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Original

# Behavioral responses to methylphenidate and apomorphine in rats exposed neonatally to bisphenol-A

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**Abstract: Neonatal exposure of rats to bisphenol-A, an endocrine disruptor, has recently been proposed as a possible animal model of attention-deficit hyperactivity disorder, because such rats exhibit motor hyperactivity and impaired habituation to a novel environment 4-5 weeks after bisphenol-A pretreatment. To extend the original experiments, the present study additionally analyzed the effects of neonatal exposure to bisphenol-A (20 and 40 μg) on rat habituation to a novel environment and drug-induced behavior particularly at a later stage after bisphenol-A pretreatment. Single intracisternal administration of bisphenol-A (20 μg) into 5-day-old male Wistar rats did not cause any significant changes in habituation, as assessed in terms of locomotor activity, rearing, sniffing and grooming in rats at 8 weeks of age. Methylphenidate (1 and 3 mg/kg, i.p.), a psychostimulant, dose-dependently enhanced locomotor activity in both vehicle- and bisphenol-A (20 μg)-pretreated rats at 8 weeks of age, whereas other behaviors, i.e. rearing, sniffing and grooming, were not significantly affected. Additional challenge with apomorphine (1 mg/kg, i.v.), a dopamine receptor agonist, in vehicle- and bisphenol-A (20 and 40 μg)-pretreated rats at 10 weeks of age elicited a similar level of repetitive jaw movements measured by a magnet-sensing system under freely moving conditions during both the dark and the light phases. Thus, the effects of apomorphine did not differ between bisphenol-A-pretreated and vehicle-pretreated rats. It is concluded that, though some behavioral changes are evident in rats at an early stage (4-5 weeks) after neonatal treatment with bisphenol-A, the pretreatment does not induce any behavioral changes in habituation to a novel environment or in response to methylphenidate and to apomorphine at a later stage (8-10 weeks). (J. Oral Sci. 49, 311-318, 2007)**

Keywords: neonatal endocrine disruptor; ADHD; habituation to a novel environment; dopaminergic agents; jaw movement; rodent.

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## Introduction

Neonatal exposure of rats to bisphenol-A, an endocrine disruptor, has recently been proposed as an animal model of attention-deficit hyperactivity disorder (ADHD) because such rats exhibit motor hyperactivity (1). More specifically, rats that are treated with bisphenol-A on postnatal day 5 show spontaneous motor hyperactivity in their home cage, particularly during the early dark phase, at 4-5 weeks of age when compared with vehicle-pretreated rats (2-4). Since ADHD is characterized by an impaired habituation response to novelty (5-7) and since motor hyperactivity

seen in ADHD patients is attenuated by methylphenidate treatment (8-14), we have recently studied the habituation response to a novel environment and responsivity to methylphenidate during the dark as well the light phase. We demonstrated that single intracisternal administration of bisphenol-A (20 and 40 μg) into 5-day-old male Wistar rats impaired habituation to a novel environment by measuring motor behavior, rearing, sniffing and grooming in the light but not the dark phase, without any change in the response to methylphenidate at 4 weeks of age (unpublished results).

Rats with neonatal exposure to bisphenol-A have been reported to show a reduction of tyrosine hydroxylase immunoreactivity in the substantia nigra at 8 weeks of age (2), suggesting that postsynaptic dopamine receptors may be functionally up-regulated because of a reduced dopamine concentration at the dopaminergic neuron terminals. Therefore, it would be expected that the habituation response to a novel environment and responsivity to methylphenidate in bisphenol-A-pretreated rats aged around 8 weeks would also be enhanced.

We have previously reported that jaw movements occurring in response to stimulation of dopamine receptors is a convenient parameter for examination of dopamine receptor function (15-18). On the basis of these findings, we hypothesized that the function of postsynaptic dopamine receptors in the brain structures that are involved in the elicitation of jaw movements would be enhanced in bisphenol-A-pretreated rats relative to vehicle-pretreated rats at around 8 weeks of age. In order to investigate this hypothesis, we further analyzed the effects of systemic injections of the dopamine receptor agonist, apomorphine (1 mg/kg, i.v.), in bisphenol-A pretreated rats. This dose of apomorphine was chosen because it is known to readily produce repetitive jaw movements in rats when administered intravenously (16,17).

## Materials and Methods

### Animals

Pregnant Wistar rats (Ishikawa Laboratory Animals, Saitama, Japan) were housed for at least 1 week in cages (42 × 26 × 20 cm) that were kept at constant room temperature and relative humidity (23 ± 2°C and 55 ± 5%, respectively) under a 12-h light/dark cycle (lights on at 07:00 h), with free access to food and water. Then, 8-15 pups born from pregnant dams were housed in the same environment and weaned at 3 weeks of age.

### Measurement of behavior

To measure locomotor activity, rearing, sniffing and grooming, rats at 8 weeks (262.8 ± 3.5 g) of age were placed singly in experimental boxes (30 × 30 × 35 cm) with Perspex sides at 09:00 h (light phase observation). Locomotor activity was measured with a battery of infrared photocells set 2 cm above the floor (Opto-Varimex, Columbas Instruments Ltd., Ohio, USA) and the number of beam interruptions during a 30-min observation period was automatically registered as locomotor activity. During light phase observations, the number of episodes of other types of behavior, i.e. rearing, sniffing and grooming, was also counted visually by a trained observer who was blind to the treatments (15,19-22): each number (rearing) or episode (sniffing or grooming) was scored as a "1". Rearing is characterized by rats standing up (including use of the cage wall) on the two hindlimbs. A sniffing period consists of distinct movement of the snout and vibrissae lasting 2-5 s, and a grooming period consists of movements related to grooming of the face, snout and/or trunk with the forelimbs and lasting 2-5 s.

### Surgical procedures for measurement of jaw movements

The surgical and recording procedures employed were as described previously (16-18). Briefly, male rats at 9 weeks of age were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a neodymium magnet (5.0 mm diameter, 2.0 mm thick, 350 mT; N-39SH, Niroku Seisakusho, Shiga, Japan) was fixed to the mandible with dental acrylic cement. Then the rats were placed in a stereotaxic frame to fix a Hall-effect transducer (HW-300B, Asahi Kasei Electronics, Tokyo, Japan) to the skull with stainless steel screws and dental acrylic cement. The rats were allowed to recover from the operation for one week. On the day of behavioral observation, the rats at 10 weeks of age (319 ± 6.3 g) were placed individually in an activity box (40 × 40 × 40 cm) with Perspex sides and a wire-mesh floor at least 30 min before commencing observation, i.e. at 09:00 h (light phase observation) and at 21:00 h (dark phase observation).

Jaw movements were recorded on a tape recorder (RD-180T, TEAC, Tokyo, Japan) for automatic off-line analysis with a spike trigger that counted vertical jaw movements during every 5-min period for 60 min.

The experiments were approved by the Animal Experimentation Committee of Nihon University School of Dentistry and were performed in accordance with institutional guidelines for the care and use of experimental animals, based on the UK Animals Scientific Procedures Act 1986. All efforts were made to minimize animal suffering and to minimize the number of animals used.

### Drugs

Bisphenol-A (Wako Pure Chemical Industries, Tokyo, Japan) was suspended in a minimal amount of 50% ethanol and made up to the required volume with olive oil (Nakarai Tesque, Tokyo, Japan). The chemical (87 nmol/10 µl/rat, = 20 µg/rat; 174 nmol/10 µl/rat, = 40 µg/rat) was injected intracisternally into 5-day-old male pups. For intracisternal injection, each pup was held manually and the injection needle (o.d. 0.5 mm) was lowered toward the cisterna magna. The needle was connected to a Hamilton syringe (25 µl) and the drug was slowly delivered by hand in a volume of 10 µl over 5 s, after which the needle was left in place for a further 15 s. The pretreatment was conducted only once. Control rats were injected with the vehicle for bisphenol-A (10 µl). Methylphenidate (Sigma, St Louis, MO, USA) and apomorphine (Sigma, St Louis, MO, USA) were dissolved in saline immediately before use, and injected intraperitoneally and intravenously from the tail vein, respectively. The doses of the drugs employed in this study were based on previous studies: bisphenol-A (1-4), methylphenidate (23), apomorphine (16,17).

### Data analysis

All values are expressed as means ± S.E.M. The time-course data for behaviors counted during a 30-min observation period were analyzed using two-way (treatment × time) analysis of variance (ANOVA) for repeated measures (time), followed by *post hoc* Dunnett's multiple comparison tests when appropriate. The summed data for jaw movements counted during a 60-min observation period were analyzed using three-way (pretreatment × phase × challenge drug) ANOVA. Differences at $P < 0.05$ were considered to be statistically significant.

## Results

### Effects of intracisternal bisphenol-A administration on locomotor activity, rearing, sniffing and grooming during the light phase

Immediately after the rats had been placed into a novel environment (the observation cage), they showed a high level of locomotor activity, rearing, and sniffing that gradually decreased over the 30-min observation period (Fig. 1). Though grooming was maintained at a low level throughout the observation period, early postnatal pretreatment with bisphenol-A (n = 23) had no significant effect on behavior habituation when compared to the vehicle control (n = 21) during the light phase at 8 weeks of age (Fig. 1).

### Methylphenidate responsivity of locomotor activity, rearing, sniffing and grooming during the light phase in bisphenol-A- or vehicle-pretreated rats

Intraperitoneal injection of methylphenidate (1 and 3 mg/kg) enhanced locomotor activity in a dose-related manner during the dark phase in both vehicle-pretreated and bisphenol-A-pretreated rats at 8 weeks of age (vehicle: $F(2,101) = 13.90$, $P < 0.0001$; bisphenol-A: $F(2,113) = 7.87$, $P < 0.001$) (Fig. 2, upper part). However, there was no difference in the responsivity to methylphenidate between bisphenol-A-pretreated and vehicle-pretreated rats.

Except for a modest enhancement of grooming in vehicle-pretreated rats ($F(2,101) = 6.44$, $P < 0.01$), methylphenidate produced no significant change in rearing, sniffing and grooming (Fig. 2, lower left). Thus, the overall effects of methylphenidate on rearing, sniffing and grooming were similar in both bisphenol-A-pretreated and vehicle-pretreated rats (Fig. 2).

### Apomorphine responsivity of jaw movements during the dark and light phases in bisphenol-A- or vehicle-pretreated rats

Intravenous injection of apomorphine (1 mg/kg) at 10 weeks of age in vehicle- and bisphenol-A-pretreated rats elicited significantly more repetitive jaw movements measured by the magnet-sensing system under freely moving conditions during both the dark and light phases than those elicited by saline injection ($F(1,72) = 250.26$, $P < 0.0001$; Fig. 3). Though induction of jaw movements by apomorphine was higher during the light phase than during the dark phase ($F(1,72) = 12.35$, $P < 0.001$; Fig. 3), there was no significant difference in the induction of jaw movements by apomorphine between rats pretreated with vehicle and those treated with bisphenol-A (20 and 40 µg) ($F(2,72) = 0.13$, $P = 0.882$; Fig. 3).

## Discussion

Neonatal exposure to bisphenol-A in the rat has recently been proposed as a possible animal model for ADHD because it elicits motor hyperactivity (1-4). To strengthen the validity of this animal model, the original experiments were replicated and subsequently extended by measuring both putative changes in behavioral habituation to a novel environment and behavioral responses to methylphenidate, two phenomena that are known to be altered in patients with ADHD (5-14). The study demonstrated that single intracisternal administration of bisphenol-A (20 and 40 µg) into 5-day-old male Wistar rats impaired habituation to a novel environment in terms of changes in motor behavior, rearing, sniffing and grooming in the light phase, but not

the dark phase, without changing the response to methylphenidate at 4 weeks of age (unpublished results).

As mentioned in the Introduction, neonatal exposure to bisphenol-A has been reported to reduce tyrosine hydroxylase immunoreactivity in the substantia nigra at 8 weeks of age (2). Therefore, it can be speculated that postsynaptic dopamine receptors will be functionally up-regulated because of a reduced extracellular dopamine concentration at dopaminergic neuron terminals. Since Masuo and colleagues (1-4) did not observe spontaneous behavior at 8 weeks of age, we examined putative changes in behavioral habituation to a novel environment and behavioral responses to methylphenidate in rats at 8 weeks of age. However, the results of the present study showed that bisphenol-A pretreatment did not elicit significant change in any of the spontaneous behaviors observed. In addition, methylphenidate produced overall changes in the behavior of bisphenol-A-pretreated rats that were more or less identical to those elicited by methylphenidate in vehicle-pretreated rats. Thus, apart from a slight methylphenidate-induced increase in grooming in vehicle-pretreated rats, the overall effects of methylphenidate on locomotor activity, rearing, sniffing and grooming were similar in both groups. These results imply that, as is the case in another rodent model of ADHD, namely the bisphenol-A-pretreated mouse (24), the response to methylphenidate is not changed in the bisphenol-A-pretreated rat.

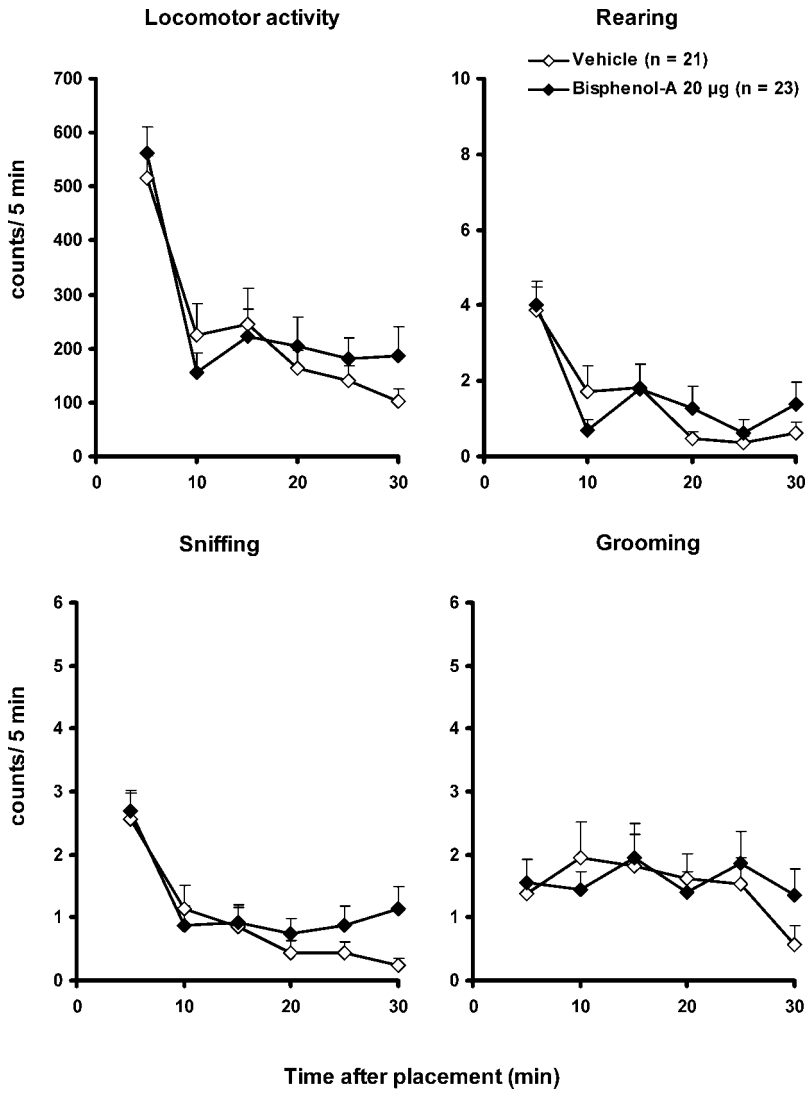


Fig. 1 Time course of locomotor activity (upper left), rearing (upper right), sniffing (lower left) and grooming (lower right) during the light phase in vehicle-pretreated (n = 21) and bisphenol-A-pretreated (20 µg, n = 23) rats at 8 weeks of age. Behavioral data were obtained immediately after placing the rats into the novel environment and expressed as the mean number of infra-red photocell beam interruptions (locomotor activity) or episodes (rearing, sniffing and grooming) counted during 5-min observation periods for 30 min. Vertical bars indicate S.E.M.

In order to investigate further the above-mentioned hypothesis, we conducted an additional experiment to study the effects of systemic injection of the directly-acting dopamine receptor agonist apomorphine, at a dose (1 mg/kg) known to readily produce repetitive jaw movements in rats when administered intravenously (16,17), in some of the bisphenol-A (20 and 40 µg)-pretreated rats at a later stage (10 weeks). In the present study, it was shown that an intravenous challenge with apomorphine in both vehicle- and bisphenol-A-pretreated rats at 10 weeks of age elicited significantly more repetitive jaw movements than those observed after saline injection.

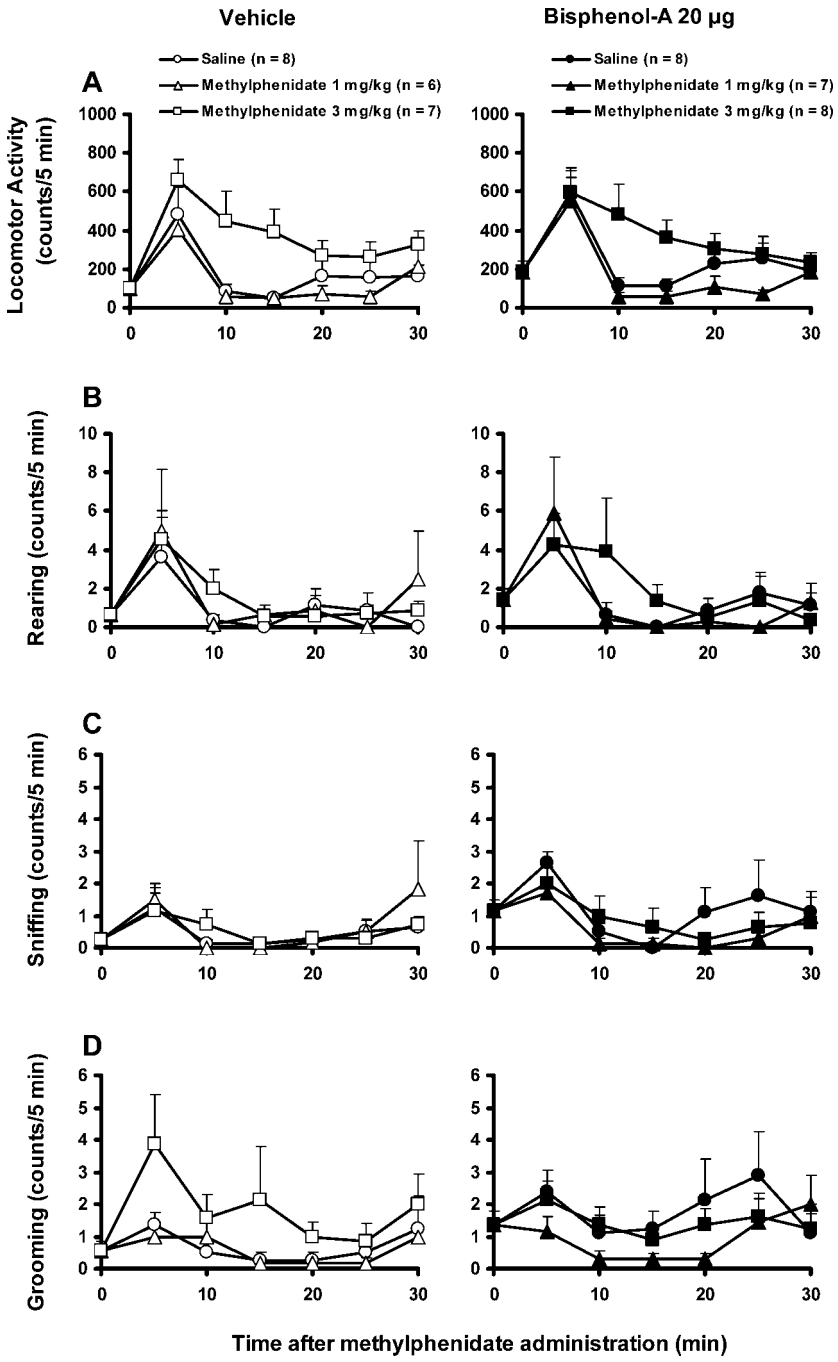


Fig. 2 Effects of intraperitoneal injections of saline and methylphenidate (1 and 3 mg/kg) on (A) locomotor activity, (B) rearing, (C) sniffing and (D) grooming during the light phase in vehicle (left)-pretreated and in bisphenol-A (20 µg, right)-pretreated rats at 8 weeks of age. The data are expressed as the mean number of infra-red photocell beam interruptions (locomotor activity) or episodes (rearing, sniffing and grooming) counted during 5-min observation periods (n = 6-8). Vertical bars indicate S.E.M.

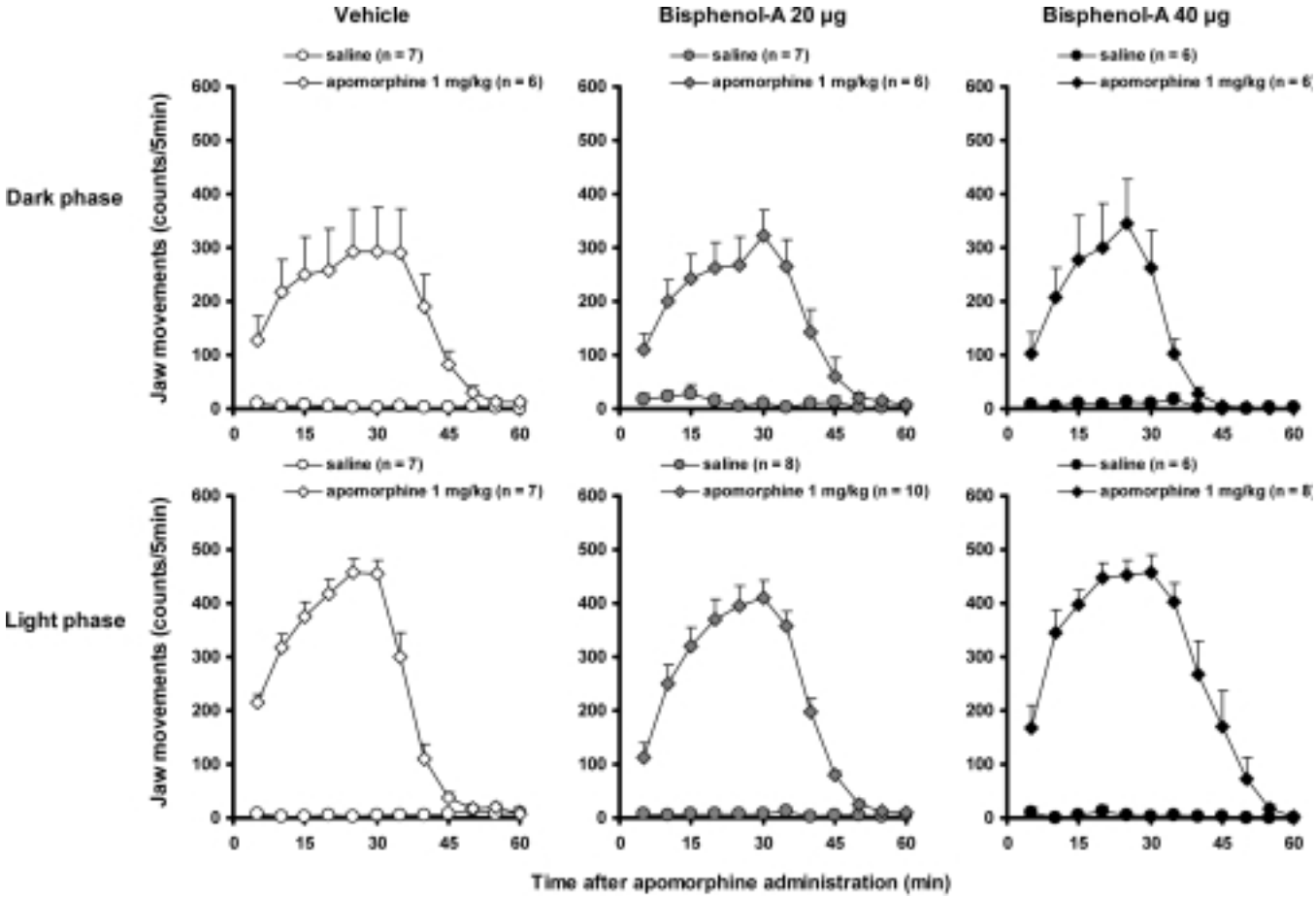


Fig. 3 Effects of intravenous injections of saline and apomorphine (1 mg/kg) on jaw movements elicited during the dark (upper part) and light (lower part) phases in vehicle (left)-pretreated and in bisphenol-A (20 μg, middle; 40 μg, right)-pretreated rats at 10 weeks of age. The data are expressed as the mean number of jaw movements occurring during 5-min observation periods for 60 min (n = 6-10). Vertical bars indicate S.E.M.

However, the induction of jaw movements by apomorphine was significantly higher during the light phase than during the dark phase. More importantly, the present study clearly revealed that there was no difference in the induction of jaw movements by stimulation of dopamine receptors between rats pretreated with vehicle and those treated with bisphenol-A. This result, together with the lack of responses to methylphenidate, suggests that no functional change occurred in dopamine receptors as a result of neonatal bisphenol-A treatment, at least at the later stage at 8-10 weeks after the pretreatment.

In conclusion, the present findings indicate that, although some behavioral changes were evident in rats at an early stage (4-5 weeks) after neonatal treatment with bisphenol-A, the pretreatment did not induce any behavioral changes in habituation to a novel environment or in response to methylphenidate or apomorphine at a later stage (8-10 weeks).

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