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Pharmacology, Biochemistry and Behavior 73 (2002) 921–927

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Neonatal toluene exposure selectively alters sensitivity to different chemoconvulsant drugs in juvenile rats

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Received 7 March 2002; received in revised form 20 June 2002; accepted 26 June 2002

Abstract

Toluene is an abused solvent widely used in several commercial products. Recent evidence indicates that this solvent is a noncompetitive inhibitor of *N*-methyl-D-aspartate (NMDA) receptors and enhances γ-aminobutyric acid$_A$ ($GABA_A$) receptor-mediated synaptic currents. Since NMDA and $GABA_A$ receptors have been implicated in seizures, this study investigated whether toluene exposure during synaptogenesis period alters the NMDA and $GABA_A$ receptor-mediated seizure susceptibility in juvenile rats. Neonatal rats were administered toluene (1 g/kg ip) daily over postnatal days (PN) 4–9. Rats were administered NMDA (10 mg/ml), picrotoxin (2 mg/ml), pentylenetetrazol, (5 mg/ml) and 4-aminopyridine (2 mg/ml) via timed tail vein infusion on PN 34–36. Toluene exposure increased sensitivity to NMDA, picrotoxin and pentylenetetrazol, but did not affect 4-aminoyridine-induced seizures in both male and female rats. These results suggest that toluene may possess a risk to the developing brain by inducing a long-term alteration in the function of NMDA and $GABA_A$ receptors.

Keywords: Toluene; Seizure susceptibility; Pentylenetetrazol; Picrotoxin; NMDA; 4-Aminopyridine

1. Introduction

Toluene is a neurotoxic organic solvent present in paints, inks, glues and thinners. One source, resulting in especially high exposure rates, is sniffing glue. A large number of abusers are adolescent and young adult women. The fetus of a pregnant toluene abuser is also exposed to this substance.

It has been shown that toluene shares certain pharmacological properties with central nervous system depressants, such as alcohol and anesthetic vapors (Evans and Balster, 1991). For example, toluene has anxiolytic (Bowen et al., 1996) and anticonvulsant (Wood et al., 1984) properties and impairs motor coordination (Moser and Balster, 1985). Furthermore, toluene has been reported to enhance $GABA_A$ receptor-mediated synaptic currents (Beckstead et al., 2000) and to affect glutamatergic neurotransmission involving NMDA receptors. Toluene produces concentration-related partial substitution for PCP (a NMDA channel blocker) in drug discrimination (Bowen et al., 1999) and dose-dependently inhibits recombinant NR1/2B NMDA receptors (Cruz et al., 1998).

The normal functioning of NMDA and $GABA_A$ receptors is critical for synaptogenesis (Bardoni, 2001; Gordon-Weeks et al., 1984; Madtes and Redburn, 1983) and cell survival (Ikonomidou et al., 2001). Temporary loss or interference with the functions of neurons with NMDA and $GABA_A$ receptors following toluene exposure during the synaptogenesis period is likely to disturb normal development of the central nervous system, resulting in long lasting changes in neurobehavioral functions.

The purpose of this study was to determine whether toluene exposure during the synaptogenesis period alters NMDA and $GABA_A$ receptor-mediated seizure susceptibility in juvenile rats. Since the synaptogenesis period occurs entirely postnatally in the rats (Dobbing and Sands, 1979), we exposed rats to toluene (1 g/kg) during early neonatal period, postnatal days (PN) 4–9. Subsequently, as a simple means of identifying sensitivity changes by drugs that act at NMDA or $GABA_A$ receptors, chemically induced convulsions were assessed 25–27 days after last injection of

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PII: S0091-3057(02)00943-7

toluene or corn oil. In addition, pentylenetetrazole (PTZ, the site of action of PTZ has not been completely elucidated; it may act as a $GABA_A$ receptor antagonist, a GABA gated chloride channel blocker and/or as an activator of excitatory amino acids) and 4-aminopyridine (4-AP, a K^+ channel blocker) were also used for comparison.

The dose (1 g/kg) used in this study was according to our preliminary study. We found that administration of toluene (500–1200 mg/kg ip) to adult female rats produced increased locomotor activity and stereotypic behaviors, which resemble behavioral signs observed in toluene abusers. Similar results have also been reported in male rats by Riegel and French (1999). In addition, the placenta penetration efficiency for toluene is greater than 90% (Shumilina, 1991). Therefore, the rats were exposed to 1 g/kg toluene by intraperitoneal injection during PN 4–9 to simulate toluene exposure in the fetus of pregnant toluene abusers.

Furthermore, seizure susceptibility for NMDA and picrotoxin has been reported to be gender-specific (Standley et al., 1995; Thomas, 1990). Therefore, both males and females were studied to detect any gender-specific differences in long-term effects of neonatal toluene exposure.

2. Methods

2.1. Animals

Sprague–Dawley rats were supplied from the Laboratory Animal Center of Tzu Chi University (Hualien, Taiwan). The experimental protocol was approved by Review Committee of the Tzu Chi University for the Use of Animal Subjects. The day of birth was considered to be PN 0. On PN 4, the litters were culled to 10–12 pups and each litter was randomly assigned to toluene or control group. The toluene animals were received 1 g/kg toluene (HPLC grade, Mallinckrodt Baker, Kentucky, USA, 0.1 g/ml dissolved in corn oil) by intraperitoneal injection and the control animals received corn oil (0.1 ml/10 g) daily over PN 4–9. Injections were given at 10–11 a.m. The pups were left with their biological dam and weaned on PN 21. Six litters were used for each treatment group. One animal for each gender was randomly selected from each litter for each chemoconvulsant. Each animal was only used for one drug test.

2.2. Seizure induction

On PN 34–36, control and toluene exposed rats were taken from their home cages for seizure induction. The experimenter was blinded to the neonatal treatment at the time of seizure induction. Convulsants were administered via a lateral tail vein. The infusion pump (L-1800, Kd Scientific, USA) was used for infusion and the infusion rate was 0.5 ml/min. The convulsants and concentrations infused were NMDA (10 mg/ml in saline), picrotoxin (2 mg/ml in saline), PTZ (5 mg/ml in saline) and 4-AP (2 mg/ml in saline), which were all purchased from Sigma (USA). The animals were weighed and placed in an acrylic chamber with numerous holes for ventilation. The tail of the rat was warmed for 1 min in warm (45 °C) water. A 25-gauge butterfly infusion needle was inserted in the lateral tail vein and correct placement was verified by the appearance of blood in the infusion tubing. The needle was fixed to the tail with an adhesive tape and the animal was then released into a $23 \times 8 \times 6$ cm^3 ($l \times w \times h$) plastic cage to allow free movement. The animal was observed throughout infusion and the time between the start of infusion and onset of four convulsion signs was record in seconds and subsequently converted to threshold convulsant dosage (i.e., milligram of drug per kilogram of body weight), based on infusion rate, body weight and latency. Threshold dosage (mg/kg) = latency (min) × infusion rate (ml/min) × convulsant concentration (mg/ml)/body weight (kg).

Timed tail vein infusion allows for observation and qualitative analysis of several different convulsion end points. Briefly, clonus indicates rapid rhythmic movements due to alternating contraction and relaxation of muscles, whereas tonus indicates rigidity due to contraction of muscles. Four convulsion signs, which occur in progression, characterize PTZ-, picrotoxin- and 4-AP-induced seizures: myoclonic (MC) twitch (sudden involuntary muscle jerk); face and forelimb (FF) clonus (rapid writhing movements of the head and neck); running and bouncing (RB) clonus (whole-body clonus, including running and jumps); and tonic hindlimb extension (THE)(extreme rigidity, with forelimbs and hindlimbs extended caudally). The two convulsion signs which reliably characterize NMDA-induced seizures are RB clonus and THE. MC twitch and FF clonus were not observed consistently in all of the animals and were therefore not reported for NMDA.

2.3. Toluene in blood

Blood samples were taken through heart puncture 1 and 3 h following the last injection of toluene. Toluene in blood was analyzed using a head-space gas chromatography-mass spectrometer (HP7649-HP6890-HP5973, Hewlett-Packard). The samples were thermostatted at 80 °C for 20 min. Pressurization and injection times were 1.5 and 0.1 min, respectively. The temperature program for the column (HP-5MS) was as follows: 3 min at 50 °C, then 10 °C/min to 60 °C, then 30 °C/min to 200 °C, then hold for 0.5 min. The flow rate of helium carrier gas was 0.6 ml/min. Toluene concentration was determined by comparing toluene to *o*-xylene peak area ratios to a calibration curve determined from prepared standards.

2.4. Statistical analysis

Seizure thresholds for male and female rats were analyzed by two-way ANOVA with treatment and gender as two grouping factors. A significant Treatment × Gender

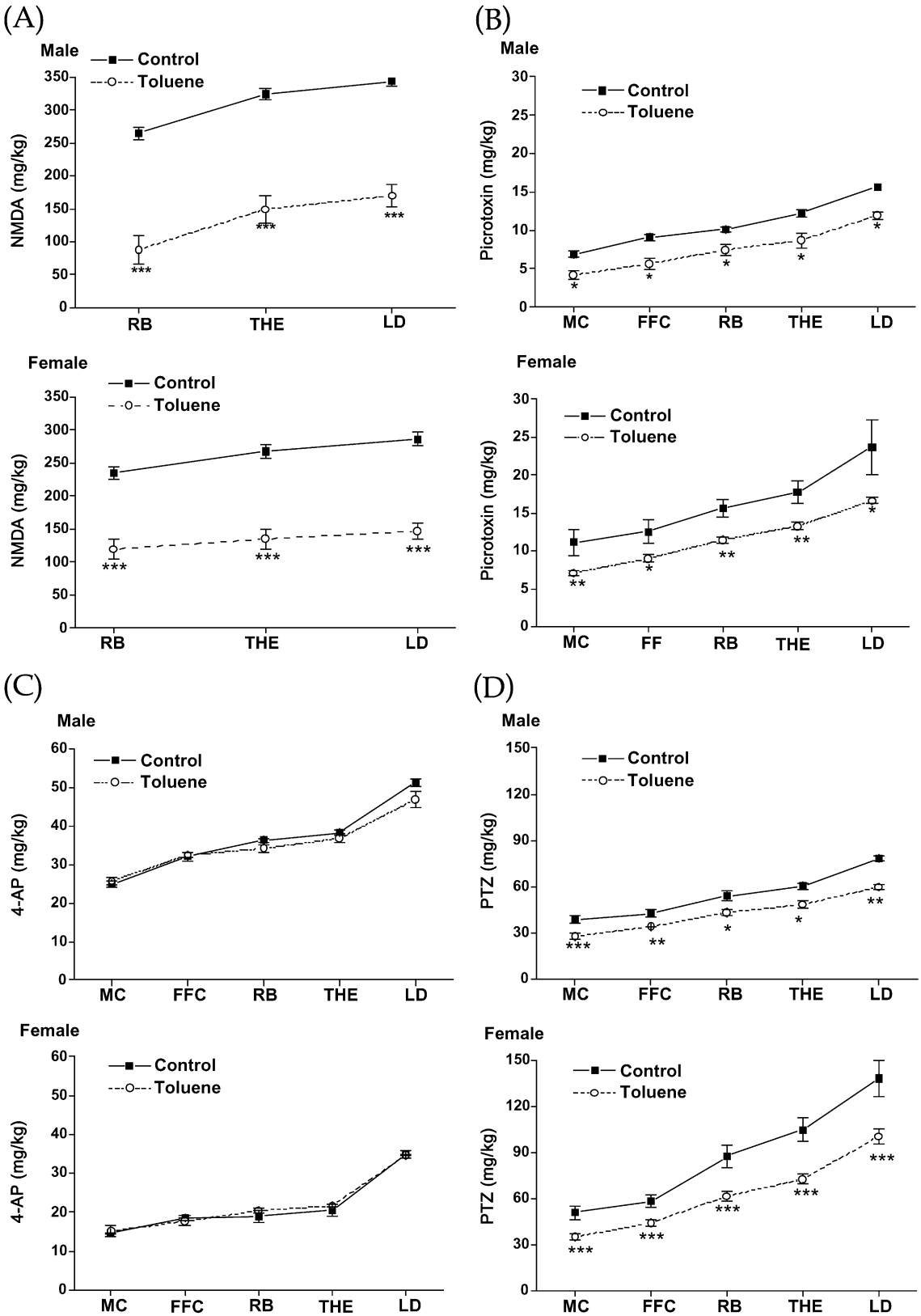

Fig. 1. Seizure thresholds for four different chemoconvulsants, (A) NMDA, (B) picrotoxin, (C) PTZ and (D) 4-AP on PN 34–36 as a function of neonatal treatment and gender. Definition of myoclonic twitch (MC), face and forelimb clonus (FFC), running and bouncing clonus (RB), tonic hindlimb extension (THE) and lethal dose (LD) were described in Section 2. Control rats (solid square) were received corn oil (intraperitoneally) and toluene exposed rats (open circle) received 1 g/kg toluene dissolved in corn oil (intraperitoneally) daily over PN 4–9 ($n=6$ males and females). Data is presented as mean ± S.E.M. * $P<.05$, ** $P<.01$, *** $P<.001$ compared to control levels, according to two-way ANOVA followed by Bonferroni test.

interaction would indicate that the effect of treatment group depended on gender. Bonferroni test was used for post-hoc comparisons. Statistical significance was attained when $P < .05$.

3. Results

3.1. Body weights and blood toluene levels

During the time of toluene administration (PN 4–9), the neonatal body weight gain from the toluene-treated rats was lower than the corn oil treated rats. Whereas corn oil treated males and females gained 14.0 ± 1.2 and 13.8 ± 1.3 g from PN 4–10, respectively, rats injected with 1 g/kg toluene gained an average of 84% of that amount (male, 12.0 ± 1.5 g; female, 11.6 ± 1.8 g). Body weights of toluene-treated rats did not differ from controls at the time for seizures induction (control: male, 129.9 ± 1.2 g; female, 105.8 ± 2.4 g; toluene, 127.6 ± 2.5 g; female, 104.8 ± 3.2 g at PN 34).

Blood samples were taken 1 and 3 h following the last injection of toluene. The blood toluene concentrations were determined as 67.38 ± 6.8 and 16.8 ± 0.5 μg/ml ($n = 4$), respectively. Toluene in the blood samples from controls was not detected.

3.2. Behavioral seizure thresholds

As shown in Fig. 1, rats that had been exposed to toluene as neonates demonstrated that all seizure thresholds and lethal doses for NMDA, picrotoxin and PTZ were declined in males and females. However, there was no neonatal toluene effect on the seizure thresholds for 4-AP. This was confirmed by two-way ANOVA (Table 1), which revealed significant differences in the treatment effects on seizure thresholds induced by NMDA, picrotoxin and PTZ, but not 4-AP. In addition, seizure thresholds induced by picrotoxin, 4-AP and PTZ depended on gender. It was reflected the higher seizure thresholds induced by picrotoxin, and PTZ for females, and 4-AP for males. A significant interactions between treatment and gender was exhibited in NMDA and PTZ-induced all seizure manifestations. The toluene effect on NMDA-induced seizure thresholds was more pronounced in male rats. On the other hand, the toluene-induced reduction in seizure thresholds for PTZ was more pronounced in female rats.

4. Discussion

In this study, rats were exposed to 1 g/kg toluene by intraperitoneal injection daily over PN 4–9. Blood toluene concentrations were determined from blood samples taken from toluene-exposed pups 1 and 3 h after the last injection and the values were 67.4 ± 6.8 and 13.6 ± 5.8 μg/ml, respectively. Since the placenta penetration efficiency for toluene is greater than 90% (Shumilina, 1991) and these levels are in the range obtained from toluene abusers (0.1–74.7 μg/ml) (Park et al., 1998), it appears that our treatment protocol is appropriate and able to mimic the toluene exposure during synapotogenesis for the fetus of pregnant toluene abuser.

The results of present study demonstrated that toluene exposure from PN 4 to 9 enhanced susceptibility to PTZ-, NMDA- and picrotoxin-induced seizures on PN 34–36, while 4-AP induced seizure susceptibility was unchanged. Moreover, there was a significant gender effect on NMDA and PTZ-induced seizure thresholds in toluene exposed rats. The reduction of seizure thresholds for NMDA in response to neonatal toluene exposure was more pronounced in male

Table 1
Statistical analysis of two-way ANOVA for the seizure responses with the factors "treatment" (control vs. toluene) and "gender" (male vs. female)

	Treatment	Gender	Interaction
NMDA			
RB clonus	$F(1,20) = 89.45$, $P < .0001$	$F(1,20) = 0.22$, $P = .64$	$F(1,20) = 7.427$, $P = .013$
THE	$F(1,20) = 72.62$, $P < .0001$	$F(1,20) = 1.621$, $P = .21$	$F(1,20) = 7.838$, $P = .011$
Picrotoxin			
MC twitch	$F(1,20) = 18.88$, $P = .0003$	$F(1,20) = 20.89$, $P = .0002$	$F(1,20) = 0.74$, $P = .39$
FF clonus	$F(1,20) = 13.23$, $P = .0016$	$F(1,20) = 12.46$, $P = .0021$	$F(1,20) = 0.022$, $P = .88$
RB clonus	$F(1,20) = 17.5$, $P = .0005$	$F(1,20) = 32.65$, $P < .0001$	$F(1,20) = 1.206$, $P = .28$
THE	$F(1,20) = 13.48$, $P = .0015$	$F(1,20) = 19.34$, $P = .0003$	$F(1,20) = 1.368$, $P = .25$
4-AP			
MC twitch	$F(1,20) = 0.9353$, $P = .35$	$F(1,20) = 196.6$, $P < .0001$	$F(1,20) = 0.009$, $P = .92$
FF clonus	$F(1,20) = 2.096$, $P = .16$	$F(1,20) = 215.1$, $P < .0001$	$F(1,20) = 0.92$, $P = .73$
RB clonus	$F(1,20) = 0.4394$, $P = .51$	$F(1,20) = 483.5$, $P < .0001$	$F(1,20) = 5.834$, $P = .02$
THE	$F(1,20) = 0.392$, $P = .75$	$F(1,20) = 265$, $P < .0001$	$F(1,20) = 7.556$, $P = .19$
PTZ			
MC twitch	$F(1,20) = 98.83$, $P < .0001$	$F(1,20) = 19.74$, $P = .003$	$F(1,20) = 5.272$, $P = .03$
FF clonus	$F(1,20) = 49.13$, $P < .0001$	$F(1,20) = 43.8$, $P < .0001$	$F(1,20) = 4.37$, $P = .04$
RB clonus	$F(1,20) = 46.85$, $P < .0001$	$F(1,20) = 102.6$, $P < .0001$	$F(1,20) = 7.181$, $P = .014$
THE	$F(1,20) = 48.31$, $P < .0001$	$F(1,20) = 138.9$, $P < .0001$	$F(1,20) = 7.908$, $P = .0108$

rats. Conversely, the effect of toluene on seizure thresholds for PTZ was more pronounced in female rats. It appears that the sex differences in seizure threshold changes in response to neonatal toluene depend on the chemoconvulsants.

Toluene has been demonstrated to inhibit NMDA receptor (Cruz et al., 1998) and enhance $GABA_A$ receptor functions (Beckstead et al., 2000) in neurophysiological experiments. Accordingly, toluene may have similar effects as NMDA antagonists and/or $GABA_A$ receptor modulators in vivo. It has been reported that neonatal administration of the NMDA antagonist PCP results in increased sensitivity to NMDA (Brooks et al., 1997). On the other hand, neonatal exposure to barbiturate (acting on $GABA_A$ receptor) increases susceptibility to audiogenic seizures (Yanai et al., 1981), a seizure type modulated by NMDA and $GABA_A$ receptors (Higashiyama et al., 1998; N'Gouemo and Faingold, 1999; N'Gouemo et al., 1996; Terra and Garcia-Cairasco, 1994). As anticipated, neonatal toluene exposure does indeed increase seizure susceptibility induced by convulsant agents acting on NMDA and $GABA_A$ receptors.

The increased NMDA-induced seizure susceptibility is possibly a supersensitivity phenomenon, resulting from an increase in NMDA receptor number or activity after termination of toluene-mediated NMDA antagonism. It is well known that the presence of multiple NMDA receptor subunits and the expression of different combinations of these subunits generate receptors with different physiological characteristics. Two classes of NMDA receptor subunit gene have been identified: NR1 and NR2. The NR2 consists of four different genes: NR2A, NR2B, NR2C and NR2D. NR1 subunit in combination with different members of the NR2 family yields receptors with different characteristics, such as differences affinities for agonists, the sensitivities for antagonists, allosteric modulators and Mg^{2+} blockade (Ishii et al., 1993). The expression patterns of NMDA receptor subunit gene show specific spatio-temporal profiles during brain development (Ishii et al., 1993). Therefore, another possible explanation is that neonatal toluene exposure altered expression pattern of NMDA receptor subunits. Actually, a parallel biochemical study is processing in our laboratory. The preliminary results show that an elevated level of NR2A receptor in the hippocampus (unpublished data).

Neonatal toluene exposure also increased picrotoxin- and PTZ-induced seizure susceptibility, which may be attributed to the changes in subunit composition of $GABA_A$ receptor complex. Picrotoxin is well known as a GABA gated chloride channel blocker. The locus of PTZ's convulsant effect is likely the $GABA_A$ receptor (Huang et al., 2001), even though interaction of PTZ with other membrane proteins, including Na^+-K^+ pump, may also play a role in its convulsant effect (Dubberke et al., 1998). The $GABA_A$ receptor is a heteroligomeric complex composed of five subunits of several classes, each class consisting of different isoforms (Barnard et al., 1998). Results from recombinant expression studies have shown that the sensitivity to picrotoxin and PTZ are influenced by the subunit composition of $GABA_A$ receptor (Bell-Horner et al., 2000; Huang et al., 2001). Similar to NMDA receptor, the expression patterns of $GABA_A$ receptor subunit gene also demonstrate specific spatio-temporal profiles during brain development (Brooks-Kayal et al., 2001; Davis et al., 2000; Roberts and Kellogg, 2000). In addition, it has been reported that $GABA_A$ receptor subunits expressed differentially in seizure-prone and seizure resistant animal models (Poulter et al., 1999). Thus, it is also highly possible that neonatal toluene exposure may alter certain subunit expression. Additional studies are necessary to determine whether toluene can induce $GABA_A$ receptor subunit changes.

Recently, Ikonomidou and his colleagues found that administration of agents that block NMDA receptors or activate $GABA_A$ receptors for only a few hours during late fetal or early neonatal life triggered widespread apoptotic neurodegeneration in the developing rat brain (Ikonomidou et al., 1999, 2000). Accordingly, it is likely that toluene exposure during synaptogenesis period blocks the NMDA receptor and activates $GABA_A$ receptors, resulting in apoptotic neurodegeneration primarily in neurons with NMDA receptor and $GABA_A$ receptor. The temporary loss of neurons with NMDA and/or $GABA_A$ receptors would possibly impact on normal neurodevelopment, leading to alterations in the functions and characteristics of NMDA and $GABA_A$ receptors and reflecting seizure susceptibility changes observed in the present study.

4-AP is a specific blocker of the voltage-dependent K^+ channels. Recent studies demonstrated that seizure activity induced by 4-AP is due to a combined action of excitatory amino acid release and direct stimulation of neuronal firing (Medina-Ceja et al., 2000; Pena and Tapia, 1999, 2000). Our results showed that the 4-AP-induced seizure thresholds were not influenced by neonatal toluene exposure. Based on the significant reduction in seizure thresholds for NMDA and complicated effects of 4-AP, it is suggested that a compensatory change in non-NMDA glutamate or other receptors may exist after neonatal toluene exposure. However, the sites of such changes are unclear at present. Further studies are guaranteed.

Classification of various distinct seizures is based on unique sites of initiation (trigger zones) and propagation of seizure activity involving specific brain structures and neurochemical systems. It is generally accepted that there are two quantitatively distinct seizure components that are mediated by separable and independent anatomical circuits (Gale, 1992). Myoclonic convulsions may reflect forebrain/limbic seizure activity whereas running and bouncing clonus or tonic hindlimb extension (generalized) seizures reflect manifestations of activity in common hindbrain pathways. Neonatal toluene exposure reduced the thresholds of all seizure components for NMDA, PTZ and picrotoxin. This suggests that the toluene-increased seizure susceptibility may be related to neurochemical changes involved in neural circuits in both forebrain and hindbrain.

Overall, these results suggest that toluene exposure during synaptogenesis does not result in a global non-specific lowering of threshold to chemoconvulsive stimuli, but rather, selective changes in CNS mechanisms associated with neural excitability may underlie toluene-related behavioral changes. Thus, reduced $GABA_A$ receptor function and increased NMDA receptor activity may become exaggerated during juvenile life as a consequence of toluene exposure during synaptogenesis.

Acknowledgements

The authors would like to thank Mr. Anren Hu and Ms. Hsiu-May Pan for technical assistance. This work was supported by a grant (NSC90-2320-B-320-012) from National Scientific Council and a grant (NHRI-EX91-9112NC) from National Health Research Institute, Taiwan.

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