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Neonatal Morphine Exposure Induces Age-Dependent Alterations in Pentylenetetrazole-Induced Epileptic Behaviors in Prepubertal Rats

ABSTRACT: *Opioids show both pro- and anti-epileptogenic effects in different experimental models of epilepsy. In the present study, the pentylentetrazole (PTZ)-induced seizure model was used to test the hypothesis that neonatal morphine administration affects seizure susceptibility in prepubertal rats. Female rats were subcutaneously injected with either morphine or saline on postnatal days 8–14 (P8–P14). To verify the long-term effect of morphine (or saline), the animals were treated second time with morphine (21 mg/kg; or saline) on either P25 or P32. Morphine administration decreased latency of myoclonic jerks and time to onset and increased tonic–clonic seizure rate at P25, but these findings were inversed at P32. Results showed a significant age difference in seizure behaviors between P25 and P32 animals. Blood corticosterone (COS) levels were significantly higher in P32 rats than in P25 rats. These findings show that neonatal morphine exposure plays an important role in increasing seizure vulnerability in P25 prepubertal rats but not in P32 rats. We conclude that early exposure to chronic morphine in infant rats might change their susceptibility to PTZ-induced seizure in an age-dependent manner. © 2012 Wiley Periodicals, Inc.* Dev Psychobiol 55: 881–887, 2013.

Keywords: *morphine; neonate; pentylenetetrazole; seizure; prepubertal; development; rat*

INTRODUCTION

Epilepsy is one of the oldest conditions known to mankind and the most common neurological disorder after stroke affecting individuals of all ages (Loscher, 2002). Intraperitoneal (IP) administrations of pentylenetetrazole (PTZ) are used to investigate the effects of acute and chronic epilepticseizures in animals (Bertram, 2007; Caspers & Speckmann, 1972). Although descending inhibitory mechanisms are not completely formed until the third week of life in humans (Nandi & Fitzgerald, 2005), morphine and other opiate agonists are effective analgesics during the early neonatal period because of the presence of spinal opiate receptors at birth (Rahman & Dickenson, 1999). Changing

Manuscript Received: 23 December 2011
Manuscript Accepted: 10 August 2012
Conflict of interest: The authors have no conflicts of interest to declare regarding the study described in this article and the preparation of the article.
Ethical Approval: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
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Contract grant sponsor: Research Council of Urmia University of Medical Sciences, Urmia, Iran.
Article first published online in Wiley Online Library (wileyonlinelibrary.com): 13 September 2012
DOI 10.1002/dev.21080 • © 2012 Wiley Periodicals, Inc.

morphine sensitivity in the post-natal period may be part of a general reorganization in the structure and function of primary afferent synapses, neurotransmitter/receptor expression and function, and excitatory and inhibitory modulation from higher brain centers (Fitzgerald & Beggs, 2001; Pattinson & Fitzgerald, 2004). For example, the density of μ-opioidreceptor binding is seen during the first 3 post-natal weeks, with peak binding at postnatal day 7 (P7) that decreases to adult levels by P21 (Rahman, Dashwood, Fitzgerald, Aynsley-Green, & Dickenson, 1998). The use of opiate analgesia has increased in neonatal intensive care units (NICUs) in recent decades as a consequence of the changes and advances in the understanding and treatment of pain in children (El Sayed, Taddio, Fallah, De Silva, & Moore, 2007). In humans, maternal opiate abuse during gestation induces permanent neurological and behavioral abnormalities in offspring (Chasnoff, 1985). Animal studies indicate that administration of morphine during gestation causes morphological and behavioral aberrations in rat offspring including changes in seizure susceptibility (Koyuncuoglu & Aricioglu, 1993; Schindler, Veliskova, Slamberova, & Vathy, 2000; Vathy, Rimanóczy, Eaton, & Kátay, 1994; Vathy, Velíšková, & Moshé, 1998; Velíšek, Velíšková, Moshé, & Vathy, 1998). In adult rats, morphine exposure during gestation days 11–18 alters seizure susceptibility in a sex- and hormone-dependent manner in the flurothyl seizure model (Vathy et al., 1998; Velíšek et al., 1998). However, an important and less well-known field of investigation is the modulatory effect of opioids on seizure susceptibility. Previous reports indicated heterogeneity of regulation of convulsive phenomena by opioids. Opioid receptor agonists such as morphine could modulate seizure susceptibility in a biphasic manner, causing dose-dependent anti- and pro-convulsant effects (Homayoun, Khavandgar, Namiranian, et al., 2002; Honar, Riazi, Homayoun, Demehri, et al. 2004; Lauretti, Ahmed, & Pleuvry, 1994; Shafaroodi et al., 2007; Yajima et al., 2000; Zhang & Ko, 2009). The biphasic effects of morphine on PTZ-induced clonic seizure threshold has been previously described (Honar, Riazi, Homayoun, Sadeghipour, et al. 2004; Lauretti et al., 1994). Morphine excites dopamine neurons in the ventral tegmental area of the brain through inhibition of gamma-aminobutyric acid (GABA) ergic inhibitory interneurons (Tzschentke, 1998). PTZ acts as a $GABA_A$ receptor antagonist (Becker, Grecksch, Thiemann, & Hollt, 2000). Therefore, both morphine and PTZ act as GABA receptor inhibitors. Although the effect of morphine on seizure thresholds has been studied in detail, very little is known about morphine exposure during the early life stage and seizure susceptibility in the prepubertal period. Neonates and infants may be exposed to opiates in medical settings, during painful procedures, or through living with parents who use opiates. The long-term consequences of neonatal morphine exposure are not known.

This study was designed to evaluate the effects of repeated neonatal morphine exposure (P8–P14) upon PTZ-induced seizures in prepubertal rats.

METHODS

Subjects

Twelve-week-old male and female Wistar rats (200–250 g) were obtained from the Urmia University of Medical Sciences, Urmia, Iran. Rats were housed in same-sex groups of 4 per cage in our animal facility under a 12-hr light/dark cycle (07:00–19:00 hr lights on), at a 22 ± 2°C, with free access to food and water. All experimental protocols and procedures complied with the guidelines of the 1975 Declaration of Helsinki as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, Iran. In addition, the Regional Medical Ethics Committee in the West Azarbyjan province of I.R. Iran approved this study. All females were mated at 13 weeks of age with a sexually experienced male of the same genotype. Each female was paired with 1 male at 9:00 AM, and we checked for the presence of plugs in females at 3:00 PM. Plugged females were immediately housed individually for the entire gestation (Ahmadzadeh, Saboory, Roshan-Milani, & Pilehvarian, 2011; Rangon et al., 2007; Sadaghiani & Saboory, 2010). If a plug was not observed, the animal was returned to her home cage for a new mating procedure. The fertilization rate was 70% in this study. In order to achieve a sufficient number of pregnant rats per group, we initially chose 30% more rats for mating in each group. Therefore, sufficient pregnant rats were available per group after mating.

Morphine Administration

After parturition, pups from different litters were mixed and evenly divided between dams. All pups were housed with their dams until weaning. Male pups were selected for another study. Neonate female rats ($n = 66$) were randomly divided into two groups: saline (S; $n = 29$) and morphine (M; $n = 37$). Pups in the morphine group received morphine subcutaneously (SC) on the back above the tail with additive doses (3, 6, 9, 12, 15, 18, and 21 mg/kg) every day between 11:00 and 12:00 hr from P8 to P14. These doses were chosen because previous studies reported similar doses (Jones et al., 2002; Rana, Mallet, Robertson, & Wainwright, 2010). Each day, the injected dose of morphine was increased by 3 mg/kg. Therefore, tolerance of the rat's body to morphine was minimized during morphine administration. Pups in the saline group received saline in a similar manner. P8 was chosen because it has been reported that rats of this age have a similar level of neurological development as that of a newborn human (Fitzgerald & Anand,

1993), and they are physiologically immature (Pattinson & Fitzgerald, 2004). Each pup received either saline or morphine on P8, once per day for 7 consecutive days. Both morphine and saline administration occurred at the same time each day (11:00 and 12:00 hr). Morphine sulfate (Temad Co., Tehran, Iran) was dissolved in .9% saline and freshly prepared for each use.

For the long-term effect experiment, rats in both groups were further divided to two subgroups: P25 and P32. In the saline group, 14 rats received PTZ only either on P25 or P32 (SP25, $n = 8$ and SP32, $n = 6$), while the remaining animals ($n = 15$) received saline followed 30 min later by PTZ (SSP25, $n = 7$ and SSP32, $n = 8$). In the morphine group, 18 rats received PTZ only (MP25, $n = 10$ and MP32, $n = 8$), and the remaining animals received morphine followed 30 min later by PTZ (MMP25, $n = 9$ and MMP32, $n = 10$). On P26 or P33 (24 hr after PTZ injection), blood samples were collected by cardiac puncture under halothane anesthesia between 08:00 and 09:00 hr. Blood was collected in EDTA-coated microcentrifuge tubes. Samples were kept on ice and later centrifuged for 20 min at 9,000 rpm and 4°C. Plasma was transferred to clean microcentrifuge tubes, and stored at −20°C until COS levels were determined (Heshmatian, Roshan-Milani, & Saboory, 2010; Rangon et al., 2007). Plasma COS was measured using a commercially available radioimmunoassay (RIA) kit (Isotope, Budapest, Hungary). Values are expressed as nanograms per milliliter (ng/ml).

Weighting and Behavior Assessment

On P7, all rats were weighed (at 08:30 hr). Weight was also measured on injection days and on P15 at 08:30 hr. On P25 and P32, prepubertal rats were injected with PTZ (60 mg/kg, IP). Following injection, the rats were placed separately in an isolated transparent glass cages and were observed for 90 min. Seizures were assessed using a previously defined scale (Becker et al., 2000). In this scale, 0 = no response; 1 = ear and facial twitching; 2 = myoclonic jerks without rearing; 3 = myoclonic jerks with rearing; 4 = turning over onto 1 side with tonic–clonic seizures; and 5 = turning onto back with generalized tonic–clonic convulsions. Other parameters monitored were clonic seizure of extremities, duration of immobility, pulling, and grooming rates. Animals were also monitored for fatal effects of PTZ until 24 hr after injection.

Statistical Analyses

Normally distributed data (weight, COS, some seizure features) were analyzed using parametric techniques. Two-group comparisons were performed using a *t*-test, whereas multiple-group comparisons were performed using the one-way analysis of variance (ANOVA). When appropriate, the Tukey test was used for post hoc analyses. Rates of grooming, pulling, tonic–clonic seizure rates, and clonic seizure of extremities were analyzed using κ^2 and Fisher's exact test. All tests used a critical significance level of $p < .05$. Results are expressed as mean ± SEM.

RESULTS

Body Weight

Effect of daily administration of morphine on pup body weight is shown in Table 1. Injection of morphine in cumulative doses did not result in significant weight changes in infant rats.

Effects of Morphine Administration on COS Levels in Prepubertal Rats

The effects of morphine administration on COS blood levels were determined at P25 and P32 from any litter in all groups. Morphine significantly increased COS levels in pups at P25 ($p < .001$). Uneven elevation of COS levels at P25 and P32 indicates an age-dependent impact of seizure activity on COS release (Fig. 1).

Effects of Neonatal Morphine Administration on PTZ-Induced Seizures

All pup rats were injected with PTZ (60 mg/kg, IP). Following PTZ administration, time to onset of first epileptic behavior (ear and facial twitching) showed an age-dependent feature. It significantly differed between similar groups of different ages ($p < .001$). Data are illustrated in Figure 2.

There were no significant differences in the rates of tonic–clonic seizures or pulling between same morphine and same saline groups. There were significant differences in grooming rates between SSP25 and SSP32 and between MP32 and MMP32 (Fisher's exact test, $p < .05$). There were significant differences in duration of immobility between SP25 and MP25, SP32 and MP32, MP32 and MMP32 (ANOVA, $p < .001$; Tukey, $p < .01$). Finally, there were significant differences in latency of myoclonic jerks between SP25, MP25, and SSP32 (ANOVA, $p < .001$; Tukey,

Table 1. Neonate Rats Received Daily Morphine or Saline for 7 Days (P8–P14)

Postnatal Day	Morphine	Saline
P7	9.58 ± .34	9.43 ± .27
P8	10.26 ± .37	10.03 ± .31
P9	11.06 ± .4	10.96 ± .33
P10	11.79 ± .44	12.06 ± .42
P11	12.6 ± .46	13.13 ± .42
P12	13.31 ± .48	13.98 ± .43
P13	13.98 ± .36	15.07 ± .49
P14	15.3 ± .5	15.86 ± .63
P15	16.44 ± .54	16.71 ± .63

Body weights (g) during the experiment did not change across treatment groups prior to weaning.

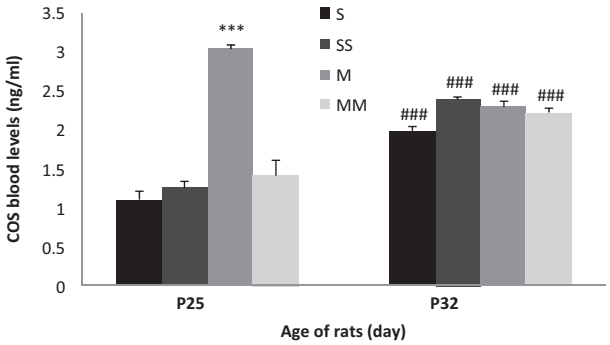

FIGURE 1 Effect of neonatal morphine administration on COS blood levels of prepubertal rats after PTZ-induced seizure. The COS levels increased in MP25 as compared with controls (SP25; ANOVA, $F = 44$, 86, $^{**}p < .001$, Tukey $p < .001$). Meanwhile, COS blood levels were significantly higher in P32 groups than in P25 groups (except MP25 that was increased compared with MMP32) ($^{*}p < .001$). There was no significant difference between P32 rats.

$p < .01$) and between SSP25, MMP25, and SSP32; SP32 and MP32; SSP32 and MMP32; MP25 and MP32; and MMP25 and MMP32 (ANOVA, $p < .001$; Tukey, $p < .01$). Data are illustrated in Table 2.

PTZ induced a sequence of events (in some cases) starting with myoclonic jerks which was then followed by a clonic convulsive phase. Early life morphine exposure significantly decreased the clonic seizure of extremities in both age groups. There was not any significant difference between the 32- and 25-day-old rats (Fig. 3).

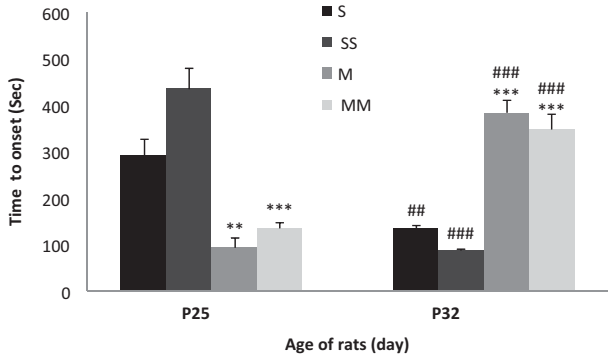

FIGURE 2 Neonatal rats received morphine or saline daily for 7 days. Then, PTZ-induced seizures were assessed on P25 and P32. Time to onset of first epileptic behavior (ear and facial twitching) was significantly different between same groups on P25 and P32. There were significant differences between animals in groups SP25, SSP25, SP32, SSP32 and MP25, MMP25, MP32, MMP32, respectively. $^{***}p < .001$ and $^{**}p < .005$ with same control group, $\#\#\#p < .001$ and $\#\#p < .01$ with same P25 group.

DISCUSSION

The present study was designed to determine whether neonatal morphine administration affects seizure susceptibility, as measured by PTZ-induced seizure characteristics and development in prepubertal rats. Neonatal rats were injected with either saline or morphine daily from P8 to P14. Seizure susceptibility was tested with PTZ-induced seizures on P25 and P32. The most important finding of this study was that the effect of morphine on seizure susceptibility was age-dependent. Morphine potentiated seizure severity in 25-day-old rats but attenuated it in 32-day-old rats.

In addition, this study showed the effects of additive doses of morphine on COS levels and body weight. Additive doses of morphine did not have significant effect on pre-weaning weight gain, as outlined in Table 1. These data are consistent with published data showing that compared with saline-exposed control rats, rats administered morphine (3 mg/kg; stable dose) SC on the back above the tail did not show a change in pre-weaning weight gain (Zhang & Sweitzer, 2008). In the present study, morphine administration decreased latency of myoclonic jerks and time to onset and increased tonic–clonic seizure rates on P25, but these results were reversed on P32, as outlined in Table 2. Our results suggest that seizure severity on P25 is greater than that on P32. These results are consistent with previous studies in developing rats, demonstrating that morphine-induced changes in seizure susceptibility are age-dependent (Vathy et al., 1998). The opioid system is one of the endogenous modulatory mechanisms that affect seizure susceptibility. Morphine exerts biphasic and dose-dependent anti-convulsant and pro-convulsant effects in the experimental model of clonic seizure induced by the GABA transmission blocker PTZ (Homayoun et al., 2002; Lauretti et al., 1994). At very high doses, morphine induces seizures by itself (Wikler & Altschul, 1950). Low doses of morphine could exert a dose-dependent anti-convulsant effect in the seizure models, in which seizures are induced by blockers of GABA transmission, such as picrotoxin, PTZ, bicuculline, and isoniazid (Becker et al., 2000; Lauretti et al., 1994; Shafaroodi et al., 2007).

Furthermore, we show that chronic exposure to morphine during the neonatal period results in age-dependent changes in susceptibility to PTZ-induced seizures. We demonstrate that susceptibility on P25 is similar to that observed with high doses of morphine and that susceptibility on P32 is similar to that observed with low doses of morphine. Previous studies have shown that chronic opiate exposure during infancy may affect the developing central nervous system (CNS) and alter the number of opioid receptors

Table 2. Classification of Seizure Parameters in Prepubertal Rats After IP Administration of 60 mg/kg PTZ

Group	Duration of Immobility (min)	Latency of Myoclonic Jerks	Tonic–Clonic Seizure Rate	Pulling Rate	Grooming Rate
SP25	48.66 ± 2.67	269.67 ± 39.95	25% (2/8)	62.5% (5/8)	37.5% (3/8)
SSP25	53.75 ± .7	781 ± 64.86	28.6% (2/7)	42.9% (3/7)	85.71% (7/8)
MP25	40.89 ± 1.146[a]	141.33 ± 25.51[a]	60% (6/10)	70% (7/10)	50% (5/10)
MMP25	55.25 ± 1.41	167.5 ± 19.25[a]	55.6% (5/9)	77.8% (7/9)	33.33% (3/9)
SP32	53 ± 1	186 ± 19.52	66.7% (4/6)	100% (6/6)	83.33% (5/6)[b]
SSP32	50 ± .80	164.8 ± 13.07	62.5% (5/8)	50% (4/8)	25% (2/8)
MP32	40.5 ± 1.68[a]	581.17 ± 44.89[a,b]	37.5% (3/8)	75% (6/8)	62.5% (5/8)
MMP32	51.25 ± .88[c]	379 ± 23.77[a,b]	30% (3/10)	40% (4/10)	0% (0/10)[c]
Test	ANOVA $F(7) = 14.811$ $p < .001$, Tukey $p < .01$	ANOVA $F(7) = 40.748$ $p < .001$, Tukey, $p < .01$	Fisher's exact test, n.s.	Fisher's exact test, n.s.	Fisher's exact test, $p < .05$

Neonate rats received morphine or saline daily from P8 to P14. PTZ-induced seizure was assessed both on P25 and P32.

[a]Significant difference with the same saline (e.g., SP25 with MP25; SSP25 with MMP25) group ($p < .01$).

[b]Significant difference between two age groups of P25 and P32 by the same name (e.g., SP32 with SP25; SSP32 with SSP25; $p < .01$; and $p < .05$ for grooming rate).

[c]Significant difference with MP32 ($p < .01$; and $p < .05$ for grooming rate).

(Thornton & Smith, 1998). Some studies suggest that the development of opioid receptors occurs within the first 2 weeks of life (Nandi & Fitzgerald, 2005). It is therefore likely that exposure to morphine from P8 to P14 leads to alterations in developing brain systems, such as the glutamate system, which in turn could change susceptibility to seizures.

COS blood levels were significantly greater in MP25 rats than in other groups. COS blood levels were significantly greater in P32 rats than in P25 rats. These results suggest that either PTZ-induced seizures are much more stressful in 32-day-old rats than in 25-day-old rats or baseline COS blood levels are age-dependent. It has been reported that a single morphine injection induces ACTH and COS release in rats (Breese, Knapp, & Overstreet, 2004; Jezova et al., 1987).

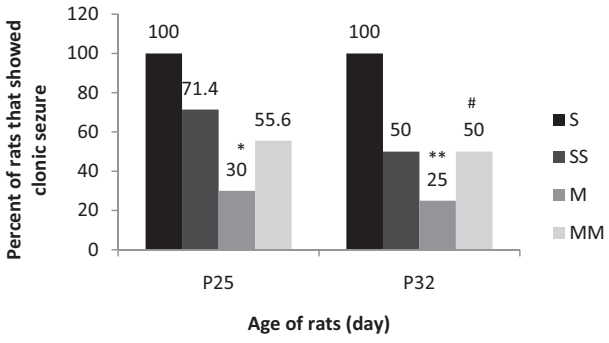

FIGURE 3 Neonatal rats received morphine or saline daily for 7 consecutive days. Then, PTZ-induced seizures were assessed on P25 and P32. Clonic seizure of extremities was significantly different between certain groups (Fisher exact test, $^{*}p = .004$, $^{**}p = .009$, and $\#p = .042$ significantly different from SP25 to SP32).

Therefore, the excitatory effect of neonatal morphine on prepubertal rats may contribute to more glucocorticoid production. Our findings are consistent with these data, as outlined in Figure 1. It has been reported that baseline COS blood levels increase with age. Hairston et al. (2001) showed an age-dependent increase in COS levels in rats tested on P12, P16, P20, and P24 (Hairston et al., 2001). Although we did not measure basal COS blood levels in the present study, our results regarding COS blood levels after seizures are consistent with those in the existing literature.

In conclusion, these data indicate that early exposure of infant rats to long-term morphine administration might change their susceptibility to PTZ-induced seizures during the prepubertal period. Our data showed that changes in seizure threshold and susceptibility are entirely age-dependent (except for clonic seizure). We also show that COS blood levels after PTZ-induced seizure are age-dependent and are consistent with basal COS levels in plasma. Furthermore, though the stage of development significantly affects the levels of peripheral COS concentrations, stress (PTZ-induced seizure) does not appreciably influence COS secretion patterns on P25 or P32. Therefore, these data provide evidence that developmental differences, independent of stress, affect circulating levels of COS and thus affect somatic growth, neuronal maturation, and neurophysiological function.

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