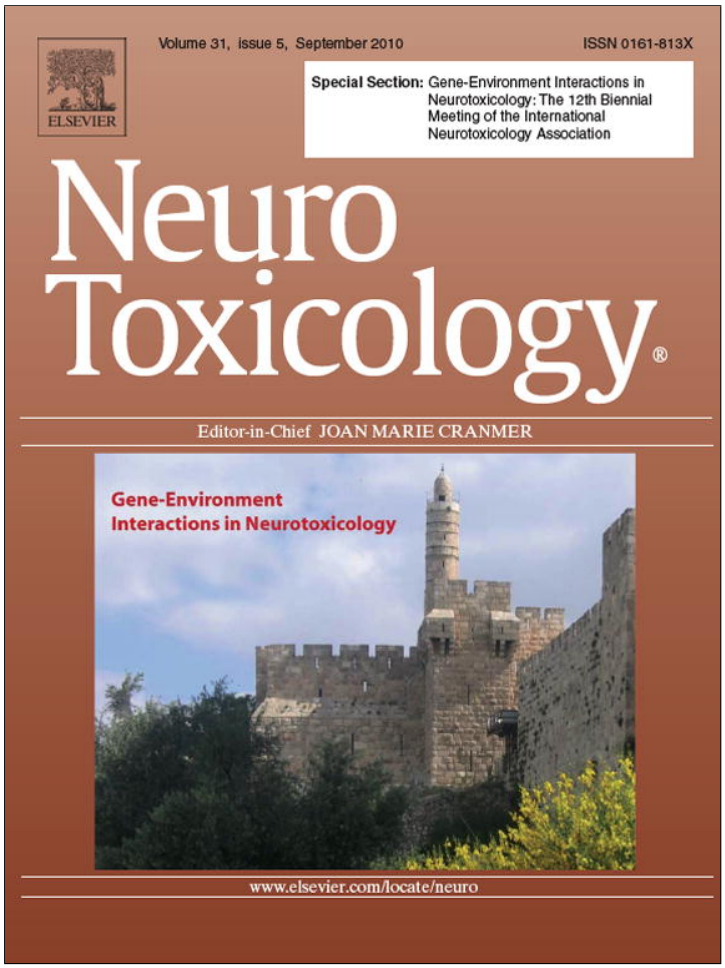

NeuroToxicology 31 (2010) 432–438

Contents lists available at ScienceDirect

NeuroToxicology

# Prenatal exposure to methamphetamine alters the mechanical withdrawal threshold and tonic hyperalgesia in the offspring

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ARTICLE INFO

*Article history:*
Received 15 March 2010
Accepted 4 June 2010
Available online 12 June 2010

*Keywords:*
Methamphetamine
Prenatal exposure
Nociception
von Frey
Formalin
Pain threshold

ABSTRACT

Maternal methamphetamine (MA) abuse during pregnancy has been proved to induce various impacts on the development of infant and child. In this study, we examined whether prenatal exposure to MA would affect the development of nociceptive system by measuring the responses to noxious stimulation in the developing rat. Adult female Sprague–Dawley rats received bi-daily subcutaneous injection of methamphetamine (5 mg/kg) or isovolumetric normal saline since the day of mating till the day of delivery. Birth profiles of the offspring including birth length, weight, and body temperature were recorded during the first postnatal month. Mechanical withdrawal thresholds were measured by von Frey filaments on postnatal day (PND) 30 and 60, and hyperalgesic behaviors following plantar formalin injection (2%, 50 μl) were evaluated on PND 60. The birth body weight and length of rats born to MA-injected dam rats (MA group) were significantly lower than those of the control rats during the first postnatal month; however, their body temperature was significantly higher than those of the control rats during the first 3 days after birth. The MA group rats had significantly lower tactile withdrawal values in von Frey test and higher pain scores in the late phase of pain in the formalin test than those of the control rats. There is a gender difference of nociceptive hypersensitivity manifested as that the female MA group rats had significantly lower withdrawal thresholds and higher pain scores in response to formalin injection than the male MA group rats. These results suggest that prenatal MA exposure could predispose an alteration in the development of nociceptive neuronal network, which leads to a long-lasting status of hypersensitivity to pain stimulations in the offspring.



## 1. Introduction

Methamphetamine (MA) is one of the most frequently used illicit drugs in the world. Increasing incidence of maternal abuse of this drug during pregnancy had raised serious public health concerns in terms of the adverse effects on the offspring (Rayburn and Bogenschutz, 2004). Clinical studies had demonstrated that intra-uterine exposure to MA caused abnormalities of the physical and neurobehavioral development in infants and children, including low birth body weight, low body length, low head circumference, poor feeding, abnormal sleep pattern and hypotonia (Smith et al., 2003, 2008; Oei and Lui, 2007). These changes in early life could last to the childhood stage, manifested as aggressive behavior, learning problems, and poor social adaptation (Smith et al., 2001; Chang et al., 2004). Animal experiments also demonstrated a variety of abnormalities in physical growth, behavior and neurocognition functions induced by prenatal MA exposure, including abnormal brain structure (Cui et al., 2006), and toxicities to nerve growth and synaptic functions (Weissman and Caldecott-Hazard, 1995; Slamberova et al., 2006; Melo et al., 2008; Pometlova et al., 2009). However, none of investigation on the influence of prenatal MA exposure on the pain perception in the offsprings has been formally reported.

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doi:10.1016/j.neuro.2010.06.002

Pain perception is indispensable for animal survival, serving as a protective alarm to prevent subjects from injuries like external threatening stimuli or internal organic damages. Pain sensation depends not only on the presence of environmental stimuli, but also on plastic controls of sensory transmission from spinal cord to brain (Woolf and Salter, 2000). Various neurotransmitter systems are involved, such as glutamate and substance P in pain transmission, serotonin, enkephalin and noradrenalin in descending inhibitory pathway, and dopamine and opioid for pain processing in limbic or cortical area. Prenatal exposure to addicted drugs can affect development of these neurotransmitter systems; thereafter alter pain sensitivity in a long-run. For instance, prenatal morphine exposure could alter kinetic properties of synaptic N-methyl-D-aspartate (NMDA) receptors in hippocampal neurons of rat offspring (Yang et al., 2000). As the effects of MA in CNS are majorly dependent on interaction with dopamine, norepinephrine and serotonin system (Weissman and Caldecott-Hazard, 1993; Gomes-da-Silva et al., 2002; Bubenikova-Valesova et al., 2009; Sofuoglu and Sewell, 2009), it is possible that prenatal exposure to MA can interfere the development of related nociceptive networks and change pain perception in the offspring (Millan, 2002; Pertovaara, 2006). To test this hypothesis, we investigated the noxious responses in the offspring born to dam rats which received bi-daily injections of MA throughout gestational period. Physical profiles including body length, weight and body temperature were recorded. The mechanical thresholds measured by von Frey test and the tonic pain evaluated by formalin injection were conducted on postnatal day (PND) 30 and PND 60. Both nociceptive assays have been proved to be reliable measures and have been widely used for pain studies (Dubuisson and Dennis, 1977; Wheeler-Aceto and Cowan, 1991; Tjolsen et al., 1992; Chaplan et al., 1994; Abbott et al., 1999).

## 2. Materials and methods

### 2.1. Animals preparation

Female Sprauge–Dawley rats (200–250 g, BioLASCO Taiwan Co. Ltd., Taipei, Taiwan) were housed individually in plexiglass cages on a 12-h light–dark cycle in the Animal Center of Taipei Medical University. The room temperature was maintained at $23 \pm 1$ °C. Food and water were available ad libitum throughout the experiment. The animal study followed the guideline of the Animal Center of Taipei Medical University. MA was purchased from the Narcotics Bureau of the National Health Administration, Taipei, Taiwan.

### 2.2. Animal model of prenatal exposure to MA

Each female was housed individually and mated with 2 adult male rats at the same time. Successful mating was proved by the presence of a copulatory plug found in the bed of housing cage (Tan, 2003). The day of finding the plug was counted as first gestational day. All female rats were randomly assigned to receive bi-daily subcutaneous injection of MA (5 mg/kg/day, 8 am and 5 pm) or vehicle (normal saline) of equal volume since the first day of gestation till the day of delivery. The dose of MA was chosen based on our preliminary study which showed that dose higher than 5 mg/kg, such as 7.5 mg/kg or 10 mg/kg, caused significant maternal death and intra-uterine fetal death, and 5 mg/kg MA produced apparent locomotor, exploratory behaviors and impairs sensory-motor coordination of dams rats without mortality and insignificance change in number of living birth. This result is consistent with other reports (Weissman and Caldecott-Hazard, 1995; Slamberova et al., 2005, 2007). Rats born to MA-exposed dams are denoted as the MA group rats, and rats born to saline-treated dam rats are denoted as the control group rats. MA group rats were separated from their biological mothers after birth, and randomly reared by the saline-treated dam rats. To keep the litter number limited, each surrogated mother raised five biological pups and the same number of MA-exposed foster pups, a method described previously (Slamberova et al., 2006). The number per litter, body length, weight and temperature, and mortality of the pups in each group were recorded from PND 1. After PND 21, the offspring of each group rats were separately reared with the same gender with 10–12 rats per cage, which were grouped by randomly picking up 2 rats from each litter. After PND 40, rats of each cage were separated equally into 2 cages for a larger living space.

### 2.3. Measurement of body length, body weight, body core temperature and postnatal mortality of the pups

The recording of physical profiles began at the first 12 h after birth. The body weights of the offspring were measured by an electronic weighting-scale with an accuracy of 0.002 g. The body length excluding the tail was measured with a standard ruler with minimal scale of 0.1 cm. Body core temperature was measured with an anal thermal sensor with sensitivity of 0.1 °C. The number of death in each group was summed at the end of the first postnatal month.

### 2.4. Determination of nociceptive sensitivity in the offspring rats

The nociceptive sensitivity was determined in the mature rats on PND 30 and PND 60 by von Frey stimulation and by formalin test. All the rats were randomly selected from different cohorts by a gender-matched and dam-equilibrated method for each group. For von Frey test, habituation to the test environment was started 3 days before experiment by placing the rats in plexiglas boxes ($11\ cm \times 13\ cm \times 24\ cm$) on an elevated metal mesh floor for 1 h without stimulation. On the experimental day, the rats were accommodated for 30 min before test. The mechanical stimulus was applied from underneath the mesh opening to the mid-plantar surface using up-down method (Chaplan et al., 1994). A series of von Frey filaments with logarithmically incremental stiffness (0.4 g, 0.6 g, 1 g, 2 g, 4 g, 6 g, 8 g, and 15 g; Stoelting, Wood Dale, IL) were chosen and each fiber was perpendicularly pressed onto the plantar surface for 5–6 s. Testing was initiated with the 2-g filament. Stimuli were presented in a consecutive fashion, whether ascending or descending. Fifty percent withdrawal threshold was calculated (Dixon, 1980), and two separated values by 5 min apart were averaged to represent the threshold measurement. The experimenter who performed the tests was blinded to the group allocation.

Formalin test was conducted on PND 60. 50 μl of 2% formalin diluted in water was subcutaneously injected into the plantar aspect of the left hind paw via a 26G needle. The rats were soon transferred to an iron-grid cage for 1-h behavioral observation. The weighted pain score, as described by Dubuisson and Dennis and modified by us (Dubuisson and Dennis, 1977; Wen et al., 2007), was assessed by computing the weighted average of the time spent for each of the following: 0 = the injected paw is not affected (i.e., foot flat on floor with toes splaying); 1 = the injected paw has little or no weight on floor with no toe splaying; 2 = the injected paw is elevated and the heel is not in contact with any surface; 3 = the injected paw is licked, bitten, or shaking. The time spent in each category was multiplied by the category weight and calculated at 1-min interval and averaged at 5-min blocks during the 1 h test period. To quantify the temporal pattern of pain behavior, two phases were identified: the early phase (initial 10 min) and the late phase (20–60 min), and the cumulative pain score (area under curve, AUC) at both phases were compared between groups. The same observer throughout the study was blinded to the group allocation.

### 2.5. Statistical analysis

All data were presented as mean ± S.E.M. (standard error of the mean). Unpaired Student *t* test was used to compare the differences in the body weight, body length, body temperature, and the birth number per little. One-way ANOVA with post hoc Bonferroni test were used to determine the difference of 50% threshold values in von Frey test and pain scores in formalin test among groups. $p$ value $< 0.05$ means statistically significant.

## 3. Results

### 3.1. Effect of prenatal MA exposure on the body weight, body length, body temperature and postnatal mortality in the offspring

During this experimental period, 72 control group rats, born by 7 dam rats, and 62 MA group rats, born by 6 dam rats, were survived at birth. In control group rats, 35 rats are male and 37 rats are female. In MA group rats, 34 rats are male and 28 are female. There is no difference in the number of pups per litter and in the mortality between the control group and the MA group rats (Table 1). The body weights of the MA group rats are significant lower than those of the control group rats since PND 1 till PND 30, and the body lengths of the MA group rats are significantly shorter than those of the control group rats since PND 1 till PND 14 (Fig. 1A and B). On the other hand, the core body temperatures of MA group rats are significant higher than those of control rats during the first three postnatal days (Fig. 1C).

### 3.2. Effect of prenatal exposure to MA on the mechanical withdrawal threshold in the offspring

Twenty control group rats (10 males, 10 females) and 24 MA group rats (12 males, 12 females). According to the rearing protocol, rats in each group are randomly chosen from different litters on PND 21. Mechanical withdrawal thresholds, as determined by hind paw responses to von Frey stimulation, of the MA group rats are 2.15 ± 0.25 g and 8.11 ± 1.08 g on PND 30 and PND 60, respectively. The thresholds of the control rats are 5.78 ± 0.69 g and 13.11 ± 0.77 g on PND 30 and PND 60, respectively. The threshold values of the MA group rats are significantly lower than those of the control group rats on both examined PND (Fig. 2A). The value of male and female rats of MA group on PND 30 is significantly lower than that of male rats or female rats of control group (Fig. 2B). However, there is no significant difference in the threshold value between male rats and female rats in either the control group rats or MA group rats. On PND 60, the threshold value of female MA group rats is significantly lower than that of male and female control group rats, whereas the threshold value of the male MA group is significantly lower than that of the male control group rats, but is significantly higher than that of female MA group rats (Fig. 2C).

### 3.3. Effect of prenatal exposure of MA on the formalin-induced hyperalgesia in the offspring

Formalin injections were performed in 12 control group rats (M/F = 6/6) and 12 group MA rats (M/F = 6/6), which are chosen by random. Both the control and MA group rats showed typical biphasic curve in pain scoring following plantar injection of formalin (Fig. 3A and B). Without considering the gender difference, the pain scores of MA group rats on 30 min and 35 min after injection of formalin are significantly higher than that of control group (Fig. 3A). However, the pain scores of the male MA group rats are not significantly different to that of control group rats in both early and late phase, whereas the pain scores of female MA group rats on 30 min and 35 min after injection of formalin are significantly higher than those of control group rats and the male MA group rats on 35 min after injection of formalin (Fig. 3B). When comparing cumulative pain scores (area under curve) regardless of the gender factor, the pain scores of late phase of MA group is significantly higher than that of control group rats (Fig. 4A). However, as the gender factor was considered, the difference between MA group and control group rats became insignificant, either in the early or the late phase, though the scores of MA group rats appears to be higher than those of control group rats (Fig. 4B).

**Table 1**
The number of birth and postnatal mortality of the control and the MA group dam rats.

| | Control dam rats ($n=7$) | MA dam rats ($n=6$) |
|---|---|---|
| No. birth/litter[a] | 10.3 ± 0.5 | 10.3 ± 0.9 |
| Mortality[b] | 4/72 (5.5%) | 3/62 (4.8%) |

[a] The data of no. birth/litter are mean ± S.E.M.
[b] The data of mortality are death/total birth with death percentage.

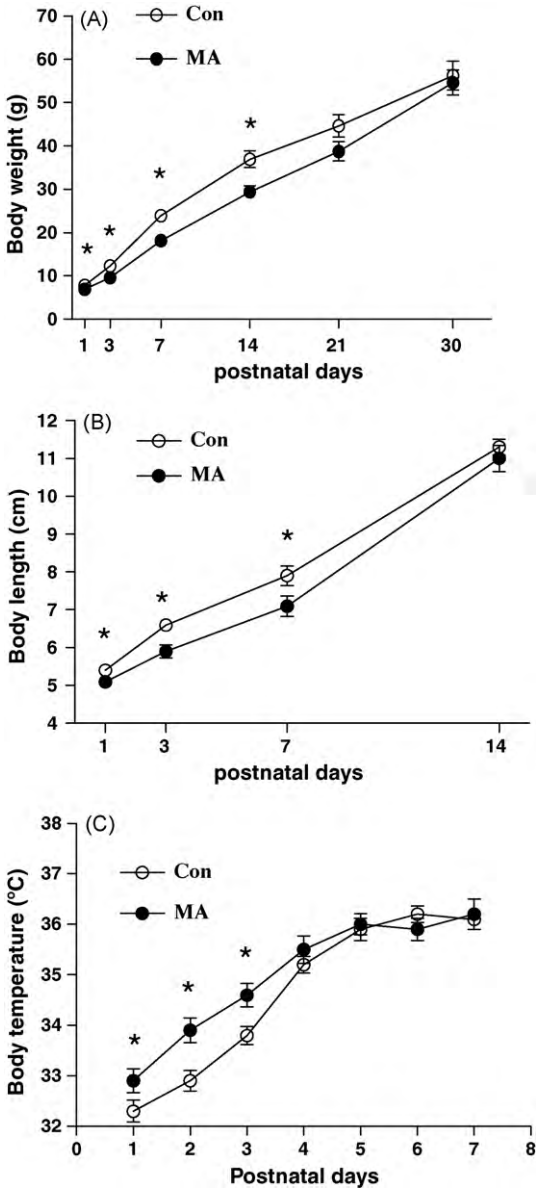


**Fig. 1.** The growth gain of body weight, body length, and the body temperature of the control (Con) and the methamphetamine (MA) group. Data are mean ± S.E.M. The numbers of control group and MA group are 36 and 37, respectively. $^{*}p < 0.05$, unpaired Student *t* test.

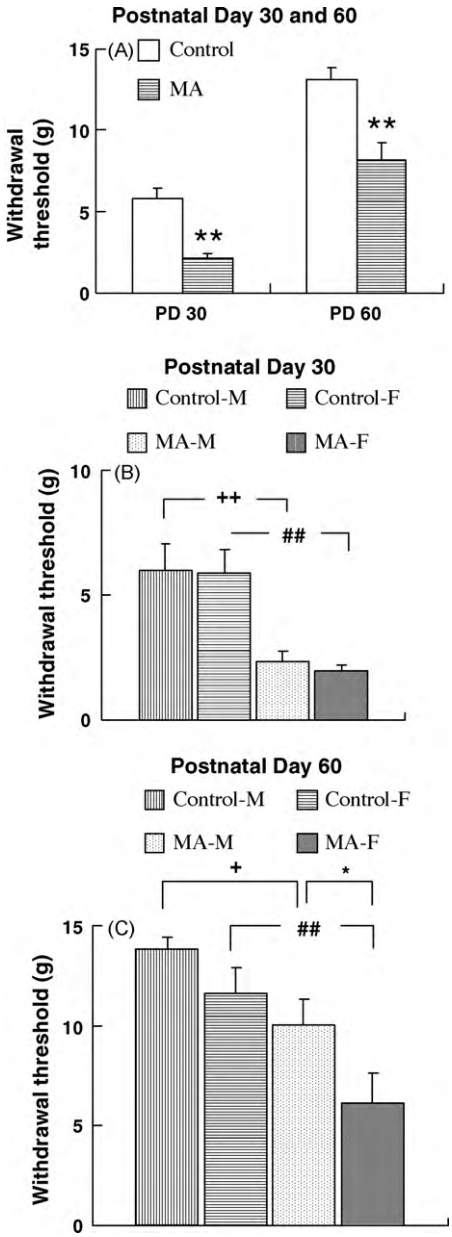


**Fig. 2.** Mechanical thresholds measured by von Frey stimulation of the control and the MA group. Data are mean ± S.E.M. The numbers of the control group and the MA group are 20 (10 males, 10 females) and 24 (12 males, 12 females), respectively. The male and female rat of the control group is denoted as Control-M and Control-F, respectively. The male and female rat of the MA group is denoted as MA-M and MA-F, respectively. (A) The result of test on PND 30 and PND 60 without gender separation. $^{**}p < 0.01$, unpaired Student $t$ test. (B) The result of test on PND 30 with gender separation. ++ means significant difference between Control-M and MA-M, $p < 0.01$; ## means significant difference between Control-F and MA-F, $p < 0.01$, all by one-way ANOVA with *Bonferroni* post hoc test. (C) The result of test on PND 60 with gender separation + means significant difference between Control-M and MA-M, $p < 0.05$; ## means significant difference between Control-F and MA-F, $p < 0.01$; *means significant difference between MA-M and MA-F, $p < 0.05$, all by one-way ANOVA with *Bonferroni* post hoc test.

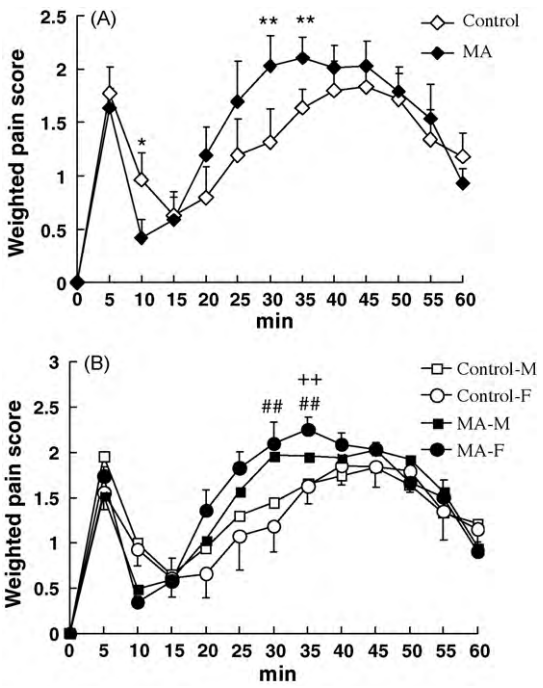


**Fig. 3.** Formalin-induced hyperalgesia of the Control and the MA group rats on PND 60 s. Data are mean ± S.E.M. Both of the control and the MA group have 12 rats (6 males, 6 females) in each group. The male and female rat of the control group is denoted as Control-M and Control-F, respectively. The male and female rat of MA group is denoted as MA-M and MA-F, respectively. (A) Pain scores between groups without gender separation. $^{*}p < 0.05$, $^{**}p < 0.01$, unpaired Student $t$ test. (B) The pain scores with gender separation. Some error bars in (B) are omitted for a better graphic clarity. ## means significant difference between Control-F and MA-F, $p < 0.01$; ++ means significant difference between MA-M and MA-F, $p < 0.01$, all by one-way ANOVA with *Bonferroni* post hoc test.

## 4. Discussion

The finding that prenatal MA exposure cause lower gains in body weight and length at the early postnatal period is consistent with evidence in neonates born to mothers with MA abuse during pregnancy (Little et al., 1988; Smith et al., 2003) and experimental pups prenatally exposed to MA (Martin, 1975; Martin et al., 1976; Slamberova et al., 2006). The growth differences between the MA group and control group disappeared gradually from PND 14 to PND 30, suggesting that the impairment of embryo growth by prenatal MA exposure could be overcome by postnatal feeding. One particular point is that MA group rats had higher body temperature during the first 3 days after birth as compared to that of control group. Such transient hyperthermia phenomenon could be interpreted as a withdrawal symptom to MA. But, it is also possible that hyperthermia was caused by residual MA in the body of MA rats after birth, as it has been shown that MA administration induced hyperthermia in adult animals (Bowyer et al., 1994; Albers and Sonsalla, 1995; Miller and O'Callaghan, 1995). The MA-induced hyperthermia effect has been attributed to results of hyperactive metabolic status, hyper-excitability, dysregulation of the thermoregulatory system (Smith et al., 2008; Paz et al., 2009). Interestingly, previous reports has proved that greater accumulation of MA by the DA transporter at higher temperatures associated with increased DA release and the production of reactive oxygen species (Xie et al., 2000). Prevention of MA-induced hyperthermia could reduce or block the neurotoxicity (Ali et al., 1996). Whether such transient postnatal hyperthermia contributes to the long-lasting change in the nociception in the MA group rats is one interesting issue to be determined.

The principle finding of this study is that the MA rats had lower mechanical withdrawal threshold values in von Frey on PND 30 and PND 60, and higher pain scores at late phase of formalin test in the hind paw when compared to the control rats on PND 60. The plantar withdrawal evoked by von Frey fibers represents the sensitivity of nociceptive reflex to tactile stimulation. In general, a decrease in the threshold value is interpreted as an increased sensitivity to acute pain stimulation, which can be attributed to hyper-excitability of peripheral conducting nerves, called the

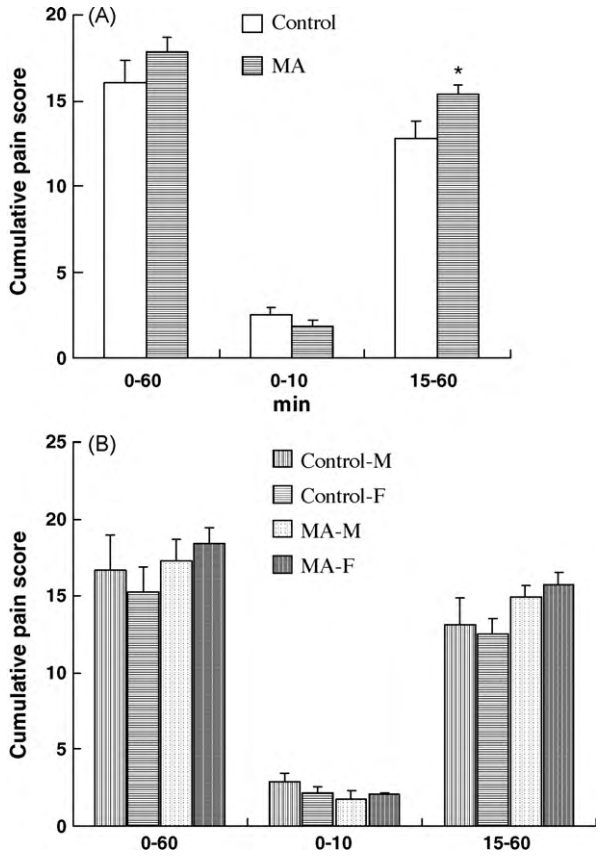


**Fig. 4.** The cumulative pain score (area under curve) at the early (0–10 min) and late (15–60 min) phases in the formalin test. Data are mean ± S.E.M. Both of the control and the MA group have 12 rats (6 males, 6 females). The male and female rat of the control group is denoted as Control-M and Control-F, respectively. The male and female rat of the MA group is denoted as MA-M and MA-F, respectively. (A) Pain scores between groups without gender separation. * Means significant difference between the Control and the MA group, $p < 0.05$, unpaired Student $t$ test. (B) Pain scores with gender separation.

tactile allodynia (Keizer et al., 2008). On the other hand, formalin injection, serving as the type of supra-threshold nociception, induces pain response through a more complicated network in the nervous system. Formalin-induced pain could be temporally divided into two phases, an early phase indicating activation of peripheral noxious afferent pathway and a late phase reflecting tonic pain by activation at spinal cord or supra-spinal level. Thus, an increase of formalin pain score at late phase suggests an increased central sensitization to a potentiate pain responses (Tjolsen et al., 1992; Abbott et al., 1995). Taking our data in both tests together, it seems that MA group rats own an genetically nociceptive hypersensitivity through developmental alterations in both peripheral and central nervous circuitry. Since the pain measurements were conducted at the ages of 1 and 2 months, it is likely that these changes may not be related to withdrawal phenomenon from prenatal MA and will persist life-long.

The mechanism underlying the increased pain perception induced by prenatal exposure to MA is not clear at present. A growing body of evidence indicates that maternal MA or amphetamine abuse during gestation causes neurological toxicity in the brain of offspring, and the most vulnerable targets are mesocortical dopaminergic pathway in the ventral tegmental area, nucleus accumben (Pontieri et al., 1990; Bjijou et al., 2002; Bubenikova-Valesova et al., 2009) and the dopamine receptor/transportor in the cortex (Gross and Marshall, 2009; Schwendt et al., 2009). Alterations in these structures could occurs as early as at the embryonic stage and could persist throughout the maturity course of the monoamine system until the first month of postnatal life (Voorn et al., 1988). Using an extracellular recording technique, prenatal exposure to amphetamine has been found to cause decreased number of spontaneously active norepinephrine cells in the locus ceruleus nucleus, a region with high level of norepinephrine and regulating the descending pain modulation system, in adult rat (Ramirez and Wang, 1986; Nasif et al., 1999). These specific toxicities on the brain region containing high activity of monoamine could be the most possible pathological mechanism responsible for the pain hypersensitivity induced by prenatal exposure to MA. However, the contribution from alteration in the neural system other than the monoamine could not be ignored. Activation of NMDA receptors in the spinal cord dorsal horn contributes to the central sensitization after persistent pain, and was found to be critical for hyperalgesia in the late phase pain of formalin test (Eisenberg et al., 1993). Interestingly, it was found that intrathecal injection of cocaine- and amphetamine-regulated transcript (CART) peptides in rats could enhance NMDA-mediated thermal hyperalgesia and depolarization in substantia gelatinosa neurons in vitro (Hsun Lin et al., 2005). Other reports also demonstrated that CART mRNA and peptides are involved in reward and reinforcement effects after the use of cocaine and amphetamine, and are closely linked to the actions of mesolimbic dopamine system (Jaworski and Jones, 2006; Rogge et al., 2008). In addition, it was shown that administration of MA could increases CART mRNA levels in the nucleus accumbens (Jean et al., 2007). By merging all these results, we tentatively propose that the activity of NMDA receptors could be up-regulated by transplacental MA during early neural development, which then cause an increase in the pain perception. Similar mechanism has been shown in rats prenatally exposed to morphine which present tolerance to morphine analgesia (O'Callaghan and Holtzman, 1976; Alleva et al., 1987; Tao et al., 2001) with increased NMDA receptor-mediated current activity (Yang et al., 2000).

It is intriguing to note that the change in the pain sensitivity in MA group rats has gender difference. Recently, sexual difference in pain responses has drawn growing attentions, with more studies demonstrating that female rats are more sensitive than males to nociception in various assays, such as tail-flick tests (Craft et al., 1999), formalin test (Gaumond et al., 2002; Gaumond et al., 2007), capsaicin-induced noxious behaviors (Lu et al., 2009), and brain reactions to visceral nociception (Wang et al., 2009). In this study, the female MA rats had a significantly lower value of withdrawal threshold as compared with the control group rats and the male MA group rats on PND 60. Agreeably, the finding at the late phase of formalin test showed that the MA females had significantly higher pain scores than the control rats and the MA males on PND 60. From this aspect, it seems that the female MA offspring retain the genetic hypersensitivity to nociception during prenatal MA exposure. Gender difference is also present in other perinatal MA-induced toxicity. For instance, postnatal MA administration during the first month of life increased the tyrosine hydroxylase (TH) activity and mRNA levels in the brain of male offspring on PND 30 but not in female offspring (Gomes-da-Silva et al., 2000). On the contrary, prenatal MA exposure caused a decrease of TH mRNA in the dopaminergic nucleuses of the female offspring but had no effect on the male offsprings (Gomes-da-Silva et al., 2002). Whether such gender difference in response to MA are related to the sex hormone, like estrogen or testosterone, is worthy for further exploration.

Estrous cycle could be another factor to affect the nociceptive alterations in the females. Fluctuation of nociceptive behaviors with estrous cycle was also reported in tail-flick test (Martinez-Gomez et al., 1994), formalin-induced pain at the paw (Mannino et al., 2007), at the temporamandibular joint (Fischer et al., 2008), and vaginal/uterine distention (Bradshaw et al., 1999; Ji et al.,

2008). Though different gonadal hormones variously modulate basal nociception and opioid antinociception (Stoffel et al., 2003), most studies agree that the female have higher noxious responses at the proestrus stage than at the estrus stage because estradiol helps to produce anti-hyperalgesic effect (Kuba et al., 2005; Mannino et al., 2007). However, there were also contradictory results showing that estrous cycle affects neither the delayed formalin-induced licking in mice (Kim et al., 1999), mechanical allodynia in carrageenan-induced pain model (Tall and Crisp, 2004) nor potentiation of 17β-estradiol to capsaicin-induced hypersensitivity (Lu et al., 2009). Taken together, female nociceptive response is multifactorially dependent and more evidence will be needed to confirm the contribution of estrous cycle to behaviors in prenatal MA-exposed female rats.

More recently, effect of prenatal MA exposure on postnatal adults' nociceptive changes was reported (Hruba et al., 2010). However, their result is in contrast to ours by showing that prenatal MA exposure did not affect nociception in the adult females but postnatal MA (i.e., continuous MA administration to lactating mothers) had pro-nociceptive effect. There are at least three reasons might explain such discrepancy. The first reason is the difference in the MA treatment protocol. In Hruba's study, the dam rats received MA injection since 3 weeks prior to mating. The second reason is that the difference in the nociceptive tests and timing of test. Nociception was determined by withdrawal responses of forelimbs, hindlimbs, and tail to noxious heat in Hruba's study, whereas, we measured mechanical threshold changes and inflammatory nociception at the hindpaws. The offspring in this study were tested at the ages younger than those of Hruba's study (30–60 days vs. 85–90 days). Younger rats are more sensitive to noxious stimulation, and may have higher chances to show subtle differences. Nevertheless, both our and Hruba's studies support the idea that early and long-term exposure to MA will alter the nociceptive development in the developing animals.

## 5. Conclusion

The present animal study demonstrated that prenatal MA exposure could cause hypersensitivity to pain stimulation in the developing offspring. Our result supports the idea that the development of nociceptive system is subjected to the MA-induced neurotoxicity. Future studies are required to determine the underlying cellular and molecular mechanisms, and to identify the long-term impact on the affected human subject to endure the different nociceptive stimulation, such as trauma, inflammation, or nerve injury.

## Conflict of interest

Nothing declared.

## Acknowledgements

This study was supported by the Grants of DOH95-NNB-1019, NHRI-EX97-9401NP, SKH-TMU-92-33 and SKH-TMU-93-41.

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