Article

Differential Contributions of Alcohol and the Nicotine-Derived Nitrosamine Ketone (NNK) to Insulin and Insulin-Like Growth Factor Resistance in the Adolescent Rat Brain

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Abstract

Aims: Since epidemiologic studies suggest that tobacco smoke toxins, e.g. the nicotine-derived nitrosamine ketone (NNK) tobacco-specific nitrosamine, can be a co-factor in alcohol-related brain disease (ARBD), we examined the independent and additive effects of alcohol and NNK exposures on spatial learning/memory, and brain insulin/IGF signaling, neuronal function and oxidative stress.

Methods: Adolescent Long Evans rats were fed liquid diets containing 0 or 26% caloric ethanol for 8 weeks. During weeks 3–8, rats were treated with i.p. NNK (2 mg/kg, 3×/week) or saline. In weeks 7–8, ethanol groups were binge-administered ethanol (2 g/kg; 3×/week). In week 8, at 12 weeks of age, rats were subjected to Morris Water Maze tests. Temporal lobes were used to assess molecular indices of insulin/IGF resistance, oxidative stress and neuronal function.

Results: Ethanol and NNK impaired spatial learning, and NNK ± ethanol impaired memory. Linear trend analysis demonstrated worsening performance from control to ethanol, to NNK, and then ethanol + NNK. Ethanol ± NNK, caused brain atrophy, inhibited insulin signaling through the insulin receptor and Akt, activated GSK-3β, increased protein carbonyl and 3-nitrotyrosine, and reduced acetylcholinesterase. NNK increased NTyr. Ethanol + NNK had synergistic stimulatory effects on 8-iso-PGF-2α, inhibitory effects on p-p70S6K, tau and p-tau and trend effects on insulin-like growth factor type 1 (IGF-1) receptor expression and phosphorylation.

Conclusions: Ethanol, NNK and combined ethanol + NNK exposures that begin in adolescence impair spatial learning and memory in young adults. The ethanol and/or NNK exposures differentially impair insulin/IGF signaling through neuronal growth, survival and plasticity pathways, increase cellular injury and oxidative stress and reduce expression of critical proteins needed for neuronal function.

INTRODUCTION

Alcohol abuse during prenatal, postnatal or adolescent development causes cognitive impairment with long-lasting adverse effects on brain structure and function. The major targets of alcohol-related brain disease (ARBD; Supplementary Table S1 for abbreviations) include corticolimbic structures, the cerebellum and white matter (de la
Monte and Kril, 2014). Previous studies established roles for brain insulin and insulin-like growth factor type 1 (IGF-1) resistance, together with increased oxidative stress, as mediators of ARBD in both mature and immature brains (de la Monte and Wands, 2002; Xu et al., 2003; Corl et al., 2005; Cohen et al., 2007; de la Monte et al., 2008a; McClure et al., 2011; Lindner et al., 2013). Mechanistically, alcohol reduces tyrosine phosphorylation of the insulin and IGF-1 receptors, decreases signaling through insulin receptor substrate (IRS) proteins, phosphoinositol-3-kinase (PI3K) and Akt, increases activation of glycogen synthase kinase 3β (GSK-3β) and impairs expression of genes and proteins needed for cholinergic function (Xu et al., 2003; Socsea et al., 2006; Ronis et al., 2007; Conti et al., 2009). Insulin signaling through PI3K-Akt promotes neuronal survival, metabolism and plasticity (Dudek et al., 1997; de la Monte and Wands, 2003; Hughes et al., 2010), and insulin resistance leads to oxidative stress, DNA damage, impairments in neuronal plasticity and deficits in energy balance (de la Monte et al., 2009a; de la Monte and Wands, 2010). Furthermore, oxidative stress and DNA damage cause mitochondrial dysfunction and stress, activating neural inflammatory pathways that exacerbate insulin resistance (de la Monte and Wands, 2001; Chu et al., 2007; Cherian et al., 2008; Haorah et al., 2008).

Variability in the nature and severity of the clinical and pathological features suggests that co-factors may contribute to ARBD pathogenesis. Since a very high percentage of heavy drinkers (up to 80%) also abuse tobacco products, typically by cigarette smoking (Romberger and Grant, 2004), and both heavy drinking and cigarette smoking adversely affect neurocognitive function (Durazzo et al., 2007) and white matter structure (neuroimaging) (Wang et al., 2009), consideration should be given to the concept that tobacco and its toxic metabolites can serve as co-factors in ARBD. Alcohol-tobacco dual effects on carcinogenesis have been well described (Johnson et al., 1996; de Boer et al., 1997; Tramacere et al., 2010; Duell, 2012), particularly with respect to the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolites (Go et al., 2005; Duell, 2012). However, potential non-carcinogenic effects of nicotine-derived nitrosamine ketone (NNK) are largely unknown, despite evidence that limited, sub-mutagenic exposures to other nitrosamines, i.e. streptozotocin (Bolzan and Bianchi, 2002; Koulimanda et al., 2003; Wang et al., 2011) or N-nitrosodimethylamine (NDEA) (de la Monte and Tong, 2009; Tong et al., 2009b), cause insulin resistance, DNA damage, lipid peroxidation, mitochondrial dysfunction, ER stress and impairments in PI3K-Akt signaling (de la Monte and Tong, 2009; Tong et al., 2009b). Furthermore, we recently demonstrated additive and interactive effects of ethanol and sub-mutagenic doses of NDEA on brain development in experimental fetal alcohol spectrum disorder (Andreani et al., 2014). The present study tests the hypothesis that sub-mutagenic doses of NNK are sufficient to cause cognitive impairment together with insulin/IGF resistance, oxidative stress and altered neuronal gene expression in the adolescent brain.

METHODS

Experimental model (See Supplementary Methods and Fig. S1)

The study goals were to generate a chronic + binge alcohol exposure model (Ramirez et al., 2014), and examine the independent and interactive effects of alcohol and NNK on brain insulin/IGF-1 signaling and cognitive function. Long Evans male rats were pair-fed for 8 weeks with isocaloric liquid diets containing 0 or 26% ethanol by caloric content (0 or 6% v/v). Rats in each group were treated with 2 mg/kg i.p. NNK and/or 2 g/kg ethanol, 3 times per week each, along with saline controls. The NNK doses were far below those needed for carcinogenesis (Rivenson et al., 1988; Trushin et al., 1994). In Week 8, rats were subjected to Morris Water Maze (MWM) testing of spatial learning and memory (de la Monte et al., 2006). Rats were housed under humane conditions with 12-h light/dark cycles and free access to food.

Enzyme-linked immunosorbent assays (ELISAs)

Direct binding duplex ELISAs were used to measure immunoreactivity (Longato et al., 2012). Target proteins were detected with horseradish peroxidase-conjugated secondary antibody and Amplex UltraRed (Invitrogen, Carlsbad, CA, USA). Binding of bionylated large acidic ribosomal protein (RPLPO) antibody (Proteintech Group Inc., Chicago, IL, USA) was detected with streptavidin-conjugated alkaline phosphatase and 4-Methylumbelliferyl phosphate (4-MUP). Fluorescence intensities were measured in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). Calculated target protein/RPLPO ratios were used for inter-group comparisons.

Competitive ELISAs

Competitive ELISAs measured Keratin-18 (USCN Life Science Inc., Hubei, PRC), protein carbonyl and isoprostane 8-iso-PGF-2α (Cell Biolabs Inc., San Diego, CA, USA). Results were normalized to protein content.

Bead-based multiplex ELISAs

Multiplex bead-based ELISAs were used to measure immunoreactivity to the insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, ribosomal protein S6 kinase (p70S6K) and glycogen synthase kinase 3β (GSK-3β) and pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT342/T344,pS6K and pS202/GSK-3β (Invitrogen, Carlsbad, CA, USA) as described (Longato et al., 2012). Fluorescence was measured in a MAGPIX (Bio-Rad, Hercules, CA, USA).

Statistics

Each experimental group included 8–10 rats. Inter-group comparisons were made by one- or two-way ANOVA with Tukey or linear trend post-hoc tests (GraphPad Prism 6, San Diego, CA, USA). F-ratios and P-values are tabulated. Significant post-test differences and trends (0.05 < P < 0.10) are shown in the graphs.

RESULTS

Systemic effects of alcohol and NNK (Supplementary Table S2)

Chronic exposures to ethanol, NNK or ethanol + NNK had no effect on body weight. In contrast, ethanol and ethanol + NNK decreased brain weight, whereas NNK had no significant effect. Blood glucose was measured because chronic ethanol or nitrosamine exposures can promote insulin resistance leading to elevated blood glucose. We detected higher serum glucose concentrations in rats exposed to NNK ± ethanol. Blood alcohol concentrations were higher in ethanol-exposed relative to NNK and control rats, and binge ethanol administration further increased blood alcohol by 3- to 4-fold. The somewhat lower blood alcohol levels in the ethanol + NNK versus ethanol-only group are not readily explained; however, the differences were not significant.

Effects of chronic ethanol and NNK exposures on spatial learning and memory

The MWM is used to assess spatial learning and memory. Latencies in arriving on the platform were measured and area-under-curve results were used for inter-group comparisons (Table 1 and Fig. 1). On trial
day 1, the latencies to locate and land on the visible platform were significantly prolonged in ethanol- and NNK-exposed rats (Fig. 1A), indicating slower learning. On trial days 2 and 3, the latencies improved for all groups, but they were still prolonged in the ethanol, NNK and ethanol + NNK groups relative to control (Fig. 1B and C). On trial day 4, latencies were significantly prolonged in NNK and ethanol + NNK relative to control and ethanol-only groups (Fig. 1D). Post-hoc linear trend analysis demonstrated that the progressive prolongation of latencies from control to ethanol, then NNK, and finally ethanol + NNK treatments was statistically significant for each trial day, with steepening slopes over time (Table 1). In other words, memory deficits were greater in the NNK and ethanol + NNK groups relative to control and ethanol only, and performance gap worsened over time, indicating that NNK (with or without ethanol) caused sustained impairments in memory.

### Table 1. Morris water maze: linear trend analysis

<table>
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<th>Trial day</th>
<th>F-ratio</th>
<th>P-value</th>
<th>Slope</th>
<th>R square</th>
<th>Linear P-value</th>
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<tr>
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<td>&lt;0.0001</td>
<td>10.64</td>
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</table>

Area-under-curve analysis was applied to each set of three daily trials. Inter-group comparisons were made using one-way ANOVA (F-ratio and P-value) with the Tukey multiple comparison post-hoc test (also see Fig. 1). Linear trend analysis tested if the increasing latencies (worsening performance) from control to ethanol, to NNK and then ethanol + NNK were statistically significant. The trend slope, calculated R square and Linear Trend P-value are indicated for each trial day.

### Ethanol and NNK effects on the insulin/IGF-IRS-Akt pathway

Insulin and IGF signaling through Akt survival and metabolic pathways can be modulated by changes in protein expression. Ethanol significantly modulated expression of the insulin R, IGF-1R and Akt protein, and produced a trend effect (0.05 < P < 0.10) on GSK-3β (Supplementary Table S3). Although there were no significant effects of NNK or ethanol × NNK interactions, trend effects were observed with respect to IRS-1 (ethanol × NNK). Post-hoc Tukey tests revealed statistical trends (0.05 < P < 0.10) for higher levels of Insulin R in ethanol versus control and NNK, and reduced levels of IGF-1R in ethanol + NNK relative to control (Supplementary Fig. 2A and B). Otherwise, the post-hoc tests were negative (Supplementary Fig. S2C–F).

### Ethanol and NNK effects on insulin/IGF-IRS-Akt pathway protein phosphorylation

We used a bead-based multiplex ELISA to simultaneously measure pYpY1162/1163-InsulinR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pS9-GSK-3β and pTnS421/424-p70S6K. Higher levels of pYpY1162/1163-InsulinR, pYpY1135/1136-IGF-1R, pS473-Akt and pTnS421/424-p70S6K reflect increased activation, whereas higher levels of pS112-IRS-1 and pS9-GSK-3β reflect inhibition of signaling. Two-way ANOVAs demonstrated significant ethanol, NNK and ethanol × NNK effects on pYpY1162/1163-InsulinR, ethanol effects on pS473-Akt, pS9-GSK-3β and pTnS421/424-p70S6K, NNK trend effects on pYpY1135/1136-IGF-1R, and ethanol + NNK trend effects on pTnS421/424-p70S6K (Supplementary Table S3). Post-hoc tests demonstrated that ethanol, NNK and ethanol + NNK significantly reduced temporal lobe levels of pYpY1162/1163-InsulinR (Fig. 2A), while ethanol and ethanol + NNK reduced pS473-Akt (Fig. 2D) and pS9-GSK-3β (Fig. 2E).

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**Fig. 1.** Ethanol and NNK effects on spatial learning and memory. Long Evans rats fed with isocaloric liquid diets containing 0 or 26% ethanol for 8 weeks were treated with NNK (2 mg/kg) or vehicle (3×/week) during the last 6 weeks, and binged with ethanol (2 g/kg) or saline (3×/week) in the last 2 weeks (Supplementary Fig. S1). The MWM test was performed over 4 days with 3 trials per day. (A) Day 1; (B) Day 2; (C) Day 3; (D) Day 4. Data were analyzed by calculating area under the curve (AUC) for latency to locate the platform. Graphs depict mean ± S.D. of results. Inter-group comparisons were made by mixed models ANOVA (Table 1). Con, control diet; EtOH, ethanol diet plus binge ethanol administration.
In addition, synergistic inhibitory effects of ethanol + NNK were observed with respect to pYpY1135/1136-IGF-1R (Fig. 2B) and pTpS421/424-p70S6K (Fig. 2F) since either exposure alone did not alter the levels of these phosphoproteins. The mean levels of pS312-IRS-1 were similar across the four sub-groups (Fig. 2C). In essence, nearly all of the inhibitory effects on insulin/IGF-1 signaling through Akt were associated with ethanol exposure, with or without NNK.

**Ethanol and NNK effects on relative protein phosphorylation through the insulin/IGF-1/IRS-Akt pathway (Supplementary Table S3 and Fig. S3)**

Relative levels of phosphorylation were calculated from the ratios of phosphorylated/total (p/T) protein to gauge whether reductions in phosphorylation shown in Supplementary Table S3 and Fig. 2 were due to changes in protein expression or impairments in phosphorylation. For example, if the phosphorylated and total protein levels declined together, the relative levels should be close to control, but if phosphorylation declined with unchanged or increased levels of protein expression, then relative phosphorylation (reflecting kinase activity) would be expected to be reduced. As observed with respect to the phosphorylated proteins, ethanol exposure significantly impacted temporal lobe levels of pYpY1162/1163-Insulin R/total Insulin R, pS473-Akt/total Akt and pS9-GSK-3β/total GSK-3β, whereas NNK significantly altered pTpS421/424-p70S6K/total p70S6K and had trend effects on pS312-IRS-1/total IRS-1 and pS473-Akt/total Akt, and ethanol + NNK had a trend effect on pYpY1135/1136-IGF-1R/total IGF-1R (Supplementary Table S3). The results of all other analyses were not statistically significant. Post-hoc Tukey significance tests demonstrated that ethanol significantly reduced pYpY1162/1163-Insulin R/total Insulin R (Supplementary Fig. S3A), while ethanol and NNK had trend effects and ethanol + NNK significantly reduced pS473-Akt/total Akt (Supplementary Fig. S3D) relative to control. In addition, ethanol and ethanol + NNK significantly inhibited pS9-GSK-3β/total GSK-3β relative to control and NNK (Supplementary Fig. S3E). None of the other post-hoc comparisons were statistically significant. In essence, most of the inhibitory effects on relative protein phosphorylation were driven by ethanol exposures.

**Fig. 2. Ethanol and NNK effects on insulin/IGF-Akt pathway activation. Temporal lobe protein homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to (A) pYpY1162/1163-Insulin R, (B) pYpY1135/1136-IGF-1 R, (C) pS312-IRS-1, (D) pS473-Akt, (E) pS9-GSK-3β and (F) pTpS421/424-p70S6K. Data were analyzed by two-way ANOVA (Supplementary Table S3). Post-hoc Tukey multiple comparison test results are depicted in the graphs (*P < 0.05; ****P < 0.0001; ξ 0.05 < P < 0.10).
Ethanol and NNK-associated increases in cellular injury and oxidative stress

Consequences of impaired insulin/IGF signaling through metabolic and cell survival pathways include increased stress marked by cellular injury, protein oxidation and lipid peroxidation, as reflected by increased levels of Keratin 18 (CK-18), protein carbonylation and 8-epimer of Prostaglandin F2α (8-iso-PGF-2α). We used competitive ELISAs to measure immunoreactivity in temporal lobe homogenates. Two-way ANOVA tests detected significant effects of ethanol on protein carbonyl and 8-iso-PGF-2α, and ethanol and ethanol x NNK interactive trend effects on CK-18 (Supplementary Table S4). In contrast, NNK did not significantly alter CK-18, protein carbonyl or 8-iso-PGF-2α. Post-hoc Tukey tests demonstrated a statistical trend for higher temporal lobe levels of CK-18 in all treated groups relative to control (Fig. 3A), significantly higher protein carbonyl levels in the ethanol and ethanol + NNK relative to control and NNK groups (Fig. 3B) and higher 8-iso-PGF-2α in ethanol + NNK relative to control and NNK groups (Fig. 3C). Therefore, both ethanol and NNK promoted cellular injury (CK-18), ethanol was the main driver of protein carbonylation and dual ethanol + NNK exposures mediated lipid peroxidation. In addition, the effects of ethanol plus NNK exposures on lipid peroxidation appeared to be synergistic.

Ethanol and NNK effects on neuronal markers of neurodegeneration

Previous studies showed that chronic ethanol and other nitrosamine exposures caused neurodegeneration with abnormalities in brain levels of tau, phospho-tau (pTau), amyloid beta precursor protein (AβPP), amyloid-beta peptide of the amyloid beta precursor protein (AβPP-Aβ), choline acetyltransferase (ChAT), acetyl cholinesterase (AChE), ubiquitin and 3-nitrotyrosine (N-Tyr). Tau is a major neuronal cytoskeletal protein, and with increased stress, pTau levels increase. AβPP is abundantly expressed in brain and has a functional role in neuronal plasticity. However, under stressed condition, its C-terminal cleavage fragment, AβPP-Aβ, accumulates in neurons. ChAT and AChE modulate synthesis and degradation of acetylcholine, one of the main neurotransmitters mediating cognition. Ubiquitin and N-Tyr served as additional stress markers because: (a) the ubiquitin-proteasome pathway is often activated with neurodegeneration (Culmsee and Landshamer, 2006; Takalo et al., 2013; Ying et al., 2013); (b) protein nitrosylation mediates neurodegeneration (Uehara et al., 2006) and (c) both ethanol and NNK can promote cell injury and death via increased protein nitrosylation (Bryan et al., 2004; de la Monte and Tong, 2009; Xu et al., 2012; Kozai et al., 2014). Immunoreactivity was measured by duplex ELISA with results normalized to an internal housekeeping protein (Longato et al., 2012).

Ethanol had significant effects on Tau, pTau, AβPP-Aβ and AChE, and a trend effect on ubiquitin (Supplementary Table S5). In contrast, NNK treatment had just a trend effect on AChE, while ethanol x NNK interaction significantly altered AβPP-Aβ and N-Tyr. Post-hoc tests demonstrated significant reductions in Tau and pTau in the ethanol + NNK relative to control group (Fig. 4A and B), decreased levels of AChE in the ethanol and ethanol + NNK relative to control and NNK groups (Fig. 4D), and decreased AβPP-Aβ levels in all three experimental groups relative to control (Fig. 4F). In contrast, ethanol produced a trend increase, and NNK significantly increased N-Tyr levels relative to control (Fig. 4H). No significant inter-group differences occurred with respect to ChAT (Fig. 4C), AβPP (Fig. 4E) or ubiquitin (Fig. 4G).

DISCUSSION

This study employed a Long Evans rat model to examine separate and additive effects of ethanol and NNK exposures on spatial learning and memory, and insulin/IGF signaling, oxidative stress and neuronal protein expression in the temporal lobe. The chronic ethanol exposures were initiated during early adolescence (4 weeks) (Spear, 2000), corresponding with the vulnerable period of hippocampal development and active plasticity (Semple et al., 2014). The NNK exposures were begun at 7 weeks of age (late adolescence), and ethanol binging was performed when the rats were 11 and 12 weeks of age. End-point studies were conducted when the rats were 12 weeks old (adults). This design mimics human adolescent exposures to alcohol and tobacco (toxins), and evaluates the adverse brain effects in young adulthood.
The chronic + binge ethanol model was used to mimic the human alcoholic scenario, as this approach causes significant steatohepatitis (Bertola et al., 2013; Mathews et al., 2014; Ramirez et al., 2014) yet the effects on the brain have not been reported. We did not examine ethanol dose effects, and instead focused on producing a robust model with a discernible phenotype. Furthermore, our intention was to compare effects of ethanol ± NNK tobacco-specific nitrosamine exposures because: (a) a very high percentage of heavy drinkers also smoke (Romberger and Grant, 2004); (b) the causes of alcoholic brain disease are complex and likely mediated in part by co-factors (de la Monte and Kril, 2014); (c) previous studies showed that sub-mutagenic exposures to other nitrosamines cause neurodegeneration (de la Monte and Tong, 2009; Tong et al., 2009b) and (d) we recently showed that alcohol and NDEA (nitrosamine present in processed foods) exposures differentially contribute to cerebellar abnormalities in experimental fetal alcohol spectrum disorder (Andreani et al., 2014). The NNK dose

![Fig. 4. Ethanol and NNK effects on temporal lobe neuronal and stress protein expression. DUPlex ELISAs were used to measure immunoreactivity to (A) Tau, (B) pTau, (C) ChAT, (D) acetylcholinesterase (ACHE), (E) AβPP, (F) amyloid-beta fragment of AβPP (Aβ-AβPP), (G) ubiquitin and (H) N-nitrosotyrosine (N-Tyr), with results was normalized to large acidic ribonuclear protein (RPLPO). Inter-group comparisons were made by two-way ANOVA (Supplementary Table S5) with the post-hoc Tukey multiple comparison test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; †P 0.05 < P < 0.10).](https://academic.oup.com/alcalc/article-abstract/50/6/670/193167)
ory. Since NNK exposures occur mainly via tobacco smoke, it was widely used to measure ethanol effects on spatial learning and memory in the presence or absence of ethanol co-exposures. Although the neuropsychological analyses revealed significant deficits in learning and memory, and that the sustained impairments in memory were similar to the first-time exposure to ethanol, our findings demonstrate for the present or absence of ethanol co-exposures. Although the neurobehavioral studies were limited to the MWM, this standard test is widely used to measure ethanol’s effects on spatial learning and memory. Since NNK exposures occur mainly via tobacco smoke, first-hand or second-hand smoke exposures in adolescents can have long-lasting adverse effects on cognitive function, and contribute to cognitive deficits that are generally regarded as consequences of alcohol abuse. These findings are consistent with human studies showing that the offspring of mothers who smoke during pregnancy have neurocognitive deficits and reduced cerebral volumes (Jacobsen et al., 2007; Rivkin et al., 2008; Eklad et al., 2015; El Marrou et al., 2014).

Insulin and IGF-1 signaling support metabolism, synaptic plasticity, myelin maintenance, neuronal survival and cellular homeostasis in the brain (de la Monte and Wands, 2005). Ethanol impairs insulin and IGF-1 signaling through survival, growth and metabolic pathways in the brain (Cohen et al., 2007; Ronis et al., 2007; de la Monte et al., 2008a, 2009b; de la Monte, 2013), as well as in other organs such as liver (Onishi et al., 2003; Ronis et al., 2007; Denucci et al., 2010; Setshedi et al., 2011; de la Monte, 2013; Longato et al., 2012; Ramirez et al., 2013). Ethanol-induced brain insulin/IGF resistance is associated with deficits in learning and memory (Duelli et al., 1994; Lester-Coll et al., 2006; de la Monte et al., 2006, 2009c, 2010; Ishart et al., 2009) because the temporal lobe and hippocampus are major targets in ARBD (Pierce and West, 1987; Zimatin et al., 1992; Silvers et al., 2006). This study generated new information about NNK as a mediator of cognitive impairment. Importantly, like ethanol, NNK’s neurotoxic effects target the temporal lobe and hippocampus. This suggests that NNK/tobacco smoke can serve as a co-factor in the pathogenesis of ARBD, and could also explain why ARBD severity is not strictly linked to alcohol dose. This concept has further relevance with respect to Alzheimer’s disease, which also is associated with impairments in brain insulin/IGF signaling (Hoyer, 1994; Rivera et al., 2005; Steen et al., 2005; Tong et al., 2009a), yet the risk of dementia is increased by smoking (Durazzo et al., 2014).

Multiplex ELISAs demonstrated multiple abnormalities in insulin/IGF-1 signaling through the Akt pathway, in which the vast majority of significant alterations in protein and phospho-protein expression were driven by ethanol. The main effects of ethanol were to increase insulin receptor and decrease IGF-1 receptor and Akt expression, decrease phosphorylation of the insulin receptor, Akt and GSK-3β and inhibit relative phosphorylation of the insulin receptor and GSK-3β. Therefore, ethanol impaired insulin/IGF-1 signaling through survival, plasticity and metabolic pathways at multiple levels in the cascade. Furthermore, since inhibition of Ser9-GSK-3β phosphorylation increases its kinase activity, ethanol likely increased oxidative stress via impairment of insulin/IGF-1 signaling. Similar effects of ethanol have been reported for other brain regions (de la Monte and Wands, 2002; Carter et al., 2008; de la Monte et al., 2009b). Although NNK’s and ethanol × NNK’s interactive effects were limited, a key point is that both ethanol and NNK inhibited insulin signaling at the most proximal point of the cascade, i.e. tyrosine phosphorylation of the insulin receptor. The adverse effects on downstream pathways were broad and likely extended beyond those investigated herein. Furthermore, the statistical trends (0.05 < P < 0.10) suggest that NNK and ethanol + NNK also negatively impacted signaling through Akt, IGF-1R and phosphorylated p70S6K.

The findings that ethanol and ethanol + NNK similarly inhibited tyrosine phosphorylation of the insulin receptor, S473-phosphorylation of Akt, S9 phosphorylation of GSK-3β and relative phosphorylation of Akt and GSK-3β indicate that ethanol rather than NNK was the major factor mediating those responses. On the other hand, the significant or trend effects of ethanol + NNK on IGF-1R, tyrosine phosphorylated IGF-1R, phosphorylated p70S6K, which did not occur with ethanol or NNK exposures alone, suggest additive or synergistic adverse effects of the dual exposures. Signaling through the IGF-1R mediates growth, plasticity and myelin maintenance (D’Ècole et al., 1996; Chesik et al., 2008; Freude et al., 2008), while p70S6K, which is downstream of Akt and connected through the mammalian target of rapamycin (mTOR) pathway, is stimulated by IGF-1 and promotes protein synthesis (Zhao et al., 2012). Consequently, ethanol + NNK-associated inhibition of IGF-1R phosphorylation correlates well with the inhibition of p70S6K activation. Since mTOR/p70S6K mediates brain-derived neurotrophic factor-induced protein synthesis and neuroplasticity (Mitew et al., 2014; Pilar-Cuellar et al., 2013), dual exposures to alcohol and tobacco would likely have profound inhibitory effects on neuronal plasticity required for learning and memory.

Previous studies showed that ethanol or nitrosamine mediated impairments in insulin signaling through Akt cause neurotoxic injury and cellular stress with mitochondrial dysfunction, cytoskeletal disruption and lipid and protein adduct accumulation (Lester-Coll et al., 2006; Tong et al., 2010). In this study, we examined ethanol and NNK effects on CK18, protein carbonyl, 8-iso-PGF-2α and 3-NTyr levels. Increased CK18 marks cellular injury (Savolainen et al., 1994) or death following toxin (Cave et al., 2011) or heavy alcohol (Gonzalez-Quintela et al., 2011; Lavallard et al., 2011; Pilar-Cuellar et al., 2013) exposures. Carboxylation is a marker of protein oxidation and stress (Videla et al., 2004). Isoprostane 8-iso-PGF-2α is formed by free radical-catalyzed peroxidation of arachidonic acid and is a marker of lipid peroxidation (Morrow et al., 1992, 1994; Soedergren et al., 2000, Yiu et al., 2007). Isoprostane mRNA levels are elevated in human livers with alcoholic or non-alcoholic steatohepatitis (Raszeja-Wysomirska et al., 2012). 3-Nitrotyrosine reflects protein nitrosylation (nitrotyrosine adducts), which can impair the function of alcohol metabolizing enzymes, including aldehyde dehydrogenase, and thereby exacerbate the toxic effects of ethanol due to acetaldehyde build-up (Moon et al., 2007).

Our studies showed that ethanol significantly altered protein carbonyl and 8-iso-PGF-2α levels, both ethanol and ethanol + NNK produced trend effects with respect to CK18, and that only dual ethanol + NNK exposures significantly altered N-Tyr levels. However, the post-hoc tests clearly demonstrated similar degrees of increased cytotoxic injury caused by ethanol, NNK or both exposures, selectively increased protein carbonyl caused by ethanol, with or without concomitant NNK exposures, increased protein nitrosylation mediated by ethanol or NNK, and additively increased lipid peroxidation following dual ethanol + NNK exposures. Therefore, although ethanol and/or NNK increased stress and adduct formation, their effects were not identical. Such differential responses could reflect how ethanol and
NNK negatively impact insulin/IGF-1 signaling through Akr and other pathways. Although increased stress can activate the ubiquitin-proteasome pathway/unfolded protein response, which is a feature of ARBD (French, 2000; Bardag-Gorce, 2010), we did not observe changes in ubiquitin immunoreactivity in relation to ethanol or NNK exposures.

Tau is a major neuronal cytoskeletal protein and p-Tau is normally present in axons. Acetylcholine is a major neurotransmitter for neuronal plasticity. ChAT regulates its biosynthesis, and AChE promotes its degradation. AβPP plays a role in neuronal plasticity, whereas the physiological functional role of AβPP-β peptide is still under investigation. Ethanol was the main factor causing significant or trend alterations in neuronal protein expression. NNK produced one trend effect with respect to AChE and ethanol + NNK, like ethanol, had a significant effect on AβPP-β peptide expression. However, post-tests demonstrated striking additive or synergistic inhibitory effects of ethanol + NNK on Tau, p-Tau and AChE, and significant reductions in AβPP-β peptide in all three experimental groups. Reduced temporal lobe levels of Tau and p-Tau could reflect degeneration of axons, collapse of growth cones and loss of synapses, which together would impair plasticity.

We also found that neither ethanol nor NNK altered ChAT, while ethanol and ethanol + NNK inhibited AChE. The finding that ethanol did not suppress ChAT contrasts with our previous studies of ethanol-exposed cerebella and frontal lobes (Soscia et al., 2006; de la Monte et al., 2008b; de la Monte and Wands, 2010), and seemingly contradicts evidence that impaired insulin/IGF signaling causes cholinergic deficits (Soscia et al., 2006). However, the observed responses could reflect regional differences in susceptibility to cholinergic pathology. For example, we showed that AChE is inhibited while ChAT is unaffected in alcoholic myopathy (Nguyen et al., 2012). Since AChE is depressed by oxidative stress (Ansari et al., 2009; dos Santos et al., 2011; Ehrlich et al., 2011; Kazi and Oommen, 2012), ethanol-associated inhibition of AChE could have been due to impairments in insulin/IGF signaling together with increased GSK-3β activation and lipid peroxidation (8-iso-PGF$_{2α}$). Since inhibition of AChE is sufficient to cause cytoskeletal collapse and degeneration (Lively et al., 1988), such adverse effects of ethanol could account for some of the impairments in learning and memory. The absence of inter-group differences with respect to AβPP expression, and reduced rather than elevated levels of AβPP-β suggest that aberrant AβPP expression and processing do not contribute to ARBD.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at Alcohol and Alcoholism online.

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**CONFLICT OF INTEREST STATEMENT**

None declared.

**REFERENCES**


