Received Date : 30-May-2016

Revised Date : 15-Sep-2016

Accepted Date : 18-Sep-2016

Article type : Original Article

Toluene inhalation in adolescent rats reduces flexible behaviour in adulthood and alters glutamatergic and GABAergic signalling

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Running title: Toluene inhalation reduces flexible behavior

Keywords: Inhalant abuse, instrumental conditioning, metabolomics, oocyte, glutamatergic dysfunction, NMDA receptor

Abbreviations

Ala: alanine
Asp: aspartic acid
Cing: cingulate cortex
CS: conditioned stimuli
DLS: dorsolateral striatum
DMS: dorsomedial striatum
GABA: gamma-aminobutyric acid
Glu: glutamic acid
Gln: glutamine
Lac: lactate
NMDA: N-methyl-D-aspartate
PFC: prefrontal cortex
PIT: Pavlovian Instrumental Transfer
PL: prelimbic cortex
PND: postnatal day
R-O: response-outcome
Tol: toluene
vm: ventromedial
VMS: ventral medial striatum

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Abstract

Toluene is a commonly abused inhalant that is easily accessible to adolescents. Despite the increasing incidence of use, our understanding of its long-term impact remains limited. Here we used a range of techniques to examine the acute and chronic effects of toluene exposure on glutameteric and GABAergic function, and on indices of psychological function in adult rats after adolescent exposure. Metabolomics conducted on cortical tissue established that acute exposure to toluene produces alterations in cellular metabolism indicative of a glutamatergic and GABAergic profile. Similarly, in vitro electrophysiology in *Xenopus* oocytes found that acute toluene exposure reduced NMDA receptor signalling. Finally, in an adolescent rodent model of chronic intermittent exposure to toluene (10,000 ppm), we found that, while toluene exposure did not affect initial learning, it induced a deficit in updating that learning when response-outcome relationships were reversed or degraded in an instrumental conditioning paradigm. There were also group differences when more effort was required to obtain the reward; toluene exposed animals were less sensitive to progressive ratio schedules and to delayed discounting. These behavioural deficits were accompanied by changes in subunit expression of both NMDA and GABA receptors in adulthood, up to 10 weeks after the final exposure to toluene in the hippocampus, prefrontal cortex and ventromedial striatum; regions with recognised roles in behavioural flexibility and decision-making. Collectively our data suggest that exposure to toluene is sufficient to induce adaptive changes in glutamatergic and GABAergic systems and in adaptive behaviour that may underlie the deficits observed following adolescent inhalant abuse, including susceptibility to further drug-use.

Introduction

Volatile solvents, such as toluene (methyl benzene), that are found in many commercial household products such as adhesives, paint products and cleaning agents, are commonly abused by deliberate inhalation resulting in euphoria, disinhibition, disorientation and hallucinations (Anderson and Loomis 2003; Brouette and Anton 2001). The ease of administration and the fact they are inexpensive, legal and readily available contribute to the popularity of abusing solvents especially by adolescents, the dominant population to misuse such products (Duncan *et al.* 2014; Bowen *et al.* 2006). Furthermore, rates of solvent abuse by adolescents are increasing and are often higher than any other illegal substance at this age, reaching 'epidemic' numbers in some countries (Bowen *et al.* 2006; Kurtzman *et al.* 2001). Abuse of inhaled solvents by adolescents is also of concern as it predicts future cognitive disturbances, mental health issues and other serious medical conditions (Yucel *et al.* 2008; Brouette and Anton 2001; Lubman *et al.* 2006; Anderson and Loomis 2003), as well as future illicit substance abuse (Johnson *et al.* 1995; Bowen *et al.* 2006).

As adolescence is a period of critical neurodevelopment it is not surprising that abuse of solvents during this period has long-term effects on the brain and behaviour (Lubman *et al.* 2008; Yucel *et al.* 2008). For example, exposure to toluene in young rats can reduce brain volume, cause white matter injury and change glucose metabolism (Duncan *et al.* 2012; von Euler *et al.* 2000; Schiffer *et al.* 2006; Dick *et al.* 2015b). Further, there is clear evidence that inhalants affect neurotransmitter signalling in the brain as levels of both glutamate and gamma-aminobutyric acid (GABA) and their receptors are altered following exposure to toluene in a region and subunit specific manner. GluN2B receptor subunits for N-methyl-D-aspartate (NMDA) glutamate receptors, for example, are up-regulated in the hippocampus following toluene exposure (Win-Shwe *et al.* 2010; Ahmed *et al.* 2007; Williams *et al.* 2005) and GluN2B- and GluN1- NMDA, and $GABA_A\alpha1$ subunits are up-regulated in the prelimbic cortex (Williams *et al.* 2005). These changes persist well beyond the period of toluene exposure (Win-Shwe *et al.* 2010; O'Leary-Moore *et al.* 2009; Perrine *et al.* 2011) suggesting long-term effects and are important as they are considered to underlie the neuroadaptations that influence behavioural changes observed following exposure to inhalants. Indeed, modulation of both NMDA and $GABA_A$ receptor function alters decision-making and flexible behaviour (Floresco *et al.* 2008; Cottone *et al.* 2013; Higgins *et al.* 2016; Yates *et al.* 2015; Popke *et al.* 2001; Wright *et al.* 2007a; Wright *et al.* 2007b; Buffalo *et al.* 1993; Cardinal *et al.* 2000; Thiebot *et al.* 1985; Butkovich *et al.* 2015) and is believed to underlie the increased likelihood of further drug-use (Lubman *et al.* 2008).

We have recently shown that chronic intermittent exposure to toluene (10,000 ppm) during adolescence results in glutamatergic dysfunction (Dick *et al.* 2014b) and alters GluN2B-containing NMDA receptor binding in the medial prefrontal cortex and striatum when assessed at the end of a

4-week exposure period (Dick *et al.* 2015a). These changes were associated with alterations in cognitive functions including reward-seeking behaviour (Dick *et al.* 2014a). Specifically, toluene-exposed animals were slower to acquire instrumental responding and reversal learning in an operant task, despite normal spatial learning and motor learning abilities (Dick *et al.* 2014a). Together these data suggest that adolescent exposure to toluene, at least in part, alters corticostriatal processing. Given that cortical output is modulated by local inhibitory interneurons and GABAergic signalling in the cortex is enhanced by toluene (Beckley and Woodward 2011), the present study investigated the impact of toluene on both glutamatergic and GABAergic systems, as well as on cognitive function and reward-seeking actions using multiple techniques. Firstly, we compared the metabolic 'fingerprint' of toluene on brain metabolism with a range of metabolomics patterns produced by ligands known to be active in glutamatergic and GABAergic systems (Moussa *et al.* 2007; Rae *et al.* 2006; Rae and Balcar 2014b; Nasrallah *et al.* 2010a), and then further characterised the nature of toluene interactions with the glutamatergic system using in vitro electrophysiological recordings. We then subsequently sought to investigate cognitive flexibility and decision-making abilities associated with reward-seeking following adolescent exposure to toluene at high concentrations using a battery of tests described previously (Bradfield *et al.* 2013; Balleine and Dickinson 1998; Corbit *et al.* 2001) and examined whether this was associated with long-term changes in both NMDA and GABA receptor subunits in selected brain regions using Western Blot analysis.

Methods

In all studies efforts were made to reduce animal numbers where possible by replacement of techniques that do not require animals such as in vitro oocyte studies. Where animals were used, processes were refined to lessen the impact of the procedure on the animals, and all experimental procedures were conducted in accordance with the Prevention of Cruelty to Animals Act, 1986, under the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia, and in compliance with international standards. Experiments were approved by the animal ethics committees of the University of NSW, University of Sydney and the Florey Institute of Neuroscience and Mental Health.

Metabolic studies

Experiments used female Dunkin-Hartley guinea-pigs, weighing 300-400g, that were obtained from South Australian Health and Medical Research (Adelaide, Australia). Guinea pigs were fed *ad libitum* on standard guinea pig/rabbit pellets, supplemented daily with fresh carrots. Animals, housed in enriched environment floor pens, were maintained on a 12 h light/dark cycle.

Guinea pig brain cortical slices were prepared as described previously (Rae and Balcar 2014a). Following metabolic recovery, slices were incubated for 40 min in a modified Krebs-Henseleit

buffer, as used in Rae and Balcar 2014a, with 2 mM sodium [3-^{13}C]pyruvate (control) and 0.3 or 3.0 mM toluene [within the range of concentrations which have been shown to be effective in culture systems previously (Cruz *et al.* 1998; Del Re *et al.* 2006)]. Slices were then removed by vacuum filtration and frozen in liquid nitrogen. Frozen slices were subsequently pulverized and extracted in methanol/chloroform (Le Belle *et al.* 2002). The aqueous phase was freeze dried and resuspended for nuclear magnetic resonance analysis in $^{2}H_2O$ containing 2 mM sodium [^{13}C]formate as an intensity reference and 4 mM EDTA as a chelating agent.

All spectra were acquired on a Bruker AVANCE III HD 600 spectrometer fitted with a cryoprobe (TCI) and refrigerated sample changer. ^{1}H spectra were acquired, both with and without decoupling ^{13}C using bilev composite pulse decoupling, across an effective bandwidth of 48000 Hz during the acquisition time, on a 30 s duty cycle, while ^{13}C{^{1}H-decoupled} spectra were acquired on a 4 s duty cycle using continuous WALTZ-65 decoupling. Total metabolite pool sizes were determined from the ^{1}H{^{13}C-decoupled} spectra and the concentration of ^{13}C-labelled compounds from ^{13}C{^{1}H-decoupled} spectra, following appropriate adjustment for relaxation and nuclear Overhauser effect made according to previously acquired fully-relaxed ^{13}C spectra, as previously described (Rae *et al.* 2000).

Electrophysiology recording

Xenopus oocytes (Dumont stage V or VI) were surgically removed from *Xenopus laevis* obtained from Nasco (Wisconsin, USA), and were prepared as described previously (Petrou *et al.* 1997). Oocytes were kept in ND96 solution and stored at 17 °C. 50 nl of capped cRNA was injected into each oocyte using Roboinject1 System (Multi Channel Systems, Reutlingen, Germany). A total of 10 ng of GluN1A and GluN2A cRNA at a ratio of 1:1 or GluN1A, GluN2A and GluN2B cRNA at a ratio of 1:1:1 was injected into each oocyte.

After 48 hr of expression, two electrode voltage clamp recording was performed using the Roboocyte2 System (Multi Channel Systems, Reutlingen, Germany). Oocytes were impaled with electrodes (0.1– 0.8 MΩ) that contained 1.5 M K-acetate and 0.5 M KCl and were voltage clamped at -70 mV and perfused with Barth's solution containing (mM) 88 NaCl, 1.1 KCl, 2.4 $NaHCO_3$, 1.8 $BaCl_2$ and 15 HEPES pH 7.4. Fresh solutions were used in all the experiments. To conform with our metabolic studies, 3mM toluene solutions were made using ethoxylated castor oil as vehicle. NMDA receptors were stimulated by switching the perfusion solution to one containing glutamate (100 µM) plus glycine (10 µM) in the presence of solvent. Recording frequency was 200 Hz and temperature was maintained between 20-22 °C.

Cognitive flexibility and decision-making

Animals

Adolescent male Wistar rats [approximately postnatal day (PND) 24 with a range of ±2 days] were obtained from the Australian Resources Centre (Perth, Australia, N=19). Rats were housed in pairs in a temperature- and humidity- controlled colony room on a 12:12 hr light-dark cycle (lights on at 7 am). Rats had continuous access to standard rodent chow and water until 3 days prior to commencement of behavioural procedures when chow was restricted to maintain body weight at approximately 90-95% of free-feeding weight.

Toluene inhalation

There were two groups of rats: rats that were exposed to toluene vapour (Tol group, n=10) and rats that were exposed to normal air as a control (Air group, n=9). Exposure to toluene or air took place in specialised boxes [37 cm (width) x 17 cm (length) and 20 cm (height)] that were constructed from toluene resistant materials and fittings (Alternative Plastics Pty. Limited, Melbourne, Vic, Australia, and Swagelok, Broadmeadows, Vic, Australia). Each box contained 2 chambers, which were connected to a system where liquid toluene (1.08389, purity >99.8%, Merck, Vic, Australia,) was vaporized with air to produce a final toluene vapour of 10,000 ppm. For control animals, replica chambers were used, and animals were exposed only to air. The concentration of toluene was regulated using gas flow meters, and monitored by means of an inline gas chromatography system (Shimadzu Corporation, Kyoto, Japan). The system was calibrated using toluene of known concentrations (BOC, Vic, Australia) and deviations greater than ±100 ppm were corrected by altering gas flow (Duncan *et al.* 2012; Duncan *et al.* 2014).

Exposure sessions began at approximately PND 27 (with a range of PND between PND 25 and 29) and continued for 4 weeks, ending at PND 55. There were three 1 hr sessions per week (either 10,000 ppm toluene or 0 ppm air, Monday, Wednesday, Friday; 12 sessions total) at approximately the same time each day (2-4 hours into the light cycle). After each session, chambers were cleaned with ethanol and rats were semi-isolated to avoid potential confounds associated with residual toluene on the rats' fur. Behavioural experiments commenced 7 days after the last exposure on PND 62.

Behavioural procedures

Note that a full description and rationale for each of the behavioural tests is provided in detail in the supplemental material and a timeline for the behavioural procedures is shown in Table 1. In brief, behavioural procedures were conducted in 16 standard operant chambers (Med Associates, VT, USA) containing two levers, speakers and a magazine receptacle. Animals underwent Pavlovian training, where two separate auditory stimuli were each paired with delivery of a different food-outcome (either a grain pellet or sucrose solution), and then instrumental training, where responding on two different levers was rewarded, each with one of these two food-outcomes.

Animals were first tested for Pavlovian Instrumental Transfer (PIT) where lever pressing was recorded in the absence of stimuli and during presentations of conditioned stimuli (CS) that predicted either the 'same' or 'different' outcomes as the lever had earned in training. Following retraining, the goal-directed nature of responding was assessed by outcome devaluation and selective reinstatement tests. Here, lever responses were recorded under choice conditions, following either devaluation of one of the outcomes by sensory-specific satiety, or following non-contingent delivery of one of the outcomes after extinction of lever press, respectively. Rats next underwent further training where the initial instrumental contingencies were reversed and the success of reversal learning examined using an outcome devaluation test. To further examine behavioural flexibility, the response-outcome contingency for one of the levers was degraded; while responding still earned the outcome, the same outcome was also delivered non-contingently (degraded condition). The response-outcome contingency of the other lever was preserved; responding still earned the associated outcome and the alternate outcome was delivered non-contingently (non-degraded condition). Sensitivity of rats to contingency degradation was then examined in a lever choice-test.

Next a progressive ratio schedule of reinforcement was used to examine the amount of effort an animal was willing to exert to earn a single pellet; the total number of lever-presses performed, break point, and latency to stop pressing were compared. Finally, a delay discounting task was used to assess impulsivity versus tolerance of delay for reward maximisation. Choice between a lever that delivered a small immediate sucrose flavoured pellet was compared to that of a lever that delivered four such pellets after increasing time delays.

NMDA and GABA receptor content

Tissue dissection and preparation

Food was unrestricted in the home-cage for 3 days following the final behavioural procedure. The brains were then taken for Western Blot analysis in adulthood (Spear 2000) at 18 weeks of age (i.e. PND 132 or approx. 4.5 months old), and approx.10 weeks after final toluene/air exposure. Rats were overdosed with sodium pentobarbital (120 mg/kg, i.p.), the brains excised and placed in ice-cold saline for 1 min. The brain was then cut into coronal sections using an ice-cold rat brain matrix and the regions of interest hand-dissected bilaterally with a scalpel blade and a dissecting microscope [according to (Paxinos and Watson 2005)]. The dorsal hippocampus was blocked between -2.0 mm and -4.0 mm from Bregma. The prefrontal cortex (PFC) was blocked between +5.0 mm and +2.5 mm from Bregma and then divided into ventromedial (vm) PFC (includes infralimbic cortex and medial orbital cortex), prelimbic cortex (PL), cingulate cortex (Cing) and lateral orbital PFC (includes ventral and lateral orbital cortices). The striatum was blocked between +2.0 mm and +0.5

mm from bregma, and then divided into ventral medial striatum (VMS, predominantly medial nucleus accumbens shell, but would include some nucleus accumbens core), and dorsomedial and dorsolateral striatum (DMS and DLS, respectively). All tissue was immediately frozen on dry ice and stored at -80 °C.

We examined changes in the expression of GABA and glutamate receptor subunits in synaptosomal tissue, which includes proteins from both the pre- and post-synaptic membranes that are available to influence communication between neurons (Ghasemzadeh *et al.* 2009a). The synaptosomal membrane fraction was obtained using a fractionation procedure modified from Ghasemzadeh et al., 2009a. Briefly, tissue was lysed using a hand-held homogeniser in 4 mM HEPES solution containing 0.32 M sucrose and 1% protease inhibitor (Sigma-Aldrich, pH=7.4, for 1 hr). Following centrifugation at 1,000 g for 10 min the supernatant was centrifuged at 10,000 g for 15 min (at 4°C). The pellet was resuspended in 4 mM HEPES solution and centrifuged at 10,000 g for 15 min, and the resulting pellet lysed hypo-osmotically for 30 min at 4°C. After centrifuging at 25,000 g the pellet (i.e. the synaptosomal membrane fraction) was resuspended in 4 mM HEPES and stored at -80°C.

Western Blot procedure

Protein concentrations of the synaptic fractions for each sample were determined using a BCA protein assay (Thermo Scientific-Pierce, Rockford, IL, USA). Each sample (10 µg/sample) was loaded and electrophoresed into 8% sodium dodecyl sulfate polyacrylamide gels, under reducing conditions. Proteins were then transferred onto polyvinylidine fluoride membranes using a semi-dry transfer apparatus (Biorad, Hercules, CA, USA). Membranes were then blocked in 5% non-fat milk power in 0.1 M Tris-buffered saline containing 0.1% Tween-20 (TBS-T, 1 hr at room temperature, RT) and incubated in primary antibody in 5% milk in TBS-T for 24 hr (at 4°C). After washing in TBS-T (4 x 10 min) the membrane was incubated in HRP-secondary antibody for 2 hr (at RT) also in 5% milk in TBS-T).

Each membrane was first probed for rabbit-anti-PSD95 (Cell Signalling, 1:5000), then mouse-anti- GluN2B (Millipore, 1:1,000) and rabbit-anti-αβ-tubulin (Cell Signalling, 1:1,000), before stripping (Restore, Thermo Scientific-Pierce) and re-probing with rabbit-anti- $GABA_A\alpha1$ (Abcam, 1:1,000) then rabbit anti- GluN2A (Millipore, 1:10,000). The secondary antibodies used were either goat-anti-mouse or goat-anti-rabbit (Millipore, 1:20,000). Finally, a chemiluminescence method was used to visualise each blot on Kodak photographic paper (Amersham ECL Prime Detection Reagent, GE Healthcare). The band density of each protein was quantified from scanned photographs using Image-J software. Each protein of interest for each sample was individually corrected for αβ-tubulin loading control.

Statistical analysis

For the metabolic studies, all data were imported and analysed using ANOVA followed by Scheffe's post hoc test using SPSS (v22), as described previously (Rowlands *et al.* 2015). Groups that reached statistical significance ($\alpha \leq 0.05$) on these tests were then subjected to group-wise Mann-Whitney U test to calculate a P value. Only those variables passing all of these tests were considered to be significantly different. Multivariate analysis was conducted in SIMCA P+ (Umetrics, Umeå, Sweden). Specifically, we used principal components analysis (PCA), an unsupervised method used to identify variance in the data. PCA classifies the data into groups based objectively on the variables that show the most change. The robustness of the resultant model is assessed by a goodness of fit algorithm, with $R^2 > 0.60$ representing a model which accounts for the majority of variance in the dataset (Eriksson *et al.* 1999). This is further cross-validated by generating a value, Q^2, which is the fraction of the total variation in the data which can be explained by a component. A rule of thumb is that values of $Q^2 > 50\%$ are considered a good fit (Eriksson *et al.* 2006). In this work $R^2 > 0.82$ %. The data were imported from the toluene condition with each variable expressed as a ratio relative to the control mean (N = 4). Data were then subjected to unit variance scaling to ensure that each variable contributed equally to the model. Two PCA models were constructed containing toluene metabolomic data. The first model contained data from a range of experiments using ligands active in the glutamatergic system (for list of ligands and experiments, see supplementary data). The second model contained data from a range of experiments using ligands active in the GABAergic system (Nasrallah *et al.* 2011; Nasrallah *et al.* 2007; Nasrallah *et al.* 2010b; Rae *et al.* 2015; Rae *et al.* 2009; Rae *et al.* 2014; Nasrallah *et al.* 2010a).

For the electrophysiology study, data were analysed using AxoGraph (AxoGraph Scientific, Sydney, Australia). Student's *t*-test was used to test statistical significance using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Behavioural data were analysed using mixed model ANOVAs and simple effects analyses were used to examine any significant interactions, and Western blot data using one-way ANOVAs.

Differences were considered significant when $p<0.05$. All data are presented as Mean ± SEM.

Results

Metabolic studies

The metabolic outcomes of incubating guinea pig cortical brain tissue slices with 0.3 and 3.0 mM toluene are shown in Figure 1. Incubating slices with 0.3 mM toluene resulted in significant increases in incorporation of ^{13}C label into Glu C2, GABA C2, Asp C2 and C3, with no significant change in Glu C4 and citrate C2/C4. Less ^{13}C was incorporated into Gln C4, Lac C3 and Ala C3 (Figure 1A). The total metabolite pools of all measured metabolites were reduced with the exception of

aspartate, which was unchanged (Figure 1B). Increasing toluene concentration by a factor of ten to 3.0 mM resulted in a similar level of increased incorporation of label into Glu C2, GABA C2 and Asp C2 and C3, but had contrasting effects at Gln C4, resulting in a significant increase in label, along with increased label in citrate C2/4 (Figure 1A). Significantly less ^{13}C was found in Lac C3 compared to control and 0.3 mM toluene. Similar to 0.3 mM toluene, less ^{13}C was incorporated into Ala C3. The metabolite pools of lactate, GABA and alanine were significantly smaller than control pools (Figure 1A). There were no significant changes in the intracellular levels of taurine, creatine or *myo*-inositol (data not shown).

When data from the experiments testing the effect of toluene on brain metabolism of [3-^{13}C]pyruvate were incorporated into a model with data from experiments conducted using ligands active in the glutamatergic system and subjected to principal components analysis, the data generated a three component model accounting for 55%, 24% and 6% of the variance in the data, respectively with a Q2 (cross-validation score) of 68% (Figure 2A). Toluene (3.0 mM) clustered with exogenous glutamate (50 µM), with 5 µM ketamine also in the vicinity, whereas the lower concentration of toluene (0.3 mM) clustered near to exogenous glutamate (10 µM) and riluzole (10 and 100 µM).

When data from toluene experiments were combined with data from experiments using ligands active in the GABAergic system and subjected to principal components analysis, the data generated a three component model accounting for 82% of the variance in the data (47%, 26% and 9%, respectively) with a cross-validation (Q2) score of 69%. Toluene (0.3 mM) clustered with the GABAρ partial agonist 20 µM CACA (*cis*-4-aminocrotonic acid) (Allan *et al.* 1980; Kusama *et al.* 1993) while 3.0 mM toluene clustered with 20 µM CI-966 (1-[2-[bis[4-trifluoromethyl)phenyl]methoxy]ethyl]-1,2,5,6-tetrahydropyridine-3-carboxylic acid hydrochloride), a very potent and selective inhibitor of the GABA transporter 1 (GAT1) (Borden *et al.* 1994) and with 100 µM CACA (Figure 2B).

Oocyte studies

Application of glutamate and glycine to Xenopus oocytes expressing heteromeric GluN1A/ GluN2A- and GluN1A/ GluN2A/ GluN2B- NMDA receptor subunit combinations produced an inward current. Application of 3 mM toluene in the presence of glutamate and glycine reversibly reduced this inward current (Figure 3). Mean current amplitude following agonist application was significantly greater for GluN1A/ GluN2A compared to GluN1A/ GluN2A/ GluN2B receptor subunits ($p<0.05$; Figure 3A); however, the inhibition by toluene was similar for both subunit configurations tested (Figure 3B).

Cognitive flexibility and decision-making

Body weight

At PN27, prior to any treatment, there was no difference in body weight for rats in each group (Air 81.8±1.8 g verses Tol 82.1±2.1 g). By the end of the exposure period (PN55) body weights were significantly greater in Air (313.9±7.9g) rats compared to Tol (277.2±5.6g) rats ($F_{(1,17)}$=4.58, p=0.047). Body weight was subsequently analysed across the 10 weeks following the exposure period. There was an effect of treatment group ($F_{(1,17)}$=6.40 p=0.022), and week ($F_{(1,17)}$=102.4, p<0.00), and an interaction between group and week ($F_{(1,17)}$=5.01, p=0.039). Analysis for each week revealed Tol rats remained significantly lighter than Air until week 6 ($F_{(1,17)}$>5.0, p<0.039 for all analysis). Body weights were no longer significantly different between groups from week 7 onwards ($F_{(1,17)}$<2.0, p>0.180, for all analysis. See supplementary materials).

Training

There was no difference between Air and Tol groups in corrected magazine entry across Pavlovian training or in rates of lever pressing across instrumental training. At the start of Pavlovian training, magazine entry within the CS period was low for both groups, 0.08±0.31entries/min for Air group and 0.09±0.30 entries/min for Tol group, and increased to 9.82±0.90 entries/min for Air group and 8.60±0.87 entries/min for Tol group by the last day of training. ANOVA revealed that there was no difference in overall magazine entry between the two groups ($F_{(1,17)}$=0.786, p=0.388), and that magazine entry during the CS period increased similarly across training days ($F_{(1,17)}$=45.862, p<0.001); there was no interaction between group and training day ($F_{(1,17)}$=0.968, p=0.339) indicating that the rate of acquisition of Pavlovian learning did not differ between groups.

Responding on the left and right levers was summed for each training session to provide a measure of overall operant rate. Lever-press rates on the first day of training were 5.4±0.5 presses/min and 4.7±0.4 presses/min, for Air and Tol groups, respectively, and increased to 38.3±4.2 presses/min and 42.1±3.8 presses/min by the last day of training. There was no group effect ($F_{(1,17)}$=1.045, p=0.321), but there was an effect of day ($F_{(1,17)}$=273.383, p<0.001), and no interaction ($F_{(1,17)}$=1.074, p=0.315), indicating that overall lever pressing increased across training and this increase did not differ between groups.

PIT test

Performance during the PIT test is shown Figure 4A. Both groups demonstrated specific PIT (i.e., response rates increased during the Same CS compared both to baseline and to the Different CS). Statistical analysis confirmed that there was no group effect ($F_{(1,17)}$=0.006, p=0.939), but there was an effect of stimulus indicating that rats responded more on the lever associated with the same outcome as the stimulus currently present than on the lever associated with the different outcome ($F_{(1,17)}$=100.94, p<0.0001). There was no interaction ($F_{(1,17)}$=0.222, p=0.6465), indicating that both groups demonstrated a similar degree of PIT.

Outcome devaluation test

Consumption of the outcomes during the devaluation manipulation did not differ between groups (18.3 ±0.7g and 19.2±0.8g for Air and Tol groups, respectively, $F_{(1,17)}$=0.612, p=0.612). The results of the choice test are shown in Figure 4B. There was a significant effect of devaluation ($F_{(1,17)}$=56.98, p<0.000) but no effect of group ($F_{(1,17)}$=0.159, p=0.695) and no devaluation by group interaction ($F_{(1,17)}$=0.018, p=0.895) indicating that both groups were able to acquire goal-directed actions, selecting the lever previously associated with the non-devalued outcome more often than the lever previously associated with the devalued outcome.

Selective reinstatement

Total lever presses were summed across both levers for the extinction period prior to the first outcome delivery. Both groups showed a similar level of extinction, decreasing rates of lever pressing across extinction (for the first minute: 78.3±7.4 presses for Tol and 77.1±9.6 presses for Air groups, and for the 13th minute: 1.8±0.7 presses for Tol and 1.8±0.9 presses for Air groups). Statistical analysis revealed an effect of minute, confirming extinction ($F_{(1,17)}$=143.6, p=0.00), no difference between groups ($F_{(1,17)}$=1.170, p=0.295), and no extinction by group interaction ($F_{(1,17)}$=1.00, p=0.331).

The results of the selective reinstatement test are shown in Figure 4C. There was no difference between groups during test; presentation of one of the food rewards enhanced responding on the lever that earned that same reward more than on the lever earning a different reward. Thus, on test there was an effect of lever (same vs. different; $F_{(1,17)}$=19.899, p<0.001), however there was no effect of group ($F_{(1,17)}$=0.284, p=0.601) and no interaction between these factors ($F_{(1,17)}$=0.008, p=0.930).

Reversal learning

After reversing the response-outcome (R-O) associations on which the rats had initially been trained, two separate outcome devaluation tests were used to examine the acquisition of the reversed lever-outcome contingencies. First, there was no difference in consumption of the outcomes in the free-feeding period prior to either of the tests (Test 1: 22.8±0.7g and 22.1±0.9g for Air and Tol groups, $F_{(1,17)}$=0.426, p=0.523. Test 2: 22.5±1.1g and 22.7±1.4g for Air and Tol groups, $F_{(1,17)}$=0.016, p=0.901).

The results of the two devaluation tests are shown in Figure 5A. Given the prior demonstration that both groups were able to show a typical devaluation effect (Figure 4B), insensitivity to devaluation in these tests provides evidence of difficulty updating R-O relationships. In the first test conducted after 8 sessions of training with the new R-O contingencies, the Air group had not yet successfully reversed the response-outcome relationships, and showed no preference for either lever at test (Figure 5A, left panel). In contrast, performance for the Tol group was similar to that seen prior to reversal; rats responded more on the lever that, in recent training, delivered the

currently devalued outcome, which would be expected if they were still utilising the initial rather than reversed response-outcome contingencies. These data suggest that encoding the new action-outcome associations and adjusting performance appropriately after reversal were slower in the Tol than in the Air group. In line with this view, after further training the results of the second test found that both groups were now able to display a preference for the non-devalued lever, indicating that both groups were able eventually to acquire the reversed contingencies given a further 4 training sessions (Figure 5A, right panel).

ANOVA revealed no main effect of group ($F_{(1,17)}$=0.016, p=0.901), test ($F_{(1,17)}$=0.338, p=0.569), or devaluation ($F_{(1,17)}$=3.490, p=0.079) and no interaction between group and test ($F_{(1,17)}$=1.081, p=0.313), or group and devaluation ($F_{(1,17)}$=2.304, p=0.147). However, there was a significant interaction between test and devaluation suggesting that lever choice varied across tests ($F_{(1,17)}$=27.046, p=0.000). As indicated above, inspection of Figure 5A (left panel) suggests that the Tol group was slower to adapt to the reversal of the R-O associations. This was confirmed by simple effects analyses which showed that in Test 1 there were differences in lever choice for only the Tol group ($F_{(1,17)}$=13.908, p=0.002) favouring, however, the original rather than the reversed contingencies. The Air group responded similarly on the two levers in this test ($F_{(1,17)}$=1.555, p=0.229) although, by Test 2, both groups showed a devaluation effect favouring the reversed R-O contingencies (Figure 5A right panel, Tol: $F_{(1,17)}$=9.806, p=0.006, Air: $F_{(1,17)}$=9.673, p=0.006).

Contingency degradation

During contingency degradation training each lever was presented alone in one of two daily sessions and served as either the degraded or non-degraded lever. The average lever-presses per minute on the final day of training were not different between groups (44±4 and 48±9 for the Tol group, and 49±5 and 39±8 for the Air group, for the non-degraded lever and degraded lever, respectively). This was confirmed by the statistical analysis across training days where there was no difference in overall rates of responding by either group ($F_{(1,17)}$=0.005, p=0.944) or in overall rates of responding for each lever during training ($F_{(1,17)}$=0.241, p=0.630). There was also no interaction ($F_{(1,17)}$=0.881, p=0.361) indicating that both groups responded similarly during training. However, differences emerged between the groups in a choice extinction test, shown in Figure 5B. Statistical analysis revealed there was no main effect of group ($F_{(1,17)}$=0.363, p=0.555) but a main effect of lever choice ($F_{(1,17)}$=5.469, p=0.032). More importantly, there was a significant interaction between these two factors ($F_{(1,17)}$=4.457, p=0.05), indicating that sensitivity to the change in contingency differed between groups. Simple effects analysis, comparing lever choice for each group, revealed that the Air group responded more on the lever for which the R-O relationship remained intact ($F_{(1,17)}$=9.405, p=0.007), whereas the Tol group responded indiscriminately on the two levers ($F_{(1,17)}$=0.027, p=0.871). This result is similar to that found after contingency reversal and suggests that toluene

exposure results in an impaired ability to integrate changes in the lever-outcome contingency to control performance.

Progressive ratio test

Compared to the Air group, the Tol group performed a greater total number of lever presses (Figure 6A) and obtained a greater number of outcomes (11.6±1.0 for Air and 15.4±1.7 for Tol) over the 2-hr test period. These differences were significant [($F_{(1,17)}$=4.729, p=0.044) and ($F_{(1,17)}$=4.515, p=0.049), respectively]. The breakpoint, defined as the time when lever pressed ceased for a 2 min period, was also significantly different between groups, occurring on average at 21.7±3.2 mins for the Air group but at 47.9±9.4 min for the Tol group ($F_{(1,17)}$=6.351, p=0.022; see Figure 6B). This result suggests that toluene exposure rendered instrumental performance maladaptive, separating instrumental performance from its cost, resulting in persistent responding for reward despite a substantial response requirement.

Delay discounting test

For each group, the average percentage of presses on the lever that delivered the large outcome relative to the lever that delivered the small immediate outcome is shown for each delay in Figure 6C. The baseline percentage of large lever selection is shown at the zero delay, where both groups were equivalent, displaying a preference for the large reward. From that point the preference decreased, indicating a shift to the small reward lever with increasing delays for large reward in the Air group compared to the Tol group. A group by delay [2x(9)] analysis revealed that overall there was no effect of group ($F_{(1,17)}$=1.504, p=0.237), or delay ($F_{(1,17)}$=3.272, p=0.088). However, importantly, there was a significant group x delay interaction ($F_{(1,17)}$=4.876, p=0.041). Simple effects analysis for each group revealed an effect of delay for the Air group ($F_{(1,17)}$=7.665, p=0.013) but not the Tol group ($F_{(1,17)}$=0.084, p=0.775) indicating that Tol group continued to prefer the large reward despite the increasing delay and that, again, Tol exposure rendered performance maladaptive and the rats relatively insensitive to changes in the cost of specific rewards.

Western Blots

At PND 132 (10 weeks after the last exposure to toluene or air and following subsequent behavioural testing), NMDA and GABA receptor content were quantified using Western Blot. Each protein examined was corrected using a tubulin loading control, and at no time were there significant differences between groups in tubulin loading (p>0.05, for all regions examined). Representative blots for each protein for the dorsal hippocampus are shown in Figure 7A [$GABA_A$α1 (55 kDa), GluN2A (170 kDa), GluN2B (180 kDa) and PSD95 (95 kDA)]. All proteins of interest increased after exposure to toluene compared to air controls in hippocampus (Figure 7B). Specifically, there were significant increases in PSD95 ($F_{(1,17)}$=5.185, p=0.036), GluN2A ($F_{(1,17)}$=4.683, p=0.045) and

GluN2B ($F_{(1,17)}$=9.399, p=0.007) for the Tol group compared to the Air group. However, the differences in $GABA_A\alpha1$ did not reach statistical significance ($F_{(1,17)}$=3.570 p=0.076).

In the vmPFC there were significant reductions in $GABA_A\alpha1$ ($F_{(1,17)}$=4.495, p=0.049) and PSD95 ($F_{(1,17)}$=5.688, p=0.029) for the Tol group compared to the Air group, but there were no differences between groups for GluN2A or GluN2B ($F_{(1,17)}$<0.252, p>0.622) (Figure 8A). For the PL, there was a significant increase in $GABA_A\alpha1$ only in the Tol group compared to Air group ($F_{(1,15)}$=6.110, p=0.0259). There was no significant differences between groups for any other proteins examined in the PL, the Cing or the lateral/orbital PFC (p>0.05) (Figure 8B-D). For the striatum (Figure 9) $GABA_A\alpha1$ was increased in the VMS in the Tol group compared to Air ($F_{(1,17)}$=4.946, p=0.040) and no significant differences were found for PSD95, GluN2A or GluN2B (p>0.05) nor were any differences found in the dorsomedial or dorsolateral striatum (p>0.05).

Discussion

Acute exposure to toluene induces a metabolomic fingerprint associated with changes in glutamatergic and GABAergic systems, and reduces NMDA receptor-mediated signalling. Furthermore, chronic intermittent exposure of adolescent rats to toluene, modelling abuse, had long-term consequences on the brain and behaviour persisting into early adulthood. This included a reduction in behavioural flexibility, as indicated by altered performance on the reversal learning, contingency degradation, progressive ratio and delayed discounting tasks. There were also subunit- and region-specific changes in the expression of select $GABA_A\alpha1$ and NMDA receptor subunits in the hippocampus, PFC, and ventromedial striatum evident two and half months after the last toluene exposure (and subsequent behavioural tasks). Collectively, our data suggest that toluene may act acutely to influence glutamate and GABA function and that long-term exposure results in persistent changes to these systems.

Acute effects of toluene on brain metabolism

Our data showed a significant metabolic effect of the acute application of toluene (0.3 and 3 mM) to brain cortical tissue slices, characterised by increased mitochondrial activity, increased glutamate/glutamine cycling and increased GABA production and release. Multivariate analysis of this metabolic fingerprint suggested that toluene produces a metabolic profile similar to that of relatively high concentrations of exogenous glutamate. Interestingly, the profile was also similar to that of riluzole, a compound known to facilitate uptake and block release of glutamate (Bellingham 2011). Although it may seem counter-intuitive that riluzole clusters with toluene given that the former restricts glutamate in the extracellular space and the latter may facilitate it, riluzole has been reported previously to enhance glucose metabolism and glutamate-glutamine cycling (Chowdhury *et*

al. 2008) as was seen here. In slices, which are relatively metabolically inactive, antagonists may often yield similar profiles to agonists under resting conditions (Nasrallah *et al.* 2011). Finally, the proximity of clustering of toluene to ketamine suggests that toluene may be acting either directly, or indirectly via glutamate release, at NMDA receptors. Toluene's ability to act at NMDA receptors was further supported by the results of our *in vitro* studies where toluene decreased the mean current amplitude at GluN2A and GluN2A/2B containing NMDA receptors. Cruz and colleagues (1998) assessed GluN2 subunits in isolation and in the absence or presence of glycine, and found GluN2B to be the most sensitive to toluene followed by GluN2A and then GluN2C (Cruz *et al.* 1998). We examined the response to GluN2A alone and in combination with GluN2B and noted that toluene had a similar effect at both receptor configurations which is consistent with the ability of toluene to inhibit NMDA receptor mediated currents in the brain. Indeed, whole-cell patch clamping of rodent medial prefrontal cortex neurons confirmed that toluene rapidly and dose-dependently inhibits NMDA-mediated currents in cortical slices (Beckley and Woodward 2011). This latter study also showed that toluene enhances GABA-mediated currents in cortex, while others have confirmed toluene enhances signalling via $GABA_A\alpha1$ subunit containing $GABA_A$ receptors in hippocampus (Beckstead *et al.* 2000). Comparison of toluene's metabolic profiles with those of ligands active in the GABAergic system showed similarities between the profiles generated by toluene and those from CACA, a partial agonist at GABAρ receptors, which are likely to be located extrasynaptically (Alakuijala *et al.* 2006). While the suggestion of activity at GABAρ is interesting and will form the basis for future experiments, our data also indicated clustering of toluene with the potent GAT1 inhibitor Ci966. This suggested a mechanism by which toluene could influence activity and expression of GABAA receptors, particularly GABAAα1 (Ohnuma *et al.* 1999; Volk and Lewis 2002). In contrast to the case of GABAρ there is an existing literature on GABAAα1 and performance in relevant behavioural tasks (Butkovich *et al.* 2015; Thiebot *et al.* 1985; Cardinal *et al.* 2000) and toluene enhances GABAAα1-mediated currents in brain (Beckstead *et al.* 2000). At the concentration used (20 µM) CI-966 also inhibits glycine re-uptake (Giusti *et al.* 1990) drawing attention to the known interaction between glycine and $GABA_A$ receptor function and inhalants (Beckstead *et al.* 2000). In summary, our metabolomics and electrophysiological analyses suggest that toluene may lead to increased glutamate and glycine release, with concomitant effects on NMDA and $GABA_A$ receptors.

The effect of binge toluene exposure on goal-directed action

After chronic intermittent exposure to toluene during adolescence, adaptive behaviour was impaired in adulthood as assessed by instrumental reversal and contingency degradation tasks. These tasks require updating of goal-directed action: specifically, the response-outcome associations encoded during the acquisition of such actions and the ability to use these associations to guide

action selection. In the reversal task, the Tol group was able to reverse the response-outcome relationships, but performance in the initial test suggests that they required more training than controls to do so. Similar deficits in reversal-learning have been reported in instrumental performance for sucrose after toluene exposure (Dick *et al.* 2014a) as well as in the Morris water maze, where animals were slower to learn the new location of a submerged platform but were able to do so with more training trials (Win-Shwe *et al.* 2010; Hass *et al.* 1999). In the contingency degradation task, air-exposed controls decreased performance of a response that was no longer causal in producing its associated outcome, but maintained performance of the response where the response-outcome contingency was intact. The Tol group was insensitive to this manipulation showing no differential responding at test. Similar response patterns have been demonstrated following lesions of the PL, hippocampus and the major afferent to hippocampus in the entorhinal cortex (Corbit and Balleine 2003; Corbit and Balleine 2000; Corbit *et al.* 2002; Coutureau *et al.* 2012; Devenport 1979; Devenport and Holloway 1980), regions where we observed changes in GABA and NMDA receptor content in the current study, respectively.

The toluene group also performed differently to the Air group in the progressive ratio and delay discounting tasks. Here animals were willing to exert more effort and to tolerate a longer delay to obtain reward than controls. As there were no group differences in initial training, consumption during devaluation tests, or body weight at this time, it seems unlikely that these effects can be explained by group differences in motivation or appetite. This is further supported by the fact that, despite chronic intermittent exposure to toluene during adolescence resulting in attenuated weight gain (as shown previously Dick 2014), body weight had normalised prior to when progressive ratio, delay discounting and contingency degradation tasks were performed. Although our previous studies did not show an effect of adolescent toluene exposure on a progressive ratio task (Dick *et al.* 2014a), there are some major differences in the schedules of reinforcement used in these studies that may influence responding, including the training schedules (random-ratio-20 vs fixed ratio-3), the increment used in the progressive ratio task (a step of 10 vs. 1 lever presses) and time point when the test was performed. Therefore, it is possible that the higher schedules of reinforcement used in the current study are needed to detect toluene influences on performance. Alterations to break-point in the progressive ratio task occur after lesions of the dorsal hippocampus, vmPFC and VMS (Gourley *et al.* 2010; Schmelzeis and Mittleman 1996; Hamill *et al.* 1999; Schneider and Koch 2005), and continued preference for the lever delivering the large reward in the delay discounting task after lesions of the vmPFC or hippocampus (Mar *et al.* 2011; Mariano *et al.* 2009; McHugh *et al.* 2008; Cheung and Cardinal 2005). Again, these are regions where differences were observed in GABA or NMDA receptor content following toluene exposure in the current study. It is also of note that the VMS is involved in tasks that enable an animal to overcome work-related response costs (Salamone

et al. 2012; Walton *et al.* 2006). Hence, a deficit in VMS function may have led to greater tolerance for delay and effort in these tasks, because of a discrepancy in the calculation of effort expenditure relative to outcome value

While our findings support those reported following chronic low-dose exposure to toluene (Beasley *et al.* 2012) they do not support our previous study where toluene exposed animals were slower to acquire lever pressing (Dick *et al.* 2014a). Again this may be due to the differing schedules of reinforcement, as discussed above, or alternatively that the Pavlovian training that occurred prior to the instrumental training in the current study may have made acquisition less taxing and more easily acquired by the Tol group. Of further importance is that toluene exposure did not impact performance on the outcome devaluation task. Other drugs of abuse have been shown to accelerate the transition to habitual behaviour using this task; specifically, prior exposure to cocaine, amphetamine or alcohol leads to insensitivity of lever-press performance to outcome devaluation, an indication of stimulus-driven or habitual responding (Nelson and Killcross 2006; Nordquist *et al.* 2007; Corbit *et al.* 2012; Corbit *et al.* 2014). Performance on this task is known to be reliant of the dorsal striatum (Yin *et al.* 2004; Corbit *et al.* 2014; Zapata *et al.* 2010; Corbit *et al.* 2012), and prior exposure to these drugs also affects the expression of glutamate receptors in this region (Loftis and Janowsky 2000; Ghasemzadeh *et al.* 2009b; Ghasemzadeh *et al.* 2009a). However, there was no evidence of such changes in the dorsal striatum after toluene exposure in the current study, suggesting differences between the effects of toluene and other drugs of abuse, at least under the current Tol exposure regimen [but see (Williams *et al.* 2005)].

NMDA and GABA subunit expression

When assessed 10 weeks after the last exposure to toluene and following subsequent behavioural training, we observed increased expression of GluN2A and GluN2B receptor subunits in the hippocampus, as well as PSD95, which is co-localised with NMDA receptors, increasing their surface expression and opening rate at excitatory post-synaptic neurons (Lin *et al.* 2006). Our findings are consistent with the effects of toluene on these NMDA subunits in hippocampus with shorter toluene abstinence periods (Ahmed *et al.* 2007; Win-Shwe *et al.* 2010; Williams *et al.* 2005) and thus, suggest that short-term effects of toluene on these receptor subunits are maintained. This increased density of NMDA subunits may contribute to altered NMDA receptor function in hippocampus. For example, it has previously been shown that exposing adolescent rats to toluene enhances NMDA-mediated excitatory postsynaptic currents (EPSC) in hippocampus which is indicative of enhanced NMDA receptor function (Chen *et al.* 2011). Similarly, prolonged toluene treatment of primary cultures of hippocampal neurons from rat pups not only increases

NMDA-mediated EPSCs, but also increases sensitivity to exogenous NMDA while increasing NR2A and NR2B subunit expression in the same tissue (Bale *et al.* 2005). Thus, toluene alters both the composition and functioning of NMDA receptor channels (Bale *et al.* 2005). This idea is also supported by our metabolic and electrophysiological data, as well as that of others (Beckley and Woodward 2011; Cruz *et al.* 1998) illustrating that toluene alters glutamatergic signalling and is suggestive of enhanced neuronal excitability following long-term exposure. We have also previously shown that NMDA receptor binding is altered at the end of the toluene exposure paradigm (Dick *et al.* 2015a) resulting in long-term changes in behavioural sensitivity to MK801 (Duncan *et al.* 2014). Notably, the hippocampus is important for progressive ratio and delay discounting (as discussed above), and manipulation of NMDA receptor function alters performance on these tasks (Floresco *et al.* 2008; Cottone *et al.* 2013; Higgins *et al.* 2016; Yates *et al.* 2015; Popke *et al.* 2001; Wright *et al.* 2007a; Wright *et al.* 2007b; Buffalo *et al.* 1993). Thus, altered hippocampal glutamate function following toluene may contribute to the observed behavioural changes.

We also observed changes in $GABA_A\alpha1$ subunit expression, which was reduced in vmPFC but increased in PL and VMS, consistent with Williams et al. 2005. These findings were also consistent with our metabolic data where alterations to GABA function were observed. Of particular interest was the clustering of toluene with the GAT1 inhibitor, CI-966, given that alterations to GAT1 activity change $GABA_A\alpha1$ expression in cortical tissue (Ohnuma *et al.* 1999). Overall, the observed changes in GAT1 and $GABA_A\alpha1$ in the current study are suggestive of reduced GABA transmission in PL and the VMS (Ohnuma *et al.* 1999; Volk and Lewis 2002). Not only have PL and VMS been shown to have an important role in the behavioral tasks where deficits were observed in the current study (as discussed above), but $GABA_{A,}$ and specifically $GABA_A\alpha1$ in PL, are important for delayed discounting and contingency degradation tasks, respectively (Cardinal *et al.* 2000; Thiebot *et al.* 1985; Butkovich *et al.* 2015). Thus, changes to GABA function in these regions following toluene exposure may play a role in the observed behavioural deficits.

The proposed neurocircuitry for flexible behaviour is likely to involve excitatory projections from vmPFC, PL, and hippocampus to VMS (Hayes *et al.* 2014; Cardinal 2006; Everitt and Robbins 2005). Hence, changes to protein content across these regions may interact to alter performance on the tasks used in the current study. Overall, our collective findings suggest altered activity at both excitatory and inhibitory synapses in this circuitry because NR2A and NR2B expression were increased in hippocampus, whereas $GABA_A\alpha1$ expression was increased in PL and VMS. Notably, vmPFC and PL subdivisions of the cortex are recognized to have different, and sometimes opposing, roles in cognitive and behavioural flexibility (Everitt and Robbins 2005). Thus, the differential regulation of $GABA_A\alpha1$ in these regions suggests alterations in the contribution of each structure to

observed behaviour. Further research is warranted to determine the intricate associations between the observed protein changes across these regions and the resulting behavioural outcomes. This is particularly true given that, at this stage, we cannot determine whether persistent alterations in protein content following toluene exposure directly affects behavioural performance, or whether the differences in protein content are due to toluene's ability to alter experience dependent synaptic plasticity that parallels behavioural testing. Although prior findings suggest that the initial effects of toluene on these subunits were maintained in the current study (Ahmed *et al.* 2007; Win-Shwe *et al.* 2010; Williams *et al.* 2005), long abstinence periods in behaviourally naive animals would be required to make this determination with certainty.

Conclusions

We demonstrated that toluene affects the metabolomic fingerprint for glutamatergic and GABAergic systems and can inhibit NMDA receptor signalling. Chronic intermittent exposure to toluene in adolescence leads to deficits in behavioural flexibility including problems with updating response-outcome associations and in effort-outcome analysis. This was associated with alterations in $GABA_A\alpha1$ and GluN2A- and GluN2B- NMDA glutamate receptor subunits in regions associated with altered behaviour and are suggestive of increased glutamatergic excitability in hippocampus, and altered GABAergic signalling in mPFC and VMS. Together these data suggest that exposure to toluene is sufficient to induce adaptive responses in both the glutamatergic and GABAergic systems which may underlie the observed behavioural changes following repeated exposures. Such deficits would serve to reduce flexible decision-making and promote maladaptive behaviour including susceptibility to further drug-use.

ARRIVE guidelines have been followed:
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Acknowledgements
The research reported in this paper was supported by the National Health and Medical Research Council of Australia, of which BWB is Senior Principal Research Fellow (1079561), AJL a Principal Research Fellow (1020737) and CDR a Senior Research Fellow (630516), contributions from project grant (568767); by grants from the Australian Research Council (FL0992409 to BWB and DP110100379 to JRD) and of which JRD was a Future Fellow during the time of the study

(100100235); and by the Victorian Government's Operational Infrastructure Support Scheme. The funding sources had no involvement in the design, analysis and decision to publish.

Conflict of interest statement

The authors declare no conflicts of interest.

Task	Treat Tol/Air	Break	Pav. train.	Instrum. train.	PIT	Outcome deval.	Selective reinstat.	Rev. 1	Rev. 2	Conting. Degrad.	Pro. ratio	Delay discount	Food given
PND	27-55	55-61	62	70	81	82	87	88	99	106	117	119	132
Day	28	7	8	11	1	5	1	11	7	11	2	13	3

Table 1: Timeline of behavioural procedures. Task: shows each of the behavioural tasks performed following Toluene or Air treatment. PND: shows the approximate post-natal day in which the task commenced. Day: shows the number of days required to complete each task (including retraining that was required before the task commenced).

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Figure 1: Effects of toluene on ^{13}C incorporation and on total metabolite pool sizes in brain cortical tissue slices incubated for 40 min with 2 mM sodium[3-^{13}C]pyruvate. (**A**) Total incorporation of label into each measured carbon position of metabolites Glu, GABA, lactate, Gln, Asp, Ala and citrate. (**B**) The total metabolic pool sizes of each measured metabolite. N = 4. Statistically significant changes are indicated by * ($p<0.05$, different to control) and # ($p<0.05$, different to other toluene concentration). Data presented as Mean ± SEM. Ala: Alanine; Asp: Aspartic acid; Cit: Citrate; GABA: gamma-aminobutyric acid; Glu: glutamic acid; Gln: glutamine; Lac: Lactate

Figure 2: PCA of labelled and total metabolite concentrations for toluene and A, Ligands active in the glutamatergic system and B, Ligands active in the GABAergic system. (**A**) Shows the first two principal components (PCs) of the multivariate analysis. This plot represents a metabolic "footprint" of the glutamatergic system and demonstrates separation of metabolic profiles according to metabolic activity. Red dots (unfilled) 0.3 mM toluene; filled red dots, 3.0 mM toluene; blue diamonds, exogenous glutamate; green squares, ketamine or riluzole, as labelled; grey squares, other glutamate drugs. (**B**) Shows the first two PCs of the multivariate analysis and represents a metabolic "footprint" of the GABAergic system. Red dots (unfilled) 0.3 mM toluene; filled red dots, 3.0 mM toluene; green diamonds (unfilled) 20 µM cis-aminocrotonic acid (CACA), filled green diamonds 100 µM CACA; blue diamonds 20 µM CI-966; grey squares, other GABA drugs. The large outer ellipse represents the 95% confidence interval (Hotellings score). Grey ellipses show data which cluster with toluene in 3D space and are not representative of any statistical construct.

Figure 3: Effects of toluene on heteromeric GluN1A/2A and GluN1A/2A/2B N-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus* oocytes. Representative responses to application of glutamate (100 µM) plus glycine (10 µM) without (blue) and with (red) toluene (3 mM) for (**A**) GuN1A/2A and (**B**) GluN1A/2A/2B NMDA receptors. Glutamate plus glycine responses are separated by 5 min. (**C**) Averaged peak currents in the absence and presence of toluene (3 mM). The peak currents in the presence of toluene were compared to those in the absence of toluene. Toluene significantly reduced current for both GuN1A/2A and GluN1A/2A/2B NMDA receptors. **(D**) Averaged percent inhibition in the presence of toluene (ns = no significant difference). (**$p< 0.01$;***$p<$ 0.0001). Data presented as Mean ± SEM.

Figure 4: Lever presses per min for PIT, outcome devaluation and selective reinstatement tests. (**A**) Both the Air and Tol groups demonstrated PIT as indicated by greater selection of the same lever over the different lever at test. (**B**) Goal-directed behaviour was demonstrated by both the Air and Tol groups during the outcome devaluation procedure where the lever associated with the non-

devalued outcome was selected more often than the lever associated with the devalued outcome. (**C**) A selective reinstatement was demonstrated by both groups by a preference for the lever that was associated with non-contingent outcome (Same lever) over the Different lever. * Indicates $p<0.05$. Data presented as Mean ± SEM, n=9 Air; n=10 Tol. CS: conditioned stimulus; PIT Pavlovian-to-instrumental transfer.

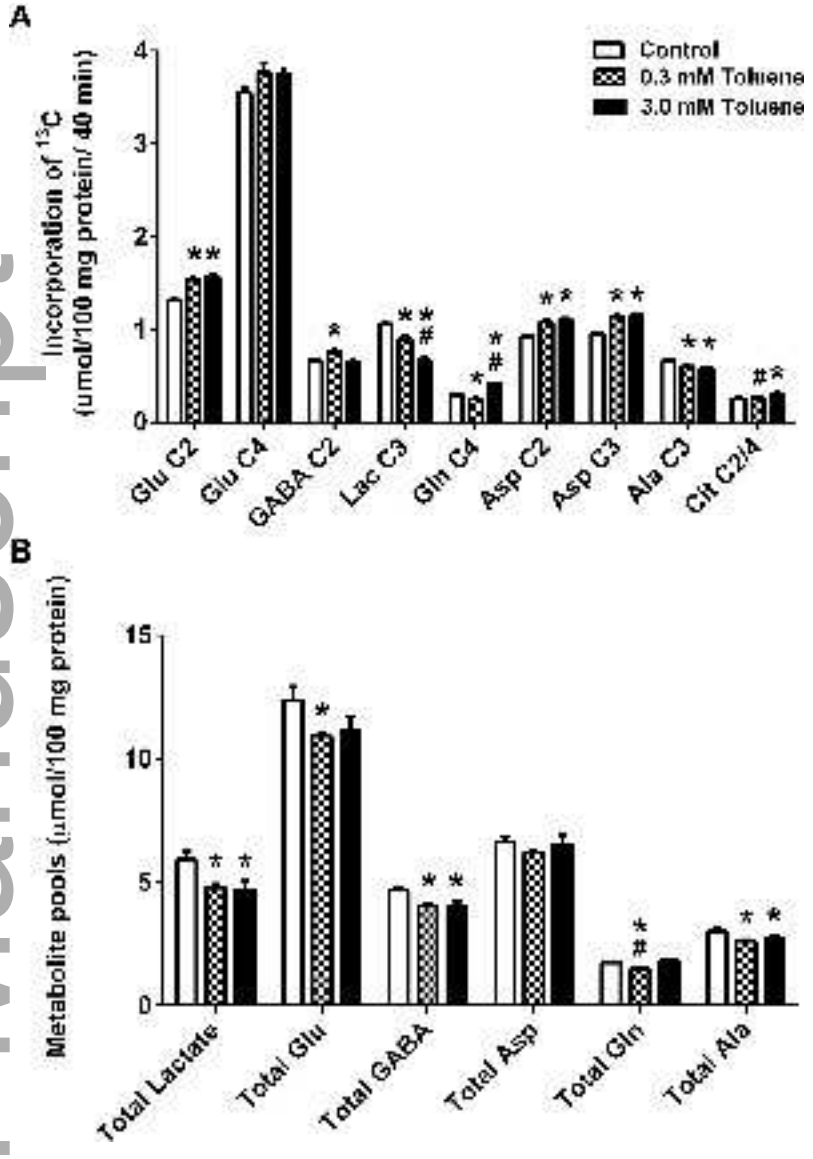
Figure 5: Lever presses per min for the reversal learning and contingency degradation tests. (**A**) A lack of preference for the non-devalued lever over the devalued lever in test 1 (left panel) suggested that the new reversed contingencies were yet to be learned by either group. However, a significant preference for the devalued lever by the Tol group was consistent with the original contingencies suggesting this group was slower to learn reversed contingencies than the Air group at this stage. After further training, both groups show reversal learning as indicated by preference for the non-devalued lever (right panel). (**B**) For the contingency degradation test, the Air group selected the non-degraded lever more often than the degraded lever indicating that delivery of a free outcome at training degraded the contingency of the associated lever. The Tol group, on the other hand, selected both levers equally demonstrating persistent selection of the degraded lever despite degradation of the lever-outcome contingency. * Indicates $p<0.05$. Data presented as Mean ± SEM, n=9 Air; n=10 Tol.

Figure 6. Progressive ratio and delayed discounting tasks (**A**) The total number of lever presses reached by the Tol group during the progressive ratio test was significantly greater than the Air group. (**B**) The time to reach breakpoint, where lever pressing ceased for a 2 min period, was also increased for the Tol group compared to the Air group indicating that the Tol group persisted for longer at this task. (**C**) Preference for the lever that delivered the large outcome over the lever that delivered the small outcome is shown for each delay. A decrease in this preference, and hence a shift to the small immediate outcome, was significantly greater for the Air compared to the Tol group with increasing delays. * Indicates $p<0.05$. Data presented as Mean ± SEM, n=9 Air; n=10 Tol.

Figure 7. Protein content for dorsal hippocampus. (**A**) Representative Western Blots for each protein examined in the dorsal hippocampus. Specifically, $GABA_A\alpha1$ (55 kDa), GluN2A (170 kDa), GluN2B (180 kDa) and PSD95 (95 kDA) are shown for the Air and Tol groups. (**B**) In the dorsal hippocampus significant differences between Air and Tol exposed groups was seen for GluN2A, GluN2B, PSD95. Each protein was corrected for loading control, αβ-tubulin (52kDa). * Indicates $p<0.05$. Data presented as Mean ± SEM, n=9 Air; n=10 Tol.

Figure 8. Protein expression for $GABA_A\alpha1$, GluN2A, GluN2B and PSD95 in the prefrontal cortex (PFC). (**A**) For the ventromedial PFC the Tol group had reduced $GABA_A\alpha1$ and PSD95 protein expression. (**B**) For the prelimbic cortex $GABA_A\alpha1$ was increased in the Tol group compared to Air. There were also no differences between groups for any of the proteins examined for cingulate cortex (**C**) or lateral/orbital PFC (**D**). Each protein was corrected for loading control, αβ-tubulin (52kDa). * Indicates $p<0.05$. Data presented as Mean ± SEM, n=9 Air; n=10 Tol.

Figure 9. Protein expression for $GABA_A\alpha1$, GluN2A, GluN2B and PSD95 in the striatum. (**A**) For the ventromedial striatum there was a significant increase in $GABA_A\alpha1$ in the Tol group compared to Air group. No differences were found between groups for any of the proteins examined in dorsomedial striatum (**B**), or dorsolateral striatum (**C**). Each protein was corrected for loading control, αβ-tubulin (52kDa). * Indicates $p<0.05$. Data presented as Mean ± SEM, n=9 Air; n=10 Tol, except for the PL n=9 Air; n=8 Tol.



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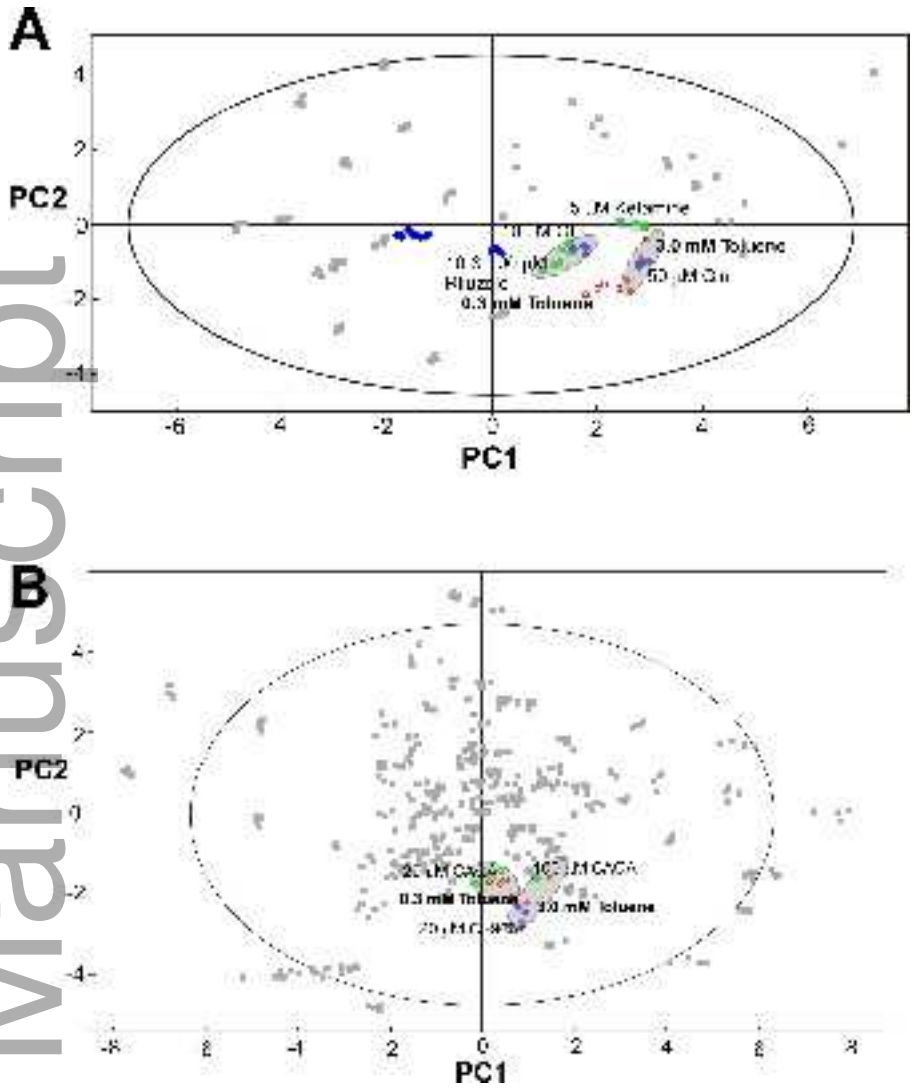

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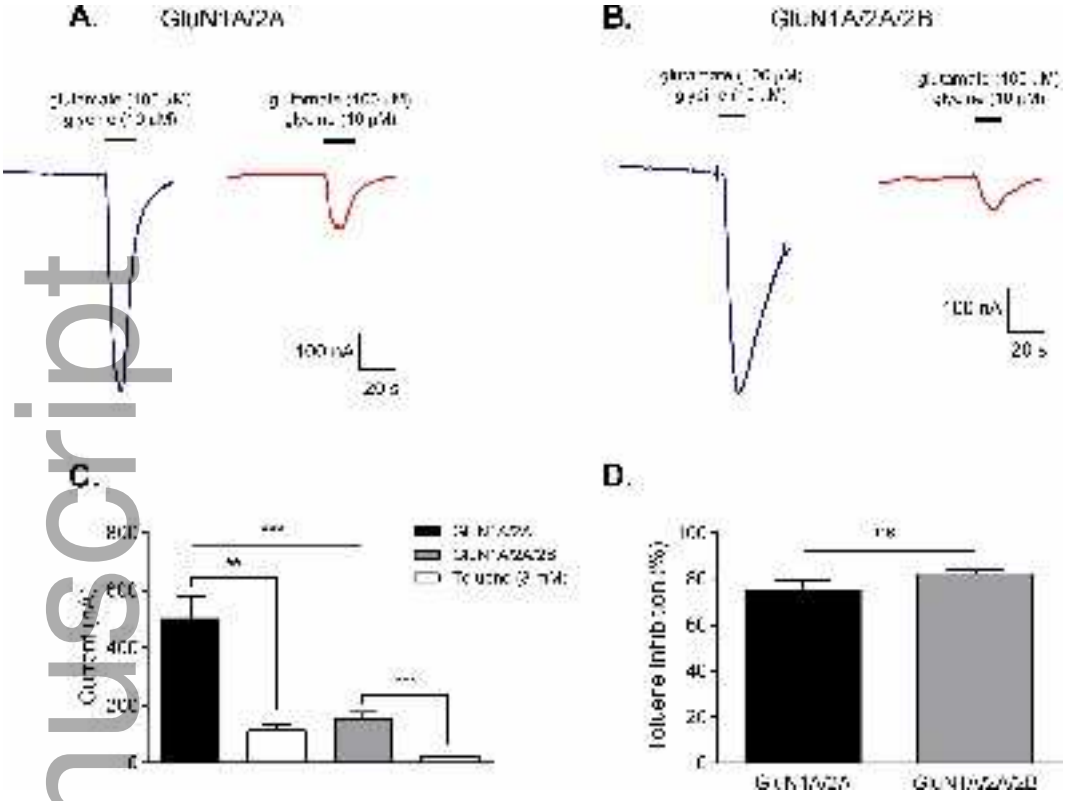

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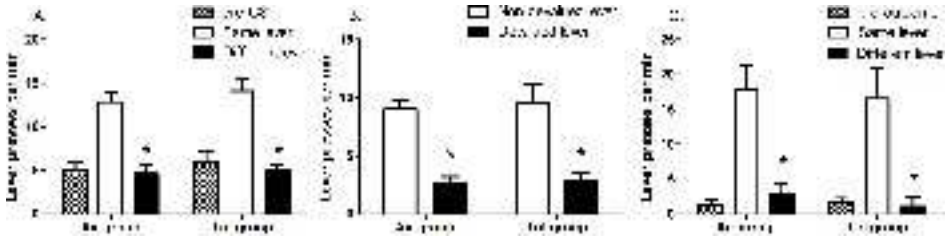

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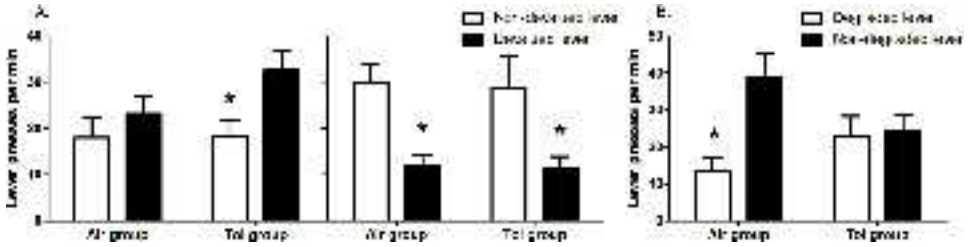

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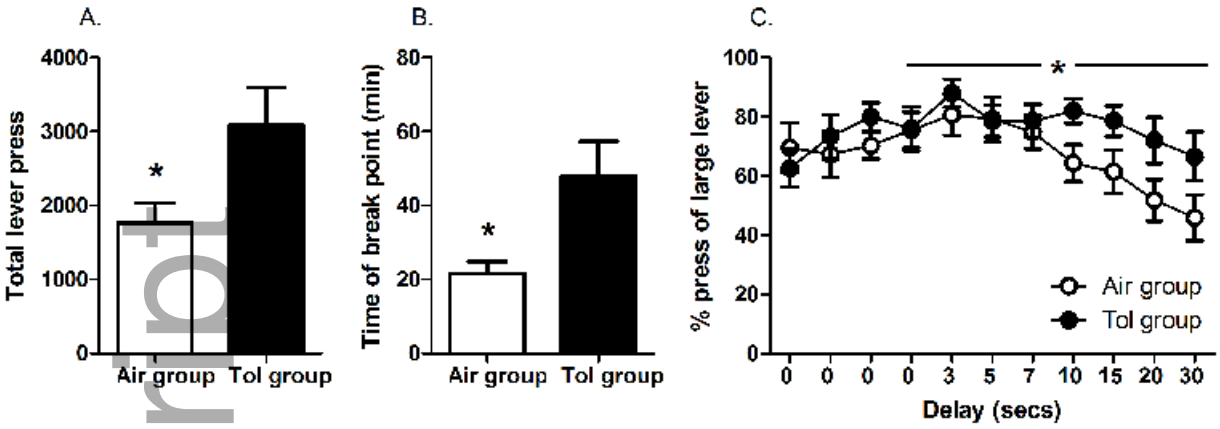

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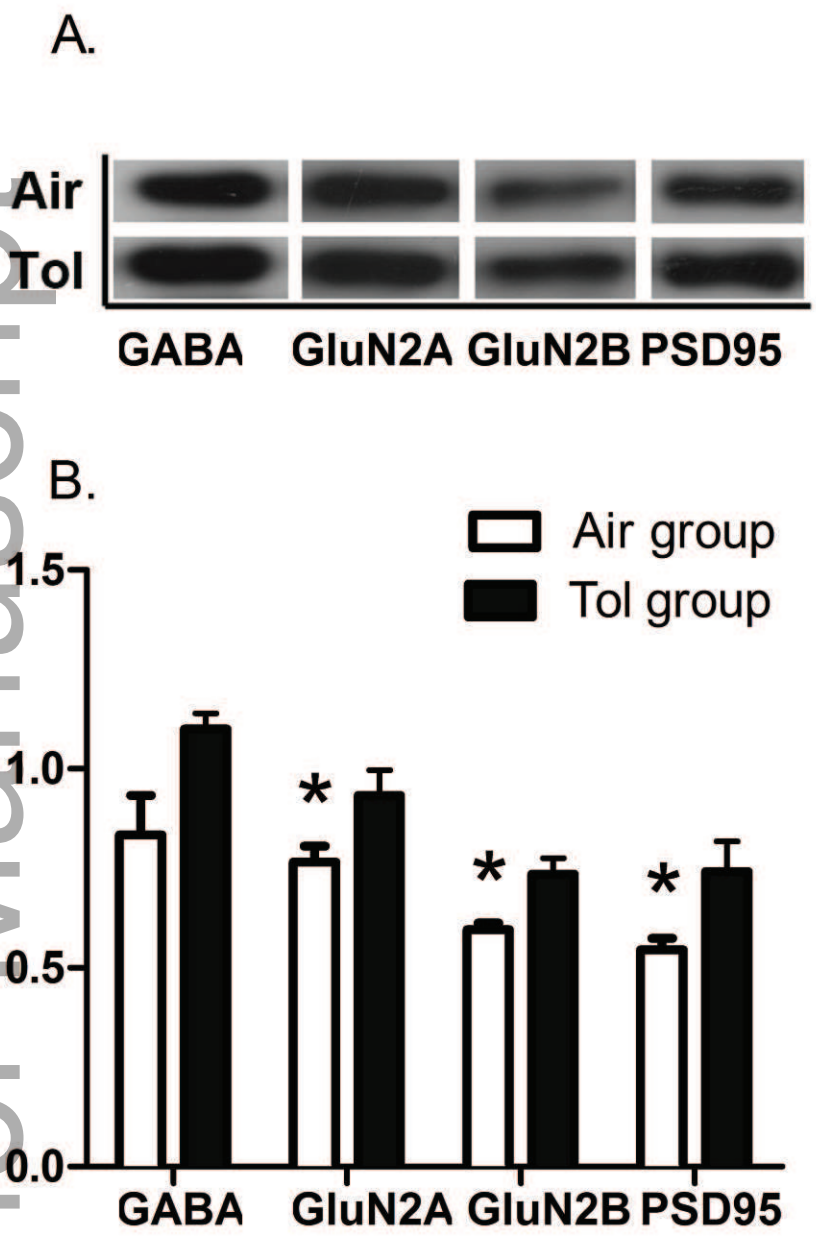

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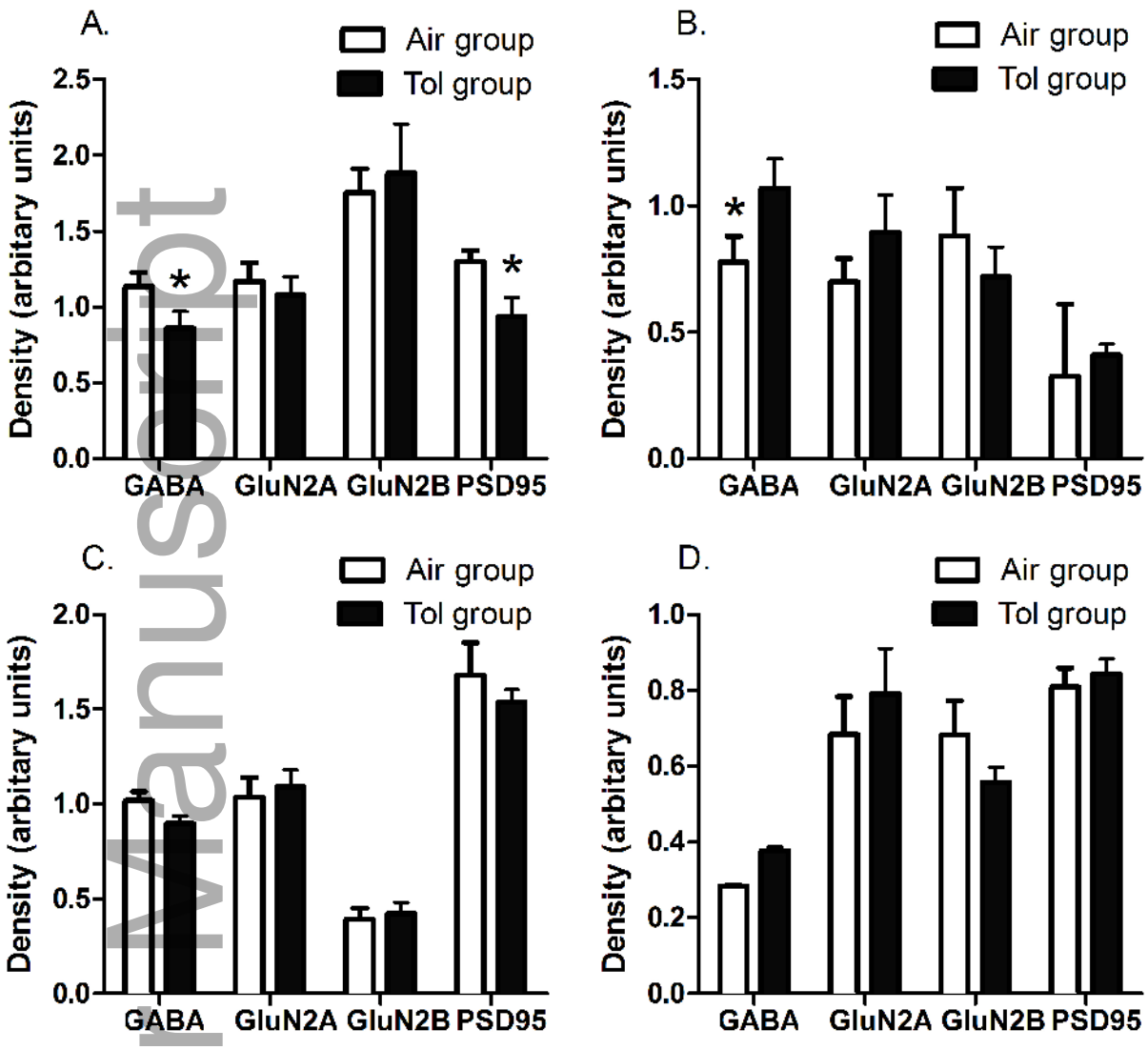

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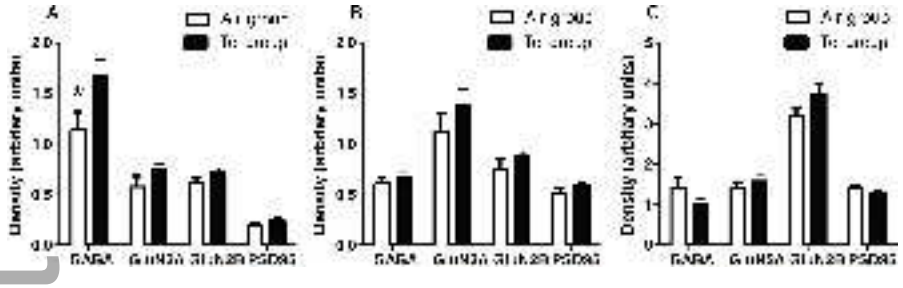

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Title:
Toluene inhalation in adolescent rats reduces flexible behaviour in adulthood and alters glutamatergic and GABAergic signalling

Date:
2016-12

Citation:
Furlong, T. M., Duncan, J. R., Corbit, L. H., Rae, C. D., Rowlands, B. D., Maher, A. D., Nasrallah, F. A., Milligan, C. J., Petrou, S., Lawrence, A. J. & Balleine, B. W. (2016). Toluene inhalation in adolescent rats reduces flexible behaviour in adulthood and alters glutamatergic and GABAergic signalling. JOURNAL OF NEUROCHEMISTRY, 139 (5), pp.806-822. https://doi.org/10.1111/jnc.13858.