Early weaning augments the spontaneous release of dopamine in the amygdala but not the prefrontal cortex: An in vivo microdialysis study of male rats

Running head: Early weaning drives amygdalar dopamine

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Abstract

Our early weaning schedule was associated with the emergence of trait anxiety in male rodents performing an elevated plus maze but not an open-field test. We previously reported that early weaning weakened excitatory neurotransmission to the amygdala from the prefrontal cortex, where the mesocorticolimbic dopaminergic (DAergic) fiber terminates on each. In this study, we investigated DAergic transmission in both these brain regions. The extracellular levels of amygdalar DA in adulthood were two times higher in rats weaned at 16 days compared to those weaned at 30 days in both the home cage and the open-field. This difference in extracellular DA levels was not apparent in the prefrontal cortex. The concurrently measured locomotor and rearing behaviors did not vary according to the weaning period and the probe-implanted region, respectively. These results suggest that the effects of early weaning on DA tone appear to be specific to the amygdala and do not represent ubiquitous upregulation as these changes were not observed in the prefrontal cortex.

Key words: dopamine, maternal environment, microdialysis, open-field, locomotion
**Introduction**

Using an early weaning model of rodent mother–infant bonding disorders that was developed by our research group, we found that mice that had been weaned early demonstrated alterations in the myelination in the anterior part of the basolateral amygdala (BLA), as well as changes in anxiety-related behaviors (e.g., in an elevated plus maze) [11], compared to controls that were weaned normally [15]. In terms of the neural projections from the prefrontal to the anterior part of the BLA [13], application of this model revealed a weakening of the paired-pulse facilitation in the prefrontal–BLA pathway in urethane-anesthetized adult rats [19]. This would be expected to result in the attenuation of pulsed information in the descending neural pathways during normal behavior.

In highly social animals, there are significant alterations in the mesocorticolimbic system during brain development [17]. These changes are driven by a variety of factors, including mother-infant interactions, emotional development, and social nurturing in the post-weaning setting [11]. Recently, it was shown that dopamine (DA) neurons in the ventral tegmental area (VTA) are involved in social interactions within large social groups of rodents [21]. Additionally, the mesocorticolimbic neuronal system and DA transmission have been key research targets in studies of social stress [1,3,7,8,10,12]. Thus, the present study aimed to determine whether early weaning would have similar influences on dopaminergic transmission in the amygdala and prefrontal cortex, which are two DA terminal sites that ascend from the VTA.

Amygdalar DA is an important component of the mesocorticolimbic neural system and is related to emotional control. It has been suggested that amygdalar DA directly strengthens sensory-promoted amygdalar output during emotional expression and weakens the inhibitory influence of the prefrontal-BLA pathway on sensory-promoted
output [5]. Thus, we propose a working hypothesis that amygdalar DA could decrease in parallel with the already-weakened transmission of the prefrontal-BLA pathway in early weaning rats [19]. For this analysis, we traded analytical sensitivity for temporal resolution of DA detection. The present in vivo study aimed to determine whether early weaning would similarly influence the DA systems in the amygdala and the prefrontal cortex.

**Materials and Methods**

*Animals*

Adult male and female Sprague-Dawley rats were purchased from Japan Clea Co. Ltd. (Yokohama, Japan) and then pair-housed in a large breeding cage (450 × 350 × 350 mm) in a temperature-controlled room (24 ± 1°C) on a 12-h light-dark cycle. All experimental procedures and care of the laboratory animals were approved by the Animal Ethical Committee of the National Institute of Advanced Industrial Science and Technology (AIST) in accordance with EC Directive 86/609/EEC.

*Weaning procedure*

The weaning procedure was performed as described previously [19]. The day of birth was designated as PD0, and on PD3 the pups were culled to standardize litter sizes to a total of eight; six male and two female pups in each litter. Until weaning, exposure to humans was minimized except for a brief cage cleaning each week. On PD16, three male pups were separated from the dam and housed in a rat-farming cage (410 × 260 × 180 mm; early-weaned group) while the remaining three male pups were weaned on PD30, which is the natural weaning time (control-weaned group). Subsequently, the early- and control-weaned animals were separately housed. After PD30, the food pellets
were changed from a breeding formula to normal feeding (i.e., from F-1 to MM-3; Funabashi Farm Co. Ltd., Chiba, Japan). Two separate cohorts of rats were used in experiments examining the amygdala and prefrontal cortex. In each experiment, the early-weaning \( (n = 12) \) and control-weaned \( (n = 12) \) groups were tested as follows.

**Extracellular dopamine (DA) measurement using in vivo microdialysis**

The microdialysis and DA measurement procedures used in the present study have been described previously [6,14,18,20]. Briefly, male Sprague-Dawley rats (approximately 400 g) were anesthetized with pentobarbital (10–15 mg/kg, intraperitoneal) and sevoflurane (1.5–2%) and their body weights were measured and analyzed with reference to PDs on the surgery day. The microdialysis probe implants were directed into the BLA (coordinates from bregma [in mm]: AP = 2.3, ML = 5.2, DV = 7.5; [16]) or the medial prefrontal cortex (AP = -3.2, ML = 0.6, DV = 3.5), as described previously [14,20].

Each I-shaped microdialysis probe consisted of a dialysis component (1 mm) and an epoxy-lapped component (approximately 7 and 3 mm for the amygdala and the prefrontal cortex, respectively) with a hollow fiber membrane (outer diameter: 0.24 mm) in a stainless shaft (outer diameter: 0.55 mm); the shaft was connected to the lapped component to maintain the distance to the dialyzed tissue. One day after surgery while the subjects were in an optically opaque home cage centered within an open-field, a microinfusion pump (CMA102; Carnegie Medicine, Gothenburg, Sweden) was used to perfuse Ringer’s solution into the implanted probe through Teflon tubes (inner diameter: 0.1 mm) at a rate of 2 µL/min using a liquid swivel (TCS2-23, two channels; Eicom, Kyoto, Japan). All microdialysis-relevant preparations, including tubing, were performed on the day of surgery in an opaque home cage with food and water provided *ad libitum,*
and then perfusion was initiated on the first experimental day. Microdialysates were collected for at least 30 min while the rat remained in the opaque home cage (basal sample) inside an open-field box. The cage was then tilted to allow the rat to voluntarily leave the box for behavioral testing and further collection of microdialysates. After the experiment, the tilted cage was brought close to the rat so that it could voluntarily return to the cage.

To minimize any handling-related stress effects, the bodies of the rats were not directly touched for the 2 consecutive days. The DA content in the dialysate was analyzed using high-performance liquid chromatography coupled with an electrochemical detector (HTEC-500; Eicom). After the experiments, all probe positions were histologically verified in 70-μm serial brain sections stained with thionin.

**Behavioral testing in the open-field**

The behavioral procedure used in the open-field test has been previously described [19]; however, in the present study, a rubber sheet was also used to cover the shock grids. Briefly, DA measurement was performed on 2 successive days (age range PD49–PD63) in an open field (a square transparent acrylic box, 438 × 438 × 395 mm). The locomotor and rearing behaviors were measured using an open-field analysis system equipped with two sets of infrared photo beam sensors at different heights (SCANET MV-20IP for counting beam brakes; Melquest Ltd., Toyama, Japan).

**Statistical analysis**

All results are presented as a mean ± standard error of the mean (S.E.M.). The data were analyzed by analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD) as the post-hoc test, or by Student’s *t*-test. All data
were analyzed using commercial software (StatView J-5; Hulinks Inc, Japan) and \( p \)-values < 0.05 were considered to indicate statistical significance.

**Results**

**Basic experimental condition**

There were no significant differences between the early-weaning \((n = 12)\) and control-weaned \((n = 12)\) groups in terms of body weight or postnatal day (PD) on the day prior to the simultaneous measurements of open-field behaviors and DA. The average weights and PDs were 401.5 ± 13.6 g on PD 58.8 ± 2.4 in the early-weaning group and 402.9 ± 17.1 g on PD 59.3 ± 2.1 in the control-weaned group \((t\)-test; \( p > 0.8\) for weight and PD).

**In vivo extracellular DA levels in the amygdala**

On the first experimental day (challenge to a novel field), *in vivo* extracellular DA concentrations in the amygdalar dialysate were measured for two successive 30-min periods; the first period was taken in the opaque home cage, and the second was taken in the open-field apparatus. A two-way repeated-measures ANOVA revealed a significant main effect of place \((F_{[1, 22]} = 4.631, \ p < 0.05)\). The early weaning group showed a significantly higher DA level in the home cage and the open-field compared with control; 6.78 ± 1.98 vs. 2.55 ± 0.31 pg/30 min in the home cage, 7.44 ± 2.04 vs. 2.90 ± 0.37 pg/30 min in the open-field \((p < 0.05\) and \( p < 0.04\), respectively by Fisher’s PLSD; Fig. 1). In contrast, on the next experimental day (challenge to a familiar field), the amygdalar DA levels did not differ according to weaning period length \((F_{[1, 22]} = 2.729, \ p > 0.1\), Fig. 1). The early weaning group showed no statistical significant differences between the first and second days. In the home cage, the early weaning group showed a 7.3% reduction in
mean amygdalar DA levels on the second day, relative to the first day, compared with a 6.2% reduction in the control-weaned group. In the open-field test, the mean amygdalar DA levels showed a 14.7% reduction on the second day in both the early weaned and control-weaned groups.

**In vivo extracellular DA levels in the prefrontal cortex**

To assess whether the DA elevations observed in the amygdala in the early-weaning group would be reflected across the whole brain, the DA content in the prefrontal cortex was measured as a regional comparison (Fig. 2). Two-way repeated-measures ANOVA revealed no significant effect on prefrontal DA levels over time when switching from the home cage to open-field on the first experimental day ($F_{[1, 22]} < 0.0000007, p > 0.99$). For reference, the prefrontal DA levels for the early-weaning and control-weaned groups were $1.89 \pm 0.32$ and $1.91 \pm 0.29$ pg/30 min, respectively, in the home cage, and $2.37 \pm 0.38$ and $2.35 \pm 0.44$ pg/30 min, respectively, in the open-field. In the home cage, the mean prefrontal DA level of the early weaning group on the second day (challenge to a familiar field) was 101.2% of the level on the first day, while that of the control-weaned group was 99.0% of the level on the first day. In the open field, the mean prefrontal DA level of the early weaning group on the second day was 100.6% of the level on the first day, while that of the control-weaned group was 95.8% of the level on the first day. Based on comparisons with prefrontal cortical DA, these data indicate that the elevation of amygdalar DA in the early-weaning group was a regionally specific phenomenon and did not occur across the whole DA system.

*Behavioral indexes in the open-field test*

The open-field behaviors of the rats during microdialysis collection from the
amygdala and prefrontal cortex (Figs. 1 and 2, respectively) were assessed in the present study: The positions of the probe were plotted by referring the stained sections (Fig. 3). As shown previously by our research group [9], differences in the weaning period did not have significant effects on the behavioral indexes, even in duration of stay in the center one-ninth area of an open field on D1 or D2, (p > 0.9 on each day and either surgery position). A three-way repeated measure ANOVA assessing locomotion and rearing in the open-field apparatus did not reveal significant differences in locomotion (F[1, 44] = 0.002; p > 0.9) or rearing (F[1, 44] = 0.056; p > 0.8). Indeed, the open-field behaviors did not vary across surgery position of the probe implantation (p > 0.3 in either case).

Taken together, these findings demonstrate that the extracellular DA levels in the amygdala of the early-weaning group were approximately double those of the control-weaned group, whereas there were no significant differences in the prefrontal cortical group. Additionally, the weaning period did not have a significant influence on the behavioral indexes in the open-field test.

Discussion

The present study demonstrated that early weaning augmented extracellular DA levels in the amygdala but not the prefrontal cortex in terms of either spontaneous basal release or reactive release following a challenge in the open-field test on the first experimental day. However, this difference was no longer significant on the second experimental day. Considering the relationship between early weaning and trait anxiety [9,10,15], the effect of augmented DA levels on the amygdala were not apparent in the open field behaviors and may be affected by anxiety. In fact, a previous study from our research group [14] showed that extracellular amygdalar DA levels are correlated with particular mood changes such that in operant learning tasks extracellular DA levels tend
to increase in conjunction with correct response increments in young rats but decrease with error trials in old rats; however, there was no relationship with learning milestones. It is possible that amygdalar DA generally regulates preferred active behaviors rather than obligatory passive behaviors. The present findings (e.g., no behavioral change in the open-field despite the DA increase in the amygdala) indicate that innate increases in amygdalar DA may selectively control behaviors following situational judgments about external environments.

The biological mechanisms underlying this augmentation in amygdalar DA remain unclear because previous studies did not observe histological differences in amygdalar DA terminals across various weaning periods [8]. In the present study, the effects of early weaning on the DA levels in the amygdala and the prefrontal cortex differed (i.e., effect and no effect, respectively; Figs. 1 and 2, respectively); that is, the effect of early weaning on the DAergic neurons varied by brain region in the present model. Mesocorticolimbic DA neurons originate in the VTA and separately project to limbic and cortical areas [2], suggesting that regional DA terminals transmit different information via respective dopaminergic pathways. On the other hand, these respective presynaptic DA terminals may be controlled by different regional neural properties [4], or, conversely, regional DA neurotransmission may control regional neurotransmission.

This basolateral output receives indirect inhibitory control from the prefrontal cortex via amygdalar inhibitory interneurons and direct excitatory control from the sensory cortex. Interestingly, amygdalar DA appears to simultaneously weaken inhibitory control and strengthen excitatory control (i.e., dual upregulation of the basolateral output). The effects of dual upregulation on early weaning were not observed in the open-field test, and it is possible that the role of dual upregulation allows control of only explicit conditioned fear. The size of the field in the present study seemed to be more suitable for
differentiating neutral behavior from anxiety-related behavior.

Early weaning also promotes the precocious formation of myelin in the amygdala [15], which attenuates pulsed information in the prefrontal-amygdalar pathway [19]. As reported previously [9], the present study reconfirmed that different weaning periods do not have an influence on locomotor or rearing behaviors in an open-field. Furthermore, it is possible that the attenuation of amygdalar pulsed information resulted in the highly compensatory open-field behaviors that appeared to be sufficient to mask changes in the brain following early weaning. Initially, it was predicted that amygdalar DA would not increase to avoid reducing inhibitory control of the basolateral output through the prefrontal-amygdalar pathway.

In this study, we hypothesized that early weaning may lead to behavioral anxiety in adolescent rodents, relative to control weaning [9-11,15,19]. When studied under conditions known to induce this type of anxiety, we found that early weaning was associated with significant increases in extracellular DA levels in the amygdala but not the prefrontal cortex. Although early weaning alters the relationships among anxiety traits, sociability, and sensitization to drugs and substance abuse, these factors may be associated with distinct anatomical roles within the mesocorticolimbic DA system [21,22]. Indeed, the early-weaning group exhibited superficial behavioral robustness in the open field; however, the neurological alterations found in the present study suggest a vulnerability to stress and could attenuate behavioral redundancies to future events.

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Figure legends

Figure 1. Extracellular amygdalar (AMY) DA levels and open-field behavior in the early-weaned and control groups; closed and open columns, respectively (n = 12 in each). Dots beside each column at the top left represent the individual DA data. DA is represented as pg/30 min in the home cage or an open-field where locomotion and rearing were simultaneously recorded. Asterisks indicate p < 0.05 (post-hoc after an ANOVA); all values are presented as mean ± standard error of the mean (S.E.M.).

Figure 2. Extracellular prefrontal cortical (PFC) DA and open-field behavior in early-weaned and control groups; closed and open columns, respectively (n = 12 in each). Dots beside each column at the top left represent the individual DA data. DA is represented as pg/30 min in either the home cage or an open-field where locomotion and rearing were simultaneously recorded. Asterisks indicate p < 0.05 (post-hoc after an ANOVA); all values are presented as mean ± S.E.M.

Figure 3. The positions of the probe are illustrated in the case of the basolateral amygdaloid nucleus (AMY) and the medial prefrontal cortex (PFC) using a 1 mm scale (lower right). Probes are represented by solid and dashed lines in the early weaning and control groups, respectively.
Figure 1.
Figure 2.
Figure 3.