeneous MPTP inhibitor, prevents intracellular oxidation by TH. (C,D) Expression of full length, wild type, mouse Bcl2 inhibits TH-induced differentiation, reverting clonal composition from mostly oligodendrocyte only clones, seen in the empty vector infected cells, to mostly precursor containing clones.

*2.2.9.2 TH-induced generation of oligodendrocytes requires activation of the redox/Fyn/c-Cbl pathway*

Having established that MPTP opening is critical for TH-induced generation of oligodendrocytes, we next investigated the molecular mechanisms that lead from exposure to TH to cessation of cell division.

Although most studies on how TH suppresses division and induces differentiation have focused on regulation of proteins that represent key cell cycle components (e.g., retinoblastoma (Rb) protein, cyclin D1) (e.g,. (Tokumoto et al, 2001)}(Casaccia-Bonnefil et al, 1997; Durand et al, 1998; Huang et al, 2002), our previous studies on the effects of environmental toxicants on OPCs raise the very different possibility that the critical effect of pro-oxidative changes is to cause a cessation of mitogenic signaling by activating c-Cbl induced degradation of receptor tyrosine kinases (RTKs) required for continued division (Li et al, 2007). These studies showed that exposure of OPCs to low levels of methylmercury, lead or paraquat (which share the property of making these progenitor

cells more oxidized) caused activation of Fyn kinase, which in turn activated the c-Cbl E3 ubiquitin ligase. This led to ubiquitylation and degradation of c-Cbl targets, including PDGF receptor-α (PDGFRα), causing reduced levels of all downstream signaling It is not known, however, whether activation of the redox/Fyn/c-Cbl (RFC) pathway and consequent loss of PDGFRα following exposure to environmental toxicants represents a cellular response to these specific chemicals, to physiological stressors in general or whether this pathway also might be of relevance to understanding signaling pathway regulation in response to normal developmental signals.

Exposure of dividing OPCs to TH caused a 25% increase in levels of phosphorylated c-Cbl, which was prevented by co-exposure to CysA (**Figure 23A**) or over-expression of Bcl-2 (**Figure 23B**), as predicted if these changes were initiated by TH-induced increases in oxidative status. Activation of c-Cbl was required for TH to induce oligodendrocyte generation . When OPCs were modified to express the dominant-negative (DN) 70Z mutant of c-Cbl (DN(70Z)-c-Cbl; (Andoniou et al, 2000)) and exposed to TH for 6 days, there was an 80% reduction in the proportion of oligodendrocytes observed. Expression of shRNA for c-Cbl was similarly effective at preventing oligodendrocyte generation. Moreover, expression of DN(70Z)-c-Cbl or c-Cbl shRNA decreased the spontaneous generation of oligodendrocytes in cultures dividing in the presence of PDGF but not exposed to TH, demonstrating that altering activity of this pathway can alter the probability of self-renewal even in cultures undergoing differentiation regulated by cell-intrinsic mechanisms.

Further evidence that TH-mediated induction of oligodendrocyte generation required activation of the RFC pathway was provided by pharmacological and genetic perturbation of Fyn kinase activity. Co-exposure to the Src family kinase inhibitor PP1 reduced c-Cbl phosphorylation (**Figure 23**) and also prevented TH-induced generation of oligodendrocytes in cultures of dividing OPCs. As PP1 also inhibits other Src family members, we further perturbed Fyn kinase function by genetic knockdown via expression of Fyn-targeted shRNA. Cells expressing non-targeting shRNA constructs showed no difference from uninfected cells in respect to the proportion of oligodendrocytes generated, while cells expressing Fyn shRNA showed a >90% reduction in the proportion of oligodendrocytes present in TH-treated cultures.

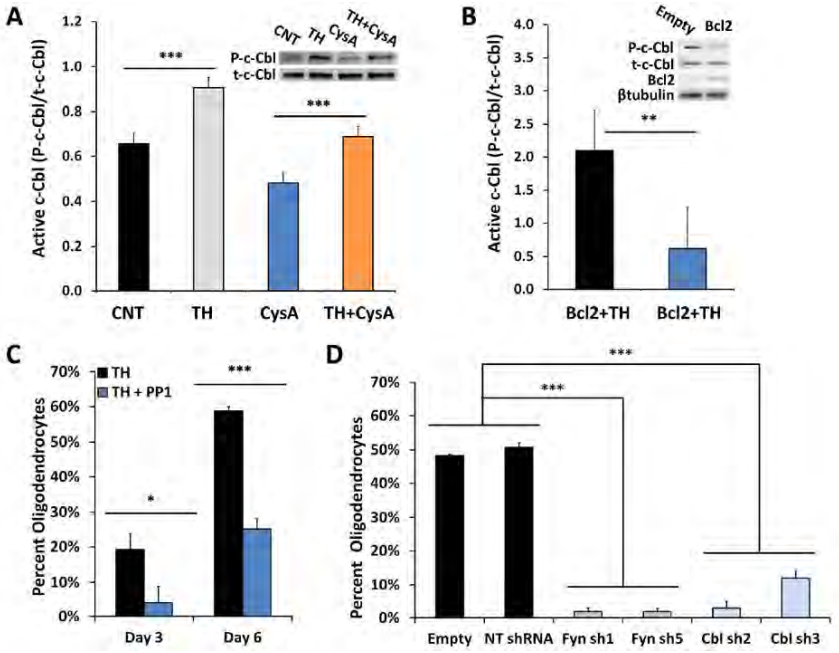



*Figure 23. Mitochondrial permeability transition pore (MPTP) inhibition prevents TH-induced O-2A/OPC differentiation by blocking the activation of the redox/Fyn/c-Cbl pathway.* (A) Western blotting shows that MPTP inhibitor CysA blocks c-Cbl activation by a 3-day TH treatment. The increase in the ratio of active phosphorylated c-Cbl (P-c-Cbl) to total c-Cbl seen with TH is abolished by CysA. (B) Expression of an endogenous MPTP inhibitor Bcl2 completely blocks c-Cbl activation by TH. (C) Flow cytometric analysis shows that a 3-day TH treatment leads to c-Cbl activation, as indicated by the increase in phosphorylated c-Cbl (P-c-Cbl) level. (D) c-Cbl activation can be block with the Src-family kinase inhibitor PP1 (E) PP1 blocks TH-induced differentiation. (F) Expression of Fyn and c-Cbl shRNA constructs inhibits the ability of TH to induce O-2A/OPC differentiation.

*2.2.9.3 c-Cbl activation is required for TH-mediated regulation of PDGFRa and cell cycle protein expression*

Previous studies on the effects of TH on dividing OPCs indicated that part of the means by which TH induces cessation of OPC division and induction of oligodendrocyte generation is through modulating expression and/or activity of proteins critical in cell cycle progression (e.g, (Casaccia-Bonnefil et al, 1997; Durand et al, 1998; Huang et al, 2002; Tokumoto et al, 2001)). Proteins previously found to be altered in their expression by exposure to TH include cyclin dependent kinases (Cdks) 2 and 4, cyclin D1, cyclin E, and levels of phosphorylated retinoblastoma (Rb) protein, Akt and ERK1/2.

We found that along with previously identified changes, TH exposure also caused reductions in levels of PDGFRα and that such effects were prevented by expression of c-Cbl shRNA (**Figure 24**) and DN(70Z)-c-Cbl. When cells were examined 8 hrs after exposure to TH (a time point at which progenitor cells deprived of PDGF signaling show a >50% reduction in the proportion of Ki67+ cells), we observed falls in levels of Cdk2 (19%) and Cdk44 (40%), cyclin E (38%) and cyclin D1 (54%) and phosphorylated retinoblastoma (Rb) protein, Akt (35%) and ERK1/2 (68%). We also found that TH caused an 86% reduction in levels of PDGF receptor α (PDGFRα). As predicted by

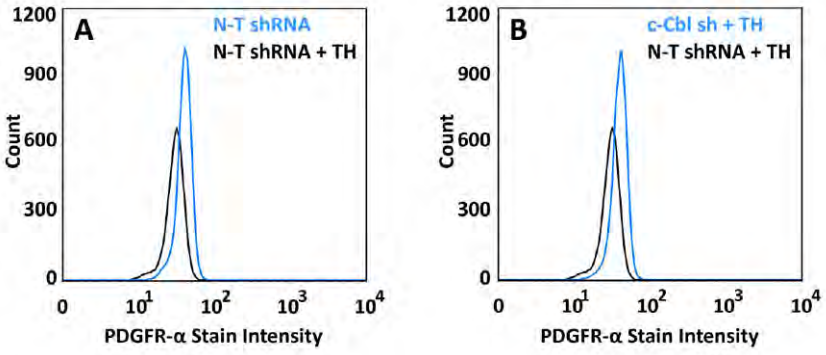
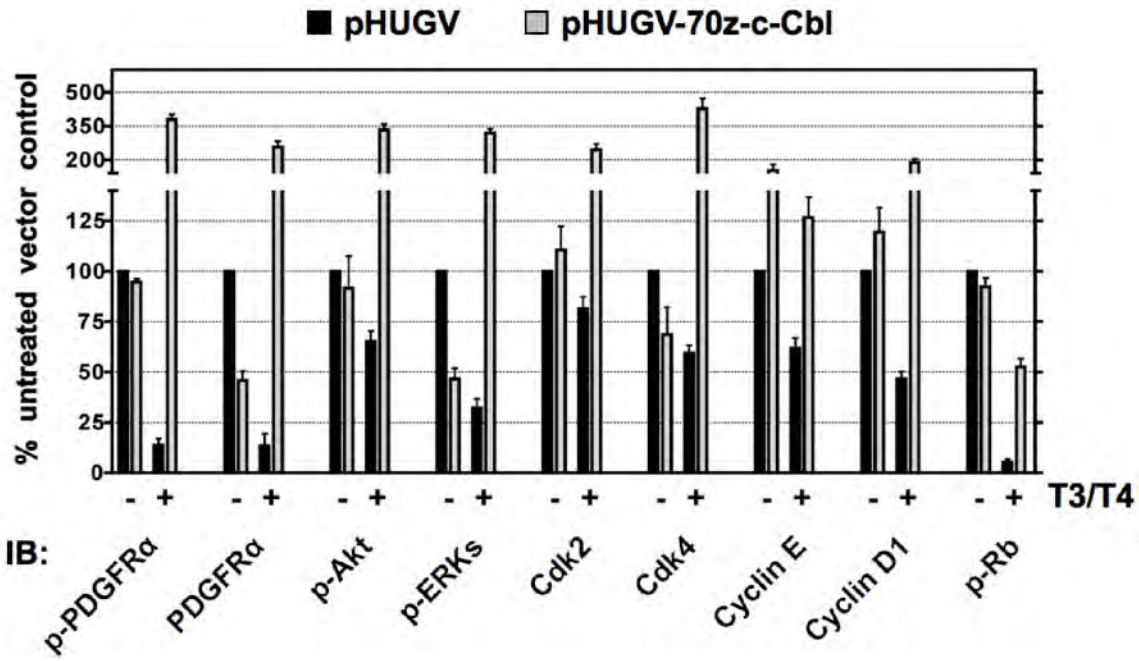



*Figure 24. c-Cbl is required for the TH-induced decrease in PDGFR-α and in multiple proteins required for cell cycle progression.* (A) Flow cytometric analysis shows that a 3-day TH treatment leads to a decrease in PDGFR-α level. (B) Expression of c-Cbl shRNA prevents the reduction in PDGFR-α level by TH. (C) Expression of DN(70Z)c-Cbl prevents TH-induced reductions in multiple proteins required for cell division



the hypothesis that these changes were dependent on c-Cbl activation, all of these effects were prevented by expression of DN(70Z)-c-Cbl (except that levels of phosphorylated Rb were only rescued to 50% of control levels) and often were at higher levels than controls (consistent with the increases in levels of PDGFRα and p-PDGFRα above control values).

*2.2.9.4 Analysis of the effects of BMP on OPCs suggests c-Cbl activation controls division rather than differentiation*

Do our findings on TH reveal general insights into how pro-oxidative signaling molecules promote differention of OPCs? To address this question, we next determined whether the RFC pathway is also involved in the regulation of differentiation induced by exposure of dividing OPCs to BMP-4 (which also causes these progenitor cells to become more oxidized (Smith et al, 2000). This was of particular importance, as BMP-4 (and also BMP-2) induces OPCs to differentiate into astrocytes rather than oligodendrocytes (Mabie et al, 1997), thus enabling us to examine a distinct pathway of differentiation.

We found that although the pattern of c-Cbl phosphorylation induced by exposure to BMP4 was like that caused by exposure to TH, the RFC pathway did not seem to be involved in causing differentiation of OPCs into astrocytes. Examination of c-Cbl phosphorylation at Y700, Y731 and Y774 showed increased phosphorylation at all three sites 6 hrs after exposure to BMP. We also noted that at 48h, both TH and BMP exposure were associated with particularly enhanced phosphorylation at Y731. However, we found that c-Cbl knockdown had no effect on BMP-induced expression of glial fibrillary acidic protein (GFAP, an intermediate filament that is one of the major identifiers of astrocytic differentiation (Eng et al, 2000; Eng et al, 1971)). Similarly, Fyn knockdown had no effect on BMP-induced expression of GFAP (**Figure 25**). In addition, antagonizing the pro-oxidant effects of BMP by co-exposure of cells to NAC did not have any significant effect on the proportion of cells differentiating into GFAP+ astrocytes.

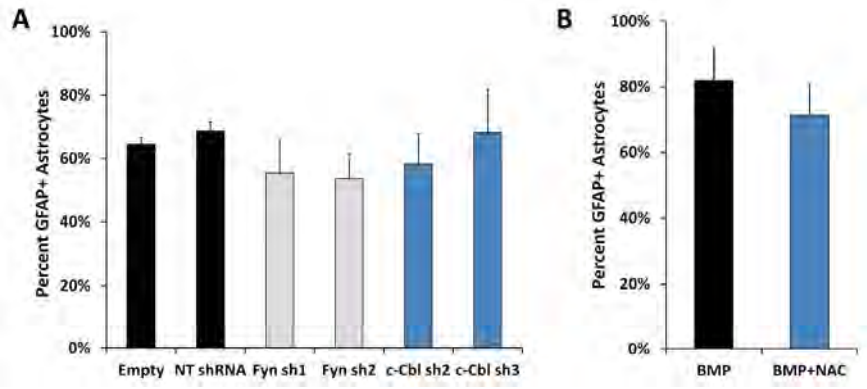



*Figure 25. Redox/Fyn/c-Cbl pathway inhibition does not prevent BMP-induced O-2A/OPC differentiation into astrocytes.*(A) Expression of Fyn and c-Cbl shRNA constructs does not prevent O-2A/OPC differentiation into GFAP+ astrocytes when exposed to BMP4 for 3 days. (B) Treatment with the anti-oxidant NAC does not inhibit O-2A/OPC differentiation into GFAP+ astrocytes when exposed to BMP4 for 6 days.

Despite the lack of effect of RFC pathway inhibition on BMP-4 mediated induction of GFAP expression in O-2A/OPCs, activation of c-Cbl did appear to play a critical role in the ability of BMP-4 to modulate expression of proteins critical for cell division (**Figure 26**). At the same 8 hr time point used to analyze the effects of TH exposure, there were multiple similarities between the effects of TH and BMP-4. BMP-4 exposure caused decreases in levels of cyclin E (48%) and

cyclin D1 (95%) and of phosphorylated retinoblastoma (Rb) protein (89%), Akt (65%) and ERK1/2 (50%). BMP exposure also caused a 92% reduction in levels of PDGFRα. The effects of BMP differed from TH in effects on Cdks, and BMP induced a larger (75%) fall in levels of Cdk2 but also caused a substantial increase in levels of Cdk4.

Almost all of the effects of BMP on proteins required for cell division were prevented by expression of DN(70Z)-c-Cbl (except that levels of phosphorylated Rb were only rescued to 50% of control levels) and often were at higher levels (consistent with the increases in levels of PDGFRα and p-PDGFRα above control values (as for TH)

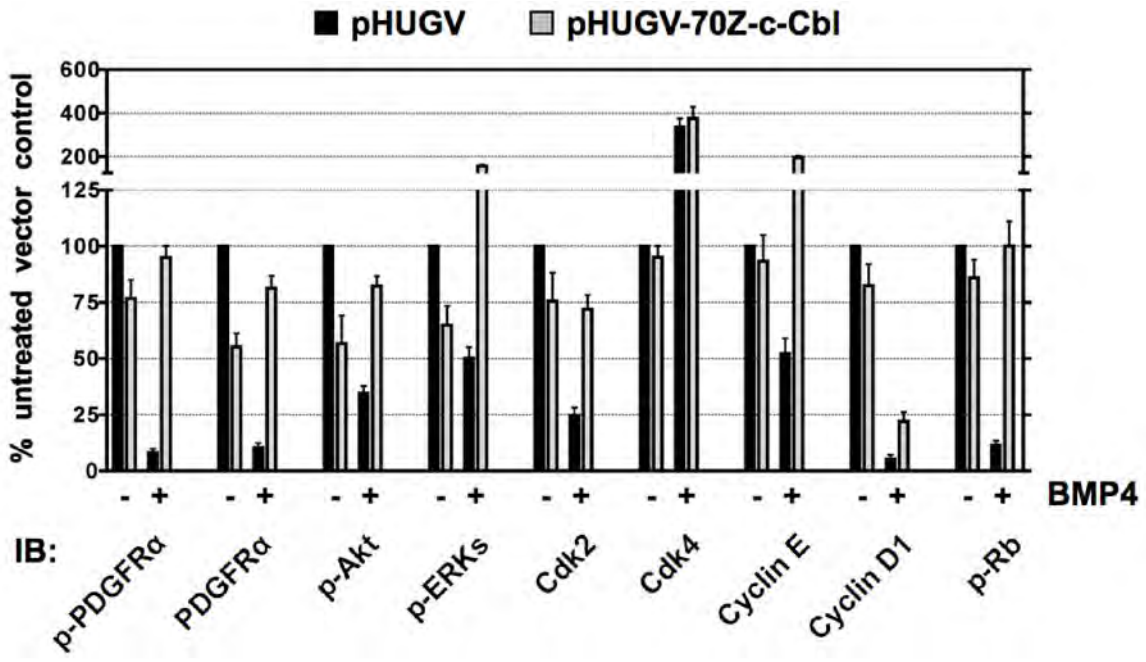



***Figure 26**. c-Cbl is required for the BMP-induced decrease in PDGFR-α and in multiple proteins required for cell cycle progression.* Expression of DN(70Z)c-Cbl prevents TH-induced reductions in multiple proteins required for cell division

*2.2.9.5 Signaling molecules that enhance O-2A/OPC self-renewal decrease c-Cbl activation*

If the induction of cell cycle exit requires activation of the RFC pathway, then it is of particular interest to determine whether agents that enhance self-renewal suppress c-Cbl activation. We previously showed that activation of the RFC pathway by environmental toxicants is prevented by exposure to NAC(Li et al, 2007). Self-renewal of O-2A/OPCs is enhanced by fibroblast growth factor-2 (FGF-2, also referred to as basic FGF) or NT-3 when these proteins are applied to cells dividing in the presence of PDGF (Bogler et al, 1990; Ibarrola et al, 1996). FGF-2 and NT-3 also both caused a more reduced intracellular redox state in dividing O-2A/OPCs, and the ability of NT-3 to enhance self-renewal was prevented by inhibition of glutathione synthesis with buthionine sulfoximine (BSO, an inhibitor of gamma-glutamylcysteine synthetase, the enzyme required in the first step of glutathione synthesis) (Smith et al, 2000).

NT-3 or FGF-2 exposure decreased the levels of phosphorylated c-Cbl in dividing O-2A/OPCs, and caused increased levels of PDGFRα and of c-Met (the receptor for hepatocyte growth factor), both of which are targets of activated c-Cbl (Li et al, 2007) (**Figure 27**). In contrast, no changes were seen in levels of the NT-3 receptor, TrkC, which is not a c-Cbl target (Li et al, 2007). As also predicted by our other observations, O-2A/OPCs co-exposed to PDGF + [FGF-2 or NT-3] had higher levels of Erk1/2 phosphorylation than those exposed to PDGF alone, as contrasted with the lower levels of Erk1/2 phosphorylation in cells exposed to TH or BMP-4. As it was previously reported that FGF-2 caused increased transcription of the PDGFRα (McKinnon et al, 1990), we also examined the effects of FGF-2 on levels of PDGFRα when cells were grown in the presence of actinomycin D. No effect of this transcriptional inhibitor was seen, indicating that the increases in levels of PDGFRα were due to post-transcriptional regulation.

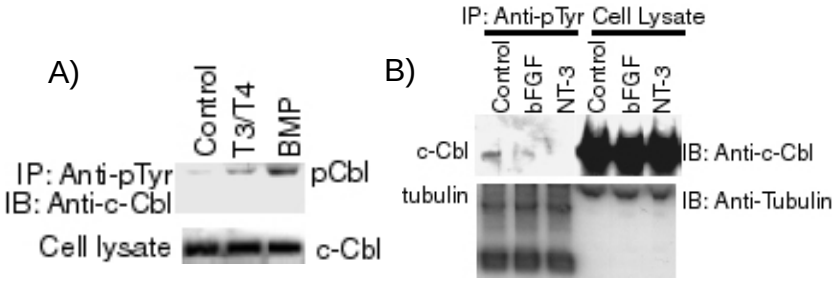



**Figure 27**: Rat O2A cells were exposed to TH (1:1000), BMP4 (10ng/ml), FGF-2 (10ng/ml) or NT-3 (10ng/ml) for 3-4 hr. Cells were lysed for immunoprecipitation using anti-phospho-tyrosine antibody, and the immunoprecipitated samples were run on a SDS-PAGE gel and blotted with anti-c-Cbl antibody. *Immunoprecipitation assay.* For the co-immunoprecipitation assay, anti-p-Tyr monoclonal antibody (Santa Cruz) was added into the pre-cleared cell lysates (250 µg of total protein) and the mixtures were gently rocked for 2 hr at 4°C. 30 µl of protein A/G agarose was then added into the mixture followed by rotating at 4°C for overnight. The protein A/G agarose was then spun down and washed thoroughly three times. The precipitates were resolved on an 8% SDS-PAGE gel and subjected to Western blot analysis using an anti-Cbl antibody (BD Pharmingen).

The ability of agents that enhance self-renewal to make cells more reduced predicts that they also will inhibit the effects of TH exposure. In accord with this prediction, exposure to FGF-2 suppressed TH-induced activation of c-Cbl and prevented TH-induced reductions in levels of PDGFRα (**Figure 28**). These changes were dependent upon the ability of FGF-2 to make cells more reduced. As seen previously for effects of NT-3 on self-renewal (Smith et al, 2000), co-exposure to BSO prevented the effects of FGF-2 and restored TH-induced changes in c-Cbl phosphorylation and levels of PDGFRα.

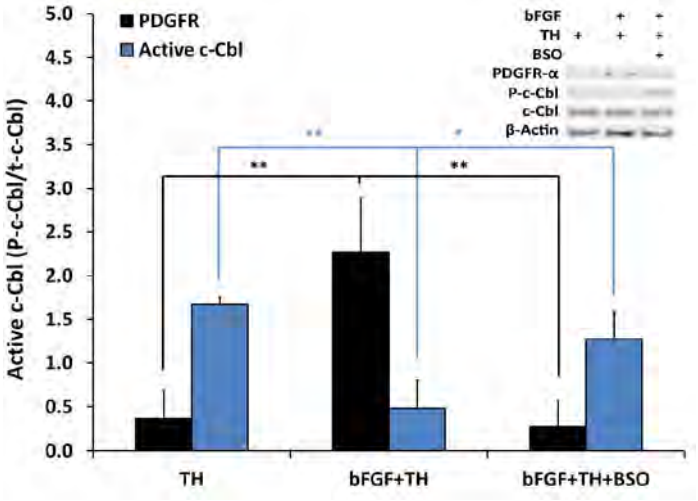



***Figure 28**. Extracellular signaling molecules that promote self-renewal prevent c-Cbl activation by TH.*(A) Western blotting shows that bFGF prevents c-Cbl activation seen with a 3-day TH treatment. This effect was reversible by the co-treatment with the pro-oxidant BSO, demonstrating that the decrease in c-Cbl activation is redox dependent (B).

## 3. Reportable Outcomes

We are now working to integrate all findings together into a new understanding that will stretch from the identification of individuals with oxidative imbalances to implications of oxidative imblanace on the response to cellular stress to molecular mechanisms underlying such altered responses and on to mechanisms that may even explain the failure of oxidized cells (as are present in individuals with ASDs) to repair their redox balance to a more physiologically normal level.

As there are sequential elements that need to be provided in order that this work will have the desired effect on the scientific community, our current plan is to publish this work in the following order:

Paper 1 (currently in preparation for submission) places the redox/Fyn/c-Cbl pathway at the center of understanding how the balance between self-renewing division and differentiation into a non-dividing terminally differentiated cells is regulated in oligodendrocyte progenitor cells. This paper also places mitochondrial regulation of reactive oxidative species production and release at the center of this regulation. This paper thus integrates redox regulation of progenitor cell function with other key cellular components, and provides the foundation needed to attract other researchers to this problem.

Paper 2 (draft text in progress) is focused on the regulation of the redox balance in progenitor cell populations that have cell-intrinsic regulation of this balance. As these progenitor cells are isolated from different regions of the developing CNS of the same animals there are no concerns about whether outcomes are due to strain differences. This paper will place the Nrf2 pathway in a primary regulatory position for normal development rather than simply as a response to oxidative stress. Such a developmental understanding of the function of Nrf2 is required for the next studies demonstrating what happens when this pathway does not function properly.

Paper 3 (draft text of progress) will provide the first paper on dysregulation of Nrf2 function in the context of human disease. Although this paper is focused on the disease ataxia telangiectasia, and the work has been conducted with funding from other sources, the experiments have often been informed by the knowledge gained from the DOD-sponsored work. Thus, it will be appropriate to to recognize the DOD support in this paper.

Paper 4 (additional experiments being conducted) will report the effects of redox status on development of dendritic complexity.

Other outcomes of significance are as follows:

1. As discussed in the following section, our studies on alterations in redox biology led to parallel studies on redox alteration in the genetic disease ataxia telangiectasia.
2. Not discussed in this report, but of great importance, is the outcome that our work attempting to understand whether vulnerability to physiological stressors could be predicted differences in cellular physiology related to developmental CNS disease has led to a series of remarkable findings related to diseases of lysosomal dysfunction. In these studies we have found that the toxic lipids accumulated in several different lysosomal storage disorders all cause more than 15% of all FDA-approved drugs to become highly toxic for OPCs. As

lysosomal dysfunction has been implicated in a wide range of neurological diseases (including Parkinson's disease, Alzheimer's disease and macular degeneration), we are now pursuing the hypothesis that these same drugs may hasten the progress of these other important diseases.

3. In both of the above cases, the attention paid to these other disorders has led to funding for expanded studies, with funding received from the SPARKS Foundation (UK) for our work on ataxia telangiectasia and from the Legacy of Angels (US) for our work on lysosomal storage disorders.

**4. Key Research Accomplishments**

• We have identified a wide range of redox-related factors that can be used to distinguish cells with different basal redox states, including differences in levels of glutathione, in the ratio of ATP:ADP and the ratio of reduced:oxidized pyrimidine nucleotides, and differences in levels of bcl-2 and superoxide dismutase-1 and in the levels of γ-glutamyl-cysteinyl-synthase heavy chain (γ-GCS) , the rate-limiting enzyme in glutathione biosynthesis.

• Analysis of multiple cell populations from the bloodstream shows that both B lymphocyte and T lymphocyte populations show the same differences in redox status as detected in oligodendrocyte progenitor cells. In contrast, these differences are not as reliably detected in myeloid populations, such as macrophages and neutrophils.

• Analysis of the levels of the c-Met protein in the hippocampus of SJL mice (which are more oxidized) shows that these protein levels are much reduced compared with that found in the hippocampus of CBA mice (which are more redudced). This demonstrates the predictive value of our redox/Fyn/c-Cbl pathway analysis. As SJL mice are intrinsically more oxidized than CBA mice, our prediction was that this would lead to lower levels of c-Met, which is a c-Cbl target.

• We next tested the hypothesis that SJL mice would have impaired neuronal dendrite generation, as has been observed in autism. This was our prediction due to the role of c-Met in dendritic regulation. Our analyses revealed several striking differences between hippocampus dendritogenesis in CBA vs SJL mice. Dendritic complexity in SJL mice was markedly reduced as compared with CBA mice of the same age. The quantity of dendrites was markedly lower in the hippocampi of SJL mice. This was reflected in a grouping of the dendritic numbers towards the lower number of values , as compared with the broader distribution of values for CBA mice. Similar outcomes were obtained for analysis of dendritic nodes, which were more numerous in the hippocampus of CBA mice than in SJL mice. As predicted from such outcomes, the number of dendritic ends was also lower in SJL mice.

• To integrate the descriptive analysis of Aim 1 with the goals of Aim 2 it was critical to identify molecular mechanisms that underlie the differences in redox state between different cell populations. The hypothesis that integrates many of the components of this research effort into a single pathway, stretching from changes observed at the descriptive level to the mechanisms responsible for these changes, can be stated as follows: Epigenetic changes in Nrf2 activity are responsible for fundamentally different basal redox states and in the ability to respond to agents that perturb redox state. When Nrf2 activity is set at a lower level, cells are intrinsically more oxidized and more susceptible to agents that further increase oxidative status. These changes cause activation

of the redox/Fyn/c-Cbl pathway, leading to increased degradation of important receptor tyrosine kinases (RTKs) at the cell surface. This leads to decreases in NF-kB activity, which in turn leads to reduced levels of bcl-2 and also of γ-GCS. Reductions in levels of bcl-2 further work to keep cells more oxidized by allowing greater efflux of reactive oxidative species from mitochondrial permeability transition pores. Thus, cells with reduced Nrf2 activity are maintained in a chronically oxidized state that is responsible for increased activity of the redox/Fyn/c-Cbl pathway, and activation of the redox/Fyn/c-Cbl pathway reduces NF-kB

## 5. Conclusions and Synthesis

This research has now brought us to a point at which we can offer a mechanism-based synthesis of how increases in oxidative status, whether induced by environmental toxicants, genetic factors, or the both working together, could alter development in the CNS in ways that potentially have significant relevance for understanding the pathogenesis of ASDs. The key pathways involved in this synthesis are summarized in **Figure 29**.

First, to recapitulate the potential links to ASDs that motivated our research:

- It has been reported that children with ASDs are more oxidized than age-matched controls.
- MRI studies suggest that children with ASD show peculiar myelination abnormalities in which major myelinated tracts of the CNS have what appears to be a larger-than-normal white matter volume early in development, with a smaller-than-normal white matter volume later in development.
- Other studies indicate that children with ASDs show ongoing inflammation in the CNS.
- Our studies on the control of development by intracellular redox balance predict that increased oxidation would make OPCs more responsive to inducers of myelination, leading to an increase in early myelination but also causing dividing progenitor cells to prematurely cease division and differentiate. This would lead to a decreased pool of the progenitor cells, followed by a decreased level of myelination later in life.
- Substances increased in the CNS during myelination are toxic for OPCs and oligodendrocytes, and thus could further contribute to myelination decreases as the CNS matures.
- Being more oxidized increases susceptibility to the cytotoxic effects of substances released during inflammation.

The above observations raised several questions that were the targets of our research: But many are

- What are the mechanisms that control cell-intrinsic differences in intracellular redox state and would lead to a more oxidized basal redox status?
- What are the molecular mechanisms by which increased oxidative status would alter development so as to cause the alterations in the time course of myelination that have been reported to occur in ASDs.
- How can you non-invasively analyze redox state in an individual so as to identify children at increased risk for vulnerability to physiological stressors of potential relevance in ASD pathogenesis?

Based on the research conducted with this funding, we now propose the following answers to the questions of what are the molecular mechanisms that control differences in intracellular oxidative status and that are involved in the translation of increases in oxidative status into changes in CNS progenitor cell function. Aspects of this discussion addressing molecular mechanisms are summarized in **Figure 29**.

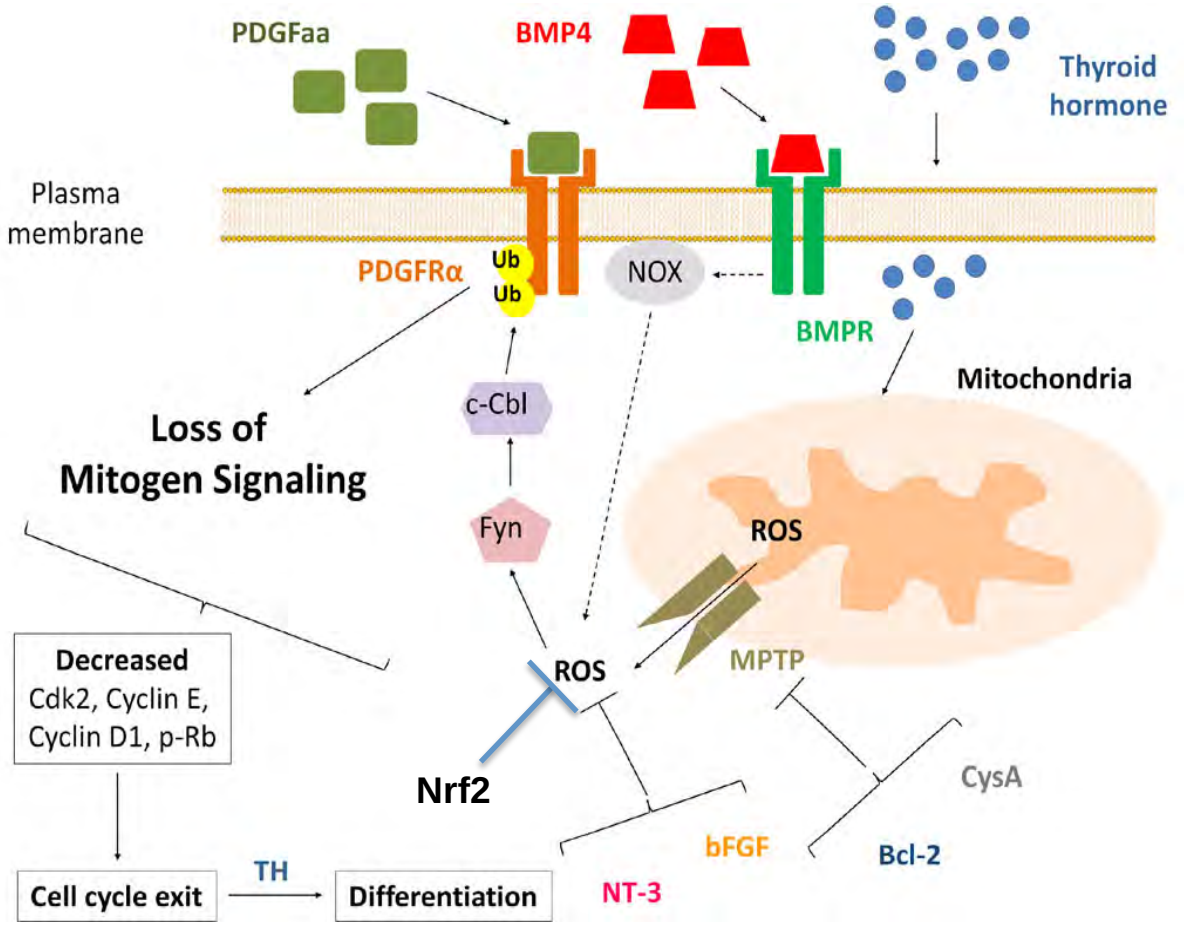



**Figure 29. Summary of molecular mechanisms by which alterations in intracellular redox status alter responsiveness to inducers of differentiation** :
• Cells with different basal redox states have different levels of Nrf2 expression, and higher levels of Nrf2 (or lower levels of Keap1) makes cells more reduced, providing one means of antagonizing the effects of ROS production. One source of ROS is via MPTP opening. ROS also can be produced by activation of NADPH-oxidases (NOX), which can be activated by inflammation but also by BMPs.
• If ROS levels are sufficient to make cells more oxidized, this activates the redox/Fyn/c-Cbl pathway, leading to cessation of OPC division and induction of oligodendrocyte division.
•OPCs that are more oxidized .thus show earlier differentiation and less self-renewal, which could lead to an earlier onset of myelination but a decrease in developmentally appropriate levels of myelination at later stages of development (as seen in ASDs).
•Activation of c-Cbl causes degradation of receptor tyrosine kinases required for cell division, with suppression of downstream signaling and reduced levels of cyclins and cyclin-dependent kinases. This can lead to falls also in levels of the c-Met receptor, which modulates dendritic complexity in the hippocampus.
•Receptor tyrosine kinases that are downregulated by c-Cbl also provide important survival signals, and a loss of this signaling would increase cellular vulnerability to physiological stressors, such as tumor necrosis factor and glutamate.

***Conclusions***

The goals of our work were to identify means of identifying cells, and individuals, that present with a more basal oxidized redox state and to identify molecular mechanisms that might functionally integrate such an oxidized state with observations that the multiple environmental insults that have been suggested to be involved in autism pathogenesis occur in many more children than those that develop ASD. This suggests that there is an underlying vulnerability phenotype that makes some children more vulnerable to such stressors. Taken together with our studies on the central importance of redox status in controlling cellular response to the environment, the observations that many children with ASD are more oxidized raises the possibility that this shift towards a more oxidative status provides a unifying principle that would explain such an increased vulnerability. Our molecular analyses of the mechanisms by which redox status controls the function of cells critical in development of the central nervous system (CNS) further suggests that it is now possible to identify the reasons for this increased vulnerability at the level of detailed analysis of signaling pathway function.

More specifically:

(i) We predicted that cells that are more oxidized for epigenetic reasons (i.e., are not due to strain/genetic differences) will show less ARE activity than cells that are more reduced for epigenetic reasons. • *Outcome:* OPCs isolated from the corpus callosum (and which are intrinsically more oxidized) show lower levels of ARE activity than progenitor cells isolated for the cortex (and which intrinsically are more reduced).

(ii) We predicted increased expression of Nrf2 will cause oxidized cells to become more reduced, including having higher levels of gluathione, and to alter their biological properties to now behave like cells that are more reduced for epigenetic reasons. • *Outcome:* Increasing the levels of Nrf2 activity by overexpressing Nrf2 in corpus callosum OPCs makes these cells more reduced and reduces the tendency of these cells to undergo differentiation into oligodendrocytes.

(iii) We predicted that inhibition of Nrf2 will cause reduced cells to become more oxidized, including having lower levels of glutathione, and to alter their biological properties to now behave like cells that are more oxidized for epigenetic reasons. • *Outcome:* Inhibiting Nrf2 activity by expression of one of the inhibitory binding partners Keap1 (which keeps Nrf2 from entering the nucleus and thus prevents Nrf2- mediated activation of the ARE promoter) makes OPCs more oxidized and increases the early spontaneous generation of oligodendrocytes.

(iv) We predicted that elevation of levels of bcl-2 will reduce the ability of pro-oxidants to activate the redox/Fyn/c-Cbl pathway. • *Outcome:* Elevation of levels of bcl-2 inhibits the ability of the pro-oxidant thyroid hormone (thought to be the major physiological regulator of oligodendrocyte differentiation) to induce OPCs to become oligodendrocytes.

(v) We predicted that pro-oxidant inducers of differentiation work through activation of the redox/Fyn/c-Cbl pathway, and can be blocked by increased activation of Nrf2 or by increased expression of bcl-2. • *Outcome:* Increasing Nrf2 activity (by overexpression of the Nrf2 protein) or increasing levels of bcl-2 both decrease the ability of thyroid hormone to activate the redox/Fyn/c-Cbl pathway and induce differentiation of dividing OPCs into oligodendrocytes.

*Increased oxidative status is associated with loss of the c-Met receptor and reduced complexity of*

*hippocampal dendrites*

The period of time when redox differences between SJL and CBA mice were reliably observed enabled us to test the predictions that brains of mice that were more oxidized would show reduced levels of the c-Met receptor. This was a key prediction of the redox/Fyn/c-Cbl hypothesis as c-Met is a c-Cbl target. Although studying dendritogenesis was not one of initial goals, and this work was conducted with other funding, analyzing dendritic complexity offered another test of the potential relevance of our studies to ASDs. We found that mice that were more oxidized showed reduced dendritic complexity in the hippocampus, as predicted by the decreased levels of c-Met.

*Non-invasive analysis of redox status by using lymphoid cells as a surrogate for analysis of the CNS*

Our work on this problem was a mixed success. During the first segment of our work we were able to reproducibly observe differences in T-cell and B-cell redox status that mirrored different redox states also observed in OPCs isolated from the brains of developing mice of the strains used to study this problem. In addition, these differences predicted that we would see lower levels of c-Cbl targets in the brains of more oxidized SJL mice. The observation that levels of c-Met were reduced predicted we would find less complex dendritogenesis in the hippocampus of SJL mice than in CBA mice, which we also observed.

Complicating these successes, however, was the fact that when we returned to work on these strains to test hypotheses emerging from our analyses of control of redox state differences between callosal and cortical OPCs, and the mechanistic implications of such differences, we were unable to detect inter-strain redox differences. This was not due to changes in our analytical techiques, as differences between cortical and callosal OPCs did not change. The reason for the changes in strain behavior are not known.

Nonetheless, the idea that using analysis of bloodstream components to obtain insights into potentially systemic redox alterations is clearly worthy of further pursuit, as revealed by the studies in Project 1.

*Why do cells have different redox states? Answers from studies on developmental redox regulation and parallel analysis of mice that are pathologically more oxidized*

The integration of our analyses with the Nrf2 pathway provides molecular mechanisms that may not only provide better markers of a more oxidative state (our Aim 1) but would have the added value of explaining why the more oxidized cells of an individual with ASD do not simply reset themselves to create a normal redox balance. This would provide new targets for resetting these metabolic problems, as there are some very interesting Nrf2 regulators (with potential clinical application) to be considered in this regard. We believe that there is a very good chance that the continuation of this work is going to identify agents that could be used to address the oxidative abnormalities that look increasingly likely to be of relevance to the understanding of ASD pathogenesis. If we are correct, this would have profound implications for understanding how to reset the redox balance in individuals with ASDs. If we are correct that such redox abnormalities are responsible for the subtly different CNS development in children with ASDs, this will bring us to the point of being able to potentially recognize such children early and to provide protective interventions.

The combined goals of Aims 1 and 2 were to provide an understanding of how differences in redox state can be recognized, what their effects are on cell function and how these processes were regulated at the level of molecular mechanism. Why do different cells have different redox states? What pathway is in control?

By taking advantage of the stable redox differences between OPCs of the developing cortex and the developing corpus callosum we were able to making several discoveries that help us to understand how these differences are regulated.

Several of the indicators of redox state that we studied are regulated by a single pathway called the anti-oxidant response element (ARE) pathway, which itself is regulated by the Nrf2 transcription factor. Oxidation has the potential to have such a plethora of negative consequences, that cells have to possess means of restoring a more appropriate redox balance. This is indeed the case, and evolution has provided entire pathways dedicated to this, of which the most important one seems to be the Nrf2/ARE pathway. Nrf 2 is activated by oxidation and causes expression of a wide range of enzymes that have the consequence of making cells more reduced. Moreover, Nrf2-mediated regulation of Phase II detoxifying enzymes provides the central means by which cells respond to toxic insults. Glutathione content is regulated in large part by the Nrf2 pathway, and multiple enzymes related to glutathione biosynthesis are controlled by this pathway.

The second major contributor to cellular redox state that is relevant to ASD is mitochondrial function. Abnormal mitochondrial function is of increasing interest as a risk factor for ASD.

We also found that cells can have different Nrf2 set points, leading to different redox states and responses to inducers of OPC differentiation, and also found that changing levels of the mitochondrial pore regulator Bcl-2 also modulates redox states and response to inducers of OPC differentiation. Both the Nrf2 pathway and mitochondrial function are relevant to the indicators of redox state specified in our original proposal. For example, in Year 1 we showed that OPCs that are isolated from the cortex of young rats, and that are more oxidized because of developmental differences in their redox regulation, have high levels of bcl- 2, gamma-glutamyl-cysteinyl synthethase (γ-GCS, the rate limiting enzyme in glutathione synthesis) and glutathione. In contrast, OPCs isolated from the corpus callosum of the same animals have an intrinsically more oxidized basal redox status than do the cortical progenitors, and have lower levels of bcl-2, g-GCS and reduced glutathione. These observations are of particular interest as bcl-2 controls mitochondrial function by blocking mitochondrial permeability transition pore and thus helps to keep cells more reduced.

It is also important to stress that by examining the differences in redox regulation in different cell populations isolated from the same animals we are able to focus attention on differences in redox status that are due to epigenetic modification of gene expression. The hypothesis that there is epigenetic regulation of redox balance is central to the hypotheses of Project 1. Because our experimental observations are not influenced by genetic differences between cells from different strains of mice, the outcomes can only be due to epigenetic differences in regulation of redox balaance – and perhaps also of the ability to respond to oxidative stress.

We tested, and found to be correct, predictions that support the following integrating hypothesis: Epigenetic changes in Nrf2 activity are responsible for fundamentally different basal redox states

and in the ability to respond to agents that perturb redox state. When Nrf2 activity is set at a lower level, cells are intrinsically more oxidized and more susceptible to agents that further increase oxidative status. These changes cause activation of the redox/Fyn/c-Cbl pathway, leading to increased degradation of important receptor tyrosine kinases (RTKs) at the cell surface. This leads to decreases in NF-kB activity, which in turn leads to reduced levels of bcl-2 and also of γ-GCS. Reductions in levels of bcl-2 further work to keep cells more oxidized by allowing greater efflux of reactive oxidative species from mitochondrial permeability transition pores. Thus, cells with reduced Nrf2 activity are maintained in a chronically oxidized state that is responsible for increased activity of the redox/Fyn/c-Cbl pathway, and activation of the redox/Fyn/c-Cbl pathway reduces NF-kB activity and levels of bcl-2 and of g-GCS, thus helping to maintain the chronically oxidized phenotype.

*The redox/Fyn/c-Cbl pathway is a critical regulator of the function of normal developmental pathways that is modulated by alterations in oxidative status*

In our original Aim 2 we proposed that changes in redox status would cause aberrant activation of the redox/Fyn/c-Cbl pathway. Our previous studies on the effects of environmental toxicants on O-2A/OPCs raise the very different possibility that the critical effect of pro-oxidative changes is to cause a cessation of mitogenic signaling by activating c-Cbl induced degradation of receptor tyrosine kinases (RTKs) required for continued division, and also for cell survival (Li et al, 2007). These studies showed that exposure of O-2A/OPCs to low levels of methylmercury, lead or paraquat (which share the property of making these progenitor cells more oxidized) caused activation of Fyn kinase, which in turn activated the c-Cbl E3 ubiquitin ligase. This led to ubiquitylation and degradation of c-Cbl targets, including PDGF receptor-α (PDGFRα), causing reduced levels of all downstream signaling. It is not known, however, whether activation of the redox/Fyn/c-Cbl (RFC) pathway and consequent loss of PDGFRα following exposure to environmental toxicants represents a cellular response to these specific chemicals, to physiological stressors in general or whether this pathway also might be of relevance to understanding signaling pathway regulation in response to normal developmental signals.

In order to understand the importance of activation of the redox/Fyn/c-Cbl pathway by environmental toxicants it was necessary to understand whether this pathway was a component of normal development or a response to physiological insults. Our studies on the Nrf2 pathway provided one example of a pathway originally identified as a means of responding to enivornmental insults as a key regulator of normal development. Our studies on the redox/Fyn/c-Cbl pathway further amplify on this paradigm.

Thus, in sum, these studies demonstrate that control of the redox/Fyn/c-Cbl pathway is central to understanding the action of signaling molecules vital to normal development of the central nervous system. These studies further indicate that the changes in oxidative status caused by exposure to environmental toxicants may amplify activity of such agents in altering normal developmental processes.

*Consideration of next steps. I. Treatment of ASDs with anti-oxidant therapy*

One of the tenets of ASD treatment in some portions of the ASD care community is the use of anti-oxidants. This has recently been tested in a study by Hardan et al. (2012), who tested the use of N-acetyl-L-cysteine in the treatment of children with ASD. Results of a 12-week double-blind,

randomized, placebo-controlled study showed that treatment with NAC resulted in significant ($p<0.001$) improvements on the ABC irritability subscale.

The double-blind placebo-controlled nature of this study is a welcome addition to the literature in demonstrating that addressing redox imbalances in children that already have been diagnosed with ASDs can provide benefit.

Our work, in contrast, suggests that early treatment to restore a normal redox balance might prove even more beneficial. As indicated by the developmental nature of the redox-related alterations in differentiation we discovered, we suggest that initiating treatment as early as possible – and potentially in mothers who exhibit redox abnormalities during pregnancy – would prove of even greater benefit.

In addition, as environmental toxicant exposure can increase oxidative status, the introduction of treatment with NAC early may also reduce risks caused by such exposures.

*Consideration of next steps. II. Potential clues identified in a genetic disease characterized by region-specific increases in oxidative status*

Our studies on the effects of altered redox state on development inspired our colleague Dr. Margot Mayer-Proschel to initiate collaborative studies with us on the disease ataxia telangiectasia (A-T), a neurological disease characterized by both defective DNA repair and an increased oxidative status. After Dr. Mayer-Proschel obtained funding for this work, studies focused on the cerebellum as this is the part of the brain that shows the earliest abnormalities in A-T. We found that the astrocytes of the cerebellum, but not of the cortex show severe redox abnormalities and that these redox abnormalities prevent them from supporting morphological development and survival of cerebellar neurons.

Analysis of cerebellar astrocytes in a mouse model of A-T revealed a new type of redox abnormality that appears to be caused by inadequate levels of the XcT cysteine importer protein. This transport protein is required to bring cystine into astrocytes as a source of cysteine for glutathione biosynthesis. When XcT levels are low, the astrocytes do not generate or secrete adequate glutathione. Genetic overexpression of XcT repairs this redox defect.

Although the work on A-T was not funded by DOD, having the A-T work going on in parallel provided 4 benefits for our work on ASDs:

(i) It confirmed that the lack of strain differences in redox status was due to the specific strains worked with, as there was no problem in continuously finding such differences in A-T mice compared with wild-type littermates.
(ii) This work demonstrated that astrocytic differences in redox state alter neuronal function, a finding of particular interest due to astrocytic abnormalities in Rett syndrome.
(iii) This work also showed that a germ-line mutation that alters redox state can alter redox state in a regionally-specific manner.
(iv) This work identified XcT as a potential target for future studies.

These ideas provide a much richer complexity to our thinking about the analysis of redox state abnormalities relevant to ASDs.

*Consideration of next steps. III. Is the combined study of iron deficiency and environmental insults the logical next step for ASD research?*

One of the unexpected benefits of the DOD-funded research was that of encouraging the interest of Dr. Margot Mayer-Proschel (University of Rochester Medical Center) in the problem of ASD pathogenesis. Dr. Mayer-Proschel has been studying the effects of sub-clinical maternal iron deficiency on the development of the CNS. This is a very common micronutritional disorder in which maternal iron levels are below normal but are not low enough to cause anemia. The previous work in the Mayer-Proschel laboratory had shown that even this low level of maternal iron deficiency was sufficient to cause a 40% reduction in iron levels in the brains of developing embryos and to cause subtle changes in CNS development.

As a result of her interactions with us, Dr. Mayer-Proshcel observed that the behavioral and neurological symptoms caused by iron deficiency showed a remarkable overlap with changes associated with ASDs, as summarized in the accompanying table. These observations have been made in multiple laboratories and appear to be quite robust. On the level of behavioral and cytological pathology they appear to offer an animal model that has one of the most complete phenocopies of ASDs of any animal model. became noticgestatdng he problem f In this proposal we focus on gestational iron deficiency (ID).

These observations are particularly intriguing in light of the study from UC Davis in which it was found that there was a markedly reduced risk of ASDs when intake of prenatal vitamin supplements was initiated during the 3 months prior to pregnancy (Schmidt et al, 2011).* While the authors focused on folic acid as the main protective factor, prenatal vitamin supplements also contain iron.

Due to the findings shown in the Table and their preliminary data, the Mayer-Proschel laboratory hypothesizes that iron supplementation in females with inadequate iron stores could just as much have been responsible for the decreased risk for developing ASDs as the folic acid supplementation. Together, we are aware of the overall conflicting data from the autism community about the role of iron in ASDs (i.e (Dosman et al, 2006)), and we suggest that this conflict is due to the fact that patient studies are only a reflection of the acute iron status at a given time and are not a refection of iron availability during gestation. This is highly problematic, as it is well known that the effect of ID on brain development is most profound during gestation at a critical window of vulnerability. It may therefore be inadequate and even misleading to draw conclusions on the impact of ID on autism from acute iron measurements in children.

| ID | Symptoms | ASD |
|---|---|---|
| X | Vast range of impairment: Subclinical > lethal | X |
| X | Multisystem impact: Immunsystem, GI tract, CNS | X |
| | Complex pathology: | |
| X | ABR latency defects | X |
| X | CNS maturation impairment | X |
| X | Initially few signs of degeneration | X |
| X | Intractable seizures | X |
| | Altered behavioral patterns: | |
| X | Anxiety, hyperactivity | X |
| ? | Repetitive behavior | X |
| X | Impaired social interaction | X |
| X | Sleep disorders | X |
| X | Language impairment | X |
| X | Food preferences | X |
| ? | Inheritability | X |
| ? | Gender bias | X |
| X | Window of vulnerability | ? |
| X | Unpredictable progression | X |
| X | Lower birthweight | X |

Based on the large body of cellular and mechanistic data from the Mayer-Proschel laboratory and from others that focus on impacts of gestational ID on brain development, we propose the hypothesis that gestational iron deficiency is a risk factor for developing ASDs and might even be a primary causative event. Moreover, nutritional iron deficiency during pregnancy has a remarkably high prevalence. Factors that contribute to the high prevalence of ID even in North America, alone bearing 1.4% of the global burden of ID and IDA, are complex and persist in many

social environments despite an adequate diet (Stoltzfus, 2003). They include intake of certain contraceptive drugs, frequent blood donation, vegetarian and vegan diets paired with frequent fasting, excessive physical activity, low compliance rate (50%) of taking iron supplements leading often to low birth weight independent of other pregnancy care factors and a rise in type-2 diabetes and obesity. All these diverse factors have the potential to interfere with iron uptake despite adequate food intake [4(Rao & Georgieff, 2007). Taken together, ID is highly prevalent and can be caused by a variety of factors many of which are not obvious and in the absence of a clinically obvious anemia will remain undetected.

It is also critical that while iron defiency can occur during all stages of pregnancy and hence can affect the fetus at various stages during development, the work of the Mayer-Proschel laboratory suggests that it is the lack of iron during the earliest stages of CNS development that is critical. Such observations are in strong agreement with the results of epidemiological studies on effects of vitamin supplements before and during pregnancy on the risks of ASD development in offspring.

Continuing studies in the Mayer-Proschel laboratory are strengthening the understanding of the ASD-like features seen in iron deficiency. Reduced dendritic complexity and ASD-like behavioral impairments are robust outcomes, and current work is showing defects in sonic hedgehog signaling and interneuron development that resemble outcomes seen in ASDs (Marín, 2012).
*As for our work with oxidative regulation, the treatment implications of the observations of the effects of iron deficiency on CNS development speak strongly in support of early intervention and identification of these metabolic and nutritional abnormalities as early in pregnancy as possible. The best way to treat ASDs is to reduce their prevalence, and studies such as ours and those of the Mayer-Proschel laboratory provide, for the first time, molecular and metabolic pathways that demonstrate how such changes in cellular function can cause ASD-like pathologies. Unlike strategies to target genetic defects, modification of nutrition and oxidative status are relatively benign and rapid therapeutic approaches, making their implementation readily feasible.*

In addition, the Mayer-Proschel laboratory has recently discovered a disturbing association between iron deficiency and uptake of certain heavy metals, opening up the possibility that iron deficiency will lead to increased oxidative stress due to increased uptake of environmental toxicants that cause increases in cellular oxidative status.

This work would not have occurred without the DOD funding of our autism-related research, and we are currently seeking funding to enable such research to be carried out.

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