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***Title page**

Antagonising TLR4-TRIF signalling before or after a low-dose alcohol binge during adolescence prevents alcohol drinking but not seeking behaviour in adulthood

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***Abstract**

Adolescents frequently engage in risky behaviours such as binge drinking. Binge drinking, in turn, perturbs neurodevelopment reinforcing reward seeking behaviour in adulthood. Current animal models are limited in their portrayal of this behaviour and the assessment of neuroimmune involvement (specifically the role of Toll-like receptor 4 (TLR4)). Therefore, the aims of this project were to develop a more relevant animal model of adolescent alcohol exposure and to characterise its effects on TLR4 signalling and alcohol-related behaviours later life. Balb/c mice received a short (P22 – P25), low dose alcohol binge during in early adolescence, and underwent tests to investigate anxiety (elevated plus maze), alcohol seeking (conditioned place preference) and binge drinking behaviour (drinking in the dark) in adulthood. Four doses of alcohol during adolescence increased alcohol-induced conditioned place preference and alcohol intake in adulthood. However, this model did not affect basal elevated plus maze performance. Subsequent analysis of nucleus accumbal mRNA, revealed increased expression of TLR4-related mRNAs in mice who received alcohol during adolescence. To further elucidate the role of TLR4, (+)-Naltrexone, a biased TLR4 antagonist was administered 30 mins before or after the adolescent binge paradigm. When tested in adulthood, (+)-Naltrexone treated mice exhibited reduced alcohol intake however, alcohol seeking and anxiety behaviour was unaltered. This study highlights that even a small amount of alcohol, when given during a critical neurodevelopmental period, can potentiate alcohol-related behaviours and TLR4 activation later in life. Interestingly, attenuation of TLR4 before or after adolescent alcohol exposure reduced only binge alcohol intake in adulthood.

1. Introduction

Adolescence is a unique neurodevelopmental period characterized by an increased sensitivity towards rewarding stimuli and an attenuated sensitivity to aversive stimuli (Spear 2011). This phenotype causes adolescence to engage in risk-taking behaviors such as unprotected sex, reckless driving and binge drinking (Johnston et al. 2015; Hingson et al. 2009; Hingson et al. 2003). Binge drinking in turn profoundly perturbs neurodevelopment causing a retention of adolescent-like phenotypes such as reward-sensitivity, into adulthood (the "locked-in" hypothesis) (Crews et al. 2016; Doremus-Fitzwater & Spear 2016). Consequently, individuals that consume alcohol during adolescence are more likely to develop problems associated with alcohol use in adulthood (see Spear 2011 for review). This finding is reinforced by the link between age of first use and alcohol dependence later in life (DeWit et al. 2000). Crucially, these phenomenon are readily translatable to rodents (Spear 2011). Adolescent rodents exposed to alcohol exhibit potentiated alcohol-reward behaviors in adulthood as inferred by increased conditioned place preference, self-administration or two bottle choice drinking (Pandey et al. 2015; Alaux-Cantin et al. 2013; Maldonado et al. 2008; Rodd-Henricks et al. 2002). However, the magnitude of this potentiation is variable owing to differences in sex, genetic background, age and the model of adolescent alcohol exposure (Strong et al. 2010; Walker & Ehlers 2009; Blizard et al. 2004; Siciliano & Smith 2001). The model of alcohol exposure is a particularly important variable. To reach high blood ethanol concentrations (BECs) researchers often use methods that bypass the natural route of administration (for example (Gass et al. 2014; Gilpin et al. 2012). This in turn, influences the molecular and behavioral responses towards alcohol (Osterndorff-Kahanek et al. 2015; Osterndorff-Kahanek et al. 2013; Gilpin et al. 2012) and consequently, it is unclear how much these models reflect the human condition (Ward et al. 2014).

Despite different exposure methodologies rodent studies have identified multiple mechanisms underlying adolescent alcohol-induced reward sensitivities in adulthood with particular emphasis placed upon the molecular and cellular alterations within the nucleus accumbens and amygdala (Spear & Swartzwelder 2014). For example, adolescent alcohol exposure reduces the expression of plasticity-related genes (BDNF, ARC and CREB), negative regulators of dopaminergic function (dopamine D2 receptor and GABA receptors) and alters dopaminergic firing and tone in

adulthood (Sakharkar et al. 2016; Philpot et al. 2009; Pascual 2009a; Pietrzykowski et al. 2008). These alterations enhance an individuals sensitivity towards dopamine-inducing experiences such as alcohol use, and reduced the ability to alter learnt behavior (Vetreno 2015; Alaux-Cantin et al. 2013; Maldonado-Devincci et al. 2010).

Recent research has additionally highlighted the importance of the neuroimmune system in contributing to the adverse neurodevelopmental consequences of adolescent alcohol exposure (Crews et al. 2016; Montesinos et al. 2016). Particular emphasis has been placed on Toll-like receptor 4 (TLR4), a pattern recognition receptor broadly expressed throughout the central nervous system (Akira & Takeda 2004; Bsibsi et al. 2002). Following activation, TLR4 signals via the MyD88 or TRIF pathways culminating in the expression of classical pro-inflammatory cytokines and type 1 interferon's respectively (see Akira & Takeda 2004 for review). Alcohol indirectly activates TLR4 recruiting MyD88 and TRIF *in vitro* (Crews et al. 2013; Fernandez-Lizarbe et al. 2009). However, whether both pathways are activated *in vivo* remains to be determined. Alcohol-induced recruitment of these adapters causes a signaling cascade resulting in the translocation of immune-related transcription to the nucleus. This in turn increases the expression of inflammatory proteins from both microglia and astrocytes (Fernandez-Lizarbe et al. 2009; Blanco et al. 2005). Importantly, $TLR4^{-/-}$ mice display reduced levels of cytokines, chemokines and inflammatory transcription factors immediately following adolescent alcohol exposure and later in adulthood compared to wildtype mice (Montesinos et al. 2016; Pascual et al. 2016; Kane et al. 2013). This coincides with reduced synaptic and myelin derangements, long-term aberrant synaptic remodelling, decreased histone acetylation at BDNF and FosB (Montesinos et al. 2016). Behaviourally, $TLR4^{-/-}$ mice do not exhibit long-term cognitive impairments (Montesinos et al. 2015), display less anxiety-like and drug seeking behaviour in adulthood compared to wildtype following adolescent exposure (Montesinos et al. 2016). While the precise neuroanatomical area underlying the long-term actions of adolescent alcohol-induced TLR4 activation remains to be determined, studies using morphine (another TLR4 agonist) have identified the nucleus accumbens as a key substrate (Schwarz et al. 2013).

However, TLR4 is pivotal to normal neurodevelopmental processes (see Okun et al. 2011 for review), therefore, studies using TLR4$^{-/-}$ animals are inherently confounded. For example, TLR4$^{-/-}$ mice have higher levels of neurons and relatively fewer glia compared to wildtype mice (Rolls et al. 2007). Further, the use of TLR4$^{-/-}$ mice does not enable researchers to investigate the relative contribution of the MyD88 or TRIF pathways in the behavioral and molecular response to alcohol. Lastly, studies investigating the TLR4 often use excessive doses/treatments of alcohol exposure which may exaggerate endpoints. Therefore, the aims of this study were to determine whether a more relevant model of adolescent alcohol exposure alters reward-related behavior and mRNA and the TLR4 pathway later in life and secondly, to determine the whether pharmacologically attenuating TLR4 prevents any alcohol-induced reward alterations later in life. These alterations were assessed using conditioned place preference, drinking in the dark and the elevated plus maze with the transcription of a selection of gene targets relating to reward (dopaminergic, opioidergic, gabaergic and glutamaterigic processes) and alcohol-induced neuroadaptions (BDNF and CREB) within the nucleus accumbens additionally assessed.

2. Methods

2.1 Subjects

Pregnant female Balb/c mice (10 – 15 days into their gestation cycle) were obtained from the University of Adelaide Laboratory Animal Services, Adelaide, SA, Australia. Following their arrival to the animal facility, mice were housed in light/dark (12/12h, lights on/off at 7am/7pm respectively) and temperature (23 ± 3°C) controlled rooms. Food and water was available *ad libitum*.

After the dams had given birth, their offspring developed undisturbed until postnatal (P) day 22 at which point they began the adolescent alcohol exposure paradigm (figure 1a - b). <The young age selected for this study was designed to reflect the age at which individuals are particularly sensitive to the effects of alcohol (DeWit et al. 2000).> After the completion of the paradigm, mice were weaned and separated into single sex housing (P25) and were left undisturbed until P51. At beginning of adulthood (P56) mice began behavioural testing. Mice undergoing conditioned place

preference or elevated plus maze remained group housed. Mice undergoing drinking in the dark were separated into individual cages.

Adult mice were handled by the experimenter for five days prior to testing. Conditioned place preference and elevated plus maze occurred during the light phase of the mouse's light/dark cycle. Drinking in the dark (2 – 4 h access alcohol drinking) began three hours into the mouse's dark cycle. Both male and female mice were used for behavioural experiments. Statistical analysis determined sex was not a significant variable for behavioural experiments and consequently, both male and female animals were pooled together for data analysis. A comparison of sex differences can be found in supplementary materials.

All animal care and experiments complied with the principles of the Australian Code of Practice for the care and use of animals for scientific purposes and was approved by the University's Animal Ethics Committee.

2.2 Drugs

Ethanol (99.5%) (herein referred to as alcohol) was purchased from Chemsupply (Gliman, SA, Australia). Alcohol was administered as an oral gavage (10 – 30 per cent v/v). The dose of alcohol ranged from 0.5g/kg to 3.5g/kg for adolescent alcohol exposure paradigm and 1.5g/kg for conditioned place preference. Saline oral gavages or intraperitoneal injections were volume-matched.

(+)-Naltrexone, a pharmacological TLR4 antagonist was synthesised and supplied by Dr Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute of Alcohol Abuse and Alcoholism, Bethesda, MD, USA). (+)-Naltrexone was administered via intraperitoneal injections at a dose of 60mg/kg (dose volume 10 ml/kg).

2.3 Adolescent alcohol exposure

2.3.1 Rational

Consuming alcohol during adolescence can impair neurodevelopment, reinforcing an underdeveloped, immature brain. In adulthood, these individuals are at risk of developing anxiety and alcohol-drinking disorders indicating alcohol specifically

alters the development of brain regions governing hedonia, reward, motivation and emotion (Doremus-Fitzwater & Spear 2016). This phenomenon is translatable to animal models with adolescent mice and rats exposed to alcohol exhibiting potentiated alcohol preference and anxiety later in life (for example, Sakharkar et al. 2016). However, generalising the magnitude of effects is difficult owing to differences in experimental design. For example, current rodent models by-pass the natural oral route of administration (Gass et al. 2014; Gilpin et al. 2012) to produce greater blood ethanol concentrations and are prolonged/chronic in nature (Vetreno 2015). Consequently, the behavioural and molecular responses attributable to alcohol are either exaggerated, minimised or clouded (Ward et al. 2014). To circumvent these confounding variables, a shorter model was utilised.

2.3.2 Alcohol exposure model

Mice received an oral gavage of alcohol (0.5, 1.5, 2.5, 3.5g/kg) or saline (volume matched) for four consecutive days (P22 – 25). An hour after the last oral gavage, tail blood was collected and blood ethanol concentration (BEC) was quantified. Mice were then weaned (P25), separated into single sex cages and allowed to mature undisturbed until P51(Figure 1a).

For studies assessing the role of TLR4 on the neurodevelopmental outcomes following adolescent alcohol exposure, a similar protocol was used. However, thirty minutes pre- or post adolescent alcohol exposure, mice received an intraperitoneal injection of (+)-Naltrexone or saline (Figure 1b). The objective of using both a pre- and post-treatment paradigm was to ascertain the mechanism by which (+)-Naltrexone works (pre-treatment) and to ascertain its efficacy once the pathology has commenced (post-treatment). Mice in this experiment received 2.2 g/kg of alcohol rather than a range of doses to minimise the number of rodents used in this study. The dose of alcohol was calculated by determining the effective dose 50 (ED_{50}) from conditioned place preference later in life (figure 3a). An hour after the last gavage of alcohol, blood was harvested from the tail to quantify BEC.

2.4 Adult behavioural tests

At the beginning of adulthood (P56) mice underwent elevated plus maze, conditioned place preference or drinking in the dark (P63).

2.4.1 Conditioned place preference

Conditioned place preference was used to infer alcohol-reward behaviour and the ability to form an alcohol-associated memory (Bardo & Bevins 2000).

2.4.1.1 Apparatus

The conditioning apparatus consisted of two conditioning chambers (10.9 (length) x 9.3 (width) x 35 (height) cm) separated by a neutral chamber (16.6 x 4.8 x 35 cm). The neutral chamber contained black walls with grey flooring. The conditioning chambers differed in tactile and visual cues. The flooring of the conditioning chambers were either black plexiglass perforated holes (5mm apart) or black plexiglass grids (5mm apart). The walls of each chamber were white or black. The combination of floor texture and wall colour were altered for each cohort to prevent any inherent bias the rodents have for a specific texture/colour combination.

During conditioning, a sliding partition restricted access to only one chamber. Movement and time spent in each chamber was recorded using Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA).

2.4.1.2 Procedure

Pre-test (day 1): Mice were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

Conditioning (day 2 – 9): Mice received an oral gavage of alcohol (1.5 g/kg) and placed within their conditioning chamber for 30 min on days 1, 3, 5, 7. On days 2, 4, 6 and 8, mice received an oral gavage of saline and placed within the unconditioned chamber for 30 min. Mice received a total of four conditioning sessions with each drug (alcohol or saline).

Test (Day 10): Mice received an oral gavage of saline and were placed into the neutral chamber and allowed to explore all three chambers for 30 min.

To infer whether the conditioning was successful, the time spent in the conditioned chamber during the post-test was subtracted from the time spent in the conditioned chamber during the pre-test.

2.4.2 Drinking in the dark

Binge-like consumption of alcohol was assessed using the drinking in the dark procedure (Thiele & Navarro 2014). At P56 mice were individually housed and acclimatised to their new environment for one week prior to experimentation. Three hours into the mouse's dark cycle, the bottle of water was removed and replaced with a bottle of 20 per cent (v/v) alcohol for two hours (P63 – 65). After two hours, the alcohol bottle was removed, weighed and replaced with a bottle of water. On the fourth and final day of testing (P66), mice received alcohol for four hours.

2.4.3 Elevated plus maze

To infer basal anxiety-like behaviour mice underwent the elevated plus maze (Carola et al. 2002). The elevated plus maze consisted of two areas characterised by high walls and a relatively dark environment and an open area.

2.4.3.1 Apparatus

The maze is made of black PVC and consists of four arms: two open and two closed. All arms were 30 cm long and 5 cm wide. The two enclosed arms had walls 25 cm high. The maze was elevated 1.2 m off the ground.

2.4.3.2 Procedure

Mice were moved into the behavioural testing room 30 minutes prior to testing to acclimatise them to a new environment. Mice were subsequently placed into the centre of the elevated plus maze with their head facing towards the open arm and allowed to explore the apparatus for five minutes. The time spent, number of exits, distance travelled and the number of immobile episodes was recorded using a Logitech Quickcam Pro 5000s and AnyMaze (Stoelting co., Wooddale, IL, USA).

2.5 RNA isolation, reverse transcription and quantitative PCR (qPCR)

The nucleus accumbens was examined owing to its pivotal importance in reward behaviour, and previous studies highlighting the adverse effects of adolescent

morphine exposure on TLR4 expression and reward behaviour later in life (Schwarz et al. 2013). The nucleus accumbens region was isolated using micropunches (Kai Medical, Seki City, Japan) from whole brains and submerged in RNA*later*® ICE (ThermoFisher Scientific, Waltham, MA, USA) prior to performing RNA isolation. RNA was isolated using Maxwell® 16 LEC simply RNA Tissue Kit (Promega, Madison, WI, USA) as per manufacturer instructions. RNA was quantified using spectrophotometric analysis, with the quality of RNA verified by the OD260/280 ratio. Isolated RNA (900ng) was reversed transcribed into cDNA using iScript™ cDNA reverse transcription kit (BioRad, Hercules, CA, USA) as per manufacturer instructions.

Gene expression was assessed using iTaq™ Universal SYBR® Green Supermix as per manufacturer instructions. Real time PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Mouse *BDNF*, *CCL2*, *CD14*, *CREB1*, *DRD1*, *DRD2*, *GABRA1*, *GABRA2*, *GAPDH*, *GRIA1*, *GRIN1*, HMGB1, *IFNβ*, *IL-1β*, *IL-10*, *MD2*, *MyD88*, *NTRK2*, *OMPR1*, *TH*, *TLR4* and *TRIF* forward and reverse primers were synthesised by Integrated DNA Technologies Pty. Ltd. (Baulkham Hills, NSW, Australia). For primer sequences refer to supplementary materials. The genes assessed were based upon previous studies demonstrating differences in dopaminergic, opioidergic, gabaergic and glutamaterigic processes following adolescent alcohol exposure (Alaux-Cantin et al. 2013; Pascual et al. 2009).

The relative difference in expression level of each of the genes of interest were normalised to the C_T of GAPDH for both the test and control sample. The ΔC_T of the test sample was normalised to the ΔC_T of a control sample (a equal amount of cDNA from all the different groups), and then expressed as a ratio ($2^{-\Delta\Delta CT}$).

2.6 Statistical Analysis

Experiment 1: Conditioned place preference (chamber x dose), elevated plus maze (arm x dose) and drinking in the dark (day x dose) were analysed using a two-way ANOVA with repeated measures and Tukey post hoc.

Experiment 2: qPCR was analysed using a one-way ANOVA with multiple comparisons and Bonferonni post hoc.
Experiment 3: qPCR analysed using a two-way ANOVA with Bonferonni post hoc (intervention x gavage).
Experiment 4: Conditioned place preference (chamber x intervention x gavage x order), elevated plus maze (arm x intervention x gavage x order) and drinking in the dark (day x intervention x gavage x order) was assessed using a four way ANOVA with Tukey post hoc. Results were considered significant if $p < 0.05$.
All data is presented as mean±SEM.

3. Results

3.1 Experiment 1: Can a short alcohol exposure during adolescence potentiate anxiety and alcohol-seeking behaviour in adulthood?

An important consideration when examining the effects of adolescent alcohol exposure on later life behaviour is the relative rise in blood alcohol following the initial alcohol experience. One hour after the last gavage tail blood was isolated and BEC was quantified. The gavage model produced a dose dependent increase in blood ethanol ranging from 57 to 431mg/100mL at the lowest (0.5 g/kg) and highest (3.5 g/kg) doses respectively (effect of dose, $F_{(3.\ 32)} = 319.8$, $p < 0.0001$). The precise statistical information and figures can be viewed in the supplementary material (figure s1).

Basal anxiety-behaviour in adulthood (P56) was assessed using the elevated plus maze. A two-way ANOVA determined alcohol exposure during adolescence did not influence the time spent, number of exits, distance travelled or immobile episodes in the elevated plus maze in adulthood (effect of dose; time, $F_{(4,\ 36)} = 1.1$, $p = 0.37$; exits, $F_{(4,\ 36)} = 1.0$, $p = 0.42$; distance, $F_{(4,\ 36)} = 1.18$, $p = 0.34$; and immobile episodes, $F_{(4,\ 36)} = 0.57$, $p = 0.68$, respectively) (figure 2a – d). Post-hoc analysis did not reveal any significant differences between the treatment groups with respect to the dose of alcohol. However, there was a significant effect of maze arm (open or closed) with respect to time, number of exits, distance travelled and time immobile (effect of maze arm; time, $F_{(1,\ 9)} = 126.1$, $p < 0.0001$; exits, $F_{(1,\ 9)} = 403.8$, $p < 0.0001$; distance, $F_{(1,\ 9)} = 4.952$, $p = 0.05$; and immobile episodes, $F_{(1,\ 9)} = 135.7$, $p < 0.0001$, respectively). No interactive effects (effect of dose x maze arm) or post-hoc

differences were present for any of the variables (p > 0.05, see supplementary material for full statistical description). These findings suggest that four consecutive doses of alcohol during adolescence are insufficient to alter baseline anxiety-like behaviour in adulthood using this model in Balb/c mice.

To determine whether adolescent alcohol exposure modifies alcohol-reward behaviour in adulthood, adult mice underwent conditioned place preference (figure 3a). Irrespective of the adolescent treatment, all mice exhibited conditioned place preference towards alcohol (effect of conditioning chamber, $F_{(1,\ 11)}$ = 47.12, p < 0.001) (figure 3a). Further, there was an effect of the adolescent alcohol dose on the change in time alcohol-conditioned chamber time in adulthood (effect of dose, $F_{(4,\ 44)}$ = 4.36, p = 0.0047). The Tukey post-hoc calculated significant differences between vehicle and 0.5g/kg, 1.5g/kg, 2.5g/kg and 3.5g/kg of alcohol, with the greatest difference observed at 2.5g/kg. Interestingly, however was no interactive effect between the dose of alcohol and the conditioning chamber suggesting the differences between doses is small (interaction, $F_{(4,\ 44)}$ = 1.69, p = 0.17). These results highlight that a comparatively minor exposure to alcohol during adolescence is sufficient to potentiate alcohol-seeking behaviour in adulthood.

To verify that the adolescent alcohol model potentiates alcohol reward-behaviour in adulthood, mice underwent drinking in the dark, a limited access-drinking paradigm (figure 3b). One concentration of alcohol (2.2 g/kg) was selected for this experiment based from the ED_{50} of the conditioned place preference results in figure 3a. A two-way ANOVA determined adolescent alcohol exposure significantly influenced alcohol intake in adulthood (effect of adolescent drug, $F_{(1,\ 9)}$ = 8.18, p = 0.019) (figure 3b). There was an additional effect of testing day (effect of day, $F_{(3,\ 27)}$ = 109.9, p < 0.001 respectively) with post-hoc analysis demonstrating significant differences between saline and alcohol groups on day 2 and 4. Collectively, the results indicate that four consecutive doses of alcohol during adolescence does not influence baseline anxiety-like behaviour but increases the alcohol-seeking behaviour and intake in adulthood. Importantly, a dose-dependent effect on alcohol seeking was shown.

3.2 Experiment 2: Does adolescent alcohol exposure "prime" molecular mediators of reward and the TLR4-signalling pathway in adulthood?

The increased alcohol seeking behaviour is potentially explained by alterations in reward-related genes in adulthood caused by adolescent alcohol exposure. Thus, the expression of genes relating to alcohol reward, seeking and synaptic plasticity in the nucleus accumbens were examined in adulthood prior to behavioural testing (P56) (figure 4). The genes assessed were based upon previous studies demonstrating differences in dopaminergic, opioidergic, gabaergic and glutamaterigic processes following adolescent alcohol exposure (Alaux-Cantin et al. 2013; Pascual et al. 2009). A one-way ANOVA determined a significant effect of alcohol dose on the expression of *DRD1*, *TH*, *OMPR1*, *GABRA1*, *GABRA2* and *CREB1* mRNA in adulthood (effect of dose; *DRD1,* $F_{(4, 10)} = 3.74$, $p = 0.016$; *TH,* $F_{(4, 10)} = 3.4$, $p = 0.041$; *OMPR1*, $F_{(4, 10)} = 4.46$, $p = 0.0073$; *GABRA1*, $F_{(4, 10)} = 4.09$, $p = 0.011$; *GABRA2*, $F_{(4, 10)} = 2.89$, $p = 0.035$; and *CREB1*, $F_{(4, 10)} = 3.60$, $p = 0.014$). This effect was not consistent however, as no alcohol-dose effect was observed for *DRD2*, *GRIA1*, *GRIN1*, *BDNF* or *NTRK2* mRNA levels (effect of dose; *DRD2,* $F_{(4, 10)} = 2.04$, $p = 0.12$; *GRIA1*, $F_{(4, 10)} = 2.11$, $p = 0.10$; *GRIN1*, $F_{(4, 10)} = 0.52$, $p = 0.71$; *BDNF*, $F_{(4, 10)} = 2.34$, $p = 0.080$; and *NTRK2*, $F_{(4, 10)} = 1.01$, $p = 0.41$). Collectively, these data indicate that adolescent alcohol exposure significantly increased the expression of receptors previously associated with alcohol seeking behaviour and intake (*DRD1*, *TH*, *OPRM1*, *GABRA2* and *CREB1*), while having no effect on genes related to glutamate (*GRIA1* and *GRIN1*) or neurotrophic support (*BDNF* and *NTRK2*).

The role of the neuroimmune system in mediating the long-term consequences of adolescent alcohol exposure are of increasing interest (Montesinos et al. 2016). Therefore, the expression of the genes important in the TLR4 pathway was assessed (figure 5). A one-way ANOVA determined a significant effect of alcohol dose on the expression of *TLR4*, *MD2*, *TRIF*, *CCL2*, *IFNβ* and *HMGB1* mRNA (effect of dose; *TLR4*, $F_{(4, 10)} = 3.42$, $p = 0.016$; *MD2*, $F_{(4, 10)} = 3.25$, $p = 0.023$; *TRIF*, $F_{(4, 10)} = 3.90$, $p = 0.0090$; *CCL2*, $F_{(4, 10)} = 3.70$, $p = 0.012$; *IFNβ* $F_{(4, 10)} = 2.68$, $p = 0.044$; and *HMGB1* $F_{(4, 10)} = 3.63$, $p = 0.014$). There was no effect of alcohol dose on the expression of *CD14, MyD88*, *IL-1β* or *IL-10* mRNA (effect of dose; *CD14,* $F_{(4, 10)} = 1.72$, $p = 0.16$; *MyD88*, $F_{(4, 10)} = 1.026$, $p = 0.40$; *IL-1β* $F_{(4, 10)} = 1.50$, $p = 0.22$; and

IL-10 $F_{(4, 10)} = 2.53$, $p = 0.056$). Interestingly, adolescent alcohol exposure increased the expression of genes associated with the TRIF and not the MyD88 pathway in the nucleus accumbens of adult mice. This suggests an inherent biased of the immune system in the brains of these animals induced by adolescent alcohol exposure.

3.3 Experiment 3: Does (+)-Naltrexone attenuate the long-term increases of the TLR4 pathway induced by adolescent-alcohol?

Given that adolescent alcohol exposure potentiated the expression of *TRIF* and *IFNβ* mRNA within the nucleus accumbens, the question arose as to whether the TLR4-TRIF pathway was associative or causative in mediating alcohol seeking and intake behaviours observed later in life. Therefore, (+)-Naltrexone, a pharmacological biased antagonist of the TLR4-TRIF pathway (Wang et al. 2016) was administered either before or after exposure to adolescent alcohol exposure and later life behaviour and mRNA expression was assessed. The decision to include both pre- and post-treatment was to ascertain whether TLR4-TRIF pathways were involved in these behaviours and whether the isomer is of any benefit once the pathology has commenced. Importantly, (+)-Naltrexone did not influence BEC following adolescent alcohol exposure suggesting any alteration in behaviour was unlikely to be attributable to alterations in metabolism (figure s2).

The ability of (+)-Naltrexone to selectively attenuate adolescent alcohol induced TLR4 gene expression was investigated using qPCR. A two-way ANOVA determined a significant effect of gavage (alcohol or saline) *TLR4, IFNβ* and *HMGB1* mRNA in the nucleus accumbens of mice in the pre-treatment paradigm (figure 6a) (effect of gavage; *TLR4*, $F_{(1, 4)} = 40.51$, $p = 0.0007$; *IFNβ*, gavage $F_{(1, 4)} = 2.59$, $p = 0.015$; and *HMGB1*, gavage $F_{(1, 4)} = 8.71$, $p = 0.025$,). There was an additional effect of intervention ((+)-Naltrexone or saline) for these genes (effect of intervention; *TLR4*, $F_{(1, 4)} = 10.09$, $p = 0.019$; *IFNβ*, intervention $F_{(1, 4)} = 44.68$, $p = 0.022$; and *HMGB1*, intervention $F_{(1, 4)} = 0.035$, $p = 0.85$). There were interactive effects for *IFNβ* and *HMGB1* but not *TLR4* mRNA (interaction; *TLR4*, $F_{(1, 4)} = 0.17$, $p = 0.68$; *IFNβ*, $F_{(1, 4)} = 9.28$, $p = 0.02$; and *HMGB1*, $F_{(1, 4)} = 0.073$, $p = 0.79$).

The expression of *TRIF* was unaffected by intervention ($F_{(1, 4)} = 0.83$, $p = 0.39$) or gavage ($F_{(1, 4)} = 2.25$, $p = 0.18$). However, an interactive effect was observed ($F_{(1, 4)} = 19.57$, $p = 0.0045$).

A two-way ANOVA determined the expression of *TRIF* and *IFNβ* was influenced by gavage (effect of gavage; *TRIF*, $F_{(1, 3)} = 0.45$, p = 0.52; and *IFNβ*, $F_{(1, 3)} = 3.04$, p = 0.013) and intervention (effect of intervention; *TRIF*, $F_{(1, 3)} = 17.76$, p = 0.0056; and *IFNβ*, $F_{(1, 3)} = 12.90$, p = 0.011) in the post-treatment paradigm. There was no significant interactions between gavage and intervention for these two genes (interaction; *TRIF*, $F_{(1, 3)} = 4.87$, p = 0.069; and *IFNβ*, $F_{(1, 3)} = 0.26$, p = 0.62). In contrast, to the pre-treatment paradigm however, *TLR4* mRNA was only significantly modified by intervention ($F_{(1, 3)} = 5.13$, p = 0.040) but not gavage ($F_{(1, 3)} = 2.4$, p = 0.17). There was no interaction between the two variables ($F_{(1, 3)} = 4.14$, p = 0.08). There was no effect of intervention ($F_{(1, 3)} = 1.17$, p = 0.31), gavage ($F_{(1, 4)} = 5.76$, p = 0.050) or an interactive effect ($F_{(1, 3)} = 2.35$, p = 0.16) on *HMGB1* expression. All remaining genes did not exhibit a significant effect of intervention or gavage with statistical information available in the supplementary material (figure s3 – 4).

Interestingly, both pre- and post-treatment paradigms had a signficant effect of the intervention (saline vs (+)-Naltrexone) on the expression of *GABRA2* mRNA (effect of intervention; pre-treatment, $F_{(1, 3)} = 17.84$, $p = 0.051$; and post-treatment, $F_{(1, 3)} = 15.79$, $p = 0.048$) (figure 7a and b). There was no effect of gavage on the expression of *GABRA2* mRNA in either paradigms (effect of gavage; pre-treatment, $F_{(1, 3)} = 1.63$, $p = 0.33$; and post-treatment, $F_{(1, 3)} = 1.96$, $p = 0.30$). However, a significant interactive effect between gavage and intervention was observed for both cohorts (interaction; pre-treatment, $F_{(1, 3)} = 349.1$, $p = 0.0029$; and post-treatment, $F_{(1, 3)} = 24.61$, $p = 0.038$). Tukey post-hoc determined (+)-Naltrexone significantly reduced the expression of *GABRA2* mRNA compared to saline. The expression of *TH* was significantly influenced by the intervention in the pre- but not post-treatment paradigm (effect of intervention; pre-treatment, $F_{(1, 3)} = 117.1$, $p = 0.008$; and post-treatment, $F_{(1, 3)} = 5.01$, $p = 0.15$). The expression of *TH* was not influenced by gavage (effect of gavage; pre-treatment, $F_{(1, 3)} = 6.56$, $p = 0.12$; post-treatment, $F_{(1, 3)} = 3.78$, $p = 0.19$) nor was there an interactive effect for the pre- and post-treatment paradigms (interaction; pre-treatment, $F_{(1, 3)} = 4.97$, p = 0.15; post-treatment, $F_{(1, 3)} = 3.54$, $p = 0.20$, respectively). There was no effect of intervention for any other reward pathway-related mRNA (Figure s2, see supplementary material for full list of statistical analysis).

3.4 Experiment 4: Does (+)-Naltrexone attenuate behavioural alterations in adulthood induced by adolescent alcohol exposure?

To verify that (+)-Naltrexone selectively attenuated the enhanced rewarding properties of alcohol and did not modify basal behaviour adult mice underwent the elevated plus maze (figure 8a and b). There was a significant effect of arm on performance in the elevated plus maze (effect of arm, $F_{(1, 144)} = 39.71$, $p < 0.0001$), with post-hoc analysis determining all cohorts of mice spent significantly longer in the closed arm relative to the open arm.

A 4-way ANOVA determined percent of time spent in each of the arms was not influenced by the gavage, intervention or the order in which that intervention was received (pre- or post-treatment) (effect of gavage, $F_{(1, 144)} = 0.12$, $p = 0.73$; effect of intervention, $F_{(1, 144)} = 0.12$, $p = 0.73$; and effect of order $F_{(1, 144)} = 0.80$, $p = 0.37$, respectively) – confirming the previous findings that this model exclusively augments reward/reinforcement behaviour. However, the multiple comparisons test determined that mice receiving an IP injection of saline followed by a gavage of saline (pre-treatment paradigm) exhibited an increase in open arm time compared to all other cohorts (figure 8a). This effect was not observed in the post-treatment paradigm. This finding is furthered as an interactive effect between arm and order was found ($F_{(1, 144)} = 39.87$, $p < 0.0001$). Collectively, this suggests that under specific circumstances, alcohol and (+)-Naltrexone may modify performance in the elevated plus maze. A list of all interactive effects can be found in the supplementary material.

The remaining markers of elevated plus maze performance (distance travelled, number of exits and immobile episodes) all exhibited a similar trend in their main effects. There was a significant effect of arm ($p < 0.001$) but not gavage, intervention or the order of the intervention (effect of gavage, intervention and order $p > 0.05$). Significant interactions were observed for arm x adolescent exposure x order and arm x adolescent exposure x order x intervention ($p < 0.05$) (a complete list of statistical analyses can be found in the supplementary materials).

(+)-Naltrexone's ability to attenuate the rise in alcohol-reward behaviour in adulthood was assessed using conditioned place preference (figure 9a and b). The change in

conditioning time was significantly modified by conditioning chamber but not gavage, intervention or order (effect of conditioning chamber, $F_{(1, 144)} = 56.09$, $p < 0.0001$; effect of gavage, $F_{(1, 144)} = 0.16$, $p = 0.69$; effect of intervention, $F_{(1, 144)} = 0.051$, $p = 0.82$; and effect of order $F_{(1, 144)} = 0.018$, $p = 0.89$). Thus, while mice overall preferred the alcohol-conditioned chamber compared to the unconditioned chamber, there was no overall effect of alcohol or (+)-Naltrexone on modifying alcohol-induced conditioned place preference. Post-hoc analysis demonstrated that both control cohorts (Saline IP -> Saline IG and (+)-Naltrexone IP -> Saline IG) exhibited a reduced change in alcohol-conditioned chamber time compared to Saline IP -> Alcohol IG group, supporting earlier findings that adolescent alcohol potentiates time spent in the alcohol-conditioned chamber in adulthood (figure 8a). Similarly, in the post-treatment the Saline IG -> Saline IP cohort exhibited a reduced change in chamber time compared to alcohol IG -> Saline IP. This suggests despite no main effect of gavage, there was still an effect of adolescent alcohol exposure on later life behaviour. This is further supported by the significant interactive conditioning chamber x gavage ($F_{(1, 144)} = 4.88$, $p = 0.037$). For the remaining interactive effects refer to supplementary material.

In contrast to conditioned place preference, drinking in the dark was significantly affected by gavage, intervention and testing day but not the order, (effect of gavage, $F_{(1, 256)} = 4.64$, $p = 0.032$; effect of intervention, $F_{(1, 256)} = 82.58$, $p < 0.0001$; effect of testing day, $F_{(3, 256)} = 8.81$, $p < 0.0001$; and effect of order $F_{(1, 256)} = 0.004$, $p = 0.95$) (figure 10a and b). Post-hoc analysis determined: mice that received alcohol during adolescence exhibited potentiated alcohol intake in adulthood compared to mice that received saline; mice that received alcohol and (+)-Naltrexone in the pre- or post-treatment paradigms exhibited reduced intake compared to mice that received alcohol and saline. Interactions of intervention x gavage ($F_{(1, 256)} = 38.40$, $p < 0.0001$), testing day x intervention x adolescent gavage ($F_{(3, 256)} = 2.57$, $p = 0.054$) and order x intervention x gavage ($F_{(1, 256)} = 42.14$, $p < 0.0001$) were additionally observed.

4. Discussion

Adolescence is a vulnerable stage of neurodevelopment, throughout which the brain undergoes substantial reorganisation and maturation. Exposure to drugs of abuse, in

particular alcohol, can perturb normal brain development, reinforcing an immature brain state in both rodents and humans (Spear & Swartzwelder 2014). As adults, these individuals are at risk of developing psychiatric disorders such as addiction and anxiety disorders (Spear & Swartzwelder 2014). Results from our study demonstrated four oral gavages during early adolescence potentiated alcohol-induced conditioned place preference and alcohol drinking when tested in adulthood. However, performance in the elevated plus maze was not altered. These behavioural alterations coincided with elevations in the expression of genes relating to dopamine, opioid and GABA receptors but not other neurotransmitter or neurotropic systems in the nucleus accumbens of adult mice. Furthermore, the expression of genes relating to the TLR4 pathway (*TLR4*, *MD2*, *TRIF*, *CCL2*, *IFNβ* and *HMGB1*) were also increased. Administration of (+)-Naltrexone either before or after adolescent alcohol exposure, prevented the increase in *TLR4*, *IFNB* and *GABRA2* mRNA and decreased alcohol intake later in life. However, (+)-Naltrexone failed to modify adolescent alcohol potentiated conditioned place preference, elevated plus maze performance or the increased expression of other neurotransmitter/neurotrophic receptors mRNA. Collectively, the results highlight the potential importance of the alcohol-TLR4-IFNβ axis in mediating adolescent-induced potentiation of later life drinking behaviour but not alcohol-seeking or anxiety behaviour.

Current models examining the effects of adolescent alcohol exposure are often limited in the generalisability of their effects these models use clinically irrelevant routes of administration (i.e. intraperitoneal Gilpin et al. 2012); are prolonged in nature (Vetreno 2015; Pascual, et al. 2009a); or use very high doses of alcohol (Vetreno & Crews 2012). These limitations are particularly important, as the dose of alcohol and route of administration influences the response to alcohol (for example Ward et al. 2014; Osterndorff-Kahanek et al. 2013). Consequently, an aim of this study was to evaluate the effects of a shorter model of adolescent alcohol exposure and characterise its behavioural and molecular outcomes. Similar to study's using more chronic models (Montesinos et al. 2016; Alaux-Cantin et al. 2013; Maldonado-Devincci et al. 2010), our adolescent alcohol exposure model potentiated conditioned place preference and alcohol drinking behaviour later in life. Interestingly, peak conditioned place preference was not observed at the highest dose of alcohol - an effect potentially attributable to alcohol's memory impairing

effects at higher doses (Land 2004). However, unlike chronic studies (Montesinos, Pascual, et al. 2016) this shorter model did not alter anxiety-like behaviour suggesting higher or more chronic doses of alcohol are required to engage brain regions governing anxiety (He & Crews 2008). Alternatively, the lack of difference in anxiety behaviour may be related to the mouse strain used in the study. Balb/c are an anxiety-sensitive strain of mice (Carola et al. 2002; Griebel et al. 2000; Makino et al. 1991) masking an alcohol response.

To ascertain why these rodents exhibited potentiated reward-like behaviour, the nucleus accumbens of adolescent alcohol exposed mice was collected in adulthood and genes pertaining to reward were assessed. Similar to (Alaux-Cantin et al. 2013), our study demonstrated genes pertaining to GABA and the endogenous opioid system were elevated in adulthood following adolescent alcohol exposure. The current study additionally demonstrated increases in genes relating to dopamine synthesis (*TH*) and receptors (D1). Tyrosine hydroxylase, dopamine and opioid receptors are associated with the hedonic and salient motivational properties of alcohol (Berridge & Robinson 2016). Therefore, persistent elevation in these genes is likely to increase the sensitivity of these individuals to hedonic and motivational properties of alcohol in adulthood. In contrast to (Alaux-Cantin et al. 2013), mRNA from other neurotransmitter systems such as glutamate, were not significantly altered by adolescent alcohol exposure. Closer analysis demonstrates a unique expression pattern, which would not prove statistically significant using conventional data analysis that relies on a linear change (eg ANOVA). For example, the alcohol dose response effect on GRIN1 expression is bell-shaped, highlighting the importance of examining a broad range of doses when examining adolescent alcohol exposure. Lastly, despite this shorter exposure model demonstrating increased alcohol seeking and drinking later in life, it remains to be determined whether this result is ontologically specific or can occur in irrespective of developmental stage.

This study's primary focus was to investigate the effects of adolescent alcohol exposure on the neuroimmune system. Specifically, the role of the TLR4 pathway was examined based on the recent studies implicating this receptor and its signalling pathway in alcohol-related behaviours (for example, (Blednov et al. 2017; Harris et al. 2017; Aurelian et al. 2016; Montesinos et al. 2016; Liu et al. 2011; Pascual et al.

2011). Despite its purported importance in mediating these behaviours, no study has examined how alcohol modifies the gene expression of TLR4's signalling pathways during a crucial neurodevelopment periods such as adolescence. TLR4 has two main signalling pathways (the MyD88 and TRIF pathway) with their activation leading to increased production of classical proinflammatory cytokines (IL-1β) and type 1 interferons, respectively (Akira & Takeda 2004). Results from our study demonstrate adolescent alcohol exposure resulted in the persistent elevation of *TLR4*, *MD2*, *TRIF*, *CCL2*, *IFNβ* and *HMGB1* mRNA in adulthood within the nucleus accumbens. Interestingly, alcohol exposure did not alter the expression of genes classically associated with the MyD88 pathway, suggesting that the long-term neuroimmune effects of alcohol may have a more pronounced effect on the TRIF pathway. However, studies determining whether the mRNA increases translate to protein-level differences are required to verify these conclusions.

While this study did not address the immediate effects of alcohol exposure during adolescence, published literature from *in vitro* experiments suggests acute alcohol activates both the MyD88 and TRIF pathways (Fernandez-Lizarbe et al. 2009). However, the degree of immune activation appears to be dampened compared to adults (Doremus-Fitzwater et al. 2015; Kane et al. 2013). While the mechanism underlying the limited neurokine response is unknown, it is hypothesised that this phenomenon is designed to limit neuroinflammatory responses which perturb neurodevelopment (Ismail & Blaustein 2013; Ismail et al. 2013).The rise in immune mediators has both short and long-term consequences. In the acute setting, the immune mediators act upon neighbouring neurons altering their function and behaviour (Marshall et al. 2016). This in turn is hypothesised to potentiate hedonic and anhedonic aspects of drugs of abuse (see Lacagnina et al. 2016 for review). For example, both TLR4 and CCL2 modify dopaminergic neurotransmission in the striatum (Northcutt et al. 2015; Hutchinson et al. 2012; Guyon et al. 2009). In addition, activation of TLR4 during adolescence has long-term effects on neurodevelopment (see Bilbo & Schwarz 2012 for review), causing reduced myelination, synaptic pruning, increased neuronal and astrocyte cell death and alters epigenetic processes which reinforce an adolescent brain (Montesinos et al. 2016; Montesinos et al. 2015; Pascual et al. 2014; Pascual et al. 2009b). These events may assist producing an underdeveloped, immature brain that is uniquely sensitive

to the hedonic aspects of acute alcohol exposure and may be more susceptible to develop addiction with chronic use.

In addition to the immediate and neurodevelopmental effects, this study highlighted that adolescent alcohol exposure can lead to persistent increases in the TLR4 related mRNAs. The study demonstrated mice exposed to alcohol during adolescence exhibited an increase in the expression of multiple inflammatory genes in adulthood prior to re-exposure. This is in accordance with other studies demonstrating increased expression of microglial activation markers ED1 and MHCII (McClain et al. 2011), inflammatory cytokines, chemokines and proteins (Pascual et al. 2016) and immune receptors (TLR4, TLR3 and RAGE) (Vetreno & Crews 2012) in adult mice that were exposed to alcohol during adolescence. The effects of this persistent elevation in TLR4-related genes are yet to be fully elucidated. However, it has been hypothesised that subsequent activation of the immune system by the original or a new immunogen, will result in an exaggerated inflammatory response. This exaggerated response will act on neighbouring cells influencing their function and potentially increasing the hedonic and anhedonic aspects of drugs of abuse. Crucially, TLR4-dependent signalling appears to assist in mediating the enduring upregulation of neuroimmune-related genes. However, studies examining the role of TLR4 in adolescent alcohol priming often use knock out animals and thus the model is confounded given the pivotal role of TLR4 in neurodevelopment. For example, $TLR4^{-/-}$ mice display increased neuronal differentiation, higher total neuron cell counts and relatively fewer glia compared to wildtype mice (Rolls et al. 2007). Given the pivotal role of glial TLR4 in mediating the molecular and behavioural adaptations induced by alcohol , it is interesting to speculate whether the reduced inflammatory effects observed in these studies (Montesinos et al. 2016; Alfonso-Loeches et al. 2010) is simply due to the reduced number of glial cells or whether it is a TLR4 specific event. The current study is the first to consider the signalling pathways activated by alcohol.

To investigate the relative contribution of TLR4s signalling pathways on the effects of adolescent alcohol exposure, (+)-Naltrexone was used. (+)-Naltrexone is a stereoisomer of the clinically approved (-)-Naltrexone used to treat alcohol dependence. Both isomers are thought to bind to the LPS-binding pocket of TLR4's

co-receptor MD2, however the precise binding site and mechanism remain to be fully elucidated (Hutchinson et al. 2010). Unlike the (-)-isomer, the (+)-isomer is devoid of mu opioid receptor activity. This compound has been further screened against 70 neurotransmitter, peptide, growth factor receptors, ion channels, second messengers and enzymes without any additional interactive effects (Hutchinson et al. 2010). *In vitro* experiments demonstrate (+)-Naltrexone blocks LPS-induced IRF3 phosphorylation and the production of nitric oxide, TNFα and IFNβ production in BV2 cells. It had no effect on the phosphorylation of p65, p38, JNK or ERK1/2 or the expression of IL-1β in these cells (Wang et al. 2016). Collectively, these results suggest (+)-Naltrexone is a biased TLR4-TRIF antagonist, as it failed to attenuate markers classically associated with the TLR4-MyD88 pathway. *In vivo* studies report contradictory findings as (+)-Naltrexone attenuated cocaine-induced IL-1β production (Northcutt et al. 2015). Results from our study further reinforce the concept that (+)-Naltrexone is a biased TLR4-TRIF antagonist as the drug decreased the expression of *IFNβ* but not *IL-1β* or *TNFα* mRNA in adult mice who received alcohol as adolescence.

Attenuating the rise in interferon mRNA may assist in reducing reward-like behavior in adulthood. Recent research has demonstrated that interferons share structural and functional similarities to endorphin, an endogenous opioid (Blalock & Smith 1981; Blalock & Smith 1980). Critically, interferons can bind to μ opioid receptor causing endorphin-like effects (Jiang et al. 2000). Given that activation of the μ opioid receptor contributes to generating the hedonic sensations (or “liking” of alcohol), it is hypothesized that attenuating the rise in interferons may reduce the potentiated hedonic sensation induced by alcohol later in life. While both drinking in the dark and conditioned place preference require opioidergic activity (Kamdar et al. 2007; Middaugh & Bandy 2000), conditioned place preference additionally requires the dopaminergic system (Kamdar et al. 2007; Buccafusco 2009). This may explain why a difference was observed for drinking in the dark and not conditioned place preference. Alternatively, given alcohol seeking and drinking behaviour engages different brain regions, the discrepancy in behavioural outcomes may be due to neuroanatomical restrictions in the expression of TLR4 or its required signalling components. For example, if TLR4 or related genes are not expressed to high levels

in brain regions governing conditioned place preference, it is unlikely to have a substantial effect in mediating this behaviour. This may assist in explaining why siRNA KO of TLR4 in the CeA but not ventral pallidum attenuates alcohol-binge drinking behaviour (Liu et al. 2011).

Interestingly, (+)-Naltrexone attenuated the expression of alcohol-induced *GABRA2* and *TH* mRNA. GABA A2 and tyrosine hydroxylase are associated with the molecular and behavioral effects of alcohol and are particularly important the generation of reward behavior (Harris et al. 2008). Importantly, previous studies have highlighted a link between TLR4 and the expression and function of both GABA A2 and tyrosine hydroxylase potentially providing a link between the effects of (+)-Naltrexone and the alterations in reward behavior later in life (Harris et al. 2017; Aurelian et al. 2016; June et al. 2015; Yan 2015; Bajo et al. 2014; Liu et al. 2011). However, future experiments are required to fully elucidate these links.

A limitation of this study is that the cell-type(s) responsible for the persistent rise in immune-related genes was not explored. Substantial evidence has established the role of neurons in mediating the actions of TLR4 and alcohol in adult rodents (Aurelian et al. 2016; June et al. 2015; Liu et al. 2011). However, these cells lack components of the TLR4 pathway that were elevated following our model of exposure (for example, TRIF and IFNB mRNA). It is unclear whether neurons can transcribe IFNβ with the differing results likely attributable to the different mechanism of IFN β activation. In response to LPS, a TLR4 agonist, neurons do cannot transcribe IFN β or activate JNK or NFκB raising doubts whether these cells can signal through TRIF or MyD88 (Okun et al. 2011). However, neurons actively produce IFN β in response to rabies virus infection (Prehaud et al. 2005). Given the conjecture, it is likely, that the primary immunocompotent cells (microglia and astrocytes) of the CNS are primarily responsible for mediating this effect. *In vitro* and *in vivo* experiments have demonstrated that alcohol indirectly activates TLR4 culminating in the increase expression of inflammatory cytokines, chemokines and proteins (Fernandez-Lizarbe et al. 2009; Blanco et al. 2005).

It is becoming increasingly apparent that the neuroimmune system plays a profound role in neurodevelopment, behaviour and the molecular responses towards drugs of

abuse. This study demonstrated that short low dose alcohol exposure during adolescence perturbs reward-related neurodevelopment increasing the preference for alcohol seeking and drinking later in life. In addition, this model demonstrated that alcohol exposure during adolescence increased the transcription of genes relating to the TLR4 pathway, an effect that persisted throughout adulthood. Attenuation of the TLR4-TRIF pathway, using (+)-Naltrexone, decreased adverse later life outcomes such as alcohol drinking (an effect potentially attributable to a TLR4-GABA A2 interaction), but had no effect on alcohol-seeking behaviour or basal anxiety behaviour.

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Figure legends

Figure 1 Timelines for behavioural experiments. (a) Between postnatal days (P) 22 and 25 adolescent mice received a gavage of alcohol (0.5 g/kg – 3.5 g/kg) or saline daily. On P25, mice were weaned and separated into single sex cages and left to develop undisturbed until adulthood. Mice were subsequently tested for anxiety-like, alcohol-seeking or alcohol drinking in adulthood using the elevated plus maze (on P56), conditioned place preference (P56 – 66) and drinking in the dark (P63 – P66) respectively. (b) Adolescent mice received either (+)-Naltrexone or saline 30 minutes before or after an oral gavage of saline or alcohol (2.2 g/kg) for four consecutive days (P22 – 25). Mice were left to develop undisturbed until adulthood upon which they were tested using the elevated plus maze, conditioned place preference or drinking in the dark.

Figure 2 Adolescent alcohol exposure has no effect on performance in the elevated plus maze in adult mice. Increasing the dose of alcohol does not influence the time (a), distance (b), exits (c) or immobility time (d) in each arm during a five-minute test. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=10.

Figure 3 Adolescent alcohol exposure potentiates alcohol-induced reward behaviours in adulthood. (a) Adolescent alcohol exposure dose-dependently increases the time spent in the alcohol-conditioned chamber relative to saline and (b) limited access alcohol intake. CS, conditioning stimuli; US, unconditioned stimuli. All data was analysed using a two-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=10, *$p < 0.05$; **$p < 0.01$, *** $p < 0.001$.

Figure 4 Adolescent alcohol exposure dysregulates the expression of genes associated with reward/reinforcement within the nucleus accumbens. Alcohol during adolescence increased the expression of *DRD1*, *TH*, *OPRM1*, *GABRA1*, *GABRA2* and *CREB1* but did not affect the expression of *DRD2*, *GRIA1*, *GRIN1*, *BDNF* or *NTRK2* mRNA in adulthood. All data was analysed using a one-way ANOVA with Bonferonni post-hoc. Summary values represented as mean±SEM; n=4, *$p < 0.05$; **$p < 0.01$, *** $p < 0.001$.

Figure 5 Adolescent alcohol exposure increases the expression of TLR4-related genes within the nucleus accumbens. Alcohol during adolescence increased the expression of *TLR4*, *MD2*, *TRIF, CCL2*, *IFNβ* and *HMGB1* but did not affect the expression of *CD14*, *MyD88*, *IL-1β* or *IL-10* mRNA in adulthood. All data was analysed using a one-way ANOVA with Bonferonni post-hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

Figure 6 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure prevents long-term increases of *TLR4* and *IFNβ* mRNA in the nucleus accumbens of adult mice. (+)-Naltrexone selectively reduces alcohol-induced priming of *TLR4* and *IFNβ* mRNA in adulthood but does not alter the expression of MyD88-related genes. All data was analysed using a two-way ANOVA with Bonferonni post-hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

Figure 7 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure prevents long-term increases of *TH* and *GABRA2* mRNA in the nucleus accumbens of adult mice. (+)-Naltrexone selectively reduces alcohol-induced priming of *GABRA2* mRNA in adulthood but does not alter the expression of other reward/reinforcement related genes. All data was analysed using a two-way ANOVA with Bonferonni post-hoc. Summary values represented as mean±SEM; n=4, *p < 0.05; **p < 0.01, *** p < 0.001.

Figure 8 Antagonising TLR4 signalling either before (a, c, e, g) or after (b, d, f, h) adolescent alcohol exposure has no effect on time spent (a and b), distance travelled (c and d), number of exits (e and f) or immobile episodes (g and h) in the elevated plus maze in adult mice. Adolescent alcohol and (+)-Naltrexone does not influence the time, distance, number of exits or immobile episodes (d) in each arm. All data was analysed using a four-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=10, *p < 0.05; **p < 0.01.

Figure 9 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure has no effect on preference for an alcohol-conditioned stimulus in adult mice. (+)-Naltrexone does not influence alcohol-induced conditioned place preference. All data was analysed using a four-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=10, *p < 0.05; **p < 0.01.

Figure 10 Antagonising TLR4 signalling either before (a) or after (b) adolescent alcohol exposure decreases alcohol intake in adult mice. (+)-Naltrexone reduces the intake of alcohol irrespective of whether the mice received alcohol or saline during their adolescence. All data was analysed using a four-way ANOVA with Tukey post-hoc. Summary values represented as mean±SEM; n=10. All post-hoc differences presented in comparison to Saline I.P -> Alcohol I.G (a) and Alcohol I.G -> Saline I.P (b).

* Saline I.P -> Saline I.G; • Naltrexone I.P -> Saline I.G; ^{x} Naltrexone I.P -> Alcohol I.G (a)

• Saline I.G -> Naltrexone I.P (b)

**p < 0.01; ****p < 0.0001; •p < 0.05; ••p < 0.01; •••p < 0.001; ^{xxx}p < 0.001; ^{xxxx}p < 0.0001;

Figure 1

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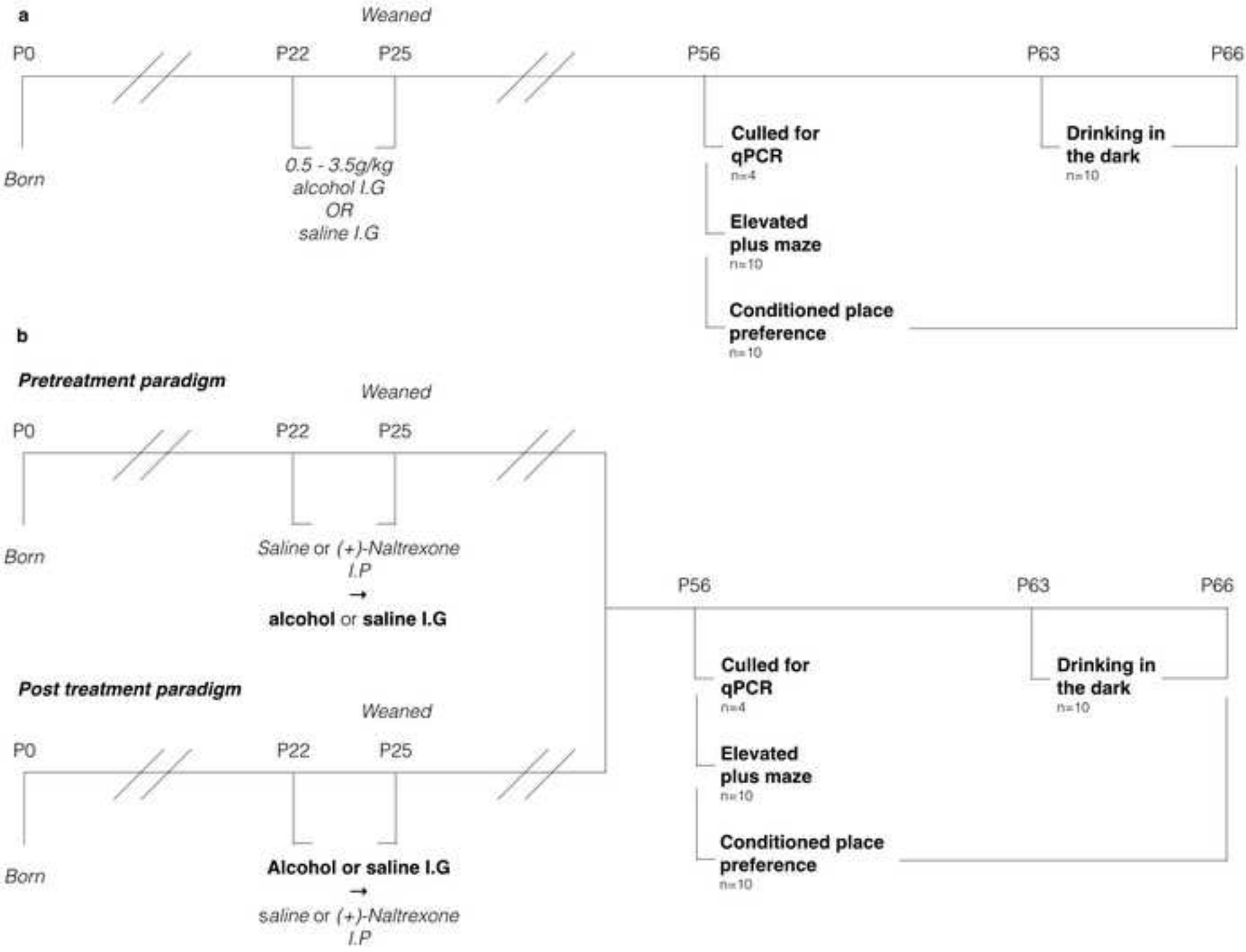

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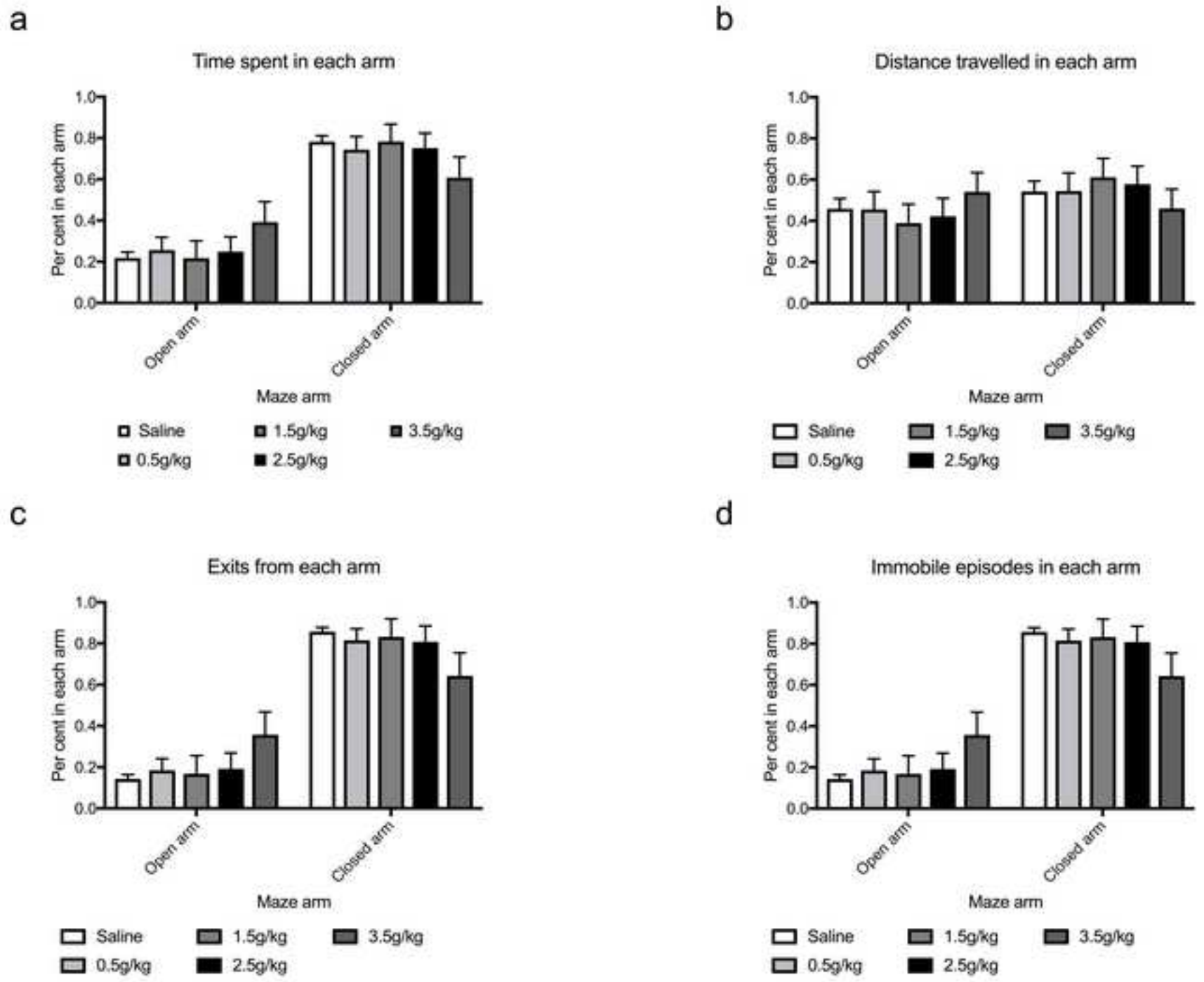

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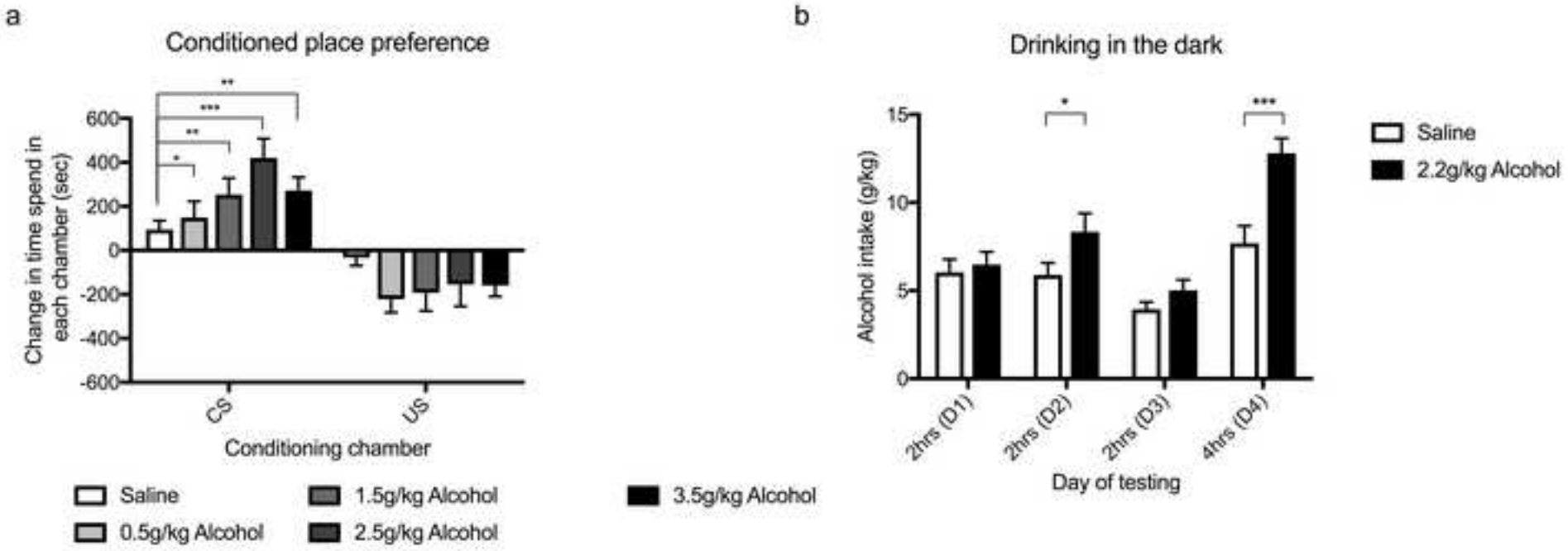

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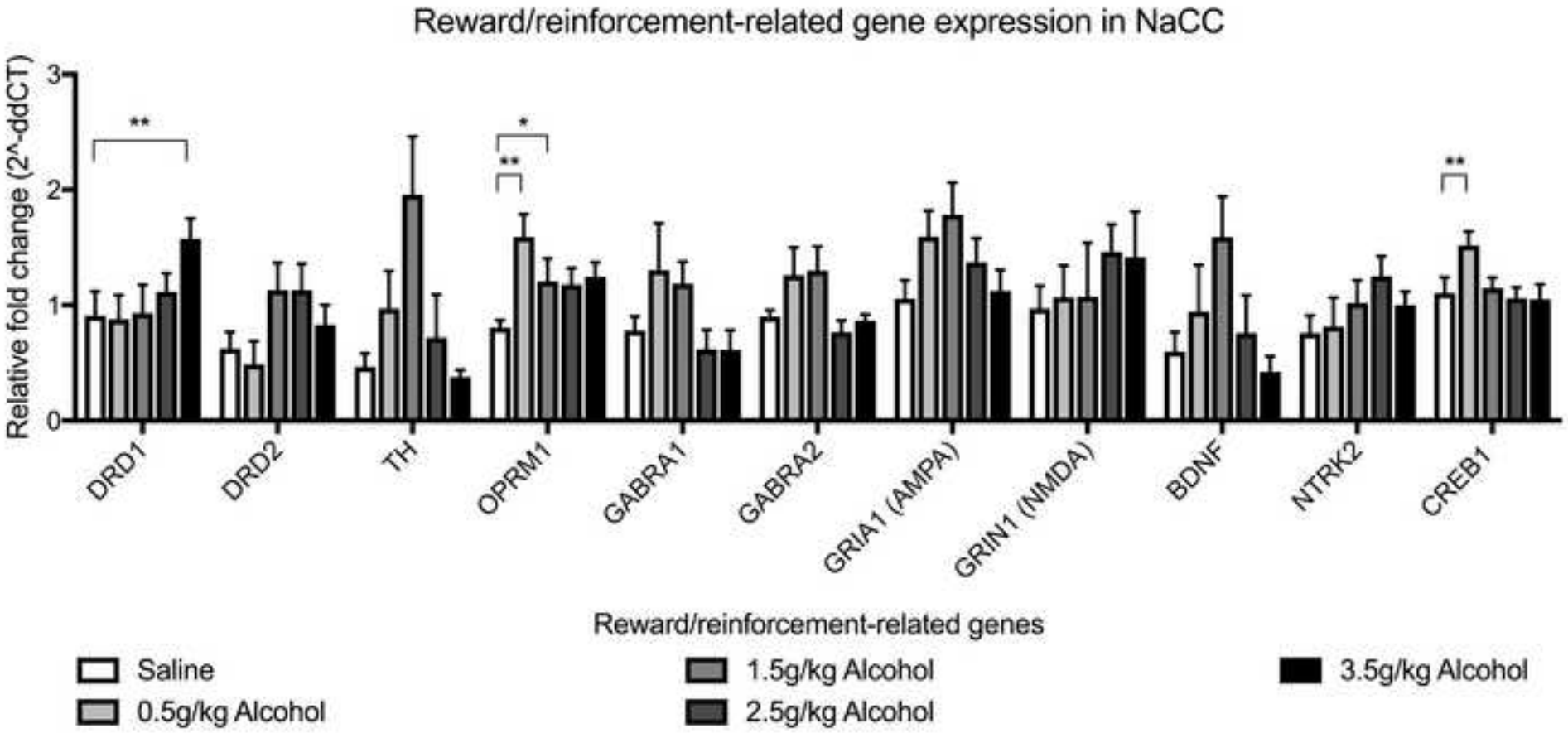

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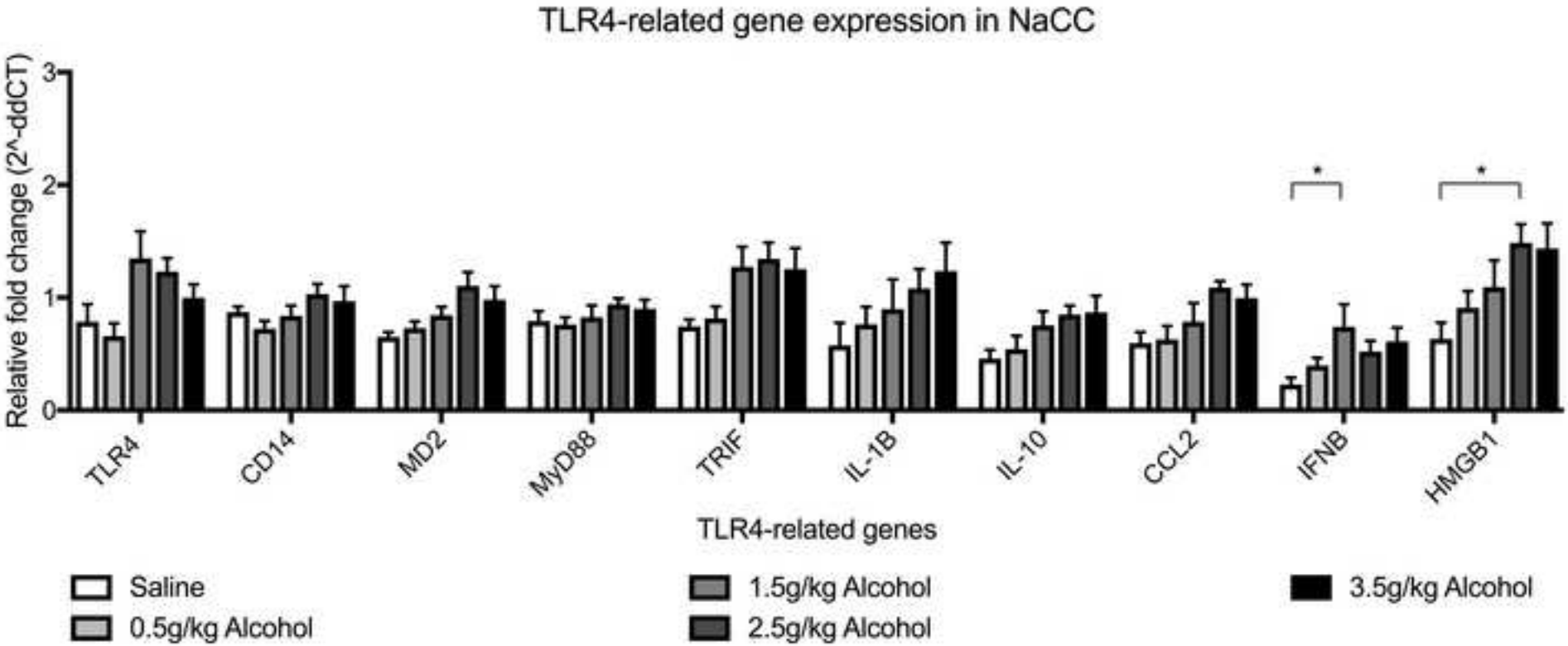

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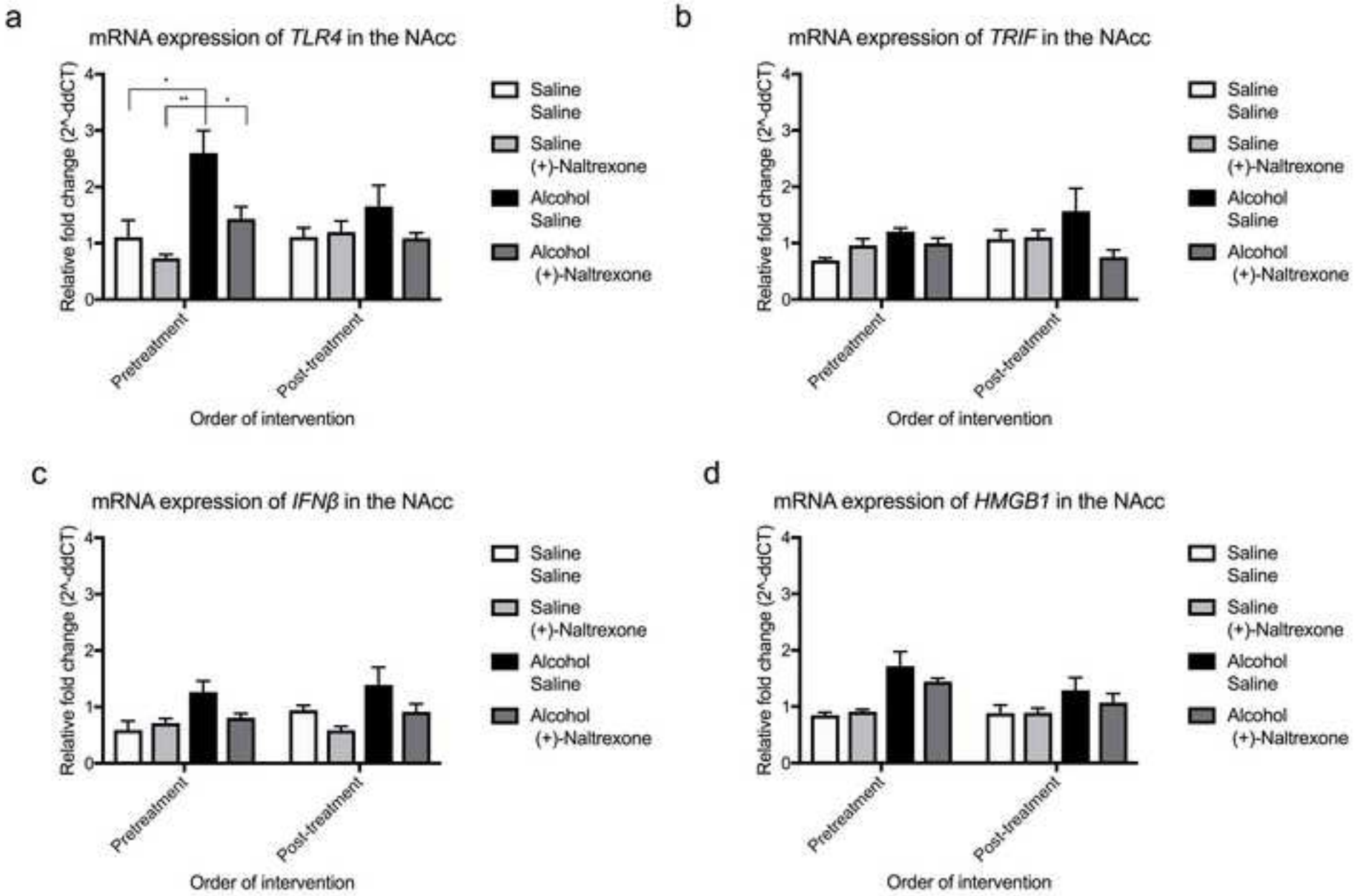

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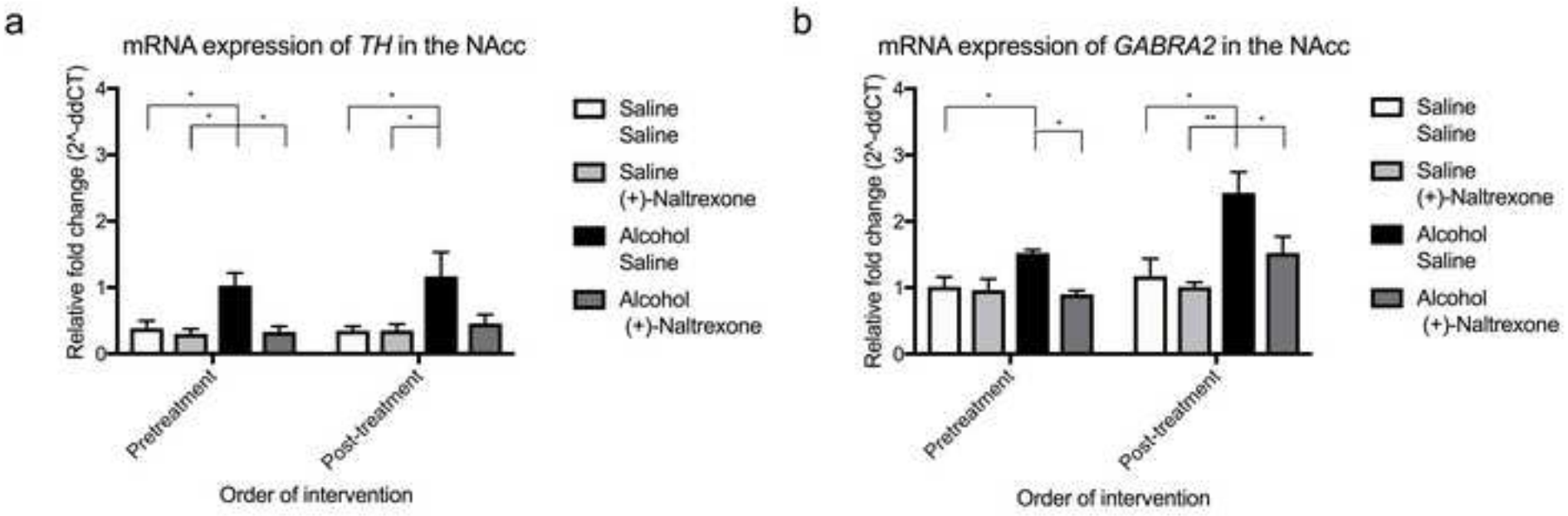

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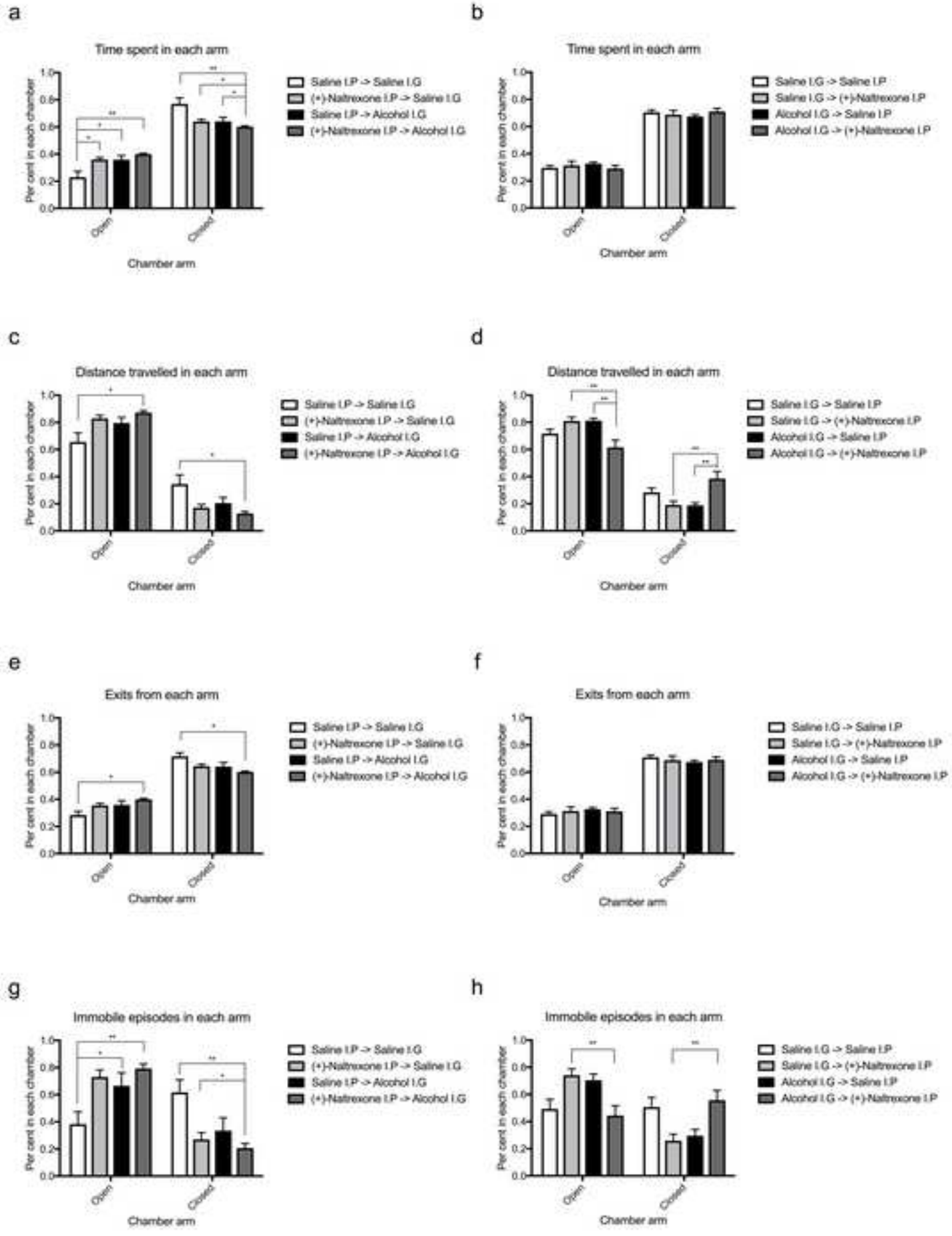

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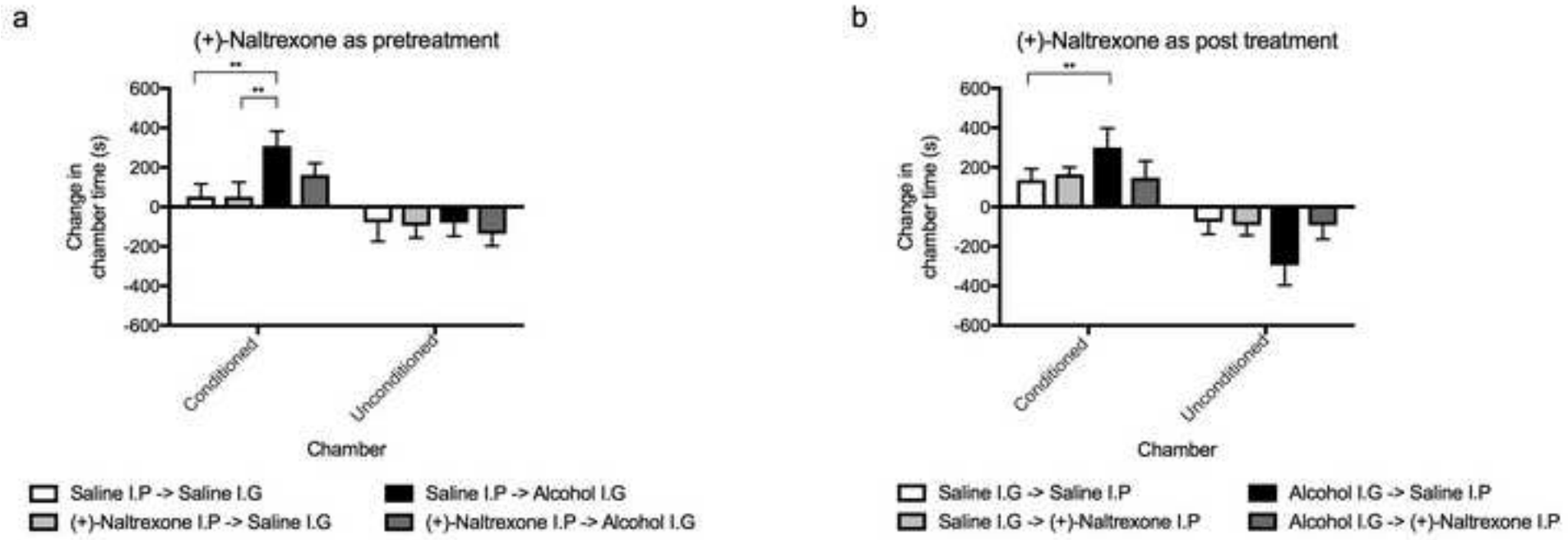

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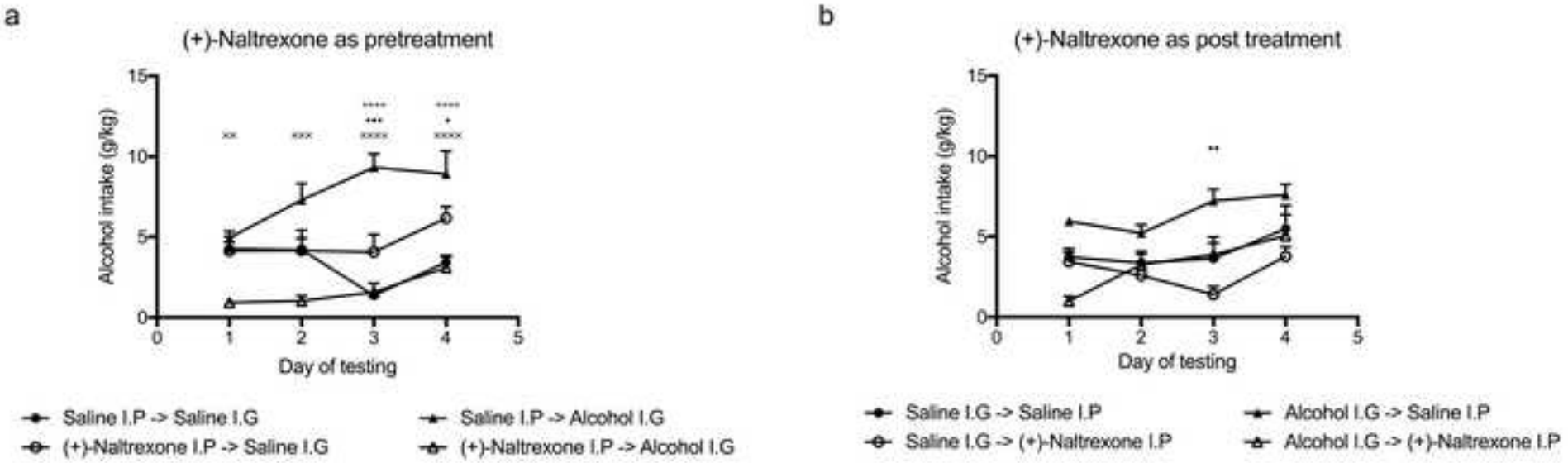

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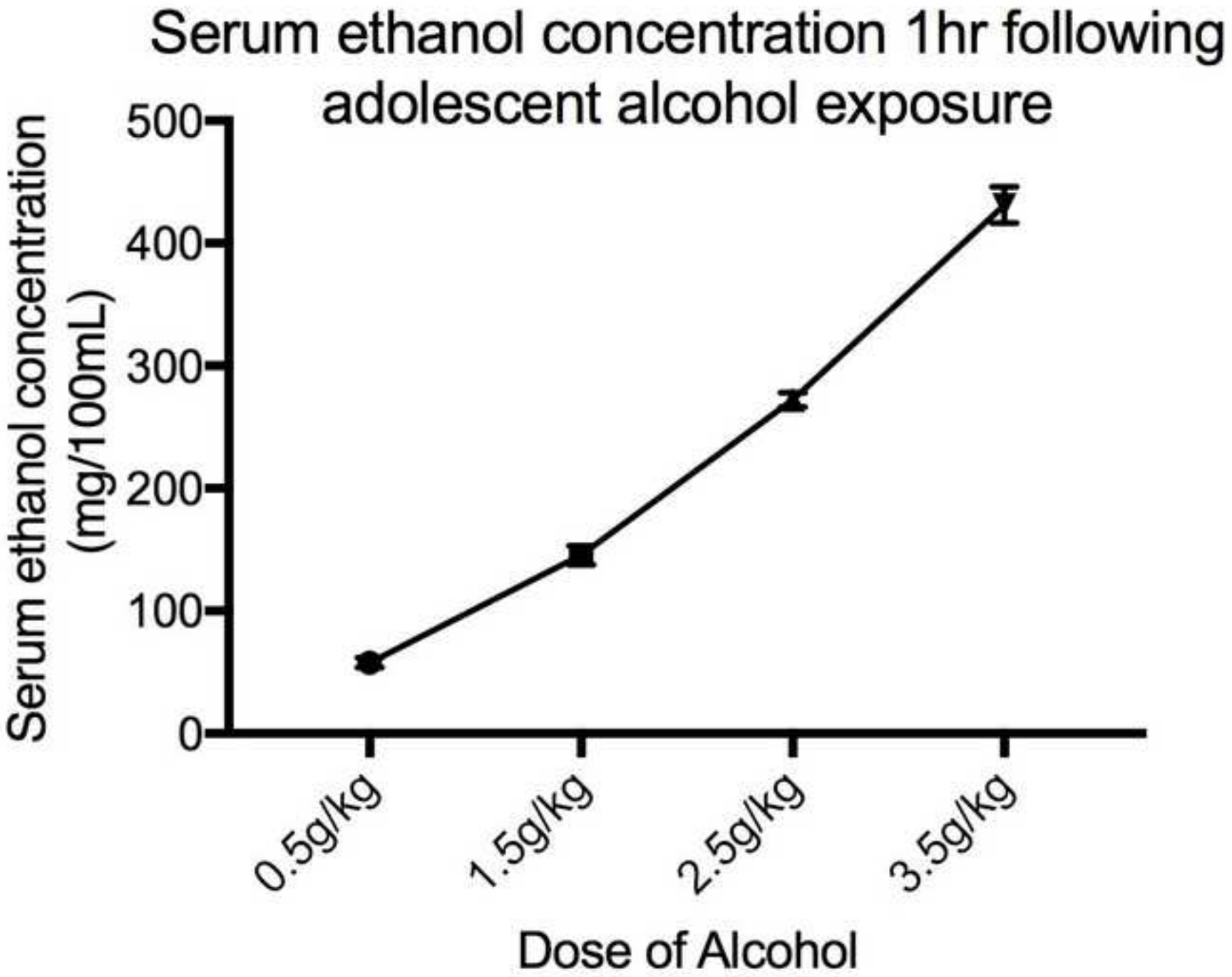

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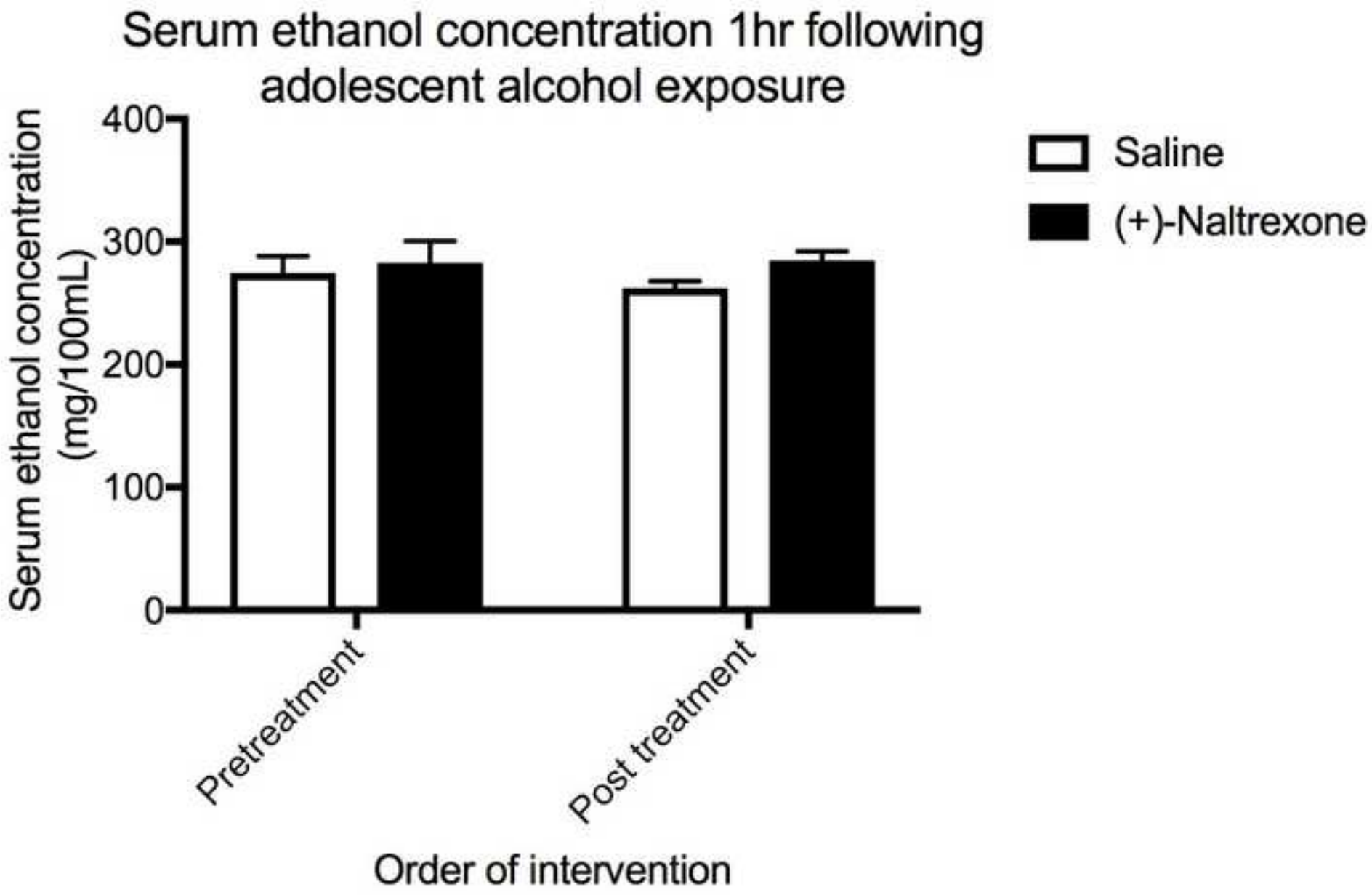

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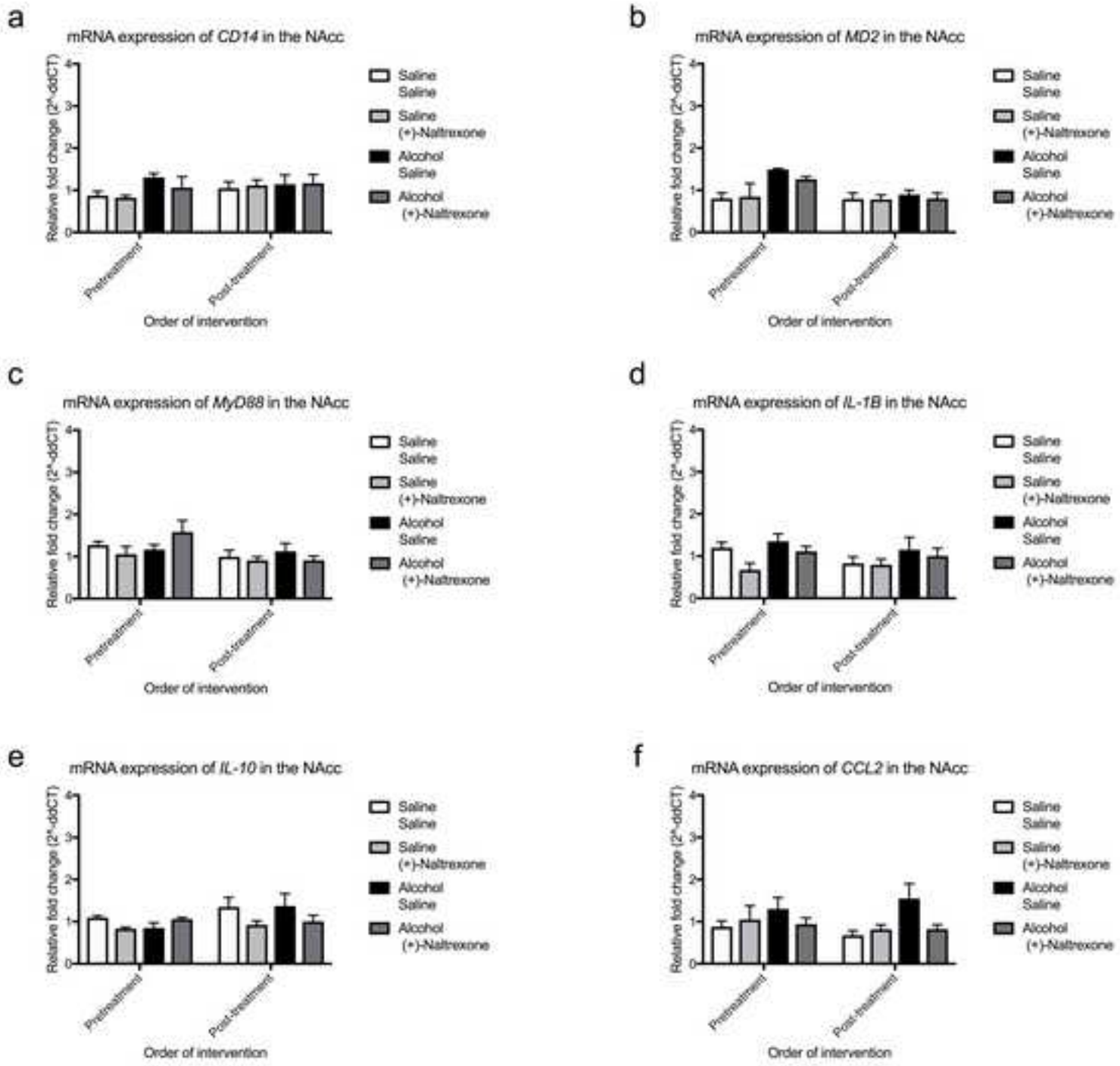

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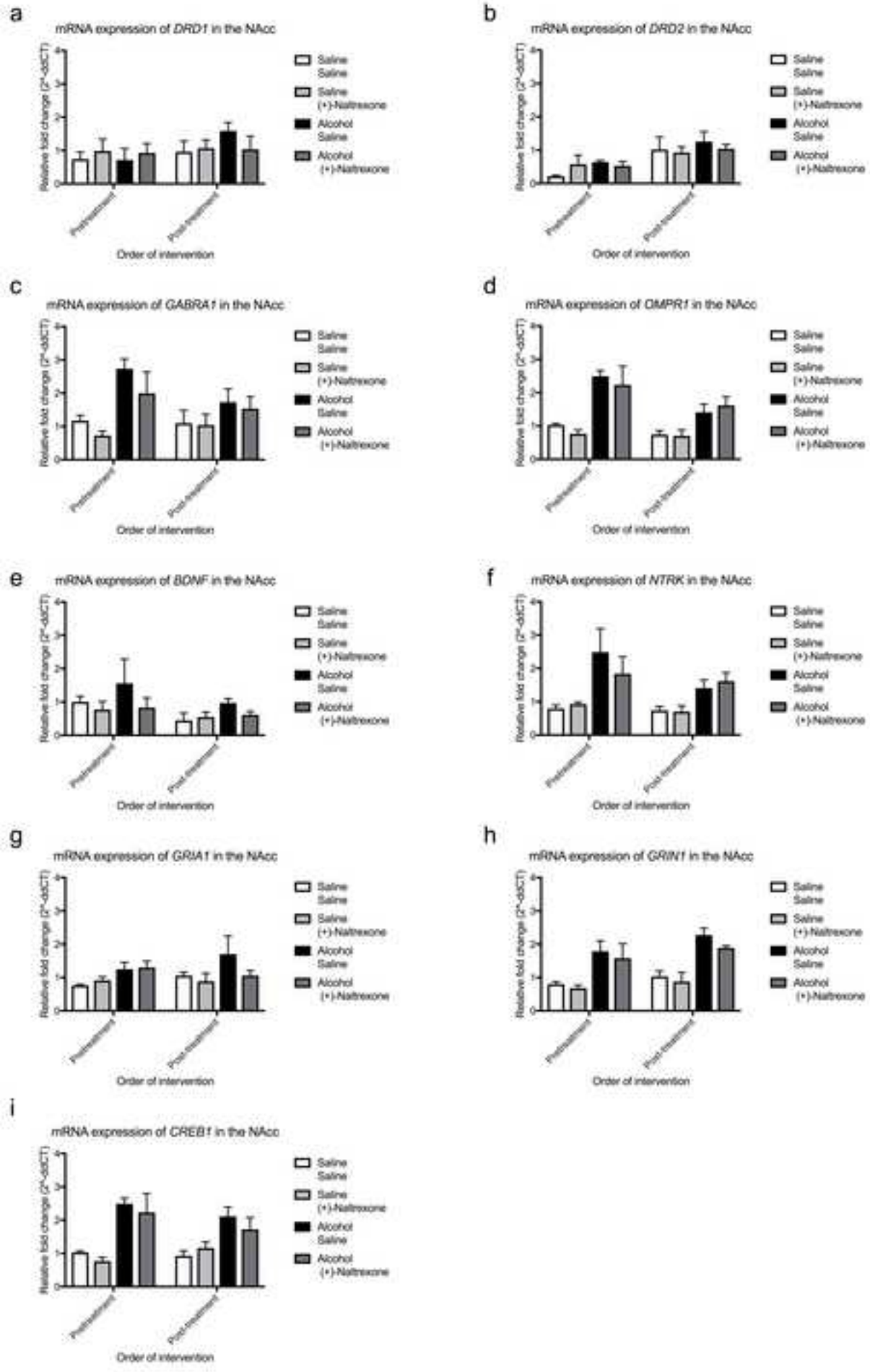

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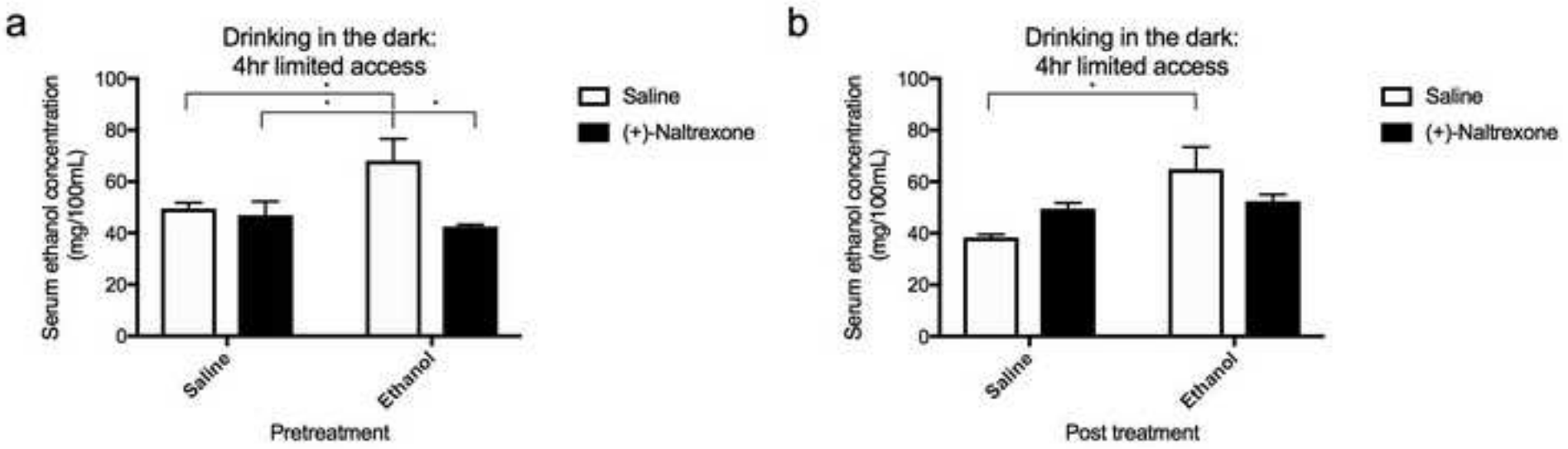

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