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RECOVERY FROM ACUTE TOLUENE INTOXICATION IS FACILITATED BY THE NMDA RECEPTOR CO-AGONIST D-SERINE, BUT NOT THE $GABA_A$ RECEPTOR ANTAGONIST PICROTOXIN

by

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THESIS

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ABSTRACT

Toluene is a volatile solvent found in many household products and when intentionally inhaled results in intoxication. In rats, acute inhalation of toluene results in sedation and neurological impairments, with marked increases in ambulation and vertical behaviour during the recovery period. Previous *in vitro* research has shown that toluene may exert its effects by inhibiting NMDA receptors, and / or by activating $GABA_A$ receptors. To test whether modulation of these receptors are also implicated in the changes in motor behaviour and neurological impairments resulting from toluene vapour inhalation, rats were injected with the NMDA receptor co-agonist D-serine (1000 mg/kg i.p.), the $GABA_A$ antagonist picrotoxin (0.05 mg/kg i.p.), or saline, and then received whole-body exposures to either 15 or 30 min of an abuse-relevant concentration of toluene vapour (~ 5000 ppm). Open field behaviours including locomotion, rearing, and grooming as well as neurological impairments were quantified before and after toluene vapour inhalation. The results indicate that D-serine increases the speed of recovery from ambulatory and neurological impairments following 30 min (but not 15 min) exposure to toluene, suggesting an important role for NMDA receptors in the behavioural impairments induced by prolonged toluene intoxication. In contrast, picrotoxin did not affect recovery from toluene intoxication, suggesting that $GABA_A$ receptors are not implicated in the effects of toluene vapor inhalation, at least at the dose of toluene and exposure durations tested.

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ABBREVIATIONS

ABD	agonist-binding domain
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ANOVA	analysis of variance
CA1	cornu ammonis area 1 of the hippocampus
CBR1	cannabinoid receptor 1
DAAO	D-amino acid oxidase
DD	dopamine deficient
EPSC	excitatory postsynaptic current
GABA	γ-Aminobutyric acid
$GABA_A$	$GABA_A$ receptor
$GABA_B$	$GABA_B$ receptor
HR-MAS ^{1}H-MRS	high-resolution magic angle spinning proton magnetic response spectroscopy
IC50	half maximal inhibitory concentration
i.p.	intraperitoneal
IPSC	inhibitory postsynaptic current
MK801	Dizocilipine
mPFC	medial prefrontal cortex
NAA	N-acetyl-aspartate
NAC	nucleus accumbens
NMDA	*N*-Methyl-D-aspartic acid

PCP	phencyclidine
PD	Parkinson’s Disease
RRN	retrorubual nucleus
THIP	4,5,6,7-tetrahyroisoxazolo[5,4-c] pyridin-3-ol
VEP	visual-evoked potential
vMPJ	ventral mesopontine junction
6-OHDA	6-hydroxydopamine

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CHAPTER 1 - INTRODUCTION

1.1. Toluene use and abuse

Inhalation of volatile solvents ("inhalants") for recreational purposes is a worldwide phenomenon. Solvents are inhaled to reach a high characterized by euphoria and are more commonly abused in adolescent populations living in isolated, remote and impoverished communities (Embleton, Mwangi, Vreeman, Ayuku, & Braitstein, 2013). Of the solvents commonly used for recreational purposes, most contain the aromatic hydrocarbon toluene (methyl benzene). Toluene is a volatile solvent found in common household products including paints, thinners, adhesives, lacquers, disinfectants and gasoline. Toluene is highly lipid soluble, giving it the ability to readily cross the blood brain barrier and making it particularly toxic for lipid rich tissues like the brain. Long-term toluene abuse can lead to deficits in cognitive function, decreased intelligence scores, personality changes, gait/motor impairments and severe brain damage including significant white matter loss (Filley, Halliday, & Kleinschmidt-DeMasters, 2004; Fornazzari, Wilkinson, Kapur, & Carlen, 1983; Rosenberg et al., 1988). Acutely, toluene vapour inhalation results in robust motor and neurological impairments which have been studied in both humans and rodents.

There are several methods for inhaling solvents including sniffing (breathing in solvents directly from the container or a heated pan), bagging (filling a bag with liquid, holding the opening of the bag around the mouth and nose, and inhaling) and huffing (soaking a cloth and inhaling the liquid while covering the mouth and nose). A typical user will inhale for tens of minutes at a time, and inhale anywhere between 5000 and 15

000 parts per million (ppm) of solvent (Brouette & Anton, 2001; Marjot & McLeod, 1989; Wilkins-Haug, 1997). Products containing volatile solvents are typically abused by young adolescents, which may be due to ease of availability and low cost. In a recent survey of Ontario students, inhalant use was highest in Grade 8 students and declined with age, contrary to all other drugs surveyed, where use increases with age (Boak, Hamilton, Adlaf, & Mann, 2013). The same survey also noted that although solvent abuse is on the decline, solvents are still more often used by students in grade 7-12 over the past year in comparison to salvia, OxyContin, synthetic cannabis and non-prescription use of "ADHD"-related medications (Boak, et al., 2013). The prevalence of toluene use may be even higher in school-aged children not currently attending a formal educational institution.

Although solvent use occurs around the world and at a relatively high prevalence in some communities, our understanding of how these solvents affect the brain has lagged behind other recreational drugs.

1.2. Toluene-induced behavioural impairments in humans

Studies of the acute behavioural effects of toluene in humans are limited due to a lack of controlled manipulations and difficulty accessing populations of recently intoxicated users. The studies conducted with human participants have shown that toluene intoxication is characterized by robust motor and neurological deficits. One such study looked at children recently hospitalized for glue sniffing and noted a series of impairments including staggering gait, dysarthria, nystagmus and intention tremors (King, 1982). Similarly, adults pulled over while driving under the influence of toluene

had high blood toluene concentration levels and were exhibiting behavioural symptoms including slurred speech, staggering gait and appeared to be in a ‘twilight state’ in which actions were performed without conscious awareness and immediately forgotten (Capron & Logan, 2009). Another study saw similar impairments in drivers which were characterized by the same ‘twilight state’ and staggering gait and slurred speech, although tremors were also evident (Miyazaki, Kojima, Yashiki, Chikasue, & Tsukue, 1990). Acute toluene-induced tremors are not active at rest, suggesting that the origin of these tremors may be caused by striatal, pyramidal or cerebellar dysfunction and appear to be similar to tremors produced by Parkinson’s disease (PD) (Miyagi, Shima, Ishido, Yasutake, & Kamikaseda, 1999). Chronic toluene abuse can lead to resting tremors, present even while the user is sober. These tremors have been described as either postural tremors (tremors seen when an individual voluntarily holds a position against gravity, for example, when arms are outstretched), or intention tremors (tremors present during goal-directed movement). Along with tremors, other peripheral behavioural disruptions including myoclonus (the involuntary jerking of a muscle or muscle group) have been noted in long-term toluene users (Arai, Tokumaru, Yagishita, Hirayama, & Iwasaki, 1986; Sugiyama-Oishi et al., 2000). These behavioural manifestations are similar to levodopa-induced dyskinesia seen in PD patients. Both PD dyskinesia and toluene-induced deficits resulting from cerebellar atrophy and decreased white matter in limbic structures were treated with amantadine (a non-selective NMDA receptor antagonist) suggesting that these behaviours may result from glutamatergic dysfunction (Deleu & Hanssens, 2000; Fox, 2013).

Neurological examinations have found robust neurological deficits in youth admitted to a substance abuse rehabilitation clinic, where more than half of the solvent users showed pathological findings in a variety of tests including a neurological assessment which measured pyramidal, peripheral and sensory nerve involvement (Uzun & Kendirli, 2005). This study employed several techniques including; electroencephalography (EEG), nerve conduction studies and somatosensorial (SEP), visual (VEP) and brainstem auditory (BAEP) evoked potentials (Uzun & Kendirli, 2005). These findings may be the result of brain stem changes, as more participants showed changes in brain-stem evoked potentials compared to somatosensory evoked potentials (Uzun & Kendirli, 2005). In another study, adult long-term toluene users were rated based on performance in a neurological test battery and results showed that cranial nerves I, II and III were impaired in a significant number of users (speaking to decreased olfaction, optic atrophy and sensory-neural hearing loss) (Fornazzari, et al., 1983). These patients also exhibited cerebellar symptoms, and peripheral tactile deficits as evidenced by decreased distal touch sensation and decreased ankle jerk responses (Fornazzari, et al., 1983).

Taken together, it is clear that toluene use results in robust motor and neurological impairments, both acutely and over time, although these studies are limited due to lack of experimental manipulation. Therefore animal models of acute toluene intoxication have been developed to further understand these behavioural manifestations and their biological underpinnings.

1.3. Toluene-induced behavioural impairments in rodents

Acute toluene exposure results in a series of behavioural impairments in rodents, but is most characterized by hyperlocomotion during the recovery period. Locomotor behaviour was not shown to be altered following 20 min exposure to 2000 or 8000 ppm toluene, while exposure to 4000 ppm toluene did increase locomotion, indicating a potential dose-response relationship between toluene and locomotor behaviour (Bowen, Charlesworth, Tokarz, Wright, & Wiley, 2007). Similarly, mice exposed to 30 min of 0, 100, 2000, 8000 or 10 000 ppm toluene revealed increased locomotor activity when doses of toluene were < 8000 ppm, whereas following exposures ≥ 8000 ppm toluene, a biphasic response where locomotion was first increased and then followed by hypoactivity was noted (Batis, Hannigan, & Bowen, 2010). A comparison of adolescent and adult rats in a motor behavioural and neurological assessment assay showed that adolescent, young adult, adult and older adult rats all exhibit increased locomotion, impaired beam crossing, impaired gait and increased neurological impairments following an acute exposure to 15 min or 30 min of 5000 ppm toluene (Samuel-Herter, Slaght, & McKay, 2014). Following injection of 250-750 mg/kg (i.p.) toluene, rats exhibited hyperlocomotor behaviour and motor deficits in a rotarod task; the hyperlocomotion and motor coordination deficits were attenuated by the NMDA receptor co-agonist D-serine (Lo, Wu, Sue, & Chen, 2009). Rats injected with 600-1200 mg/kg (i.p.) toluene exhibited an inverted U-shape dose response curve to the locomotor stimulating effects of toluene, when behaviour was tested every 20 min for 3 hr following toluene injection, and these hyperlocomotive effects were blocked by the selective D_2 dopamine antagonist remoxipride (Riegel & French, 1999). A 6-hydroxydopamine (6-OHDA) lesion of the

nucleus accumbens (NAC) or pre-treatment with the metabotropic mGlu2/3 receptor agonist LY379268 also attenuated toluene-induced increases in locomotion (Riegel, Ali, & French, 2003).

Open field behaviours other than locomotion have also been quantified during and following toluene exposure and include findings of decreased rearing behaviour (Bowen, Batis, Paez-Martinez, & Cruz, 2006; Duncan et al., 2012; Tegeris & Balster, 1994). Decreased rearing behaviour following toluene inhalation may be the result of decreased balance and thus an inability to stand on the hind legs, as toluene-exposed animals do display difficulty in maintaining balance in balance beam and rotarod tasks (Chan, Chung, Stoker, Markou, & Chen, 2012; Chan, Lee, Lin, Wu, & Chen, 2012; Lo, et al., 2009; Samuel-Herter, et al., 2014). D-serine was able to attenuate the motor incoordination effects produced by toluene in a rota rod task suggesting that decreased balance behaviour may be the result of NMDA receptor hypo function (Lo, et al., 2009). Grooming behaviour also decreased following toluene exposure, which may be similarly affected by balance issues, or may indicate an anxiolytic effect of toluene (Samuel-Herter, et al., 2014). Toluene has been shown to act as anxiolytic in rats, as exposure to 1000-6000 ppm toluene increased time spent in the open arms of an elevated plus maze (Bowen, Wiley, & Balster, 1996). Similarly in mice, toluene dose-dependently (0, 1000, 2000 or 4000 ppm toluene for 30 min) decreased measures of anxiety in both a burying behaviour and plus-maze task (Lopez-Rubalcava, Hen, & Cruz, 2000).

The presence of stereotypic (repetitive) behaviours have also been identified including hindlimb myoclonus, (the rhythmic moving of the hind limb in a kicking/scratching motion) (Himnan, 1984). A neurological assessment battery

comparing the effects six alkylbenzenes on mice behaviour showed that 20 min exposure to between 2000 and 8000 ppm toluene decreased rearing, arousal, sensorimotor reactivity, mobility and increased gait abnormalities and clonic movements (Tegeris & Balster, 1994). Using an adapted neurological assessment battery, toluene has been shown to dose-dependently and age-dependently increase the presence of behaviours such as salivation, lacrimation, tremor and myoclonus either during exposure or during recovery following exposure to toluene (Samuel-Herter, et al., 2014). Salivation and lacrimation were only present within the exposure chamber, likely as a result of the irritant effects of toluene (Balster, 1987).

The behavioural profiles of acute toluene exposure (including hyperactivity at low doses, decreased activity at high doses, tremor and myoclonus) are similar to other drugs including the NMDA receptor antagonist phencyclidine (PCP) and the GABA receptor agonist ethanol. These similarities have led to studies seeking to understand the pharmacological profile of acute toluene inhalation.

1.4. The role of NMDA receptors in toluene intoxication

1.4.1. Behavioural evidence

Toluene produces a behavioural profile of effects similar to NMDA receptor antagonists that are characterized by hyperlocomotion, ataxia at higher doses, salivation, nystagmus and circling behaviour (Willetts, Rice, & Balster, 1990). These similarities are supported by the action of toluene as a partial substitute for PCP in a drug discrimination task where toluene dose-dependently acted as a partial substitute for mice trained to discriminate 2 mg/kg (i.p.) PCP (Bowen, Wiley, Jones, & Balster, 1999; Cruz,

Gauthereau, Camacho-Munoz, Lopez-Rubalcava, & Balster, 2003). As such, it is reasonable to suggest that toluene may exert its actions in a similar manner to NMDA antagonists like PCP. Following a 30 min exposure to 4000 or 8000 ppm toluene, mice were given a seizure-inducing dose of NMDA and behaviour was measured (Cruz, et al., 2003). Toluene dose dependently reduced the occurrence of seizures, increased the latency to reach an occurrence of seizure, decreased NMDA-induced neurological impairments and protected against NMDA-induced lethality indicating a potential antagonist effect of toluene at the NMDA receptor (Cruz, et al., 2003). There is further evidence to suggest that toluene acts specifically to inhibit the NMDA receptor as steady state pattern-elicited visual-evoked potentials (VEPs) were blocked by injection of the NMDA antagonist MK801 prior to toluene inhalation, and VEPs were unaffected when MK801 was injected post-exposure to toluene (Bale et al., 2007). Further, it was recently shown that administration of the NMDA receptor co-agonist D-serine (1000 mg/kg, i.p.) prior to toluene injection (250-750 mg/kg, i.p.) resulted in a decrease in the motor incoordination, hyperactivity and memory impairments typically seen following acute toluene injections (Lo, et al., 2009). There were some limitations to this study, including the difference between behaviour following injected and inhaled toluene, as well as the quantification of motor behaviour over 90 min of recovery. It will be necessary to replicate this study using an inhalation paradigm, and investigate in more detail the effects of D-serine on behaviour. Similarities between toluene and NMDA antagonists have also been demonstrated by studies completed *in vitro* at the cellular level.

1.4.2. Evidence from cellular recording

There is evidence to suggest that toluene does have direct effects on NMDA receptors. In *Xenopus laevis* oocytes, toluene dose-dependently and subunit-dependently inhibited recombinant NMDA receptors (Cruz, Mirshahi, Thomas, Balster, & Woodward, 1998). Specifically there was a high affinity for the NR1/2B subunit combination with an IC_{50} value for toluene-induced inhibition of 0.17 mM, while the NR1/2A and NR1/2C subunit combinations were 6 and 12 fold less sensitive respectively (Cruz, et al., 1998). Toluene did not significantly affect non-NMDA receptors indicating that toluene is specifically inhibiting ion channel gating, and not compromising the cell membrane as once postulated (Cruz, et al., 1998). In a whole cell patch clamp experiment of cultured hippocampal neurons, toluene dose-dependently inhibited NMDA receptor-mediated responses (with an IC_{50} of 1.5 mM) but did not affect AMPA or kainate receptor responses indicating specificity for NMDA receptors (Bale, Tu, Carpenter-Hyland, Chandler, & Woodward, 2005). Further, this study noted that prolonged toluene treatment (1 mM over 4 days) increased NR2A and NR2B, but not NR1, subunit expression, and this increased subunit expression led to greater whole-cell responses when NMDA was applied (Bale, et al., 2005). Although NR1 subunit levels were not increased following prolonged toluene exposure as measured by immunoblotting, an immunohistochemical analysis of cultured cells showed increased NR1 subunit density (Bale, et al., 2005). In a similar experiment looking at medial prefrontal cortex (mPFC) neurons, whole-cell patch clamp experiments showed that toluene dose-dependently (0.1 – 3 mM) inhibited NMDA-mediated excitatory post-synaptic currents (EPSCs) (Beckley & Woodward, 2011). This effect was cannabinoid receptor 1 (CBR1) sensitive indicating a potential

effect of toluene on CBR1 receptors, although whether this effect is direct or not has yet to be determined (Beckley & Woodward, 2011). When toluene was injected at 0, 200, 500 and 1000 mg/kg (i.p.) in neonatal rats from postnatal day (PN) 4 to PN 7, toluene dose-dependently decreased intracellular Ca^{2+} signals in response to exogenous glutamate/glycine and NMDA/glycine in cultured cerebellar granule cells (Chen, Wei, Lin, Chien, & Chan, 2005). This effect was attributed to the NR2B subunit as toluene had no effect on the inhibition produced by Mg^{2+} or MK801 but did decrease the potency of the NR2B preferring antagonist, ifenprodil (Chen, et al., 2005). Following 10 days of exposure to 8000 ppm toluene for 30 min/day, a Western blot analysis showed that the expression of the NR1, NR2B and GluR2/3 subunits were all increased in the mPFC, while the NR1 subunit alone showed increased expression in the substantia nigra compacta, and the NR2B subunit alone showed increased expression in the nucleus accumbens (Williams, Stafford, & Steketee, 2005). This increase in subunit expression may drive changes in glutamate levels in the brain.

1.4.3. Evidence from microdialysis and magnetic resonance spectroscopy

In vivo microdialysis experiments showed that the extracellular glutamate levels in the hippocampus of freely moving mice were rapidly and reversibly increased within 30 min following an acute injection of 150 or 300 mg/kg (i.p.) toluene, as measured by liquid chromatography (Win-Shwe et al., 2007). In a high-resolution magic angle spinning proton magnetic response spectroscopy (HR-MAS ^{1}H-MRS) study, young adult rats were found to be the most sensitive to acute (2 x 15 min) exposure to 8000-12000 ppm toluene as compared to adolescent and adult rats (O'Leary-Moore et al., 2009).

Toluene exposure resulted in decreases in choline and GABA levels in the frontal cortex and striatum and decreased glutamine and N-acetyl-aspartate (NAA) levels in the frontal cortex (O'Leary-Moore, et al., 2009).

1.5. NMDA receptors and D-serine

The above evidence suggests that toluene acts as an NMDA antagonist, similar to PCP. The NMDA receptor complex is well-characterized as a glutamate-gated ion channel with a voltage-sensitive Mg^{2+} block. D-serine has been identified as a potentially useful therapeutic for combating NMDAR hypo-function as it can be administered exogenously. In a clinical trial of patients with schizophrenia, D-serine ($\geq$ 60 mg/kg/day) was an effective treatment for the persistent symptoms and neurocognitive dysfunction (Kantrowitz et al., 2010).

Depletion of endogenous D-serine in serine-racemase knock-out mice led to behavioural changes including hyperlocomotion and increased anxiety (as measured by decreased exploration of the centre area in an open field task) (Basu et al., 2009). Studies have shown that the glycine site on NMDA receptors is not saturated *in vivo*, and that the introduction of exogenous co-agonists glycine or D-serine attenuated the hyperlocomotive behaviour caused by injection of MK 801 (0.2 mg/kg, i.p.) indicating a direct effect of glycine site activation on motor behaviour (Nilsson, Carlsson, & Carlsson, 1997). D-serine injected intracerebroventricularly (1.0 μmol/rat) attenuated PCP and MK 801 induced stereotypy and ataxia, indicating an ameliorative effect of D-serine on a variety of the behavioural effects of NMDA antagonists (Contreras, 1990). Further, MK 801 induced stereotypy and ataxia were reduced in mice lacking in D-amino acid oxidase

(DAAO) activity indicating that increasing levels of endogenous D-serine by limiting the regulatory break-down enzymes may combat drug-induced NMDA receptor antagonism (Hashimoto, Oka, & Nishikawa, 1995). Exogenously injected D-serine has been targeted as a potential therapeutic for disorders characterized by NMDA receptor hypofunction, and has been shown to attenuate the hyperlocomotion, motor incoordination and memory deficits induced by toluene injection (Lo, et al., 2009). Sarcosine (100 or 300 mg/kg, i.p.), an NMDA receptor co-agonist at the glycine site and a GlyT1 inhibitor, was shown to similarly reduce motor incoordination in a rota rod task, cognitive deficits in a novel object recognition task and toluene-induced hypothermia in mice (Chan, Chung, et al., 2012). This evidence suggests that D-serine may act to attenuate the increased ambulatory behaviour and motor incoordination resulting from toluene intoxication, and as it attenuates stereotypic behaviour and ataxia caused by other NMDA antagonists including MK 801 and PCP.

1.6. The role of GABA receptors in toluene intoxication

1.6.1. Behavioural evidence

The acute effects of toluene also share key similarities with the acute behavioural effects of ethanol including motor incoordination, hyperlocomotion at lower doses and decreased locomotion at higher doses. Many of the effects of ethanol mirror those of $GABA_A$ receptor agonists, as a 15% solution of 95% ethanol (20 mL/kg, i.p.) induced effects such as sedation, which were attenuated by the $GABA_A$ receptor antagonist picrotoxin (Liljequist & Engel, 1982). Toluene (300 – 5400 ppm) acted as a substitute for pentobarbital and ethanol (both substances known to activate GABA receptors) in

drug discrimination tasks (Rees, Coggeshall, & Balster, 1985; Rees, Knisely, Breen, & Balster, 1987). Toluene-induced prolongation of nystagmus (the result of a 30 min exposure to 1000 ppm toluene) following rotary acceleration was blocked by pre-treatment of baclofen (1, 3 and 5 mg/kg i.m.) and 4,5,6,7-tetrahyroisoxazolo[5,4-c] pyridin-3-ol (THIP) (5, 10 and 15 mg/kg i.m.), a $GABA_B$ and $GABA_A$ agonist, respectively (Tham, Larsby, Eriksson, & Niklasson, 1990). These results indicate that blocking GABA receptors blocks some of the effects of toluene. Additionally, toluene-induced CPP (developed following 14 pairings of 30 min exposures to 3000 ppm toluene) was blocked by 150 mg/kg (i.p.) of gamma-vinyl GABA, suggesting a role of GABAergic transmission in the addictive potential of toluene (Lee, Schiffer, & Dewey, 2004).

1.6.2. Evidence from cellular recording

Whole-cell patch clamp recordings from mPFC neurons revealed that toluene (0.3, 1 and 3 mM) enhanced stimulus-evoked GABA-mediated IPSCs; after TTX application, toluene continued to increase the amplitude and frequency of miniature IPSCs suggesting that the effects of toluene are action potential independent, and thus occur at the level of the synapse (Beckley & Woodward, 2011). Cultured rat hippocampal neurons when subjected to prolonged toluene exposure (1 mM over 4 days), had reduced responses to exogenously applied GABA and reduced amounts of synaptically-activated GABA-mediated currents (Bale, et al., 2005). Another study using whole cell patch clamp recordings in hippocampal neurons found that GABA synapses in CA1 pyramidal cells were facilitated by 1 mM toluene (MacIver, 2009). Whole cell

voltage clamp studies looking at the effect of toluene on $GABA_A$ receptors expressed in human IMR-32 neuroblastoma cells showed that toluene (10 or 30 μM) inhibited $GABA_A$ receptors; this low concentrations of toluene is typical for occupational toluene exposure, but not the higher abuse-relevant concentrations of toluene (Meulenberg & Vijverberg, 2003). Toluene-induced motor deficits may result from increased inhibitory synaptic transmission in the cerebellum, as toluene was recently shown to dose-dependently (0, 0.1, 0.136, 1.0 and 3.16 mM) reduce the frequency of Purkinje cell action potential output in whole-cell patch clamp preparations (Gmaz & McKay, 2014). Toluene exposure for 10 days (8000 ppm, 30 min/day) increased the $GABA_A$ α1 subunit in the mPFC and striatum, and decreased $GABA_A$ α1 subunit in the substantia nigra and VTA (Williams, et al., 2005). This indicates that $GABA_A$ receptor subunit expression is particularly sensitive to toluene exposure and extracellular changes in GABA levels may be significantly affected by toluene exposure.

1.6.3. Evidence from microdialysis and magnetic resonance spectroscopy

In a rat microdialysis study, extracellular levels of GABA in the cerebellum were increased following toluene exposure (2000 ppm for 2 hr), and this effect was blocked by tetrodotoxin indicating the increase in extracellular GABA was sodium action potential dependent (Stengard, Tham, O'Connor, Hoglund, & Ungerstedt, 1993). *In vivo* microdialysis showed that following the same acute inhalation of toluene (2000 ppm for 2 hr), extracellular GABA levels decreased in the globus pallidus during and after exposure, while striatal GABA levels increased post-exposure only (Stengard & O'Connor, 1994). HR-MAS 1H-MRS showed that acute exposure (2 x 15 min exposure

to 8000-12000 ppm) toluene reduced levels of GABA in the hippocampus (O'Leary-Moore, et al., 2009).

1.7. $GABA_A$ receptors and picrotoxin

Toluene may act as a CNS depressant by increasing the activation of GABA receptors much like ethanol, and like ethanol, the behavioural impairments of toluene intoxication may be blocked or attenuated by $GABA_A$ receptor antagonists. $GABA_A$ receptors are ligand-gated, ionotropic receptors found globally throughout the brain. They function as a binding site for the major inhibitory neurotransmitter GABA. Influx of Cl- ions acts to hyperpolarize the postsynaptic neuron and thus results in an inhibitory response. $GABA_A$ receptors with specific subunit combinations also allow for the binding of the allosteric modulating benzodiazepines at specific benzodiazepine binding sites.

Picrotoxin acts as a non-competitive channel blocker for the $GABA_A$ Cl^- channel and therefore acts to block inhibition in the postsynaptic cell (Carpenter, Lau, & Lightstone, 2013). At a low dose, picrotoxin may block the behavioural effects of acute toluene inhalation as 0.5 mg/kg (i.p.) picrotoxin did not result in changes in baseline locomotion but did reduce ethanol-induced sedation by increasing locomotor activity when injected prior to ethanol injection (Liljequist & Engel, 1982). The possibility then emerges that picrotoxin may reduce the ataxic behaviour seen following exposures to toluene. In contrast, systemic injections of picrotoxin at higher doses (1-4 mg/kg) resulted in increased masticatory movements, salivation, tremors and locomotion which are similar to the behavioural effects of toluene exposure indicating that picrotoxin may

act to worsen the behavioural impairments of toluene intoxication when used in higher concentrations (Chang, Wang, & Lin, 2004). Picrotoxin counteracted chlordiazepoxide (a sedative drug and known benzodiazepine) induced decreases in rearing and locomotor behaviour but had no effect on chlordiazepoxide-induced decreases in head dipping, although picrotoxin significantly decreased rearing and locomotor behaviours when injected alone (File, 1982). Picrotoxin (1.0 mg/kg, i.p.) increased bouts of rearing but did not increase rearing duration, indicating that antagonizing the inhibitory actions of $GABA_A$ receptors may result in increased exploratory behaviour, and perhaps reduced anxiety (Garg, 1969). In contrast picrotoxin (0.6 and 1.0 mg/kg s.c.) proves to be anxiogenic, increasing corticosterone serum levels in mice and decreasing the amount of time spent in the open arms of an elevated plus maze (Stankevicius, Rodrigues-Costa, Camilo Florio, & Palermo-Neto, 2008). Therefore, lower doses of picrotoxin may decrease exploratory behaviour and reduce the anxiolytic effects of toluene. Finally, following exposure to 5700 ppm toluene, animals showed increased AMPA/NMDA ratios at synapses of the mesolimbic core VTA dopamine neurons (similar to other abused drugs such as cocaine and may reflect initiation of long term potentiation (LTP)), and this effect was blocked by pretreatment with picrotoxin (Beckley & Woodward, 2011). Although picrotoxin appears to have complex dose-dependent effects on behaviour, the balance of the evidence suggests that picrotoxin should attenuate the behavioural effects of toluene.

1.8. Hypothesis and aims

Due to the similarities in behavioural effects of acute toluene intoxication in humans and rodents, a rodent model of toluene abuse will be employed to understand the potential underlying pharmacological roots of toluene-induced behavioural changes. This study aims to describe the effects of the NMDA receptor co-agonist D-serine on the well-studied motor and neurological behavioural impairments induced by abuse-relevant concentrations of inhaled toluene. Further, this study looks to explore the effects of the $GABA_A$ antagonist picrotoxin on toluene-induced behavioural impairments, as there is currently very little behavioural evidence that the motor and neurological impairments seen following acute toluene exposure are related to GABAergic transmission, although there is much cellular evidence to suggest that $GABA_A$ receptors are implicated in toluene intoxication. Based on the similar behavioural outcomes between toluene and known NMDA receptor antagonists, and the behavioural similarities between toluene intoxication and select aspects of PD pathology, it is expected that D-serine will attenuate toluene-induced behavioural impairments. Based on the similarities between toluene intoxication and ethanol intoxication, it is postulated that the $GABA_A$ receptor antagonist picrotoxin will attenuate the motor and neurological deficits following acute toluene intoxication.

CHAPTER 2 - METHODOLOGY

2.1. Animals

Male Long-Evans rats (Charles River Laboratories, St-Constant, Quebec, ~ 3.5 months old; n=44) were pair housed and maintained on a 12 hr light/dark cycle (lights on at 0700 hr) with food and water available *ad libitum*. Rats were allowed to acclimate to the facility for one week before handling. Rats were handled for two days (~ 5 min per day) immediately prior to the onset of experiments. All experiments were approved by the Wilfrid Laurier University Animal Care Committee and were in accordance with the guidelines established by the Canadian Council on Animal Care.

2.2. Drugs

All drugs were purchased from Sigma-Aldrich (Oakville, ON) and administered intraperitoneally (i.p.). D-serine was prepared at a concentration of 500 mg/mL in physiological saline and injected at a volume of 2 mL/kg (due to poor solubility in saline) for a final dose of 1000 mg/kg. The D-serine solution was prepared daily; the solution was warmed and stirred to ensure complete dissolution and allowed to cool to room temperature prior to injection. Picrotoxin was prepared at a concentration of 0.5 mg/mL in physiological saline and injected at a volume of 1 mL/kg for a final dose of 0.5 mg/kg. The picrotoxin solution was warmed and stirred until completely dissolved and was refrigerated and stored in a tinfoil-covered bottle due to light sensitivity. The picrotoxin solution was warmed to room temperature prior to injection. Vehicle (0.9% saline, injected at 1 mL/kg) was similarly refrigerated and allowed to warm to room temperature prior to injection.

2.3. Apparatuses

Rat behaviour was measured in a transparent, plastic open field environment (45 cm by 30 cm by 34 cm; l x w x h) with a grid of six squares (15 cm by 15 cm) painted on the underside of the floor. Mounted above the chamber was a web cam (Microsoft LifeCam HD 3000) which utilized Microsoft LifeCam.Ink software to record videos of each open field session for later quantification of behaviour. Videos were analyzed on Windows Media Player 2009. Vapor exposure to either toluene (VWR; Mississauga, ON) or air took place in two, identical, custom-built, plastic chambers (~6 L in volume). Each exposure chamber was equipped with two 90 mL, fluid-filled, plastic reservoirs secured to opposite corners. In the 'Toluene' chamber, the reservoirs contained 20 mL each of liquid toluene, while in the 'Control' chamber the reservoirs contained 20 mL each of distilled water. The reservoirs were equipped with plastic lids which had holes (~3 mm in diameter, covering ~30% of the lid surface) to allow for vapor release. An external air source was connected to each reservoir via plastic tubing (0.25" inside diameter) and room air was pumped into each reservoir at a rate of ~600 mL/min. In the 'Toluene' chamber this rate of air-flow resulted in a toluene vapour concentration of ~5000 ppm toluene (as measured in a previous study by gas chromatography (Perit et al., 2012)). The concentration of 5000 ppm is a behaviourally relevant dose, as human inhalant users have been shown to inhale anywhere between 5000 and 15 000 ppm (Wilkins-Haug, 1997).

2.4. Open Field assessments

Locomotion (defined as all four of a rat's paws crossing a line in the open field environment), rearing (defined as the rat lifting both forepaws off the floor) and grooming (defined as bouts of activity where both forepaws were lifted and used to rub the face, or one hindpaw was used to scratch the side) were analyzed manually from the video recordings of each open field session.

2.5. Neurological assessment

The battery of tests selected for the neurological assessment had previously been used for the quantification of neurological impairments caused by toluene inhalation (Samuel-Herter, et al., 2014), and were based on a subset of tests from the SHIRPA (SmithKlein Beecham, Harwell, Imperial College, Royal London Hospital, phenotypic assessment) neurological test battery (Rogers et al., 2001). Tests were scored as follows: 'body position' (0 = active, 1 = not active, where rats that exhibited active body position displayed movement and normal posture), 'tremor' (0 = not active, 1 = active, where tremors were typically present in the upper torso), 'eyes' (0 = open, 1 = closed), 'lacrimation' (0 = absent, 1 = present, where rats eyes watered, or tears were noted in the fur directly below the eye), 'startle' (0 = present, 0.5 = barely present, 1 = absent, where rats positive for startling behaviour responded to a loud, unprimed auditory stimulus), 'salivating' (0 = absent, 1 = present) and 'myoclonus' (0 = absent, 1 = present, where rats positive for myoclonus exhibited involuntary, repetitive, tic-like motions with any leg). All assessments were scored in real time while the animal was in the open field

environment. A ‘total neurological score’ was computed as the sum of all individual neurological behaviour scores.

2.6. Experimental design

Rats received the following four treatments in a randomized order: saline injection followed by air exposure (saline + air), saline injection followed by toluene exposure (saline + toluene), drug injection (D-serine or picrotoxin) followed by air exposure (D-serine + air; picrotoxin + air), and drug injection (D-serine or picrotoxin) followed by toluene exposure (D-serine + toluene; picrotoxin + toluene). These treatments were administered on alternating days. Rats were also exposed to toluene for either a duration of 15 min or 30 min in order to examine the effects of toluene at different time points in the exposure paradigm. Previously it was shown that the 15 min exposure to 5000 ppm toluene resulted in increased activity immediately following exposure, while the 30 min exposure resulted in a period of ataxia followed by increases in behaviour, modelling a low and high dose respectively (Samuel-Herter, Slaght and McKay, 2014). Rats in the D-serine experiment (n = 20 total; n = 10 for 15 min vapor exposures, n = 10 for 30 min exposures) were not used in the picrotoxin experiment (n = 24 total; n = 12 for 15 min vapor exposures, n = 12 for 30 min vapor exposures); rats used in 15 min vapor exposure experiments were not used in 30 min vapor exposure experiments.

After injection with D-serine, picrotoxin or saline (see Figure 2.1) rats were placed in the centre of the open field environment and monitored for 10 min (“pretest”; divided into two 5 min blocks). Rats were then immediately placed in either the toluene or air exposure chambers and monitored for 15 or 30 min (“exposure”). Open-field

behaviours were not recorded or quantified inside the vapor exposure chamber due to its small size which restricted rat movement. Rats were then immediately removed from the exposure chamber and placed back into the cleaned open field environment ("test") and monitored 30 min (divided into six 5 min blocks).

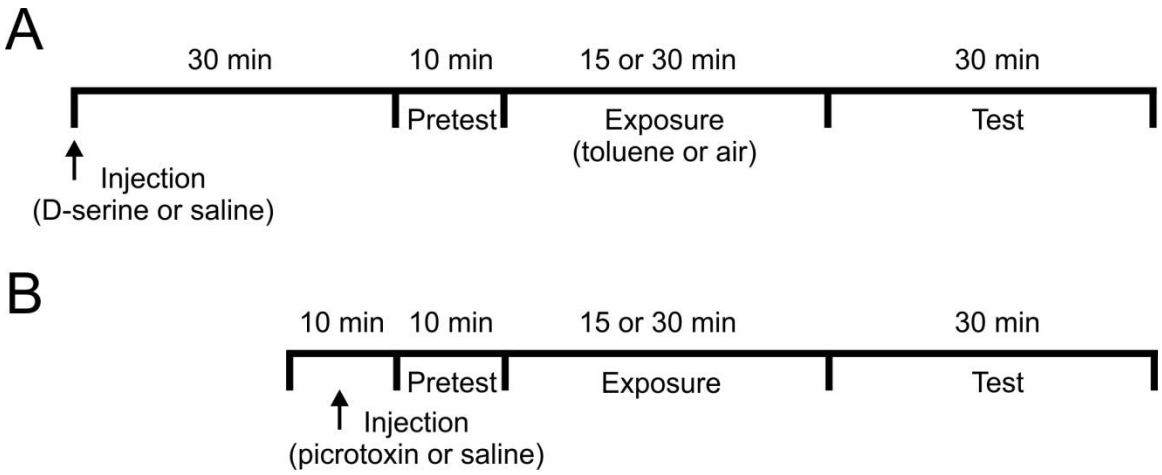

Figure 2.1. Timing of D-serine or picrotoxin injections. Figure additionally illustrates the timing of pretest, exposure, and test epochs. A) refers to the D-serine experiment, B) refers to the picrotoxin experiment.

2.7. Statistics

All data were analyzed using PASW Statistics 21 (SPSS Inc. Chicago, IL). D-serine and picrotoxin experiments were analyzed separately. Total neurological score (and each of its components), locomotion, rearing and grooming were all analyzed separately. Each behaviour was initially analyzed using a repeated measures multivariate analysis of variance with one within subjects factor (trials: eight 5 min blocks

representing two 5 min blocks during the pre-test and six 5 min blocks in the test-phase) and three between subjects factors (drug: D-serine or picrotoxin vs. saline; vapor exposure: toluene vs. air; exposure duration: 15 min vs. 30 min). *Post hoc* analyses were completed with one-way analyses of variance (ANOVAs) and paired *t*-tests, where appropriate. Statistical significance was set at $p < 0.05$.

CHAPTER 3 - RESULTS

3.1. Toluene and NMDA Receptors (D-serine)

Neurological score, ambulatory activity, vertical exploration and grooming behaviour were analyzed using an 8 x 2 x 2 x 2 four-way repeated measures analysis of variance with one level repeated (Trial) and three levels not repeated (Exposure: toluene vapour or air; Injection: D-serine or saline; Exposure Duration: 15 or 30 min). Within subjects results are shown in Table 3.1 and between subjects results are shown in Table 3.2.

Table 3.1. Within subjects statistical results for the D-serine experiment

	Degrees of Freedom	**Neurological Score**	**Ambulatory Behaviour**	**Vertical Exploration**	**Grooming Behaviour**
Trial	**7,504**	158.2***	13.4***	9.2**	39.2***
Trial x Exposure	**7,504**	165.1***	30.2***	107.4***	8.6**
Trial x Injection	**7,504**	4.2***	3.9***	1.5	3.2
Trial x Exposure Duration	**7,504**	7.7***	45.9***	168.2***	6.4*
Trial x Exposure x	**7,504**	3.0**	4.0***	5.0*	0.0

Injection					
Trial x Exposure x Exposure Duration	**7,504**	4.4***	38.1***	111.6***	0.0
Trial x Injection x Exposure Duration	**7,504**	6.8***	5.2***	2.3	3.9
Trial x Exposure x Injection x Exposure Duration	**7,504**	4.0***	5.2**	6.5*	1.4

*p < 0.05, **p<0.01, ***p<0.001.

Table 3.2. Between subjects statistical results for the D-serine experiment.

	Degrees of Freedom	**Neurological Score**	**Ambulatory Behaviour**	**Vertical Exploration**	**Grooming Behaviour**

Exposure	**1,72**	522.3***	177.2***	7.8**	1.4
Injection	**1,72**	9.0**	3.4	12.3**	0.1
Exposure Duration	**1,72**	36.9***	6.8*	25.2***	2.8
Exposure x Injection	**1,72**	3.1	0.8	0.4	0.0
Exposure x Exposure Duration	**1,72**	19.2***	23.1***	12.4**	1.4
Injection x Exposure Duration	**1,72**	1.3	0.0	0.0	0.6
Injection x Exposure x Exposure Duration	**1,72**	0.1	0.0	0.8	2.3

*p < 0.05, **p<0.01, ***p<0.001.

3.1.1. Neurological Assessment

Toluene exposure impaired neurological function as indicated by increased scores on the neurological assessment test battery, similar to previous findings (Samuel-Herter, et al., 2014). There was a significant Trial by Exposure by Injection by Exposure Duration interaction (Table 3.1). Injection of D-serine for the 15 min toluene exposure duration resulted in decreased peak impairment scores in comparison to the saline injected/toluene exposed group (Figure 3.1A); for the 30 min toluene exposure duration, D-serine injection significantly speeded up the recovery of neurological function (Figure 3.1B). When examining the individual components of the neurological assessment test battery, it was apparent that D-serine significantly facilitated the recovery of primarily tremor and myoclonus following the 30 min exposure duration (Figure 3.2).

3.1.2. Locomotion

Similar to the neurological assessment, amount of line crossing also resulted in a Trial by Exposure by Injection by Exposure Duration interaction (Table 3.1). This interaction was characterized by exposure duration differences where rats exhibited hyperlocomotive behaviour immediately following the 15 min exposure to toluene vapour, but following the 30 min exposure to toluene vapour, exhibited a period of ataxia followed by hyperlocomotion (Figure 3.1C and Figure 3.1D, respectively). D-serine reduced the latency for ambulation to return to baseline scores following toluene exposure, and this effect was exacerbated following the 30 min exposure duration in comparison to the 15 min exposure duration.

3.1.3. Rearing

Exposure to toluene vapor (either 15 or 30 min) decreased rearing behaviour during the test phase. The interaction between Trial and Injection was revealed by *post hoc* analyses to be due to pre-test differences (Table 3.1). The effect of Exposure Duration by Trial also showed pre-test differences, with rats about to receive a 30 min exposure to toluene exhibiting fewer bouts of rearing. During the test phase, the 15 min exposure group (Figure 3.1E) reared more than the 30 min exposure group (Figure 3.1F).

3.1.4. Grooming

There were no main effects of Injection, but there was an interaction between Trial and Exposure and between Exposure and Exposure Duration (Table 3.1). Following the 15 min exposure duration, toluene exposed rats groomed less while D-serine + air rats showed increased grooming behaviour initially during the test phase, and saline + toluene rats groomed the most at the end of the test phase (Figure 3.1G). Following the 30 min exposure duration, toluene-exposed rats groomed less in the first test phase time interval 0-5 min (Figure 3.1H).

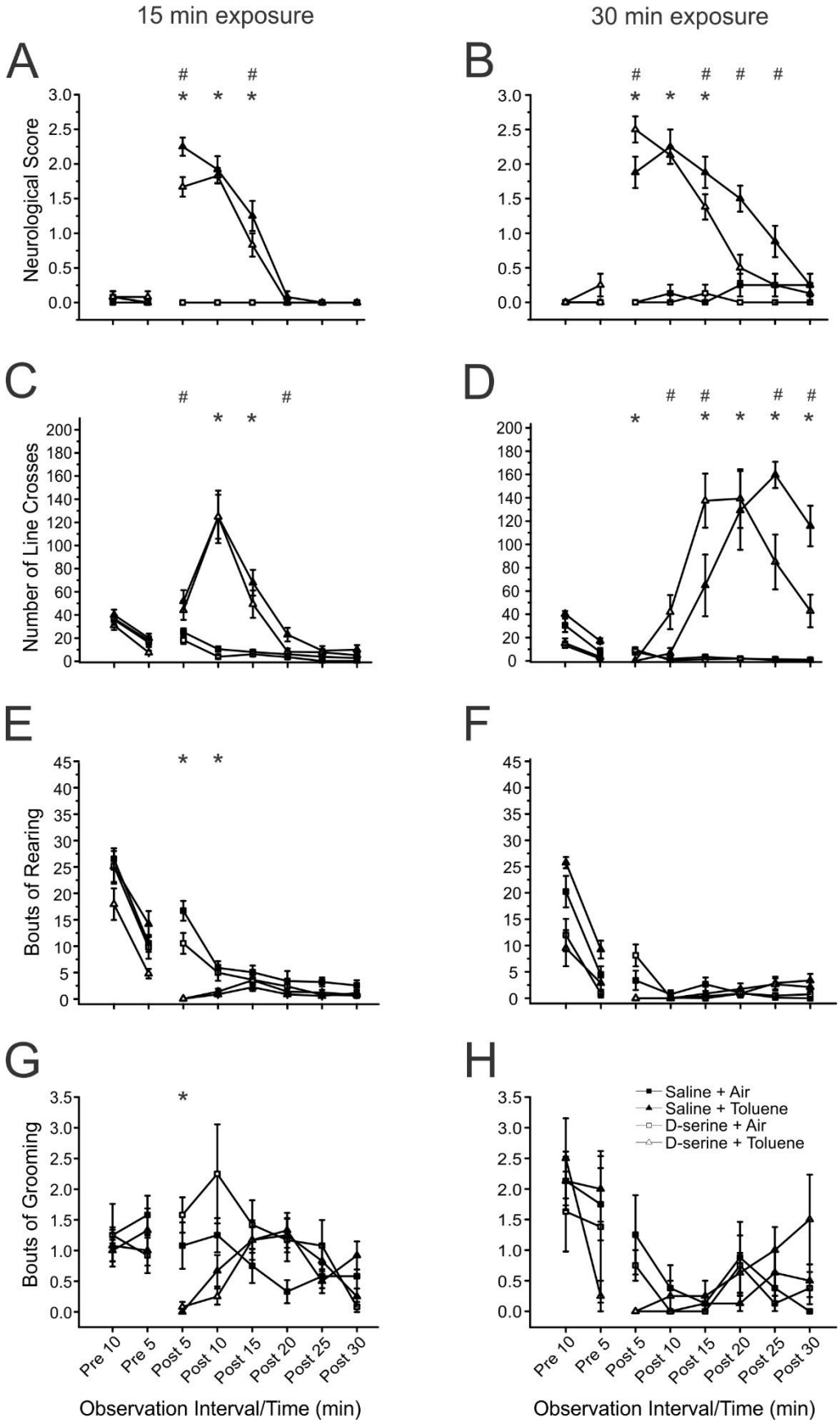
15 min exposure
30 min exposure
A
B
C
D
E
F
G
H
Neurological Score
Number of Line Crosses
Bouts of Rearing
Bouts of Grooming
Saline + Air
Saline + Toluene
D-serine + Air
D-serine + Toluene
Pre 10
Pre 5
Post 5
Post 10
Post 15
Post 20
Post 25
Post 30
Observation Interval/Time (min)
Observation Interval/Time (min)

Figure 3.1. Neurological assessment and open field measures for toluene-exposed, D-serine treated, rats. Neurological assessment score (A, B), number of line crosses (C, D), number of bouts of rearing (E, F) and number of bouts of grooming (G, H) in an open field environment following either 15 (A, C, E, G) or 30 (B, D, F, H) min exposure to toluene vapour. * $p < .05$ toluene versus control. # $p < .05$ D-serine + toluene versus saline + toluene.

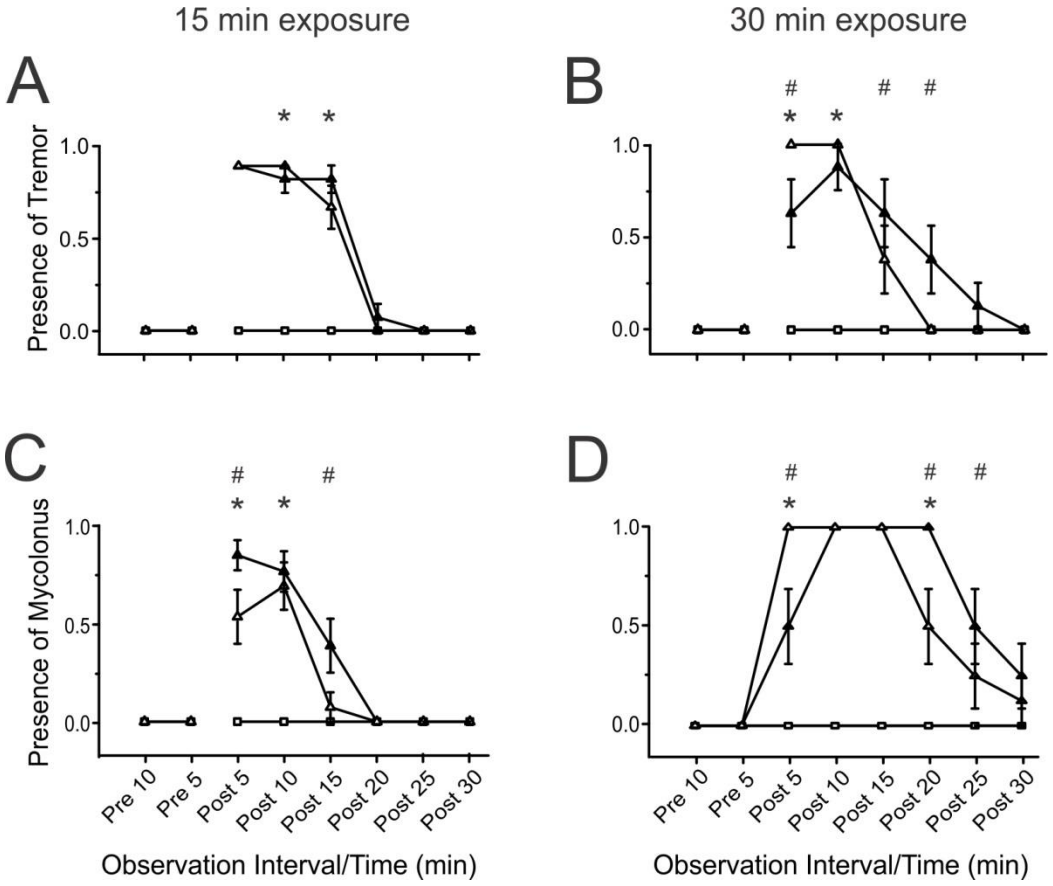

Figure 3.2. Tremor and myoclonus in toluene-exposed, D-serine-treated, rats. Presence or absence of tremor (A, B) and presence or absence of myoclonus (C, D) following either a 15 (A, C) or 30 (B, D) min exposure to toluene. * $p < .05$ toluene versus control. # $p < .05$ D-serine + toluene versus saline + toluene.

3.2. Toluene and $GABA_A$ Receptors (Picrotoxin)

Neurological score, ambulatory activity, vertical exploration and grooming behaviour were scored using an 8 x 2 x 2 x 2 four-way repeated measures analysis of variance with one level repeated (Trial) and three levels not repeated (Exposure: toluene vapor or air; Injection: picrotoxin or saline; Exposure Duration: 15 or 30 min). Within subjects results are shown in Table 3.3, and between subjects results are shown in Table 3.4.

Table 3.3. Within subjects statistical results for the picrotoxin experiment.

	Degrees of Freedom	**Neurological Score**	**Ambulatory Behaviour**	**Vertical Exploration**	**Grooming Behaviour**
Trial	**7,616**	182.7^{***}	61.7^{***}	245.2^{***}	10.7^{***}
Trial x Exposure	**7,616**	187.2^{***}	67.6^{***}	52.2^{***}	8.9^{***}

Trial x Injection	**7,616**	1.1	0.2	1.8	0.3
Trial x Exposure Duration	**7,616**	3.0**	15.2***	0.8	0.7
Trial x Exposure x Injection	**7,616**	1.2	0.5	1.6	1.0
Trial x Exposure x Exposure Duration	**7,616**	2.4	14.8***	1.8	0.6
Trial x Injection x Exposure Duration	**7,616**	0.9	0.3	1.0	0.9
Trial x Exposure x Injection x Exposure Duration	**7,616**	0.7	0.3	0.8	1.0

*p < 0.05, **p<0.01, ***p<0.001.

Table 3.4. Between subjects statistical results for the picrotoxin experiment.

	Degrees of Freedom	**Neurological Score**	**Ambulatory Behaviour**	**Vertical Exploration**	**Grooming Behaviour**
Exposure	**1,88**	511.8***	177.4***	20.0***	7.7**
Injection	**1,88**	0.8	0.2	0.7	2.2
Exposure Duration	**1,88**	1.6	15.4**	0.0	0.8
Exposure x Injection	**1,88**	0.3	0.3	1.6	2.2
Exposure x Exposure Duration	**1,88**	2.7	7.2**	4.3*	2.9
Injection x Exposure Duration	**1,88**	1.3	1.3	0.0	0.4
Injection x Exposure x	**1,88**	0.0	0.4	0.4	0.2

Exposure Duration

*p < 0.05, **p<0.01, ***p<0.001.

3.2.1. Neurological Assessment

Toluene vapor exposure, either 15 or 30 min in duration, resulted in significant impairments as indicated by increased neurological assessment scores. Following the 15 min exposure, peak neurological score and recovery to baseline was similar for both toluene exposed groups (Figure 3.3A). Pre-planned comparisons looked at differences between specific behaviours within the test battery including tremor and myoclonus. Rats exposed to toluene for 15 min trembled significantly more following exposure compared to air-exposed rats, and at the 15-20 min test phase interval, the groups exposed to toluene differed with the picrotoxin injected group trembling significantly less than the saline injected group indicating a potential effect of picrotoxin to decrease the latency to return to baseline trembling scores (Figure 3.4A). Toluene exposed rats also showed differences in recovery from myoclonus where the saline injected rats displayed myoclonic behaviour longer than picrotoxin injected rats (Figure 3.4C). Immediately following the 30 min exposure duration, the picrotoxin + toluene group had neurological assessment scores higher than all other groups, and both toluene-exposed groups recovered to baseline in a similar fashion (Figure 3.3B). The neurological impairments driving this effect were tremor and myoclonus. Trembling behaviour was increased in

toluene-exposed animals, and persisted until the 5-10 min post-test interval (Figure 3.4B). Toluene-exposed animals also exhibited myoclonic behaviours more than controls (Figure 3.4D). At the 15-20 min test phase interval, the saline + toluene-exposed rats displayed myoclonic behaviour more frequently than the picrotoxin + toluene group indicating a potential effect of picrotoxin at this time point (Figure 3.4D).

3.2.2. Locomotion

Exposure to 15 and 30 min of toluene vapor resulted in impaired locomotor behaviour as compared to air-exposed controls. Following the 15 min exposure, both toluene exposed groups were similarly impaired and recovered at the same rate with all groups ambulating more than the picrotoxin injected, air exposed group during test-phase interval (20-25) and the saline injected, toluene exposed group ambulating significantly more than all other groups during the last test phase interval (25-30 min) (Figure 3.3C). Following the 30 min exposure, there was no effect of picrotoxin as both the toluene exposed rats had similar scores on the number of line crosses and recovered to baseline at the same time (Figure 3.3D).

3.2.3. Rearing

Following the 15 and 30 min exposures to toluene, rearing was significantly decreased in toluene-exposed rats. Air-exposed control rats showed differences in rearing following the 15 min exposure in which picrotoxin injected animals reared significantly less, which indicates a potential effect of picrotoxin on decreasing vertical

behaviour (Figure 3.3E). Similarly, air control rats showed differences in rearing in the test phase following the 30 min exposure (Figure 3.3F).

3.2.4. Grooming

Grooming was initially decreased following both the 15 and 30 min exposure for toluene-exposed rats. Following the 15 min exposure, the air exposed animals differed such that the picrotoxin injected group groomed more than the saline injected group. The picrotoxin injected, toluene exposed group groomed more than all other groups at post-test interval (20-25) (Figure 3.3G). Following the 30 min exposure, there were again differences between the air exposed groups indicating an effect of picrotoxin for increasing grooming behaviour (Figure 3.3H).

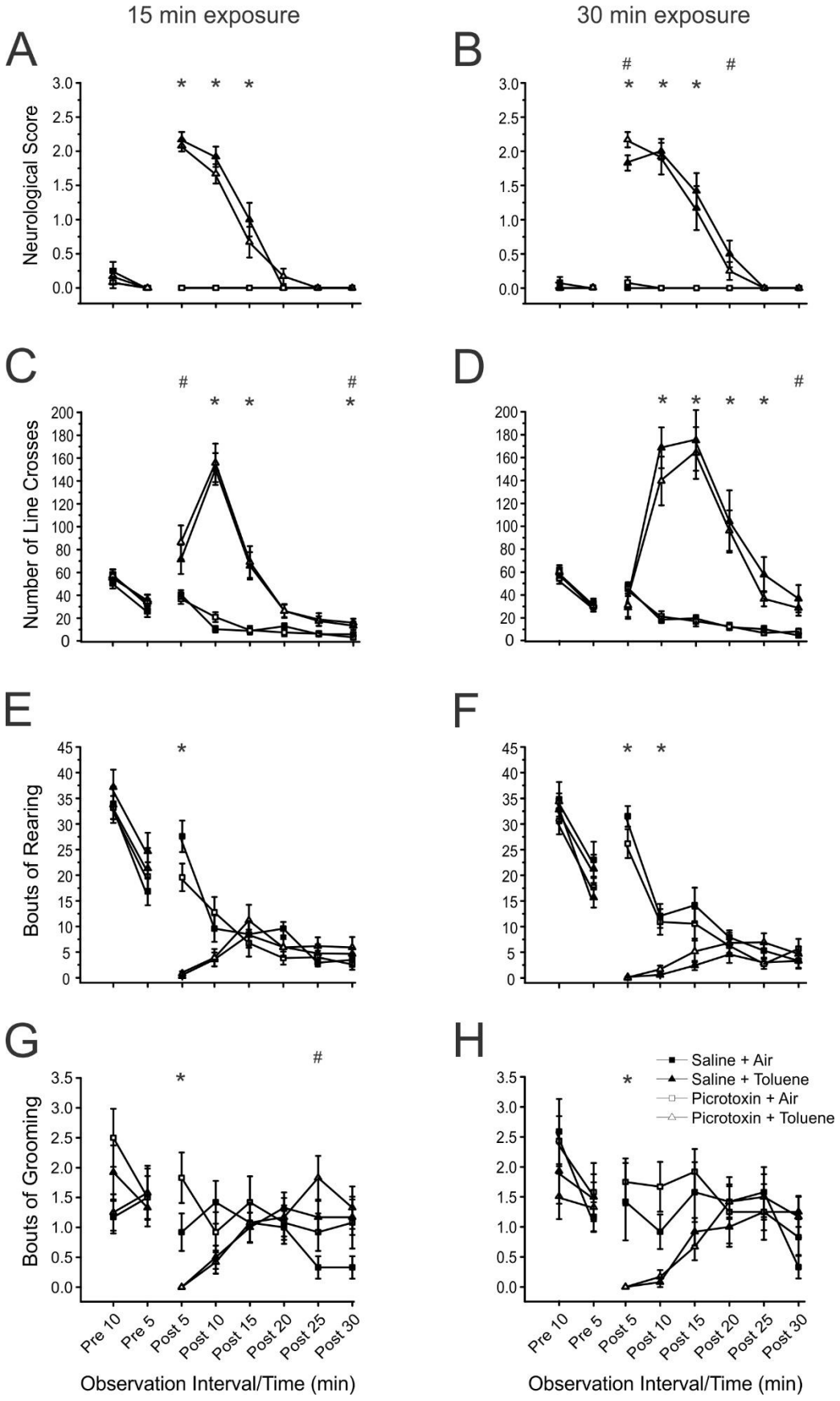
15 min exposure
30 min exposure
A
B
C
D
E
F
G
H
Neurological Score
Number of Line Crosses
Bouts of Rearing
Bouts of Grooming
Saline + Air
Saline + Toluene
Picrotoxin + Air
Picrotoxin + Toluene
Pre 10
Pre 5
Post 5
Post 10
Post 15
Post 20
Post 25
Post 30
Observation Interval/Time (min)

Figure 3.3. Neurological assessment and open field measures for toluene-exposed, picrotoxin-treated, rats. Neurological assessment score (A, B), number of line crosses (C, D), number of bouts of rearing (E, F) and number of bouts of grooming (G, H) in an open field environment following either 15 (A, C, E, G) or 30 (B, D, F, H) min exposure to toluene vapour. * $p < .05$ toluene versus control. # $p < .05$ D-serine + toluene versus saline + toluene.

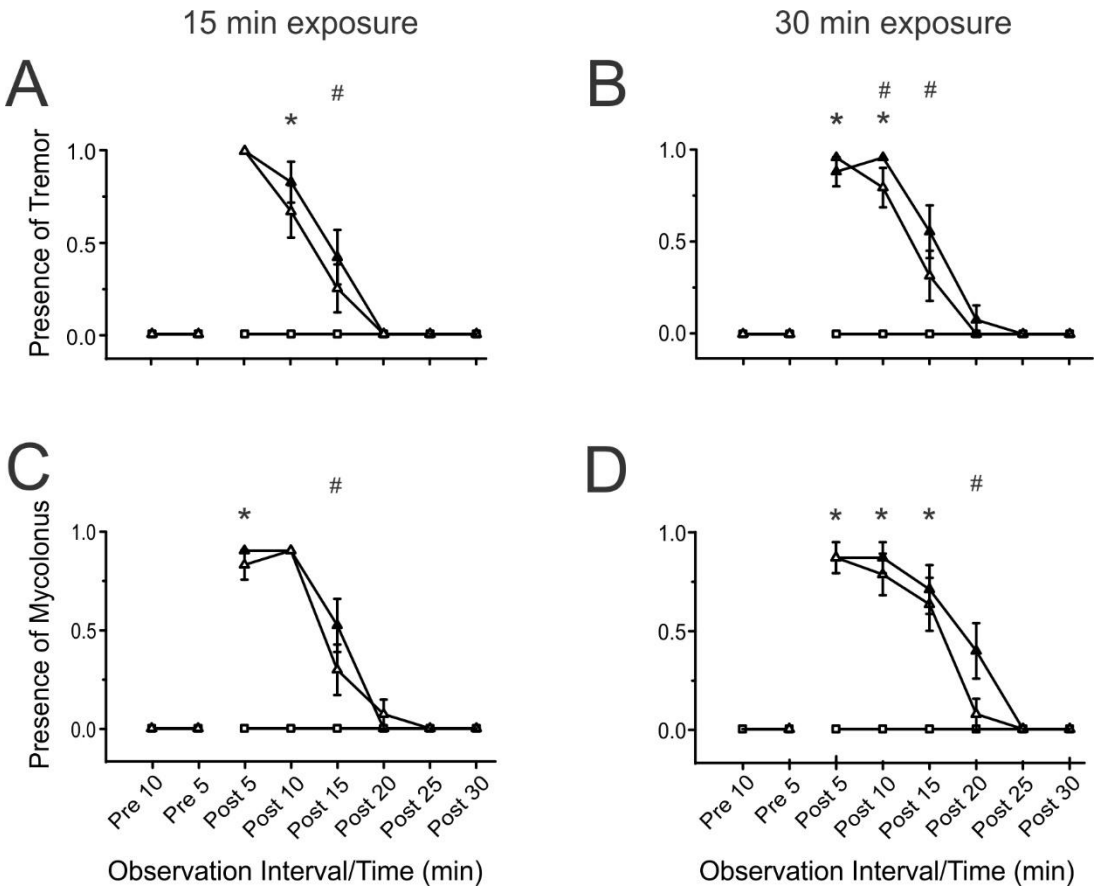

Figure 3.4. Tremor and myoclonus in toluene-exposed, picrotoxin-treated, rats. Presence or absence of tremor (A, B) and presence or absence of myoclonus (C,D) following either

a 15 (A, C) or 30 (B, D) min exposure to toluene. . * $p < .05$ toluene versus control. # $p < .05$ D-serine + toluene versus saline + toluene

CHAPTER 4 - DISCUSSION

4.1. Overview

The present study sought to quantify the behavioural effects of pre-treatment of the NMDA receptor co-agonist D-serine on an acute exposure to inhaled toluene. Further, the current study attempted to understand the role of $GABA_A$ receptors in toluene-induced behavioural impairments by blocking $GABA_A$ receptor activation with the Cl^- channel blocker picrotoxin. Toluene (~5000 ppm) exposures lasting either 15 or 30 min resulted in robust neurological, locomotor, rearing and grooming impairments as seen previously (Samuel-Herter, et al., 2014). Pre-treatment of D-serine (1000 mg/kg) reduced the time to recover from toluene-induced hyperlocomotion and neurological impairments (tremor and myoclonus). Rearing behaviour was not an accurate measure of the effects of D-serine as there were pre-test differences between the groups, and grooming behaviour was not significantly affected by pre-treatment of D-serine. Pre-treatment of picrotoxin did not significantly affect recovery from acute toluene intoxication. This result indicates that the $GABA_A$ receptor is not implicated in the behavioural effects of the dose and duration of toluene, and dose of picrotoxin, used in the current study, indicating that $GABA_A$ receptors may be employed for different behavioural measures or at different toluene vapor doses.

4.2. A potential mechanism through which toluene alters locomotor behaviour

Locomotor behaviour has been used as a measure of excitability and may reflect drug reward processes, as increased dopamine release in the NAC has been associated with the locomotor stimulating and rewarding properties of abused drugs. One potential

pathway through which toluene may increase motor behaviour is through the blockade of NMDA receptors on GABA interneurons in the VTA, which could lead to enhanced dopamine cell firing via disinhibition (Riegel, Zapata, Shippenberg, & French, 2007) . However, blockade of $GABA_A$ receptors by picrotoxin did not affect motor behaviour in our study indicating a potentially different mechanism. Interestingly, NMDA antagonists such as PCP and MK 801 have been shown to induce hyperlocomotor behaviour in dopamine deficient (DD) mice, while restoring dopamine signalling in DD mice also increased locomotor behaviour indicating an independent, as well as synergistic effect of NMDA and dopamine transmission on locomotion (Chartoff, Heusner, & Palmiter, 2005). In contrast, evidence suggests that toluene-induced changes in locomotion are dependent on dopamine transmission as the locomotor enhancing effects of injected toluene can be blocked by SCH23390, remoxipride, raclopride and nafadotride (D1, D2, D2 and D3 receptor antagonists, respectively) which may indicate a difference between toluene and other known NMDA antagonists (Lo, et al., 2009; Riegel & French, 1999). Inhaled toluene results in increased burst firing in dopaminergic cells of the VTA which is similar to the bursting pattern seen following glutamatergic activation of VTA efferents as measured by single-cell recording in anesthetized rats (Riegel & French, 1999). The use of ketamine (also a NMDA antagonist) as an anesthetic may have played a role in this change.

The evidence suggests that toluene acts as an NMDA antagonist, similar to PCP. The NMDA receptor complex is well characterized as a glutamate-gated ion channel with a voltage sensitive Mg^{2+} block. There are seven known NMDAR subunits (GluN1, GluN2A – GluN2D, GluN3A and GluN3B) which assemble into various heteromers. It

has been shown that NMDAR inhibition by toluene is subunit specific, with NMDA receptors composed of the NR1/2B subunits being the most sensitive to toluene (Cruz, et al., 1998). In the adult forebrain, this receptor combination is found predominantly in peri- and extrasynaptic sites, and the presence of the NR2B subunit makes the NMDAR particularly mobile in cultured cells further complicating understanding the potential site of action of toluene (Paoletti, Bellone, & Zhou, 2013). All subunits are made up of 4 domains including the agonist-binding domain (ABD) which binds co-agonists glycine and D-serine in subunits GluN1 and GluN3, and glutamate in GluN2 (Paoletti, et al., 2013). As D-serine did not block the effects of toluene, as it only shifted the timing of the recovery from acute toluene intoxication, we can assume that there are other actions involved in the effects of toluene beyond NMDA receptor antagonism.

4.3. D-serine and recovery from toluene intoxication

Acute toluene exposure to either inhaled or injected toluene results in robust changes in locomotor behaviour which are biphasic. At low doses acute toluene exposure results in increased ambulation, while at high doses toluene exposure results in ataxic behaviour followed by hyperlocomotion during the recovery phase (Himnan, 1984; Samuel-Herter, et al., 2014). As the longer duration exposure was more significantly affected by pre-treatment with D-serine, it may be the case that NMDA receptors are only affected when brain/toluene concentrations are higher or have been elevated for a prolonged period. The current study found that toluene acts like an NMDA receptor antagonist at concentrations high enough to produce ataxia followed by hyperlocomotion. The result of a study using the HR-MAS ^{1}HR MRS technique to quantify

neurotransmitter levels following an acute binge-like exposure pattern (2 x 15 min exposures separated by 2 hr) found that glutamate levels were only decreased in the anterior striatum following the highest toluene vapor exposure (12 000 ppm) (O'Leary-Moore, et al., 2009). This result indicates that toluene vapor may only affect glutamatergic output when brain/toluene concentrations reach a certain threshold. Due to dosing differences it is difficult to pinpoint exactly at what brain concentration this threshold may be reached.

D-serine pretreatment also reduced the latency of recovery from specific neurological impairments resulting from toluene exposure, including myoclonus and tremor. Although in the current study picrotoxin had no effect on myoclonic behaviour, hindlimb myoclonus can be induced by picrotoxin injections (0.5 – 1.5 μg in 2 μL saline) in the caudate nucleus (Tarsy, Pycock, Meldrum, & Marsden, 1978). The onset of myoclonic behaviour was delayed in this study by pre-treatment of scopolamine (a competitive antagonist of the muscarinic acetylcholine receptor) (Tarsy, et al., 1978). Tarsy and colleagues therefore noted a shift in onset of myoclonic behaviour in the direction opposite of D-serine's effect on toluene-induced hindlimb myoclonus noted in the current study. Myoclonic behaviour can also be blocked by NMDA receptor antagonists including MK 801 and S-ketamine when hindlimb myoclonic seizures are induced by opioids (Kolesnikov, Jain, Wilson, & Pasternak, 1997). These results together indicate that myoclonic behaviour is complex, and exhibited for a variety of reasons making it difficult to compare and explain the results reported in the thesis. However, it is clear that toluene produces a robust alteration in this behaviour as it has been observed and characterized in great detail (Himnan, 1984; Hinman, 1987). A potential cause of this

behaviour is alterations in brain stem function, as NMDA lesions (0.5 M, 0.5 μl) to the retrorubual nucleus (RRN) and ventral mesopontine junction (vMPJ) in cats resulted in hindlimb myoclonic behaviour (Lai & Siegel, 1997). As toluene produces high c-Fos activation in brainstem structures following 30 min exposures to 5000 ppm toluene (Perit, et al., 2012) it is reasonable to suggest that in the current study the effects of toluene in the brainstem may be responsible for the presence of myoclonic behaviour

Toluene inhalation was also shown to induce tremors, and the recovery from trembling behaviour was increased by D-serine pre-treatment. As the cerebellum is a critical structure involved in motor timing, it is a major contributor to tremor pathology. A potential underlying cause of toluene-induced trembling behaviour is cereballar dysfunction. In a whole-cell patch clamp preparation, toluene was shown to enhance inhibitory drive on Purkinje cells (Gmaz & McKay, 2014). Alterations in Purkinje cell function have been linked to trembling behaviour in clinical populations (Axelrad et al., 2008). Experimentally when Purkinje cell function was decreased by a knockout of sodium channel $Na_v1.6$, mice displayed motor impairments similar to those seen following acute toluene inhalation including splayed gait and tremors during movement (Levin et al., 2006; Samuel-Herter, et al., 2014). Importantly, D-serine is found in the molecular layer of the cerebellum of mature rats, which houses the dendritic trees of Purkinje cells (Schell, Molliver, & Snyder, 1995). Specifically, motor coordination in a rotarod test was disrupted when the D-serine binding site of the GluD2 receptor was altered, providing a mechanistic link between the presence and proper function of D-serine with motor coordination (Kakegawa et al., 2011).

4.4. Pharmacokinetics of toluene vapor inhalation and D-serine effects

It was shown that log blood and brain toluene concentrations have a linear relationship with log air toluene concentrations (up to a 3 h exposure to 1000 ppm toluene) with a ratio of brain to blood toluene levels of 1.56 (Benignus, Muller, Barton, & Bittikofer, 1984). Similarly, a test of the CNS effects of differing toluene vapor concentrations and exposure durations was quantified using a signalled bar-press shock-avoidance task (Kishi, Harabuchi, Ikeda, Yokota, & Miyake, 1988). It was found that blood and brain concentrations of toluene were closely linked to toluene vapor exposure concentration and duration, indicating that anything which may alter toluene absorption or excretion may shift behavioural outcomes. Interestingly, it has been shown that blood concentrations of toluene do not increase in a linear fashion. In a recent study, 10 min exposures to 1000 – 6000 ppm toluene produce blood concentrations of toluene which were between 64% and 81% of those seen following 20 min exposures, indicating a potential saturation point (Shelton & Slavova-Hernandez, 2009). The current study used an exposure paradigm closely related to Shelton and Slavova-Hernandez. Therefore it is likely that there are considerable differences in the blood concentrations of toluene between the 15 and 30 min exposure group, which may also be a predictor of differences in brain/toluene concentrations.

Toluene can be inhaled, consumed orally, injected or absorbed through the skin. For the purpose of the current study, we will focus on the pharmacokinetic processes as they relate to inhaled toluene. The typical trajectory for inhaled substances is as follows: a) inhaled substances are absorbed in the lungs and enter the pulmonary arteries via gas exchange b) blood travels to the heart and the inhaled substance is pumped systemically

throughout the body (with a high proportion of blood travelling to the brain) c) tissues receive the blood, the inhaled substance is metabolized and waste is diffused back into the blood stream d) venous blood returns to the lungs and waste products are expelled via respiration, or metabolites are excreted in urine. Toluene tends to settle in lipid-rich tissues such as bone marrow, kidney, liver and the brain (Barceloux, 2012). Within these tissues toluene is metabolized primarily by cytochrome P450 isoenzymes to become benzyl alcohol (Gillette, 1959). Benzyl alcohol is then oxidised by alcohol and aldehyde dehydrogenases to become benzaldehyde and benzoic acid (Lof et al., 1993). Approximately 80% of inhaled toluene is excreted as hippuric acid (which is formed by conjugation of acid by glycine) (Lof, et al., 1993). A very small proportion (<1%) of metabolised toluene is excreted as o- and p-cresol, while 7-14% of toluene is excreted by exhalation (Lof, et al., 1993).

4.4.1. Increased ventilation rates may drive D-serine-mediated increases in toluene vapor clearance

As the results here indicate that D-serine acted to shift the recovery time for an acute toluene exposure as opposed to simply reducing the effects of toluene, it may be the case that D-serine influences the pharmacokinetics of toluene, specifically by increasing the rate of clearance of toluene from the brain. In a study comparing spray painters exposed to solvents exhibiting impairments, it was noted that those painters who also smoked tobacco had higher toluene clearance from blood than non-smokers, potentially due to an enhancement of cytochrome P450 activity in smokers (Smith & Bend, 1981; Wallen, 1986). Importantly, pharmacokinetic models of toluene vapor exposure indicate

that alveolar ventilation rate is highly influential in predicting blood toluene concentrations in rats (Kenyon et al., 2008).

Co-agonists glycine and D-serine act to increase the recovery rate from receptor desensitization during synaptic activation, and this effect may be the driving force behind reductions in the time to recover from the locomotor stimulating and neurological impairing effects of acute toluene vapor exposures (Yang & Svensson, 2008). Both inhaled toluene exposure concentration and duration have been related to the increase of brain and blood toluene concentrations (Kishi, et al., 1988). A large portion of un-metabolised inhaled toluene (7-14%) is excreted in exhaled air (Lof, et al., 1993). Pre-treatment of D-serine may act to increase the rate of ventilation and thus increase the amount of un-metabolized toluene excreted via respiration. For instance, NMDA receptors are responsible for mediating the transition from inspiration to expiration, and NMDA receptor blockade by antagonists results in apneusis (a breathing pattern characterized by gasping during the inspiration phase and a shortened, insufficient expiration phase) (Haji, Okazaki, & Takeda, 2000). Although it is yet to be determined whether D-serine affects ventilation directly, it is a probable hypothesis to explain the effects noted in the current study. Future studies should look at how increasing the rate of respiration may act to decrease brain/toluene concentrations as this effect would be an excellent option for reducing the effects of acute toluene intoxication in clinical settings.

4.4.2. Considerations of the route of toluene administration

Lo and colleagues found that total distance travelled (cm) following an acute toluene injection (750 mg/kg) over a 90 min test phase was significantly attenuated by

pre-treatment of D-serine (1000 mg/kg). The current study found that rather than being attenuated, the locomotor stimulating effects of toluene were shifted such that latency to recover from these impairments was significantly shortened during a 30 min test phase, following the highest length of exposure studied here (5000 ppm for 30 min). When analyzed in the current study, total number of line crosses did not significantly differ between D-serine and vehicle-injected rats following toluene exposure (results not shown) indicating a difference between these results and those of Lo and colleagues. Behaviourally speaking the effects of injected and inhaled toluene may differ. Due to the ease of dose determination, injection of toluene has been commonly used as a rodent model of toluene intoxication, even though this route of administration is essentially unheard of in reports of human toluene abuse. Inhaled toluene results in observable changes in behaviour sooner than injected toluene (Bowen, et al., 2006). Inhaled and injected toluene groups also show a different pattern of c-Fos expression following a 30 min exposure to 5000 ppm toluene compared to injection of 1000 mg/kg toluene (Chen, et al., 2005; Perit, et al., 2012). The mechanism underlying these differences is unknown but may be due to overall differences in brain toluene concentrations. Lo and colleagues found that a dose of 750 mg/kg toluene resulted in increased locomotion but also ataxia and hindlimb abduction (or splaying) indicating that this dose was comparable to the behavioural effects of inhaled toluene noted in the current study. There may have been a delay in the behavioural effects as timing of behaviour onset was not reported.

4.5. Limitations

A potential limitation to the current study was the timing of injections and the dose of D-serine and picrotoxin. It is difficult to compare the behavioural effects of D-serine and picrotoxin as D-serine was injected 30 min prior to pre-testing and picrotoxin was injected 10 min prior. However, each injection time was chosen based on previous research. D-serine (1000 mg/kg) injected i.p. 30 min before toluene exposure had significant effects on toluene-induced motor and cognitive behaviour (Lo, et al., 2009). It was more difficult to choose an injection schedule for picrotoxin as the methodologies of the existing studies differ greatly from one study to the next. Although a recent study showed that 0.01 and 0.03 mg/kg toluene injected immediately prior to an acute toluene exposure attenuated toluene-induced hypothermia, the current study was interested in motor behaviours, including locomotion (Paez-Martinez et al., 2013). Therefore the current study employed an injection schedule and dose of picrotoxin (0.05 mg/kg, 10 min prior to toluene inhalation) based on a study which used the same schedule and found that picrotoxin significantly enhanced the locomotor stimulating effects of ethanol (Liljequist & Engel, 1982).

4.6. Future Directions

Based on the outcomes of the current study, a future study exploring the effects of D-serine pre-treatment on various toluene vapor exposure durations (ex. 15, 30, 45, 60 min) could identify a potential dose-dependent response to NMDA receptor drugs. This would potentially allow for the construction of a timeline which could ultimately be used to identify the stages of toluene intoxication and the receptors involved. A second study

focusing on the effects of D-serine following an acute toluene exposure would also be warranted, as this could ultimately lead to the development of therapeutics to be used in clinical settings for patients hospitalized for toluene use. Lastly it will be necessary to incorporate measures of rate of respiration in future studies in order to identify whether D-serine has an effect on respiration, and whether this effect drives increases in toluene clearance.

4.7. Conclusions

D-serine but not picrotoxin reduced the recovery time from an acute toluene-vapor exposure. The neurological and locomotor impairments caused by acute toluene vapor exposures may therefore more likely be due to changes in NMDA receptor function than GABA receptor function. This result adds to the growing body of literature which attempts to determine the mechanism of action of toluene vapor in the brain.

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