# The involvement of amygdala phosphorylated Erk1/2 in the anti-immobility effect of long-term desipramine HCl treatment in a forced swimming test

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**Abstract:** Desipramine (DMI), a tricyclic antidepressant (TCA), is widely used for the clinical treatment of depression. However, the understanding of DMI's mechanism of action is still inadequate. In the present study, we investigated the relationship between the extracellular signal regulated kinase1/2 (Erk1/2) and the effect of DMI treatment. The Porsolt forced swimming test (FST) was used to estimate the anti-immobility effects of DMI. Moreover, the phosphorylated levels of Erk1/2 were assessed using Western blot analysis. There was no obvious difference between treated and untreated rats in their locomotion activity. However, on day 21, the DMI-treated rats showed a significant decrease in the FST immobility time (IMT); there were no changes in IMT after 5 days of DMI treatment. The Erk1/2 phosphorylation of the amygdala was assessed; the Erk1/2 phosphorylation was significantly decreased in the amygdala after 21 days of DMI treatment. Aurintricarboxylic acid (ATA), a nonpeptide MAPK activator, was injected locally into the amygdala regions bilaterally in a group of 21-day DMI-treated rats after the FST; these rats recovered significantly from their immobility behavior. Furthermore, the amygdala Erk1/2 phosphorylation of ATA-treated rats was consistent with the behavioral test. This study is the first to demonstrate that phosphorylated Erk1/2 in the amygdala may be involved in DMI's mechanism of action with long-term treatment but not with short-term treatment.

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## 1. Introduction

DMI, a tricyclic antidepressant, is usually characterized as a norepinephrine reuptake inhibitor [1, 2]. Recent studies have not only shown that DMI's mechanism of action occurs via the acute increase of norepinephrine in the synaptic gap [3,4], but also showed following intracellular molecules to be involved in DMI's mechanism of action: brain-derived neurotrophic factor (BDNF) and Ca2+-calmodulin-dependent protein kinase kinase (CaMKK). [5, 6]. However, the involvement of the molecules, Erk1/2, in DMI's mechanism of action is still not well elucidated.

In recent years, the Erk1/2 signaling in the amygdala was revealed to be crucial for the manipulation of fear memory formation or extinction in rats [7, 8], and even revealed to be involved in mood regulation [9]. With the progress of cellular and molecular biology, it is extremely important to assess the certain specific brain region (i.e. the amygdala) that have been found to be involved in the occurrence of the symptoms of depression when antidepressants' mechanisms of action were studied [10, 11]. Therefore, in the present study, we tried to examine the role that Erk1/2 in the amygdala has in DMI's mechanism of action and to further elucidate DMI's cellular mechanism of action.

The FST, originally described by Porsolt et al. in 1977, is a widely used animal model for the evaluation of antidepressant efficacy. By recording the IMT during the FST in antidepressant-treated rats, the efficacy of antidepressants can be evaluated [12]. A shorter IMT during the FST indicates the dose of the antidepressant that is more effective. Another test, the open field test (OFT), is usually used to assess spontaneous locomotion in animals [13]. Using this test, the effects of DMI on the spontaneous locomotion of rats can also be assessed..

## 2. Material and Methods

### Animals

This study was conducted in conformity with the policies and procedures detailed in the "Guide for Animal Care and Use of Laboratory Animals". The animal experimental protocols of the "Guide" were approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Hwa University of Medical Technology. Male Sprague-Dawley rats weighing 130-150 g at the time of testing were

housed in groups at a constant room temperature (22±1°C) and humidity (55%±10% RH) with a 12-h light: dark cycle. Food and water were freely available.

**Desipramine HCl treatment protocol**

The rats were treated with DMI (Sigma Company [St. Louis, MO 63195, U.S.A]) or normal saline (saline group) given intraperitoneally. The DMI dosage was 10 mg/kg. The DMI-treated rats received once daily DMI injections for 5 days or 21 days. The 5-day DMI-treated rats were treated from postnatal day (PND) 31 to PND35; the 21-day DMI-treated rats were treated from PND15 to PND35. During the 2-day washout period prior to re-testing, DMI was not given.

**Forced swimming test**

Rats after DMI treatments were subjected to the FST 24 hr later. During the FST, the rats were immersed in Plexiglas cylinders (diameter 18 cm, height 38 cm) filled to a depth of 25 cm with water at 25°C. On the first experimental day, the rats were placed in the water for a 15-min habituation period. After removal from the water, they were dried in a Plexiglas box under a 60 W bulb for 30 min. The next day, they were placed in the cylinders again and observed for 5 min. During this period, the total time that the rats spent making the movements necessary to remain afloat was recorded. After the 5-min test, the rats were removed from the water and sacrificed for immunoreactivity analysis. However, some animals were re-tested; these re-tested animals performed two successive FST at 48-hr intervals.

**Open field test**

The rat's locomotion activity was measured using a chamber (length 120 cm, width 120 cm, height 80 cm) made from transparent acrylic. On the first experimental day, the rats were introduced to the center of the chamber for a 60-min habituation period. The next day, they were placed in the corner of the chamber for a 10-min test session during which their locomotion time was recorded.

**Western blot analysis**

The rats after the FST were decapitated within 10 min. Then, transverse slices (500 μm) were cut from the brain tissue block using a Vibroslice (Campden Instruments, Silbey, UK). The amygdala regions were examined under a stereomicroscope and were dissected from the brain slices using a scalpel. The minipieces of brain slices obtained from the amygdala were transferred to artificial cerebrospinal fluid (ACSF) solution bubbled continuously with 95% O2/5% CO2 and then prepared to be removed for subsequent analyses.

The amygdala region was sonicated transiently in ice-cold HB-II buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 10 mM EGTA, 5 mM ethylenediaminetetracetic acid, 2 mM sodium pyrophosphate, 4 mM para-nitrophenylphosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 20 μg/mg leupetin, and 4 μg /ml aprotinin). Following sonication, the soluble extract was obtained after pelleting the crude membrane fraction by centrifugation (50,000 g, $4^0$C). Protein concentration in the soluble fraction was then measured using a Bradford assay, with bovine serum albumin as the standard. Equivalent amounts of protein for each sample were resolved in 8.5% sodium dodecyl sulfate (SDS) – polyacrylamide gels, blotted electrophoretically to immobilon, and blocked overnight in TBS buffer. To detect the phosphorylated forms of Erk1/2, the blots were incubated with anti-phospho-ERK (anti-P-ERK; New England Biolabs, Beverty, MA, USA) antibody. To control the content of the specific protein per lane, the membranes were stripped with 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris-HCl (pH 6.8) for 30 min at $70^0$C and then re-probed with a mouse monoclonal anti-pan-ERK (BD Transduction Laboratories, Los Angeles, CA, USA) antibody. An enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA, USA) was used. The density of the immunoblots was determined by an image analysis system installed with BIO-ID software (Viber Lourmat, Mame-la-Valee, France). To assess the changes in Erk1/2 phosphorylation, the rats' total kinase levels and phosphorylated kinase levels were normalized by the control group levels, and then Erk1/2 immunoreactivity was expressed as a percentage.

**Stereotaxic surgery**

The 21-day DMI-treated rats treated with an intra-amygdala reagent (ACSF or ATA) underwent stereotaxic surgeries on PND30. The rats were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally (i.p.) and then mounted on a stereotaxic apparatus for surgery. Two cannula made of 23-gauge stainless steel tubing were implanted into the LA (lateral amygdala) or the BLA (basolateral amygdala) bilaterally. The coordinates were AP -2.3 mm, ML ±4.5 mm, DV –7.0 mm, based on Paxinos and Watson [14]. Three jewelry screws implanted in the skull served as anchors, and the whole assembly

was affixed on the skull with dental cement. A 28-gauge dummy cannula was inserted into each cannula to prevent clogging. After the surgical procedure, the rats were given 6 days (from PND30 to PND35) to recover prior to the FST.

**Drug microinjection**

A group of 21-day DMI and stereotaxic surgery-treated rats were subjected to the re-test protocol. In the re-test protocol, aurintricarboxylic acid, a nonpeptide MAPK activator [15], was microinjected into the amygdala regions bilaterally, 10 min prior to the second FST. The ATA dosage was 1 μl per side (3 μg dissolved in 1 μl ACSF), and the injection rate was 0.1 μl/min [16, 17].

**Statistics**

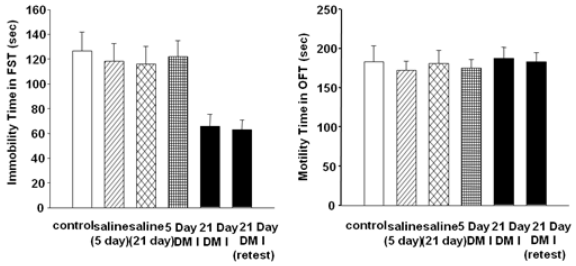
Results are expressed as mean ± SEM. Sample sizes are indicated by n. Comparisons between groups were carried out with a one- or two-way analysis of variance (ANOVA). Differences between two groups were compared using the unpaired Student's *t*-test; $p<0.05$ was considered statistically significant.

**3. Results**

**The effects of short-term and long-term treatment with DMI on normal rats during the FST and OFT**

Figure 1(a) shows the effects of DMI on normal animal behavior. The IMTs of normal rats in the control and the saline groups (5-day and 21-day saline-treated groups) were almost the same (control, 126.4±15.6 sec; saline [5 days], 118.1±14.7 sec; saline [21 days], 116±14.2 sec, n=7 each). However, compared to controls, the 21-day DMI-treated rats had a drastically decreased IMT (65.6±9.8 sec, n=7, ***$p<0.001$), while no significant change was observed in the IMT of 5-day DMI-treated rats (122.3 ± 12.8 sec, n=7, $p>0.5$). After a 2-day washout period, the IMT of the 21-day DMI-treated group did not return to its original level (63±8.0 sec, n=7).

Figure 1(b) shows the effects of DMI on rat locomotion activity. There was no significant difference among the groups. The motility time (MT) in the control group, saline groups (5-day and 21-day saline-treated groups), 5-day DMI-treated group, and 21-day DMI-treated group were 183.3 ± 2.0 sec, 172.3±11.6 sec,180.5±16.7 sec, 175.0±11.0 sec, and 187±14.6 sec, n=7 each, respectively. There was no obvious change in the locomotion activity of the 21-day DMI-treated rats after a 2-day washout period (183±12.0 sec, n=7).




(a) (b)

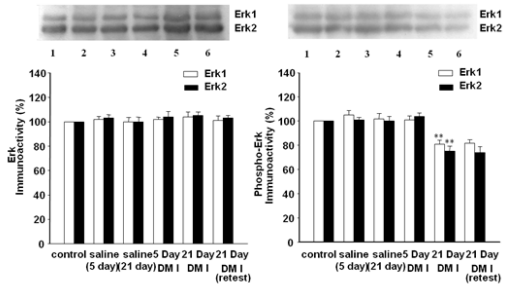
**Fig. 1** The behavioural effects of DMI treatments on normal rats DMI (10 mg/kg) was given intraperitoneally to normal rats for 5 or 21 days once daily. The IMTs in the control and saline groups (0.9% normal saline treatment for 5 day or 21 days) were almost the same. However, the IMT was significantly decreased in rats after 21-day DMI treatment (*** $P<0.001$, compared to the normal or saline control groups). No changes were seen in the IMT performance of 5-day DMI-treated rats. The reduced IMT in 21-day DMI-treated rats did not rebound to initial levels after the 2-day washout period (n=7 in each group). In the OFT, no significant changes in the MT were observed in the 5-day and 21-day DMI-treated rats.

**The effect of DMI on amygdala Erk1/2 phosphorylation**

In figure 2(b), it showed that the basal Erk1/2 phosphorylation levels were low in the control groups; nevertheless, the amygdala Erk1/2 phosphorylation levels were also significantly decreased in 21-day DMI-treated rats (Erk1, 81%± 3.1%; Erk2, 75%±4.2% versus the control or 21-day saline control group [Erk1, 102% ± 4.2%; Erk2, 100%±3.7%]; n=5, **$p<0.01$), while there were no changes in the 5-day DMI-treated group (Erk1, 101% ±3.1%; Erk2, 104%±2.6% versus the control or 5-day saline control group [Erk1, 105%±3.6%; Erk2, 101%±2.2%]; n=5, $p>0.5$). The total amounts of Erk1 and Erk2 were unchanged (Fig. 2(a)). Two days after DMI treatment was stopped, the 21-day DMI-treated rats continued to show inhibitory effects on amygdala Erk1/2 phosphorylation (Erk1, 82% ± 2.6%; Erk2, 74%±5.0%, n=5, $p>0.5$ versus 21-day DMI-treated rats).

**Behavioral re-tests for 21-day DMI-treated rats with an intra-amygdala MAPK activator in the FST and OFT**

The 21-day DMI-treated rats and the control group rats were studied here. Prior to the FST re-test, the 21-day DMI-treated rats received microinjections of ACSF or ATA, the MAPK activator, into the amygdala regions bilaterally.




(a) (b)

**Fig. 2** 21-day intraperitoneal administration of DMI inhibited basal amygdala Erk1/2 levels in rats. (a) There was no change in the total amounts of Erk1/2 in each group. (b) Desipramine was given intraperitoneally for 5 or 21 days to normal rats. The basal amygdala phosphorylated Erk1/2 levels in the 21-day DMI-treated rats were suppressed (** P<0.01 compared to normal rats) and did not rebound to initial levels after a 2-day washout period. (n=5 in each group, white bars: Erk1; black bars: Erk2).

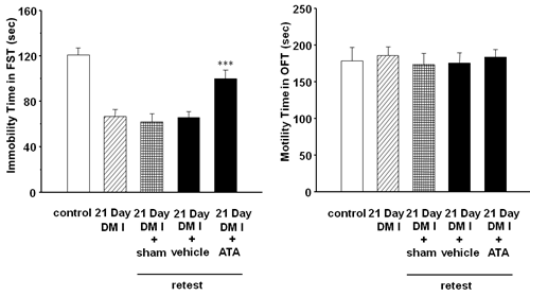



**Fig. 3** 21-day DMI-treated rats after intra-amygdala infusion of the MAPK activator, ATA, had a significant restoration in IMT in the FST; there was no effect on the MT in the OFT. The 21-day DMI-treated rats prior to the behavioral re-tests received intra-amygdala ATA. The MAPK activator-treated rats had a significant restoration in IMT on the FST; there was no effect on the MT in the OFT.

Figure 3A shows that there were no changes in the IMT in sham (21-day DMI and cannula-treated) or vehicle (21-day DMI and intra-amygdala ACSF-treated) groups compared to the 21-day DMI treatment only group (21-day DMI group, 67±5.8 sec; sham group, 62±7.4 sec; vehicle group, 66±5.3 sec; n=7 each, respectively, p>0.5). However, the ATA group (21-day DMI and intra-amygdala ATA-treated) showed a significant recovery of IMT (101±7.8 sec, n=7, ***p<0.001, versus the 21-day DMI-treated group). There were no obvious changes in the locomotion activity among the groups, as shown in figure 3B. The MT of the control group, the 21-day DMI-treated group, the sham group, the vehicle group, and the ATA-treated group were 179.0±18.0 sec, 186.0±12.0 sec, 174.0±15.0 sec, 176.0±14.0 sec and 184.0±10.0 sec, respectively; each group consisted of 7 rats. The infusion cannula tip locations are shown in figure 4.

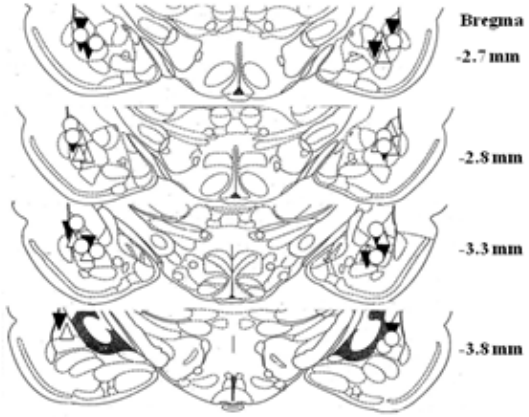
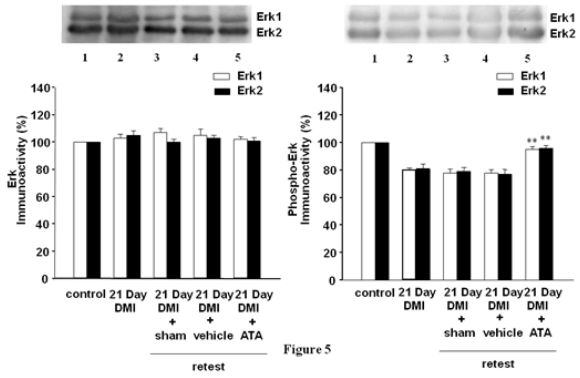



**Fig. 4** The injection sites used for intra-amygdala infusion are shown schematically.The black inverted triangles represent the distribution of the cannula tips in the amygdala of the sham group (21-day DMI-treated rats with cannula only). The white circles and the white triangles represent the distributions of the sites of the cannula tips in the amygdala of 21-day DMI-treated rats infused with ACSF and ATA, respectively.

**Western blot analysis of amygdala Erk1/2 activities in MAPK activator-treated rats**

The effects of ATA on amygdala Erk1/2 phosphorylation were assessed. The amygdala Erk1/2 activities were measured in the control group, the 21-day DMI-treated group (Erk1, 80%±1.4%; Erk2, 81%±3.3%), the sham group (21-day DMI and cannula-treated [Erk1, 78%±2.6%; Erk2, 79%±3.0%]), the vehicle group (21-day DMI and intra-amygdala ACSF-treated [Erk1, 78%±2.3%; Erk2, 77%±3.5%]), and the ATA group (21-day DMI and intra-amygdala ATA-treated [Erk1, 95%±2.1%; Erk2, 96%±1.6%]). In figure 5(b), it can be seen that, other than in the ATA group, the results of all groups were unchanged from the previous values (figure 2). In the ATA group, the amygdala Erk1/2 phosphorylation levels recovered significantly (versus the 21-day DMI-treated group; n=5,

**p<0.01). The total amounts of Erk1 and Erk2 were unchanged in all groups (Fig. 5(a)).




(a) (b)

**Fig. 5** 21-day DMI-treated rats after intra-amygdala infusion of the MAPK activator, ATA, had a significant restoration in the amygdala phosphorylated Erk1/2 levels. (a) There was no change in the total amount of Erk1/2 in each group. (b) The amygdala phosphorylated Erk1/2 levels in the 21-day DMI and ATA-treated rats showed a significant restoration (** P<0.01, compared to 21-day DMI-treated rats). (n=5 in each group, white bars: Erk1; black bars: Erk2).

## 4. Discussion

Herein we have been the first to demonstrate that amygdala phosphorylated Erk1/2 may be involved in the anti-immobility effect of long-term DMI treatment. The presence of this statement was shown by the following results. First, 21-day DMI-treated rats but not 5-day DMI-treated rats had an obvious decrease in the IMT on the FST. Second, the amygdala phosphorylated Erk1/2 levels were changed only in 21-day DMI-treated rats. Third, the amygdala phosphorylated Erk1/2 levels decreased in parallel with the IMT reduction in 21-day DMI-treated rats. Fourth, no recovery of IMT and no recovery of the amygdala Erk1/2 levels was seen in 21-day DMI-treated rats after a 2-day washout period. Fifth, when given ATA, an intra-amygdala MAPK activator, the 21-day DMI-treated rats recovered the previously decreased IMT on the FST. Lastly, the decreased amygdala phosphorylated Erk1/2 levels of 21-day DMI-treated rats were restored with ATA treatment.

Whether the different ages (day 15 and day 31) of rats receiving the DMI treatment could influence the effects of DMI on rats was a concern. In our additional age-matched control experiment, 15-day-old rats receiving DMI for 5 days expressed no significant alteration in IMT in FST compared to that of the 21-day-old control group (30±15 sec versus 43±10.0 sec, p>0.2, n=6 in each group). The results suggested that 21-day DMI treatment per se was more effective on the reduced immobile behavior of rats in the FST rather than on the early developmental effect of rats.

Einat et al. revealed that the blockade of the Erk pathway in the central nervous system may induce an anti-immobility effect on rats in the FST [14]. Similarly, our previous study also showed that the immobility behavior of rats in the FST may be modulated by the Erk1/2 phosphorylation in the amygdala [18]. The present study, moreover, could also verify the regulatory role of Erk1/2 phosphorylation in the amygdala on the immobility behavior in the FST by the anti-immobility effect of long-term DMI's treatment on rats. From the above results, not only the relationship between the immobility behavior of rats in the FST and Erk1/2 signaling in the amygdala could be identified further, but also Erk1/2 signaling in the amygdala could be a new insight into the mechanisms of antidepressants [4].

In figure3, the 21-day DMI-treated rats were given intra-amygdala ATA, they recovered from their decreased IMT significantly, although the recovery of the IMT did not reach as well as control levels. In order to confirm that the effects of the recovery of decreased IMT were due to the treatments of ATA on the amygdala region, the location of cannula tips on the lateral or basolateral amygdala were recorded and the data showed that there was no difference between the effects of ATA on the lateral and basolateral amygdala regions (Fig.4). This finding strengthens the correlation between DMI's mechanism of action and Erk1/2. Furthermore, the amygdala Erk1/2 phosphorylation noted in the ATA group provides stronger evidence that DMI's mechanism of action is related to Erk1/2 (Fig.5).

## 5. Conclusion

Consequently, this study's finding relating to the role that Erk1/2 play in the mechanism of action of DMI does not only provide new information about the pharmacology of antidepressants, but also provides a profound insight into the development of new antidepressants from compounds known to perturb Erk1/2 phosphorylation in specific brain regions.

**6. Acknowledgements**

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