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Acute ethanol increases IGF-I-induced phosphorylation of ERKs by enhancing recruitment of p52-Shc to the Grb2/Shc complex

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Abstract

Ethanol plays a detrimental role in the development of the brain. Multiple studies have shown that ethanol inhibits insulin-like growth factor I receptor (IGF-IR) function. Because the IGF-IR contributes to brain development by supporting neural growth, survival, and differentiation, we sought to determine the molecular mechanism(s) involved in ethanol's effects on this membrane-associated tyrosine kinase. Using multiple neuronal cell types, we performed Western blot, immunoprecipitation, and GST-pulldowns following acute (1 – 24 hours) or chronic (3 weeks) treatment with ethanol. Surprisingly, exposure of multiple neuronal cell types to acute (up to 24 hours) ethanol (50mM) enhanced IGF-I-induced phosphorylation of ERKs, without affecting IGF-IR tyrosine phosphorylation itself, or Akt phosphorylation. This acute increase in ERKs phosphorylation was followed by the expected inhibition of the IGF-IR signaling following 3-week ethanol exposure. We then expressed a GFP-tagged IGF-IR construct in PC12 cells and used them to perform fluorescence recovery after photobleaching (FRAP) analysis. Using these fluorescently-labeled cells, we determined that 50mM ethanol decreased the half-time of the IGF-IR-associated FRAP, which implied that cell membrane-associated signaling events could be affected. Indeed, co-immunoprecipitation and GST-pulldown studies demonstrated that the acute ethanol exposure increased the recruitment of p52-Shc to the Grb2-Shc complex, which is known to engage the Ras-Raf-ERKs pathway following IGF-1 stimulation. These experiments indicate that even a short and low-dose exposure to ethanol may dysregulate function of the receptor, which plays a critical role in brain development.

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Introduction

The insulin-like growth factor I receptor (IGF-IR) is a multifunctional membrane-associated tyrosine kinase capable of activating intracellular signaling pathways that are known to promote cell growth (Reiss et al., 1998, Morrione et al., 2000, Gualco et al., 2009, Arsenijevic et al., 2001), survival (O'Connor et al., 1997, Gualco et al., 2010), differentiation (Dentremont et al., 1999, Arsenijevic and Weiss, 1998, Hsieh et al., 2004), motility (Nakao-Hayashi et al., 1992, Drukala et al., 2010), and DNA repair (Trojanek et al., 2003). In the brain, the IGF-IR is abundantly expressed during embryonic and postnatal development, but its expression declines significantly during adolescence and adult life (Bondy and Lee, 1993). Early studies regarding the expression patterns of the IGF-IR were followed by numerous studies providing overwhelming evidence for the role of the IGF-IR in protecting neurons from oxidative stress (Heck et al., 1999, Davila et al., 2016, Davila and Torres-Aleman, 2008), high glucose (Russell and Feldman, 1999), nitric oxide (Zheng et al., 2002), and TNF α (Wang et al., 2006, Ying Wang et al., 2003). In transgenic models, mice overexpressing IGF-I demonstrated an increase in brain weight, which was significantly larger than the corresponding increase in total body weight (Mathews et al., 1988, Reiss et al., 1996, Popken et al., 2004). In contrast, transgenic mice with targeted disruption of the IGF-IR gene (*IGF-IR*^{-/-}) have reduced brain size and altered brain structures, including reduced myelination due to decreased proliferation and maturation of oligodendrocytes (Liu et al., 1993). Previous immunohistochemical analysis of *IGF-IR*^{-/-} embryos revealed high levels of apoptotic cells detected in the brain and in the dorsal root ganglia. In addition, the brain and dorsal root ganglia of the knockout mice were very small and poorly differentiated, which correlated with decreased expression of neuronal markers, and higher levels of expression of a marker of neural progenitors, nestin, compared to larger and more fully differentiated brains from age-matched non-transgenic littermates (Gualco et al., 2010). Taken together, cell culture and animal studies clearly indicate that impairment of the IGF-IR compromises the development and maintenance of the different cellular components of the CNS.

Ethanol has well-established detrimental effects on the brain and CNS (Breese et al., 1993, Torres and Zimmerberg, 1992). Although the teratogenic effects of ethanol were first characterized many years ago, rates of fetal alcohol exposure remain high (Jones and Smith, 1975, Floyd and Sidhu, 2004). Because this disorder has varying severity depending on many factors – genetics, timing, nutrition - its classification has been difficult; however, rates of the most common disorder that results from fetal alcohol exposure, fetal alcohol spectrum disorders (FASD), can reach as high as five percent of all live births in the United States (Randall, 1987), although more recent estimates put this figure closer to one percent (Centers for Disease and Prevention, 2012). Individuals affected by this disorder have pronounced difficulties in learning, speech, decision-making, and behavior, which are often accompanied by malformations within the brain and CNS (Chokroborty-Hoque et al., 2014). Further studies revealed that changes in CNS development and function are the result of multiple ethanol-dependent changes at the cellular and molecular level (Breese et al., 1993, Torres and Zimmerberg, 1992). Although the mechanism through which ethanol exerts its detrimental effects on cellular function has remained elusive, it is known that ethanol alters

signaling through the IGF-IR signaling pathway (Cohen et al., 2007, Resnicoff et al., 1993). The first experimental data showing ethanol's effects on the IGF-IR demonstrated a decline in IGF-IR receptor auto-phosphorylation, which occurred in the absence of detectable effects of ethanol on the ligand (IGF-I) binding in 3T3 fibroblasts (Resnicoff et al., 1993) and in cerebellar granule neurons (Zhang et al., 1998). Later, animal studies utilizing gestational or adult exposure to ethanol confirmed the inhibitory effects on the IGF-IR autophosphorylation in the brain. These *in vivo* studies differed from the *in vitro* results in that the ligand binding to the IGF-IR was inhibited in this context (Cohen et al., 2007). Although experimental data from multiple labs and using different cell and animal models confirmed the overall attenuation of IGF-IR signaling pathways following chronic ethanol exposure (Cohen et al., 2007, de la Monte et al., 2005, Ila and Solem, 2006, Lang et al., 2010), the exact mechanism(s) responsible for this inhibition require further investigation.

The purpose in undertaking this study was to further investigate the underlying mechanisms of ethanol-mediated effects on IGF-IR signaling. In contrast to our expectations, our initial results demonstrated that acute fibroblast exposure to 50mM ethanol actually enhanced IGF-I-induced phosphorylation of p42/p44 extracellular regulated kinases 1/2 (ERKs). This unexpected finding led us to conduct the experiments presented herein which sought to: 1) confirm the finding that acute ethanol exposure leads to enhanced phosphorylation (activation) of multiple IGF-IR signaling substrates using different neuronal models and 2) determine a mechanism through which this potentiation of IGF-IR signaling occurred. Our results confirmed that several different neuronal cell lines and primary neural progenitors, exposed to 50mM ethanol from 1 to 24 hours, had a significant increase in the phosphorylation of ERKs, without noticeable changes of the IGF-IR tyrosine phosphorylation or Akt serine phosphorylation, and highlight the importance of understanding the molecular mechanism of action of ethanol on IGF-IR signaling.

Material and Methods

Cell Culture

Culture conditions for PC12 rat pheochromocytoma cells (ATCC# CRL-1721) were as previously described (Ying Wang et al., 2003). The R- and SH-SY5Y (ATCC# CRL-2266) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). In each case, serum-starvation was achieved by incubating cells for 24 hours in basal media supplemented with 0.1% bovine serum albumin (BSA). Neural progenitors were maintained in Neurobasal media (Gibco, Carlsbad, CA) supplemented with B27 (Gibco, Carlsbad, CA), 1 µg/mL heparin (Stem Cell Technologies), 2mM Glutamax, N2 (Gibco, Carlsbad, CA), 20 ng/mL EGF, and 20 ng/mL bFGF.

Isolation of neural progenitors and neurosphere cell culture—Using a standard technique (Singec et al., 2006), neural progenitors were isolated from the brains of embryonic day 17 mouse embryos. Dissociated by gentle trypsinization (TrypleE Express, Gibco, Carlsbad, CA) single cell suspension neural cells were plated on non-adherent Petri dishes at densities ranging from 1×10^2 to 1×10^5 cells/cm². The culture medium used to support proliferation of neural progenitors and the formation of primary neurospheres

consisted of NeuroBasal Medium (Gibco-Invitrogen, Carlsbad, CA), B27 supplement (Gibco, Carlsbad, CA), N2 supplement (Gibco, Carlsbad, CA), Heparin (2 ng/ml), EGF (20 ng/ml, Invitrogen, Carlsbad, CA), bFGF (20 ng/ml, Invitrogen, Carlsbad, CA), and Glutamax (Gibco, Carlsbad, CA). Following 5 days of the continuous growth, primary neurospheres were dissociated to single cell suspension and plated at low density on non-adherent cell culture dishes in the same medium. Differentiation of neural progenitors was induced by plating secondary neurospheres in glass chamber-slides coated with poly-D-lysine (Sigma, St. Louis, MO) and laminin (Corning, Corning, NY). The media used to support differentiation was the same as that for proliferation, except that growth factors, EGF and bFGF, were removed. Images of differentiated neurospheres immunolabeled with anti- β III tubulin (mouse monoclonal; Biolegend, San Diego, CA), and anti-GFAP (rabbit polyclonal; Millipore, Billerica, MA) antibodies were taken using confocal microscope (Olympus FV1000) equipped with multi-line Argon laser (458 nm, 488 nm, 515 nm) and diode lasers (405 nm, 559 nm, 635 nm). Quantification of the content of neurons, astrocytes and nuclei was performed by utilizing Mask analysis included in SlideBook5 software according to manufacturer recommendation (Intelligent Imaging Innovations, Inc).

Western Blotting, Immunoprecipitation and subcellular fractionation (membrane rafts)

Membrane rafts fraction and detergent soluble fractions were separated according to the methodology previously described (Tai et al., 2003). Protein concentration in isolated fractions was determined by a Bio-Rad Protein Assay (BioRad, Hercules, CA), and 50 μ g of protein aliquots were separated on a 4–15% gradient SDS-PAGE (BioRad, Hercules, CA) and transferred onto nitrocellulose membranes using the a semi-dry transfer system (BioRad, Hercules, CA). Resulting blots were probed with the following primary antibodies: rabbit polyclonal antibodies against total IRS-I (Upstate Biotechnology), tyrosine phosphorylated IRS-I (pY612/pY608), rabbit monoclonal anti-phosphorylated ERKs (pT202/pY204), rabbit polyclonal anti-IGF-1R α (SantaCruz Biotechnology, Dallas, TX) and mouse polyclonal total Grb2 (BD Biosciences, San Jose, CA). Mouse monoclonal anti c-Src antibody (Santa Cruz Biotechnology, Dallas, TX) was utilized as a marker of the membrane rafts fraction, and mouse monoclonal GAPDH (Research Diagnostics Inc) was used as a marker of the cytosolic fraction. Standard IP/Western protocols were used as described in our previous work (Trojanek et al., 2003). Immunoprecipitations were carried out with anti-Grb2 (Santa Cruz Biotechnology, Dallas, TX), anti-Shc (Millipore, Billerica, MA) antibodies, or anti-IGF-1R β (Cell Signaling Technologies, Danvers, MA), and corresponding Western blots were developed with anti-Shc, or anti-phosphotyrosine (Cell Signaling Technologies, Danvers, MA) antibodies. Western blots were quantified using ImageJ image analysis software.

GST-Pulldown Assay—GST and GST-Grb2 recombinant proteins were expressed in BL21-Gold (DE3) *E. coli* strain (Agilent, Santa Clara, CA). Following 2h 100 μ M IPTG (Isopropyl β -D-1-thiogalactopyranoside) induction, bacteria were centrifuged and resuspended in PBS with protease inhibitor cocktail (Roche, Basel, Switzerland). After sonication Triton X-100 was added to 0.5% final concentration and bacterial debris were removed by centrifugation. Recombinant proteins were affinity purified on Glutathione Sepharose 4B (GE Healthcare, Chicago, IL) after 30' incubation at room temperature.

Following two PBS washes, GST and GST-Grb2 protein concentration were measured and quality of protein verified with SDS-PAGE followed by Coomassie Blue staining. For GST-pull down 10 ug of Glutathione Sepharose 4B immobilized GST and GST-Grb2 proteins were then rotated overnight (20 hours) at 4°C with 200 ug of experimental cell lysates. Sepharose beads were collected via centrifugation for 45 sec at room temperature and washed with 500 µl immunoprecipitation buffer (20mM HEPES, 150mM NaCl, 0.1% Triton X-100, 10% glycerol). After three washes, slurries were centrifuged at 10,000 RPMs for 1 minute at 4°C and supernatants removed. Beads were resuspended in Lamelli buffer containing β -mercapthoethanol and heated to 95°C for 10 minutes. Samples were centrifuged at 14000 RPMs for 1 minute and then supernatants were loaded onto 4–15% gradient gels (BioRad, Hercules, CA).

IGF-IR-GFP cloning strategy

pALS5 is retroviral construct expressing eGFP fused in frame to the C-terminus of the full length human IGF-IR. The vector was constructed in a retroviral pLEGFP-N1 (Clontech, Mountain View, CA) vector backbone. Human IGF-IR was PCR amplified with Phusion Hot Start II High Fidelity DNA polymerase (Finnzymes, Thermo-Fisher, Waltham, MA) from pcDNA3 hIGF-IR construct. **F primer:** AGAGTCGACcgccaccATGAAGTCTGGCTCCGGAGGA. Capital letters: human IGFIR 5' sequence; Capital letters: SalI restriction site; small letters: Kozak sequence. **R primer:** GCGGGATCCgcGCAGGTCGAAGACTGGGGCA. Capital Letters: human IGFIR 3' sequence without stop codon; Capital letters: BamHI restriction site; small letters: two bases added to be in frame with eGFP.

FRAP parameters

The FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) equipped with four lasers: a blue diode (405nm), a multiline argon (457, 488, and 514nm), plus green (543nm) and red (633nm) HeNe lasers with corresponding photodetectors. For FRAP, the system carries an additional scanner (SIM) which allows photoactivation or photobleaching of well-defined regions of interest at 405nm while imaging at high resolution with any of the other laser lines using the main scanner. For FRAP experiments, environmental control during imaging of live cells is achieved via a Live Cell culture dish system (Pathology Devices, Westminster, MD). Time-lapse capture, the FRAP module, and analysis are managed through the Olympus FluoView software. Imaging: 30 frames at 40µs/pixel with 488nm at 0.2% laser power; photobleaching: 1 frame at 20µs/pixel with 405nm at 16% of laser power.

Statistical analysis

The data were analyzed with paired Student's T-test and are displayed as mean $\pm$ standard deviation. The differences between the control and experimental groups were considered significant and marked with asterisk (*) for p values lower than 0.05.

Results

Effects of ethanol on IGF-I-induced signaling responses in neuron-like cultures

In early experiments conducted on the effects of ethanol on IGF-IR signaling, the presence of ethanol was shown to inhibit IGF-I-induced auto-phosphorylation of the IGF-IR, attenuate tyrosine phosphorylation of insulin-receptor substrate I (IRS-I), and reduce the phosphorylation-dependent activation of downstream kinases, Akt and ERKs1/2 (Cohen et al., 2007, de la Monte et al., 2005, Resnicoff et al., 1993). Further experimental work conducted both *in vivo* and in primary neuronal cultures, demonstrated that, in addition to its overall negative signaling effects, ethanol also decreased expression of IGF-I and the IGF-IR (de la Monte et al., 2005). Surprisingly, the results in Fig. 1A demonstrate that the pre-incubation of PC12 neuron-like cells with 50mM ethanol for 1 hour significantly increased IGF-I-induced phosphorylation of IRS-I (pY612) and ERKs (T202/Y204); however, phosphorylation of Akt (S473) was largely unaffected. In a similar manner, 24-hour ethanol exposure also resulted in a significant augmentation of IRS-I and ERKs phosphorylation, while Akt phosphorylation was again affected only minimally (Fig. 1B). We have repeated these experiments at least three times and the corresponding densitometry and statistical analyses are provided below the corresponding blots. This hyper activation of IGF-I-dependent IRS-I and ERKs phosphorylation was followed by a significant decline in the phosphorylation of both IRS-I, ERKs after 3 weeks of continuous ethanol exposure (Fig. 2A). In this experiment, similar to the acute exposure experiments, the effects of ethanol on IGF-I-induced phosphorylation of Akt were less pronounced.

Several reports in the literature suggest differential dose-dependent effects of ethanol on intracellular signaling responses (He et al., 2007). We therefore wanted to test whether this was the case in our system. Results in Fig. 2B demonstrate dose-dependent effects of ethanol on IGF-I-induced IRS-I and ERKs phosphorylation. The phosphorylation levels of these two signaling molecules increased gradually with increasing concentrations of ethanol up to 50mM, and sharply decreased in the presence of 200mM ethanol. There are also reports that the temporality of ethanol exposure can have effects on the signaling responses within the cell (Ting and Lauth, 2006). Thus, we also tested the duration of ethanol exposure necessary for the augmentation of IGF-I-dependent phosphorylation of ERKs. The results in Fig. 2C demonstrate that 5 minute exposure to 50mM ethanol actually decreases the signal, which subsequently recovers after 30 minutes, reaching the highest levels between 1 and 3 hours, and this elevated ERKs activity persists up to 24 hours of continuous ethanol exposure (Fig. 1C). Note also that ethanol did not trigger any detectable ERKs phosphorylation in the absence of IGF-I stimulation (Fig. 2C, last lane).

Since, the observed augmentation of the IGF-IR signal in the presence of ethanol had previously been largely unreported, it was possible that this unexpected response of PC12 cells was somehow unique to this particular cell line. Therefore, we also tested the effects of ethanol in monolayer cultures of SH-SY5Y human neuroblastoma cells, and in three-dimensional primary neural progenitor cultures. Results in Fig. 3A demonstrate that SH-SY5Y cells responded to ethanol with elevated phosphorylation of ERKs, similarly to PC12 cells (Fig 1A), however they did not show any obvious changes at the level of IRS-I tyrosine

phosphorylation. With respect to neural progenitors, ethanol's effect on IRS-1 and ERKs phosphorylation (Fig. 3B) was similar to what was seen in PC12 and SH-SY5Y cells *i.e.* significantly higher phosphorylation following ethanol treatment. One difference noted was that the basal phosphorylation level of ERKs was high in the neural progenitors, which was most likely due to the presence of insulin in the media used to culture the progenitor (in the absence of insulin these progenitor cells undergo apoptosis), and further stimulation with IGF-1 decreased the signal (Fig. 3B, columns 3, 4, 7, 8).

We have also tested cellular responses to 50 mM ethanol and have found that proliferation and survival of PC12 cells was not significantly affected at this concentration (not shown). However, with respect to neural progenitors we observed that the differentiation pattern was changed significantly following 24-hour exposure to 50mM ethanol (Fig. 3C). In this experiment neurosphere cultures were first exposed to ethanol for 24 hours. Neurospheres were then allowed attachment and differentiation in the absence of ethanol for 4 days (see methods). The data in Fig. 3C demonstrate that ethanol pretreatment did not affect the size of differentiating neurospheres (numbers below histogram). However, acute exposure to 50mM ethanol actually stimulated differentiation towards neurons and attenuated differentiation towards astrocytes. Results in Fig. 3C (both histogram and insets) show a significant increase of β III tubulin positive processes (neuronal marker) and a significant decrease in GFAP positive processes (glial marker) following ethanol pretreatment. Accordingly, the average astrocyte/neuron ratio was 1.263 and 0.615 in control and ethanol-treated neurospheres, respectively. This unexpected finding indicates that the observed decline in astrocyte/neuron ratio following acute ethanol exposure could deprive neuronal cells from the optimal cellular and extracellular environment likely affecting development of the CNS.

Since we had seen stimulatory effect of acute ethanol on IGF-IR signaling in three different neuronal cell types (Figs. 1 and 3), and in fibroblasts (data not shown), as well as stimulatory effects of acute ethanol exposure towards neuronal differentiation (Fig.3C), we sought to determine a possible mechanism through which this effect may occur.

Ethanol Affects the Mobility of the IGF-IR within the Membrane

Because the initial signaling steps of ERKs activation occur within or very near the cell membrane (Carter-Su et al., 2015), we asked if ethanol's effects on cell membrane dynamics (Bae et al., 2005) are responsible for this IGF-IR system hyper activation. To analyze this, we constructed an expression vector in which human IGF-IR cDNA was cloned in frame with GFP (Fig. 4A). We first tested this construct in IGF-IR-knockout mouse embryo fibroblasts (R- cells), and demonstrated that the IGF-IR-GFP fusion protein localizes preferentially in the cell membrane of R- cells (Fig. 4A), can be grossly overexpressed, and the IGF-IR-beta subunit demonstrates expected retardation in gel mobility in comparison to the wild type IGF-IR-beta subunit expressed in R508 cells (fibroblasts expressing 22,000 IGF-IR copies) (Sell et al., 1994) (Fig. 4B, lower panel). Importantly, results in Fig. 4C demonstrate that in comparison to the control R-/GFP cells, R-/IGF-IR-GFP cells fully responded to IGF-I stimulation, and again we saw an ethanol-mediated enhancement of ERKs phosphorylation (Fig. 4D). However, these R-/IGF-IR-GFP cells, responded slightly

differently to IGF-I stimulation in comparison to PC12 cells. Only one form of ERKs (p44) was phosphorylated in R- cells (compare Figs. 2B and 4D), and although phosphorylation of p44 ERK was significantly induced by ethanol, the effect was delayed. Further, IRS-I tyrosine phosphorylation was not affected by ethanol in this control cell line (Fig. 4D). We also tested the IGF-IR signaling response in the presence of ethanol in PC12 cells stably expressing this construct and found a similar augmentation of ERKs phosphorylation (data not shown). Importantly, ethanol had a similar effect on ERKs phosphorylation in these R-/IGF-IR-GFP cells as it did in PC12, SH-SY5Y, and neural progenitors.

We next used the PC12 clones stably expressing membrane-associated IGF-IR-GFP fusion protein (PC12/IGF-IR-GFP) (Fig. 4E) to test the acute effects of ethanol on the basic lateral mobility of the IGF-IR within the cell membrane. The results in Fig. 4E demonstrate an example of fluorescence recovery after photobleaching (FRAP) analysis in which a significant increase in the rate of fluorescence recovery was recorded in PC12/IGF-IR/GFP cells exposed to 50mM ethanol for 1 hour. Quantitatively, the IGF-IR-dependent average time required for 50% recovery of fluorescence was 15 sec in the control medium (0mM ethanol), while it decreased to 12.4 sec in the presence of 25mM ethanol, and decreased further to 9.3 sec in the presence of 50mM ethanol. This represents a 38% decrease in time to 50% recovery ($p=0.022$). Surprisingly, further increases in the ethanol concentration (up to 500mM) had no further effect on the IGF-IR-GFP membrane mobility; however, it remained significantly lower in all ethanol concentrations tested.

Ethanol Modulates IGF-IR Signaling through Effects on the Cell Membrane

Next we asked whether this significant change in IGF-IR membrane motility could affect its recruitment to membrane rafts, which is an early step that leads to activation of substrates within the IGF-IR pathway (Hong et al., 2004). In order to test this, we exposed PC12 cells to ethanol for 1 hour and then fractionated them into membrane rafts and cytosolic fractions. Results in Fig. 5C demonstrate very similar IGF-IR levels detected in the membrane rafts fraction in all tested conditions (i.e. +/- IGF-I and +/- ethanol), which indicates that the quantity of the IGF-IR in membrane rafts is not responsible for the observed effects of ethanol on the augmented phosphorylation of ERKs. The autophosphorylation of the IGF-IR is a necessary step in the propagation of the extracellular signal to the downstream kinases Akt and ERKs, so we also examined whether ethanol was able to perturb this molecular event. Our data indicate that the phosphorylation of the IGF-IR is unaffected by acute exposure to 50mM ethanol (Fig. 5A & 5B), explaining, at least partially, why our results do not show any ethanol-dependent changes on IGF-I-induced phosphorylation of Akt (Fig. 2A).

Following ligand binding, the IGF-IR forms complexes with signaling intermediates such as growth-receptor bound protein 2 (Grb2) and Src homology and collagen homology (Shc) (Xi et al., 2008). After these molecules are recruited into a complex with the activated IGF-IR, and subsequent to the activation of several intermediates, ERKs become phosphorylated and can translocate to the nucleus to induce their target gene expression. Thus, we sought to determine whether ethanol was able to affect the formation of this signaling complex. We tested this using two approaches; an immunoprecipitation followed by Western blot, and a

glutathione synthase transferase (GST) pull-down assay. Our results from Fig. 6A show that following IGF-I stimulation, there is an increase in the amount of p52-Shc bound to immunoprecipitated Grb2. In a separate experiment, we used GST-tagged recombinant Grb2 in order to perform a GST pull-down assay. The results of the GST pull-down indicate that following 24h ethanol exposure, the formation of the Grb2-p52-Shc complex was enhanced (Fig. 6B). The Shc protein has three isoforms that are expressed in neuronal cells, p46-, p52-, and p66- Shc (Wills and Jones, 2012). Within the context of IGF-IR signaling, p52-Shc has been shown to be required for the propagation of the signal from the activated IGF-IR downstream to ERKs (Xi et al., 2008). Thus, an increase in the amount of p52-Shc bound to Grb2 would indicate that the signal transmitted downstream to ERKs would be more robust. While it is unclear from these experiments whether the ethanol-induced changes in the mobility of the IGF-IR within the membrane and this enhanced formation of the Grb2-p52-Shc signaling complex are due to the same effect, we believe that the combination of these two events is the mechanism through which acute ethanol exposure is able to augment the activation of ERKs following IGF stimulation.

Discussion

In these experiments we have shown that acute exposure of neuron-like cells to 50mM ethanol enhances the activation of substrates within the IGF-IR pathway, and that these effects are likely the result of ethanol's effects on 1) IGF-IR mobility within the cell membrane and 2) the binding of p52-Shc to Grb2 following IGF-I stimulation. Ethanol is known to have pleiotropic effects depending on timing, concentration, and cell type, among other things (He et al., 2007, Ting and Lutt, 2006). Indeed, within the context of ethanol and IGF-IR signaling there are some contrary reports regarding signaling events including the affinity of IGF ligand for its receptor in the presence of ethanol (Cohen et al., 2007, Resnicoff et al., 1993). Additionally, even though other studies have shown that ethanol positively affects Akt phosphorylation at low doses, but inhibits Akt phosphorylation at high doses (He et al., 2007), in our experiments, we did not see robust effects on Akt phosphorylation (Figs. 1 & 2A). Therefore, although we have tested multiple cell types and seen a reproducible enhancement of phosphorylation of certain IGF-IR signaling intermediates, it is possible that in other cellular contexts or treatment regimens, our observations may more closely resemble previously published results. It is also quite possible that the differences observed in these experiments are related – our data that ethanol has a bimodal effect on IRS-I and ERKs phosphorylation (compare Fig 1 to Fig 2A) support this idea.

Several studies have provided evidence that IGF-I is able to increase the number of neural cells, including neurons, astrocytes, and oligodendrocytes, *in vivo* and *in vitro* (Arsenijevic et al., 2001, Mason et al., 2003, Aberg et al., 2003a, Aberg et al., 2003b). In addition to its mitogenic properties, IGF-I can also direct the differentiation process towards the neuronal and oligodendrocytic lineages. For instance, it has been reported that during development IGF-I promotes differentiation of neural progenitors towards neurons (Arsenijevic and Weiss, 1998), and affects the fate of multipotent adult neural progenitors by directing their differentiation towards oligodendrocytes (Hsieh et al., 2004). Further experiments demonstrated that the observed increase in the number of neurons produced by IGF-I was

not mediated by an increase of cell survival or cell proliferation, but rather depended upon induction of the differentiation program (Arsenijevic et al., 2001, Arsenijevic and Weiss, 1998). With respect to ERKs, there is also ample experimental data to show that these kinases are able to regulate neuronal proliferation as well as differentiation (Qui and Green, 1992, Walowitz and Roth, 1999), and that the prolonged activity of ERKs can lead to neurodegeneration (Colucci-D'Amato et al., 2003). Our own neurosphere data seem to support this notion. At the molecular level, acute EtOH increases the activation of ERKs, and at the cellular level, this results in an increase in the volume of neuronal immunolabeling and decrease in the volume of astrocytic immunolabeling (Fig 3). This may not seem as though it would result in cognitive dysfunction, but there are an increasing number of reports that a deficit in either the number of astrocytes or the ratio of astrocytes to neurons results in cognitive dysfunction. Multiple papers have described the phenomenon whereby brains of more evolutionarily complex organisms show increasing ratios of astrocytes to neurons as well increasing astrocytic complexity and size (Herculano-Houzel and Kaas, 2011, Oberheim et al., 2009, Pereira and Furlan, 2010). Additionally, it has been shown in multiple studies that EtOH exposure *in utero* results in a decrease in the number of astrocytes (Guerri et al., 2001, Rubert et al., 2006). This highlights the importance of a better understanding of the mechanism through which ethanol interferes with the IGF-IR signaling because of the critical nature of this pathway during *in utero* CNS development.

We are not the first to report that ethanol has effects on the properties of the cell membrane (Bae et al., 2005). Indeed, there have been multiple studies carried out regarding this phenomenon. The earliest of these studies were done at very high ethanol concentrations (500–1500mM), which, although biochemically informative, are not physiologically relevant (blood ethanol concentrations above 200mM in humans are often lethal). Results from later studies show that ethanol is able to disrupt the components found within lipid rafts (Nourissat et al., 2008), and that ethanol interferes with Na⁺/K⁺ ATPase activity through changes in membrane fluidity (Rothman et al., 1994). Our FRAP results are similar to previous reports with respect to increases in membrane fluidity in the presence of ethanol. So, although our experiments can only give us direct evidence that the IGF-IR is more mobile within the membrane in the presence of 50mM ethanol, it seems that the effect is due more to an increase in membrane dynamics in general rather a specific effect on the IGF-IR.

The IGF-IR activates multiple downstream effector molecules, some of the most well-known following IGF stimulation, are the ERKs and Akt. Because signaling through this system is so critical for proper cellular function it has been thoroughly studied. Mechanistic studies regarding the specific substrates that are activated following ligand binding the IGF-IR have shown that one of the crucial events in the activation of ERKs is the recruitment of Grb2 and p52-Shc to a signaling complex. It is critical that p52-Shc is bound to this complex, because it has been shown that when another Shc protein, p66-Shc, is preferentially recruited to this complex, the activation of ERKs is attenuated (Xi et al., 2008). Our results regarding the formation of the Grb2-p52-Shc complex show that there is an increase in the amount of Grb2 bound to p52-Shc, without a significant effect on p66-Shc. This supports our initial findings whereby ethanol was able to increase the phosphorylation of ERKs following acute exposure, and leads us to conclude that this is one of the mechanisms through which ethanol enhances the phosphorylation of ERKs.

Our results show acute increases in IGF-IR signaling in the presence of 50mM ethanol, which, at least on the surface, may contradict previous data indicating that ethanol impairs IGF-IR signaling. One potential explanation for the differences seen in our experiments as compared to those which have reported a reduction in IGF-IR signaling in the presence of ethanol is that this acute enhancement of signaling may precede the impairment of signaling seen following chronic exposure of cells to ethanol. The reasoning for this is twofold: 1) we have seen that in our system, chronic (3 weeks) exposure to 50mM ethanol resulted in a reduction in IRS-I and ERKs activation and 2) it is well-known that there are cellular mechanisms in place which are meant to shut down constitutively active or overactive signaling, which include conformational changes (ion channels) (Foster and Coetzee, 2016), downregulation of receptor expression (growth factor receptors) (Bache et al., 2004), and receptor uncoupling (G-protein coupled receptors) (Fehmann and Habener, 1991). Thus, our results do not necessarily contradict previous reports; rather, they may simply be interpreted as reflective of earlier time-point changes, which precede ethanol's chronic effects. Further studies are required to test this hypothesis, but considering the data we have presented in this manuscript, as well as what is known in the literature regarding growth factor receptor regulation, we believe that this is a plausible explanation (Bache et al., 2004).

In conclusion, our results indicate that acute (1 – 24h) exposure to 50mM ethanol leads to an enhanced IGF-mediated increase in ERKs phosphorylation, which is most likely due to effects at the level of the interaction between the IGF-IR and cellular membranes. This is significant because the IGF-IR signaling pathway is critical in the development of the fetal brain and CNS, and thus, exposure to ethanol (and the perturbations to IGF-IR signaling that result) during this critical period in development could contribute to the well-known detrimental effects of *in utero* ethanol exposure on the cognitive function of individuals with FASD (Chokroborty-Hoque et al., 2014).

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Figure 1. Effects of acute ethanol exposure on IGF-1-induced signaling responses in PC12 neuron-like cells *in vitro*

Western blot analysis showing levels of phosphorylated (activated) forms of IRS-1, ERKs, and Akt. During 24 hours of serum starvation, PC12 cells were cultured in serum-free medium (SFM) in the presence or absence of 50mM ethanol (EtOH), which was applied for either 1 hour (**Panel A**) or 24 hours (**Panel B**). In both panels control cells were treated with an equal volume of vehicle (sterile H₂O), indicated as “No EtOH”. Prior to IGF-1 stimulation, the medium containing EtOH was replaced with EtOH-free SFM and the cells

were stimulated with IGF-1 (50 ng/mL) for 0.5, 3, or 6 hours. Loading conditions were tested by reprobing the same blot with anti-Erks and Grb-2 antibodies. Histograms below demonstrate densitometric evaluation of the obtained blots using ImageJ software. Data represent average values from 3 independent experiments with standard deviation (n=3). Densitometric values for each protein were normalized by the corresponding densitometric values of the loading marker, Grb-2, and are expressed as arbitrary densitometry units $\times 100$. (*) indicates statistically significant differences (paired student t-test, $P \leq 0.05$) between controls (No EtOH) and matching ethanol treated samples (EtOH 50mM) at indicated time points following IGF-1 stimulation.

Figure 2. Time- and dose- dependent effects of ethanol exposure on IGF-IR signaling pathways
Panel A: PC12 cells were grown and passaged in the presence or absence of 50 mM ethanol (EtOH) for 3 weeks (21 days). On day 20, both EtOH-containing and EtOH-free cultures were serum-starved for 24 hours. Prior to IGF-1 stimulation, the medium containing EtOH was replaced with EtOH-free medium and the cells were stimulated with IGF-1 (50 ng/mL) for 0.5, 3, or 6 hours. Loading conditions were tested by reprobing the same blot with anti-Grb-2 antibody, as described in our previous studies. **Panel B:** PC12 cells were serum-starved for 24 hours and treated with increasing concentrations of EtOH (0, 25, 50, 200 mM)

for 1 hour at the end of serum starvation. **Panel C:** PC12 cells were serum-starved for 24 hours and then incubated with 50mM EtOH for varying amounts of time (0, 5, 30, 60, 180 minutes) at the end of serum starvation. Data represent average values from 3 independent experiments with standard deviation (n=3). (*) indicates statistically significant differences (paired student T-test, P 0.05) between controls (No EtOH) and matching ethanol treated samples (EtOH 50mM) at indicated time points. In Panels B and C (*) indicates statistically significant difference from 0 mM EtOH.

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Figure 3.

Effects of ethanol on IGF-1-induced IRS-1 and ERKs phosphorylation analyzed in human neuroblastoma cell line, SH-SY5Y (**Panel A**), and in three dimensionally-growing, primary, mouse neural progenitors (**Panel B**). Western blot analysis and ethanol treatment of SH-SY5Y were identical to the experimental protocol described in Figure 1A. For neural progenitors, however, the ethanol treatment was applied in medium containing growth factors and insulin, which are required to maintain their survival (see Materials and Methods). Data represent average values from 3 independent experiments with standard

deviation (n=3). (*) indicates statistically significant differences (paired student T-test, P 0.05) between controls (No EtOH) and matching ethanol treated samples (EtOH 50mM) at indicated time points. **Panel C:** Effects of acute (24 hour) ethanol exposure on the growth and differentiation of neural progenitors. In this experiment neurosphere cultures were exposed first to 50 mM ethanol for 24 hours, and then the neurospheres were re-plated to induce neural differentiation (see methods). The quantifications of the average number of voxels associated with neurons (β III tubulin positive), astrocytes (GAFP positive) and nuclei (DAPI positive) were based on confocal images using SlideBook5 software as described in our previous studies (Gualco et al., 2010, Wilk et al., 2011). Data represent average values with standard deviation (n=3). * indicates values statistically different from the corresponding controls (no EtOH).

Figure 4. Effects of ethanol on IGF-IR mobility within the plasma membrane

Panel A: Fluorescent image of a positive clone of R- cells [(mouse fibroblasts with targeted disruption of IGF-IR gene (Ullrich et al., 1986)] expressing IGF-IR-GFP fusion protein.

Panel B: Western blot analysis of total protein extracts from R-/IGF-IR/GFP cells depicted in Panel A. R- cells expressing GFP only (R-/GFP) and R508 cells, were used as negative and positive controls, respectively. The blots were probed with anti-IGF-IR antibodies recognizing either alpha (upper panel) or beta subunit (lower panel) of the IGF-IR. Note the significant difference in the mobility of the IGF-IRβ isolated from R-/IGF-IR-GFP cells in

comparison to the IGF-IR beta subunit from R08 cells (lower panel). **Panel C:** IGF-1 stimulation of IGF-IR-GFP signaling in R-/GFP and R-/IGF-IR-GFP cells. Anti-Grb-2 antibody was used as a loading marker. In contrast to R-/GFP cells (negative control) R-/IGF-IR-GFP cells demonstrated IGF-I-induced phosphorylation of all tested signaling molecules. **Panel D:** Effects of ethanol on IGF-I-induced ERKs and IRS-I phosphorylation in PC12/IGF-IR-GFP cells. **Panel E:** Fluorescence recovery after photo bleaching (FRAP) of PC12 cells stably expressing the IGF-IR-GFP fusion protein (PC12/IGF-IR-GFP; inset). The graph at right is a representative plot of fluorescence intensity recorded during the recovery of fluorescence after photo bleaching of PC12/IGF-IR-GFP cells in the presence or absence of 50 mM ethanol, which was applied 1 hour before the measurement. The histogram demonstrates average values of the time to 50% recovery after photo bleaching for PC12/IGF-IR-GFP cells exposed to the indicated concentrations of EtOH. (*) indicate values which are statistically different ($p < 0.05$) from control (0 mM EtOH).

Figure 5. Effects of ethanol on IGF-1-induced activation of the IGF-IR
Panel A: Western blot analysis using PC12 cell protein lysates and an anti-pY1135/1136 IGF-IR antibody (Cell Signaling). Western blot and ethanol treatment conditions were identical to the experimental protocol described in Figure 1A. **Panel B:** Immunoprecipitation (IP) Western blot (W) analysis (IP/W) in which PC12 total protein lysates were extracted following 24h EtOH exposure (50mM) and IGF-1 stimulation. For Panels A and B, histograms below the corresponding blots show densitometric analysis using ImageJ software. The data represent average densitometric values from three blots (n=3), which were normalized either with the loading marker, Grb-2 (Panel A), or with

immunoprecipitated IGF-IR (panel B), and are expressed as arbitrary densitometry units $\times 100$. **Panel C:** Effects of acute EtOH exposure on IGF-IR content in membrane rafts. The procedure for cytosolic and membrane raft protein extraction is described in Material and Methods. Following 1 hour EtOH exposure and IGF-1 stimulation (50 ng/mL for 30 min), cytosolic and membrane raft fractions were isolated and used for Western blot analysis with anti-IGF-IR α , anti-Src (membrane raft marker), and with anti-GAPDH (cytosolic marker).

Figure 6. Effects of ethanol on the formation of Grb2-p52-Shc complex

Panel A: Immunoprecipitation of Grb2 followed by a Western blot using an α -(pan)Shc antibody (Millipore). PC12 cells were exposed to EtOH for 24h and then stimulated with IGF-I. Cells were then lysed and lysates were subjected to immunoprecipitation using α -Grb2 antibody. In the presence of EtOH, there was an increase in Grb2-p52-Shc complexes, the results of which are quantified and shown in (**Panel B**). Following production of GST-tagged Grb2, a GST-pulldown was performed (**Panel C**). As in the IP/Western blot there was an increase in the amount of Grb2 bound to p-52-Shc in the presence of EtOH. The results from this GST- pulldown are quantified in (**Panel D**). Data represent average values from 3 independent experiments with standard deviation (n=3). (*) indicates statistically significant differences (paired student T-test, P 0.05) between controls (No EtOH) and matching ethanol treated samples (EtOH 50mM).