Effects of Ethanol on IGF-1R Signaling in a Neural Progenitor Model

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Abstract

Fetal Alcohol Syndrome (FAS) and Fetal Alcohol Spectrum Disorder (FASD) exemplify the most common forms of non-inherited cognitive impairments in developed nations and result from maternal consumption of alcohol during pregnancy. These disorders significantly impact the development of the central nervous system (CNS). Previous studies indicate that the insulin-like growth factor 1 receptor signaling pathway is critical for regulating various cellular processes and maintaining precise proliferation and differentiation in the CNS during embryogenesis. This study focuses on three major roles of the IGF-1R system during brain growth: inhibiting cell apoptosis, promoting neural progenitor proliferation, and supporting neural progenitor differentiation. The aims of the experiments are to study the molecular effects of ethanol on IGF-1R signaling in neural progenitors, examine the effects on the proliferation and survival of these neural progenitors, and determine how ethanol-dependent changes in IGF-1R signaling alter neural progenitor differentiation. We immunolabeled neural progenitors with markers for neurons, astrocytes, and nuclei after treatment with EtOH to assess differentiation and proliferation, and we used an MTS assay to determine cell viability following EtOH exposure. MAPK inhibitor FR 108204 (IGF-FR) was used to reverse the changes induced by ethanol. This analysis is significant in clarifying possible molecular mechanisms of IGF-1R signaling using a neural progenitor model and its relation to the mental abnormalities associated with FAS and FASD.

Background

The U.S. spends nearly $7 billion per year to study and care for the physiological, behavioral, and mental defects in children suffering from FAS and FASD[1]. Evidence has shown that ethanol disturbs the IGF-1R signaling pathway that is responsible for many aspects of brain growth including neural progenitor proliferation and differentiation. Neural progenitors are contained within the neural tube during embryogenesis. These neural progenitors specialize into neurons, astrocytes, or oligodendrocytes, or undergo programmed cell death as maturation occurs in the embryonic CNS. These three differentiated cell types form the mature CNS and are critical to its function. Neurons transmit information through action potentials and neurotransmitters to other neurons, muscle cells or gland cells, while astrocytes and oligodendrocytes work to support neuronal function and survival. A disruption in the IGF-1R system results in improper ratios of these cell types, seen in FAS and FASD. Individuals with IGF-1R mutations suffer severe physiological development and mental retardation[2]. Additionally, genetic manipulations of the IGF system in mouse models have led to major developmental irregularities and death shortly after birth[3]. Therefore, proper regulation of the IGF-1R system is critical for CNS development especially during embryogenesis.
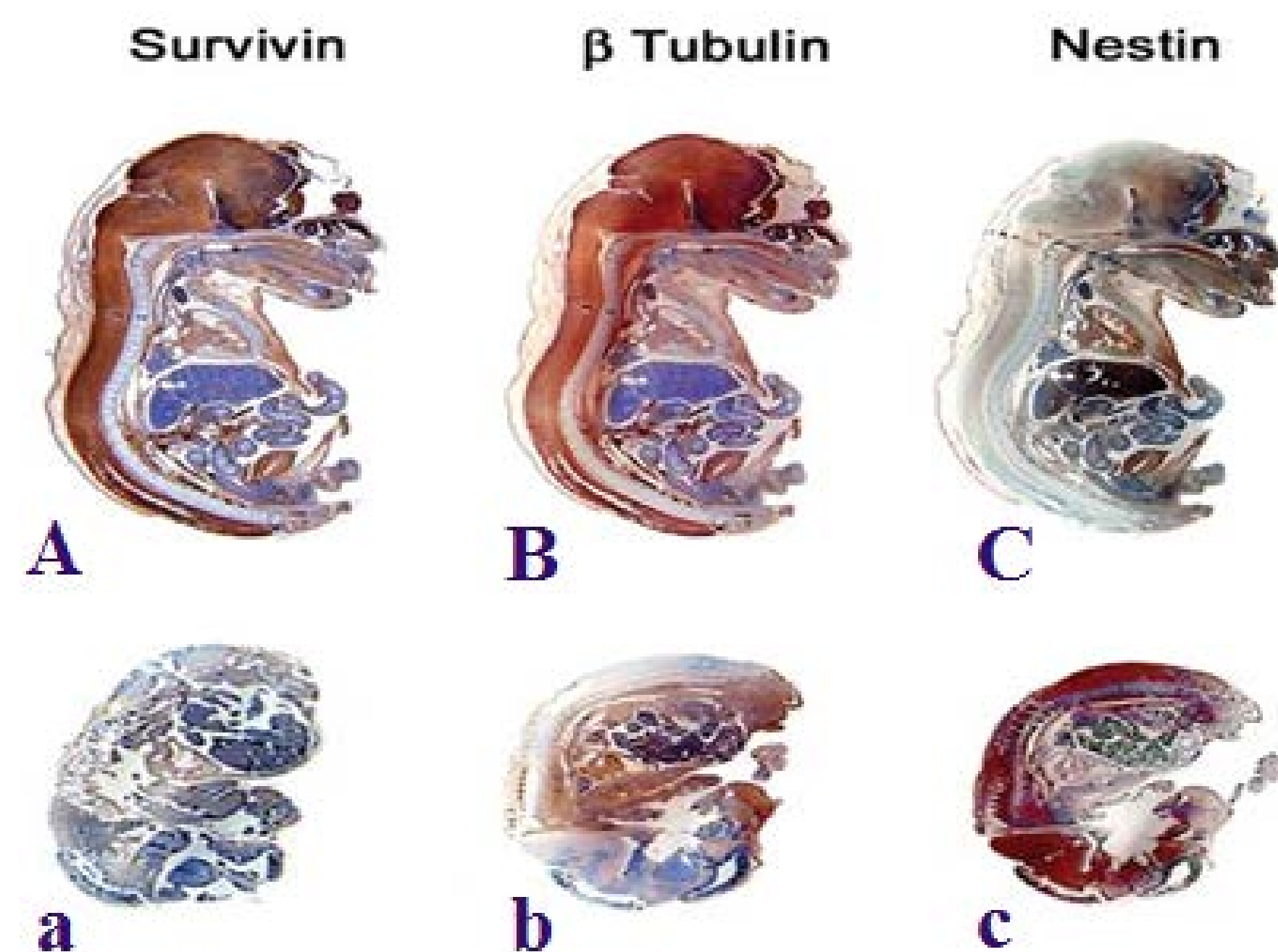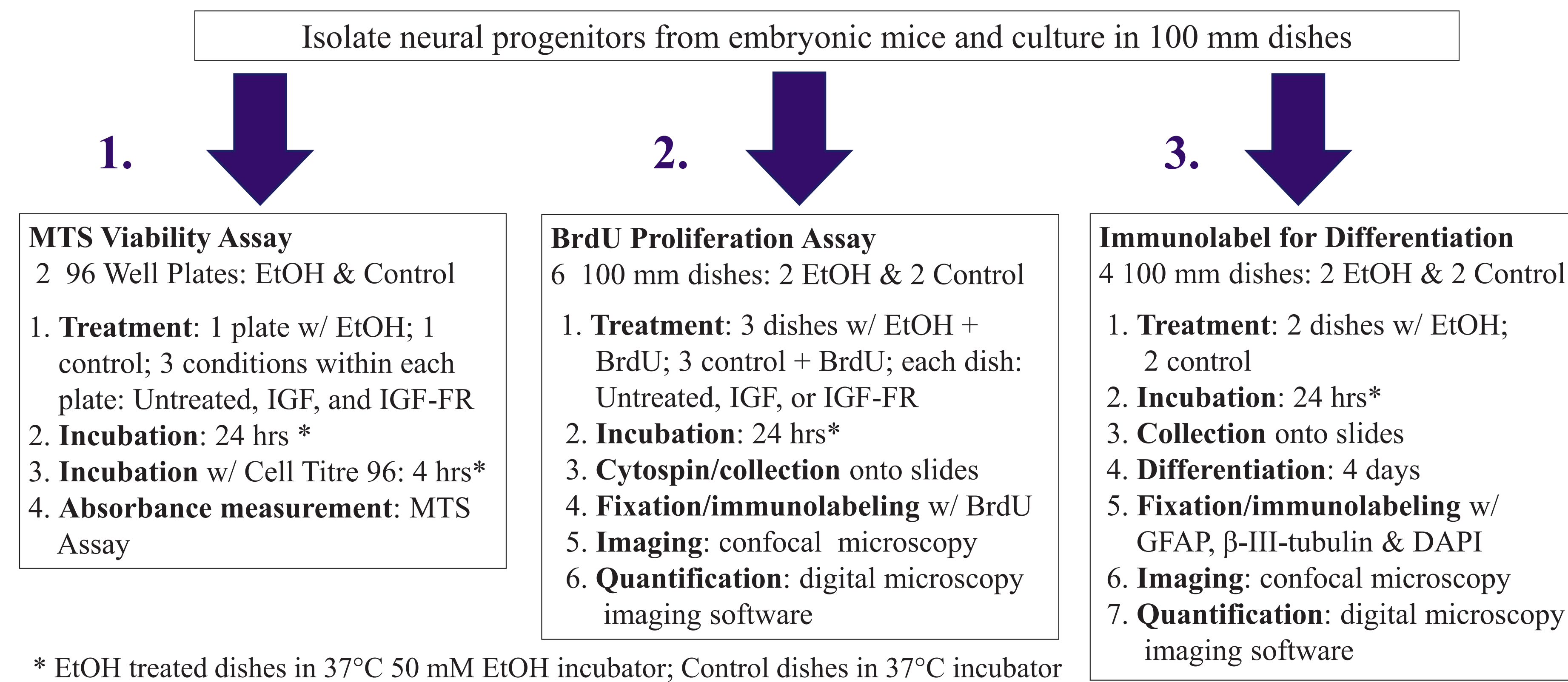

Figure 1. The IGF-1R system is critical for protecting cells against apoptosis and regulating proper differentiation. Cross sections of embryos fixed in formalin demonstrate the effect that IGF-1R has on development. The first row of three embryos shows a functional IGF-1R (A,B,C), while the bottom row represents IGF-1R knockouts (a,b,c).
A/a) Survivin is a protein of the IGF family and regulates apoptosis throughout the body. When IGF signaling is impaired, cells are not properly protected and there is a significant increase in apoptosis. **B/b)** β-III-tubulin is a marker for neurons. A dysfunctional IGF-1R system leads to imprecise neural progenitor differentiation with fewer mature neurons present in the CNS. **C/c)** Nestin is a marker for neural progenitors. More neural progenitors are present in the non-functional IGF-R system because they did not properly differentiate into neurons, astrocytes, and oligodendrocytes.
Overall, IGF-1R knockout embryos are smaller in size and display a poorly developed CNS.
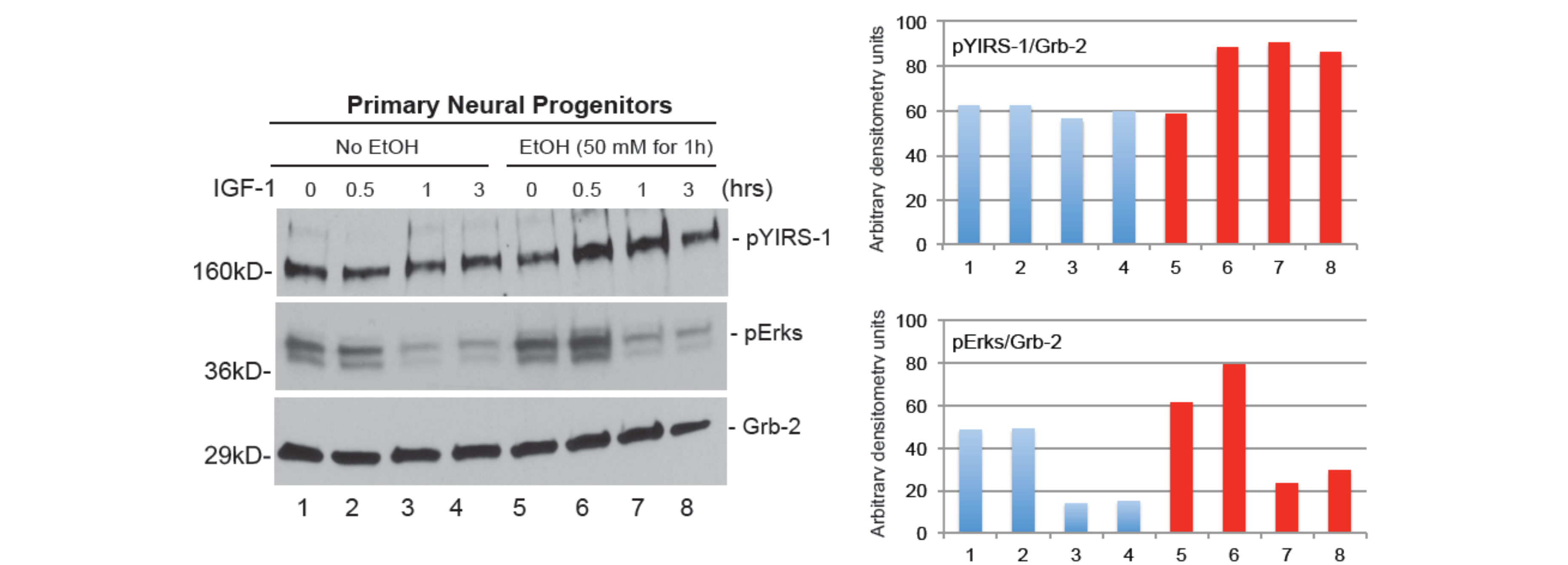
Hypotheses

Ethanol exposure interferes with the IGF-1R pathway and induce progenitor apoptosis, reduce neural progenitor proliferation, and cause abnormal differentiation. Specifically, we believe that ethanol distorts the ratios between neurons, astrocytes, and neural progenitors, and causes reductions in the number of neurons and astrocytes.

Methods

Isolate neural progenitors from embryonic mice and culture in 100 mm dishes

1.

MTS Viability Assay
2 96 Well Plates: EtOH & Control
1. **Treatment**: 1 plate w/ EtOH; 1 control; 3 conditions within each plate: Untreated, IGF, and IGF-FR
2. **Incubation**: 24 hrs *
3. **Incubation** w/ Cell Titre 96: 4 hrs*
4. **Absorbance measurement**: MTS Assay

2.

BrdU Proliferation Assay
6 100 mm dishes: 2 EtOH & 2 Control
1. **Treatment**: 3 dishes w/ EtOH + BrdU; 3 control + BrdU; each dish: Untreated, IGF, or IGF-FR
2. **Incubation**: 24 hrs*
3. **Cytospin/collection** onto slides
4. **Fixation/immunolabeling** w/ BrdU
5. **Imaging**: confocal microscopy
6. **Quantification**: digital microscopy imaging software

3.

Immunolabel for Differentiation
4 100 mm dishes: 2 EtOH & 2 Control
1. **Treatment**: 2 dishes w/ EtOH; 2 control
2. **Incubation**: 24 hrs*
3. **Collection** onto slides
4. **Differentiation**: 4 days
5. **Fixation/immunolabeling** w/ GFAP, β-III-tubulin & DAPI
6. **Imaging**: confocal microscopy
7. **Quantification**: digital microscopy imaging software

* EtOH treated dishes in 37°C 50 mM EtOH incubator; Control dishes in 37°C incubator
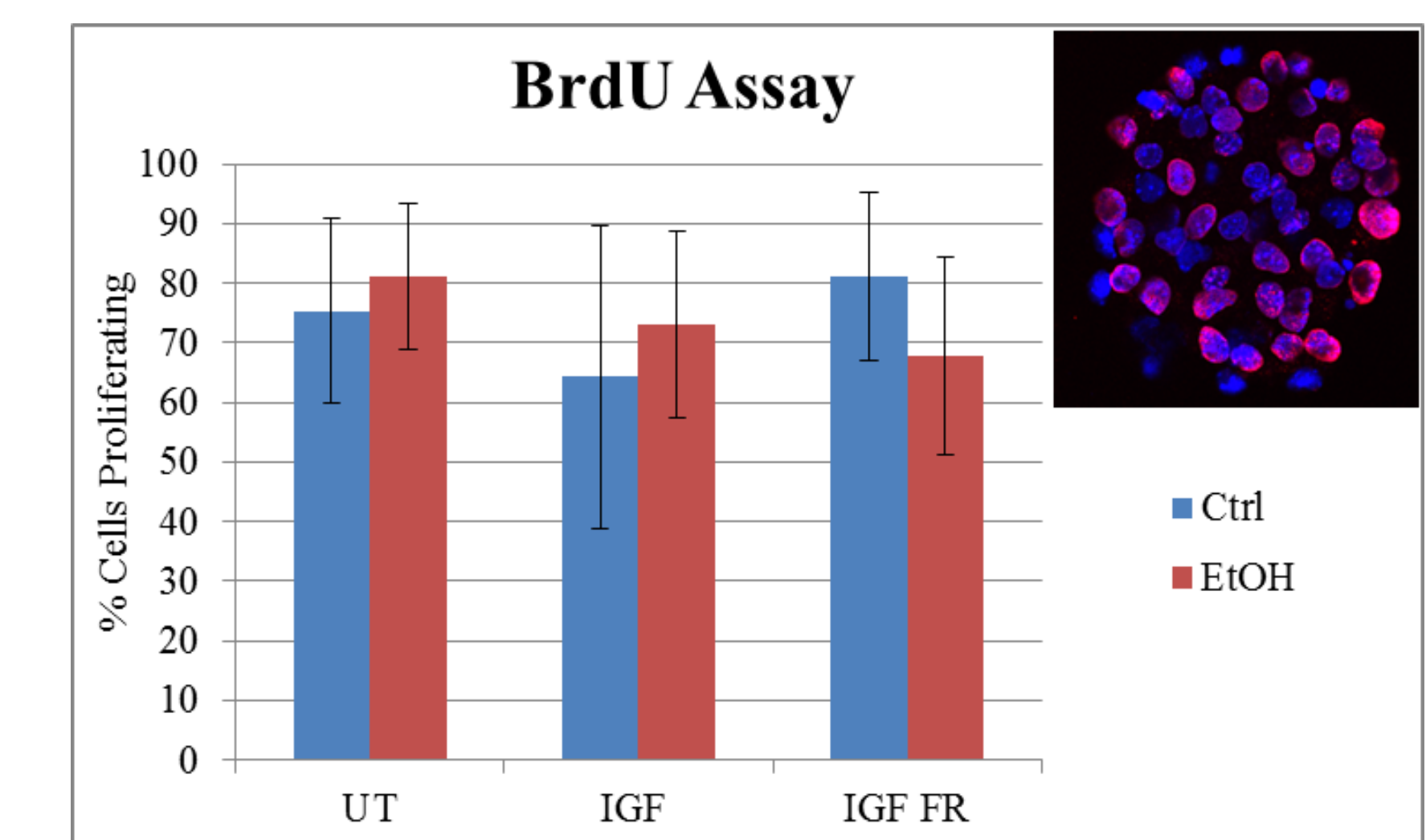
Results

Figure 2. Western Blots of neural progenitor lysates following acute exposure to EtOH (1hr). IGF stimulation time varied at 0.5, 3, and 6 hours. EtOH concentration was kept at 50mM and Grb-2 was used as a loading control. Activation (phosphorylation) of pYIRS-1 and pErks signaling substrates is analyzed. The overall trend is toward hyper-active signaling through the IGF-1R receptor system after acute alcohol exposure.
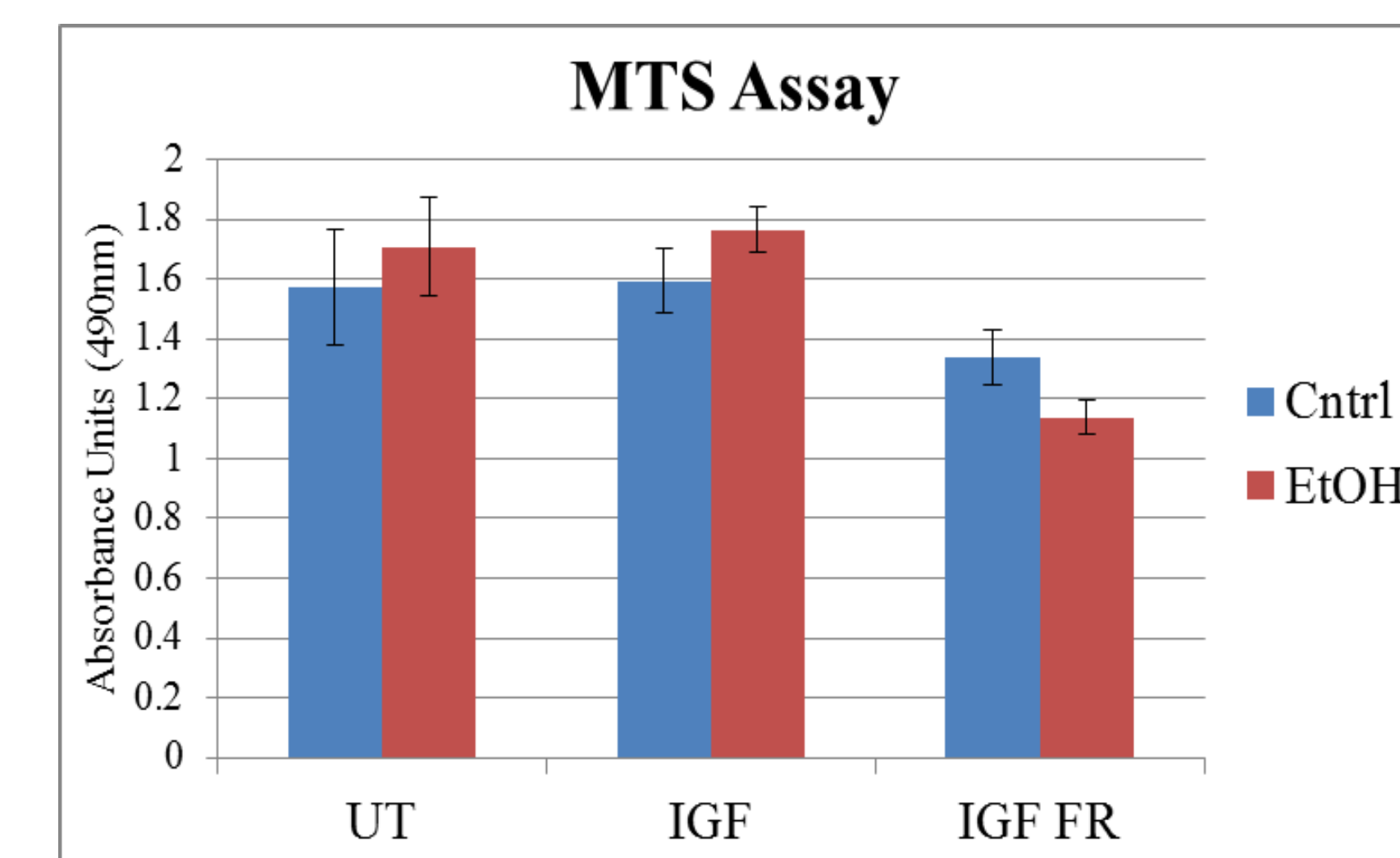

Figure 3. MTS Assay of neural progenitors following acute exposure to EtOH (24hrs) measuring cell viability. Cells are untreated, stimulated with IGF, or stimulated with IGF and IGF-FR. As acute (1hr) EtOH exposure hyper-activates IGF-1R signaling in Western blots, acute EtOH exposure also yields greater absorbance and a higher number of viable cells. When MAPK signaling is inhibited, both the control and acutely exposed EtOH neural progenitors are less viable. IGF-FR more significantly affects the number of viable cells following acute EtOH exposure than their control counterparts.

Figure 4. BrdU Assay of neural progenitors following acute exposure to EtOH (24hrs) measuring proliferation. Cells are untreated, stimulated with IGF, or stimulated with IGF and IGF-FR. Neural progenitors show greater proliferation than control cells in the untreated and IGF-stimulated conditions following acute EtOH exposure. The addition of IGF-FR seems to reverse the effect of ethanol by blocking MAPK activation .
Inset: Confocal image of neural progenitors stained with DAPI and BrdU. DAPI immunolabels nuclei and BrdU immunolabels cell division.

* Note: 1 hr and 24 hr EtOH acute exposure produced similar results and are used interchangeably

Results
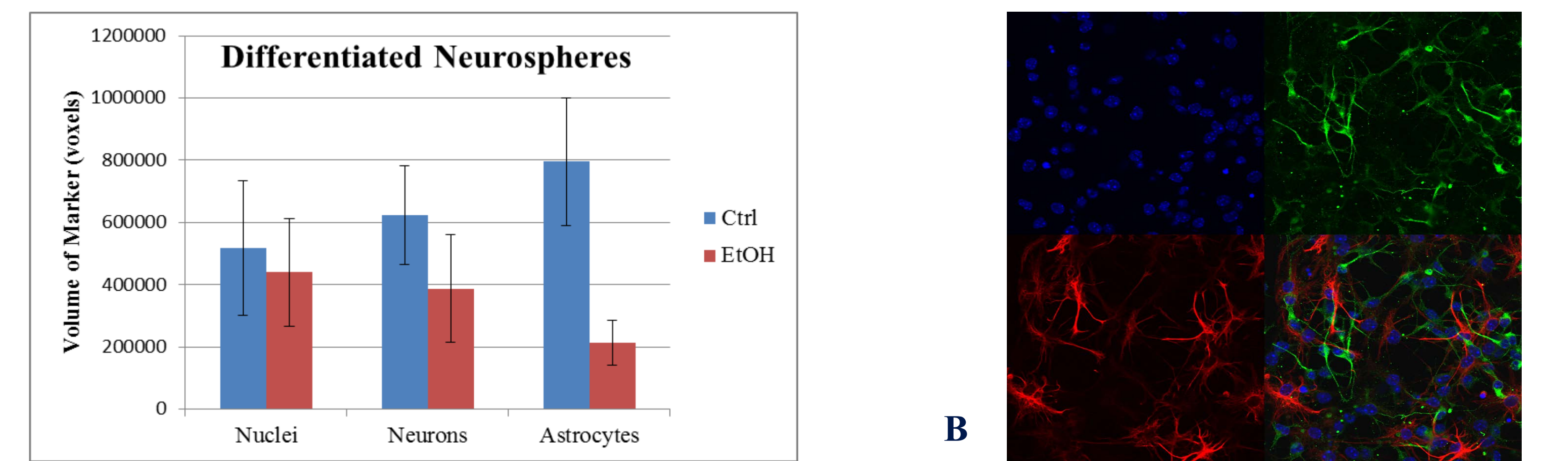
Figure 5. Immunocytochemisty of differentiated neural progenitors exposed to acute EtOH. Neural progenitors were exposed to 50mM EtOH for 24h during proliferation. Cells were then allowed to attach and differentiate. **Panel A:** Quantification of ten independent fields of differentiated neurospheres labelled with markers; DAPI (nuclei), βIII tubulin (neurons), and glial fibrillary acidic protein (astrocytes). While the total number of cells did not change in the presence of EtOH, the numbers of both neurons and astrocytes were significantly reduced. **Panel B:** Representative image of fluorescently labelled, differentiated neural progenitors. Frames represent nuclei (blue), neurons (green), astrocytes (red), and a merged image in the bottom right. These images were then quantified using SlideBook5 image analysis software to produce the graph in Panel A of this figure.

Conclusions

While previous evidence has demonstrated that EtOH inhibits IGF-1 receptor signaling, our data confirms this finding with chronically exposed cells (3 weeks), but also reveals a paradoxical effect with acutely exposed cells. One hour and 24 hour exposure of neural progenitors to 50mM EtOH resulted in a significant increase of IGF-1R signaling activation in multiple pathways. These findings using Western blot were supported by our data from the MTS and BrdU incorporation assays, which showed slight increases in cell viability and number, respectively. Although this seemingly contradicts what is known about EtOH's effects on cells, we believe that this acute upregulation of signaling causes an eventual desensitization of the IGF-1R system, which leads to the reductions in IGF-1R activation, as well as reductions in cellular viability, and proliferation seen following chronic EtOH exposure. The perturbations in IGF-1R signaling also led to abnormal differentiation, where the total number of cells was minimally affected, but the number of neurons and astrocytes were decreased. This is potentially due to EtOH inhibiting the ability of the neural progenitors to begin differentiation. Further experiments will examine the presence of the neural progenitor marker nestin to determine if EtOH inhibits progenitor differentiation at the initial stages. In summary, acute EtOH potentiates signaling through the IGF-1R pathway, which leads to increases in cellular viability and proliferation, but decreases in the number of differentiated neurons and astrocytes.

Future Implications

These analyses demonstrate how a disruption in the neuroprotective and pro-proliferative signaling caused by ethanol can initiate neural progenitor death and dysfunction, ultimately impairing the development of a fully-functioning CNS. Further experimentation is required to explain the molecular mechanisms that produce the paradoxical effect of acute EtOH exposure (up to 24 hrs) on neural progenitors. Supplemental research examining how ethanol exposure modifies substrates within the IGF signaling pathway will further develop the findings of the current study. This study reveals changes in the IGF-1R system after acute EtOH exposure which can be responsible for the cognitive defects seen in children suffering from FASD or FAS.

Acknowledgments

References

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