

Full length article

Implication of protein kinase C of the left intermediate medial mesopallium in memory impairments induced by early prenatal morphine exposure in one-day old chicks

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ABSTRACT

Previously we reported that prenatal morphine exposure during embryonic days 5–8 can cause cognitive deficits of one-trial passive avoidance learning (PAL) in one-day old chicks. Because protein kinase C (PKC) has been associated with memory capacity, we investigated the effects of prenatal morphine exposure on PKC isoforms expression in the left intermediate medial mesopallium (IMM) of chick brain at a time when memory tests were performed at 30, 120 and 360 min respectively following training in PAL paradigm. We found that the level of PKC α in the membrane fractions in left IMM was decreased but that in the cytosol fractions showed a increased trend in prenatally morphine-exposed chicks with impaired long-term memory (120 and 360 min). Moreover, the translocation of PKC δ from cytosol to membrane in left IMM was shown in prenatal morphine group which had significantly impaired long-term memory at 360 min after training. Furthermore, there were no statistical differences between the two groups regarding the expressions of PKC α and PKC δ in the membrane fraction, although their levels in the cytosol fraction of prenatal morphine group which showed impaired intermediate-term memory at 30 min after training, were quite different from that of prenatal saline group. Taken together, these results indicate that PKC α and PKC δ in the left IMM are differentially involved in the impairments of long-term memory induced by prenatal morphine exposure. Neither PKC α nor PKC δ in left IMM may be associated with the disruption of intermediate-term memory of chicks prenatally exposed to morphine.

1. Introduction

A growing body of behavioral literature has suggested that opiates abuse during pregnancy could induce cognitive deficits of offspring (Beckwith and Burke, 2015; Che et al., 2005; He et al., 2010; Lu et al., 2012; Schrott et al., 2008; Slamberova et al., 2001; Wang and Han, 2009), whose cellular and biochemical mechanisms have not been fully understood yet. Early researches have demonstrated the existence of the connection between PKC isoforms and opioid receptors (Mangoura and Dawson, 1993), which may open up a new perspective for the roles of this signal transduction system in memory impairments induced by prenatal opiates exposure. For example, prenatal exposure to heroin leads to the downregulation of membrane PKC γ in the intermediate medial mesopallium (IMM) and induces memory impairments, as shown by poor performances on the imprinting behavior of the chicks (Yanai and Metsuyanin, 2002). Furthermore, other studies on the rodents have also indicated the specific relationship of learning and

memory deficits to the disruption of cholinergic-the protein kinase C (PKC) signaling induced by prenatal heroin exposure (Huleihel and Yanai, 2006; Lu et al., 2010; Shahak et al., 2003; Yanai et al., 2000). Those findings would seem to suggest that dysfunction of PKC seems to be the most relevant for the prenatally opiates-induced neurobehavioral deficits.

At least 12 isoforms of the PKC family are classified into three subcategories based on their structure, calcium dependence and lipid activators (Olive and Newton, 2010). Among them, the activities of the conventional α/β -isoforms are the greatest in the brain (Jaken and Kiley, 1987; Kikkawa et al., 1987). Furthermore, some studies on mammalian have indicated that opiate-related behaviors has been associated with the downregulation of PKC α in the brain of heroin, morphine and methadone-dependent human (Busquets et al., 1995; Garcia-Sevilla et al., 1997) and rodents (Lu et al., 2010; Ventayol et al., 1997), suggesting that probably this might be a unique pattern of PKC α activity in behavioral impairments induced by opiates. In addition,

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PKC δ belongs to one of the novel subgroups of PKC isoforms, which is known to play a critical role in regulating the state of polysialylation of neural cell adhesion molecule (Gallagher et al., 2001), implying that PKC δ may be implicated in memory consolidation.

Our previous findings indicated that embryonic days 5–8 was a sensitive time window during development, when prenatal morphine exposure produced memory impairments of one-trial passive avoidance learning paradigm (PAL) in one-day old chicks (He et al., 2010). Given that the IMM of chick brain is known to be important for associative learning and memory in the rewarding effects induced by morphine (He et al., 2009) and in the PAL task, in which IMM especially has a trend of left lateralization (Burchuladze et al., 1990), the present study was designed to investigate the effects on levels of PKC α and PKC δ in the left IMM of prenatally morphine-exposed chicks with cognitive deficits in PAL paradigm.

2. Materials and methods

2.1. Injection of morphine into eggs

Freshly fertilized “BAU-3” eggs (60 ± 5 g) were obtained from Beijing Agricultural University (BAU) and placed at 5–10 °C for no more than 1 day before incubation. Morphine injections were once given in E5 and E8, based on our previous findings following that injections of morphine (1 mg/kg of egg) every other day at E5–8 were likely to confer the maximal risks for vulnerabilities to the breakdown of learning and memory (He et al., 2010). Morphine was dissolved in 0.9% sterile saline and injected into the chorioallantois end of the shell of each egg. The injection volume was 20 μ l/egg. Vehicle eggs received equivalent volume of 0.9% physiological saline. The total number of eggs per group was 200. After the injection holes of eggshell were sealed with paraffin wax every time, the eggs were incubated for 21 days (E0–21) in a BD Model 264 (Dezhou, ShanDong) incubator. Eggs were incubated at 37.8 °C/55–60% relative humidity and automatically turned every 2 h., with exposure to 12 h light/dark cycles.

2.2. The one-trial passive avoidance learning (PAL) task

The PAL task was carried out on the second day after hatch, and had been described by our previous work (He et al., 2010). Briefly, the chicks were placed in pairs in 20×20×25 cm aluminium cages each illuminated with a 25 W white light. Before pretraining by three 10-s presentations of a white bead (2 mm diameter), they were allowed to familiarize to the experimental environment at 28–30 °C for at least 30 min. Twenty minutes after the last pretraining trial each chick was presented with a 4 mm diameter red bead coated with 20% methylanthranilate (MeA) for 10 s. About 11.7% of chicks were not used for further analysis because of failing to peck at the training bead or having no an obvious disgust reactions (i.e. peck-and-shake, peck-and-back away) for the MeA. Tests of memory were conducted at 30, 120 and 360 min following training. On testing, chicks were presented first with a dry red bead (identical to the one in the training trial), and then a dry white bead (identical to the one in the pretraining trial) on two 10 s trials, 5 min apart. Each chick's behavior was recorded as peck or non-peck. Memory retention was calculated as a percent avoidance score (the number of chicks in each group that avoided the red bead but pecked the white on test 100×/total number of the trained chicks). Each chick was trained and tested only once. The laboratory protocol and procedure were carried out in accordance with the animal care and use guidelines enforced by the NIH (no. 8023, revised 1996), and approved by the Animal Care and Research Committee of Tianjin Medical University.

2.3. Western blot analysis of the PKC isoforms

2.3.1. Tissue dissection and preparation

As described previously (Burchuladze et al., 1990), chicks of each group were tested respectively at 28 min, 118 min and 358 min after training and decapitated immediately after testing. The left hemispheres of the chick brains were removed and placed on an ice-cold plate for dissection of the left IMM samples using the procedures described by Watson (Watson et al., 1991). The samples were frozen immediately in liquid nitrogen and then preserved at –80 °C in a refrigerator for analysis. Considering the enough quantities of material for subsequent analysis, the material from 3 identically trained chicks was combined into a single sample.

2.3.2. Immunoblotting of the PKC isoforms

The protein of cytomembrane and cytoplasm for each sample was extracted using the Cytoplasm membrane proteins extraction kit (Keygen biotech). Protein amounts were estimated via the Bradford assay using bovine serum albumin (BSA). Equivalent amounts of membrane and cytosol fractions (50 μ g) for each sample were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes (Millipore, USA) after electrophoresis. The membranes were blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) with agitation. Subsequently, the membranes were incubated overnight at 4 °C with primary antibodies (P-PKC α , 1:20000 dilution, Abcam, Cambridge, MA, USA; P-PKC δ , 1:500 dilution, LifeSpan BioSciences Inc.). On the next day, the membranes were washed three times with TBST buffer and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Boster Biotechnology, Wuhan, China) diluted 1:50000 in TBST containing 5% skim milk for 2 h at 37 °C with agitation. After incubation, the membranes were thoroughly washed in TBST. The antigen-antibody peroxidase complex was finally digitized and quantitated using an Enhanced Chemiluminescent (ECL) kit (Thermo Fisher Scientific, Massachusetts, USA) and visualized by exposure to Kodak film (eastman Kodak, Kodak, NJ). Integrated density values of each band of the PKC α or δ subunits were analyzed using Fluor Chen 2.0 software (Olympus, Yokohama, Japan) and normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The values are presented as a ratio of the membrane or cytosol to GAPDH.

2.4. Statistics

The differences of memory retention between the two groups (30 min, 120 min, 360 min after MeA training) were evaluated with Chi-square test. Results from Table 1 and the differences in the expressions of PKC membrane and cytosol fractions of the left IMM were determined by unpaired Student's *t*-test. Data are presented as mean \pm S.E.M. In all instances, a value of $P < 0.05$ was considered statistically significant. Calculations were performed using the SPSS 15.0 statistical package.

Table 1
Effects of prenatal morphine exposure on general offspring characteristics.

Group	Hatch day	Hatch weight (g)	Hatch rate (%)	Sample numbers
Saline	20.92 \pm 0.07	41.96 \pm 0.74	91	182
Morphine	21.45 \pm 0.08 ^a	42.07 \pm 0.87	85	170

^a Hatch day of the morphine group is longer than that of the saline group (a, $P < 0.01$). Data are expressed as mean \pm S.E.M. Chicks were weighed when being dried completely.

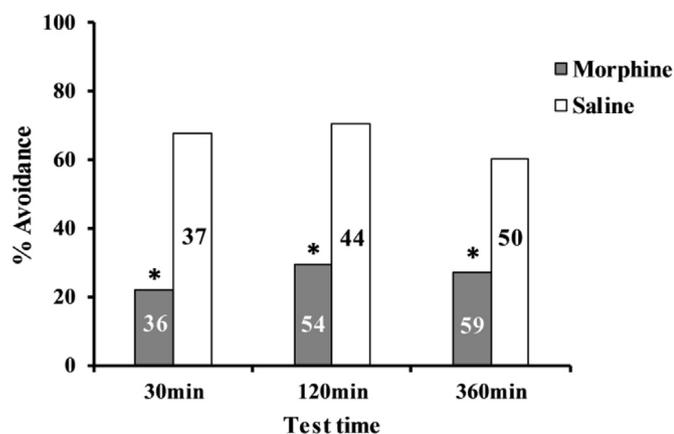


Fig. 1. The effects of prenatal morphine exposure during E5-8 on memory in one-day old chicks. Results are presented as percent avoidance for the passive avoidance learning tasks at 30 min, 120 min and 360 min after training. *, $P < 0.01$ versus prenatal saline group. Numbers of chicks in each group are presented in the relevant bars.

3. Results

3.1. Prenatal morphine exposure during E5-8 impaired memory of PAL tasks in one-day-old chicks

As seen in Fig. 1, there were significant differences in percent avoidance at 30 min ($\chi^2=15.147$, $df=1$, $P < 0.001$), 120 min ($\chi^2=16.190$, $df=1$, $P < 0.001$) and 360 min ($\chi^2=11.996$, $df=1$, $P=0.001$) between the saline and morphine group. These results demonstrated that the intermediate-term memory (ITM) and long-term memory (LTM) in one-day-old chicks were all obviously impaired by intra-allantoic

injection of morphine at E5-8.

3.2. The effects of prenatal morphine exposure on the translocation of PKC α in the left IMM of chick brain after the PAL tasks test

We determined the protein levels of PKC α in the left IMM of the chicks prenatally exposed to morphine or saline at E5-8 after tests of memory at 30, 120 and 360 min respectively following training (representative bands of Western blots in Fig. 2D–F). Unpaired Student's *t*-test indicated that the contents of PKC α in the membrane fractions of the morphine group were significantly lower than that of the saline group when determined at 120 min (membrane: $t_{10}=2.332$, $P=0.042$, as shown in Fig. 2B) and 360 min (membrane: $t_{5,571}=5.860$, $P=0.001$, as shown in Fig. 2C), whereas in the cytosol fractions, the morphine group showed an opposite trend, which meant that a higher level of PKC α was observed when compared to the saline group (120 min, cytosol: $t_{6,549}=-2.587$, $P=0.038$; 360 min, cytosol: $t_{8,009}=-3.973$, $P=0.004$; as seen in Fig. 2B and C). Although there was no significant difference in the expression of PKC α in the membrane fractions at 30 min ($t_8=0.036$, $P=0.972$; as seen in Fig. 2A) between the two groups, PKC α protein in the cytosol fractions of the morphine group presented the significantly higher level than that of the saline group ($t_8=-4.857$, $P=0.001$; as seen in Fig. 2A).

3.3. The effects of prenatal morphine exposure on the translocation of PKC δ in the left IMM of chick brain after the PAL tasks test

We also examined the protein levels of PKC δ in the left IMM of the trained chicks after the PAL tasks test (representative bands from Western blots in Fig. 3D–F). In contrast to the activities of PKC α , Fig. 3C indicated that the morphine group displayed the significant increases of PKC δ in the membrane fractions and the decreases in the cytosol fractions at 360 min (membrane: $t_{5,40}=-2.540$, $P=0.048$; cytosol: $t_{10}=4.574$, $P=0.001$), compared with the saline group.

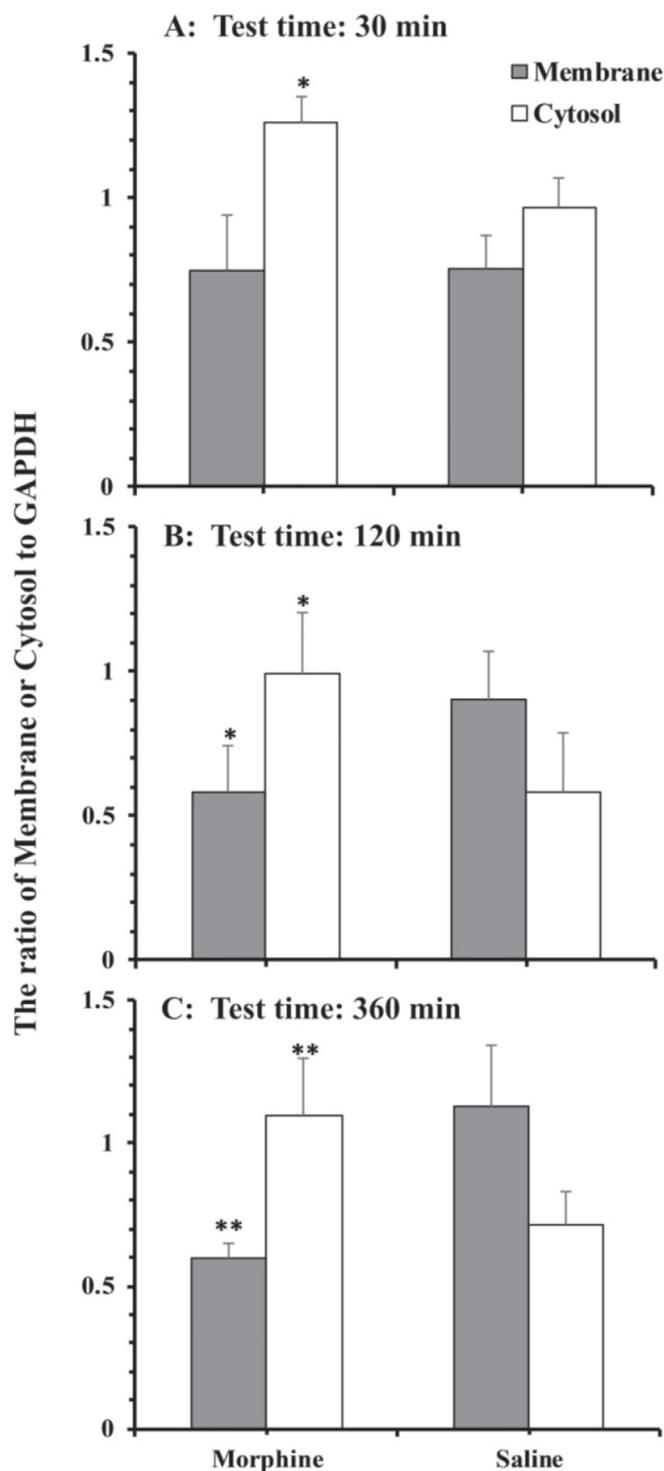
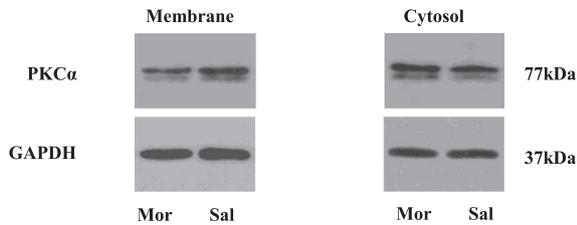


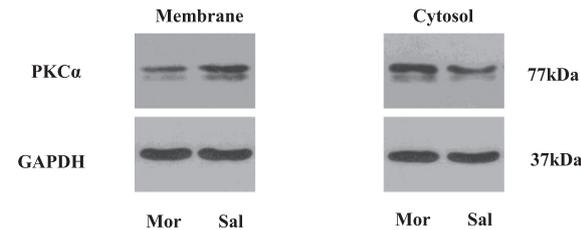
Fig. 2. Effects of prenatal morphine exposure on the protein levels of PKC α of the left IMM in the passive avoidance learning tasks. A–C. The expression of PKC α in the left IMM membrane and cytosol fractions at 30 min, 120 min and 360 min respectively after training. Data, obtained from 15 to 18 chicks in each group, are expressed as mean \pm S.E.M. *, $P < 0.05$ or **, $P < 0.01$ versus prenatal saline group. D–F. Representative bands from Western blots. Mor=morphine; Sal=saline.

Moreover, although there were no significant differences in the levels of PKC δ in the membrane fractions at 30 min ($t_6=-1.283$, $P=0.247$; as seen in Fig. 3A) or at 120 min ($t_{10}=-0.784$, $P=0.451$; as seen in Fig. 3B) between the two groups, the levels of PKC δ in the cytosol fractions of prenatal morphine group showed significant decreases when compared with prenatal saline group (30 min: $t_{3,892}=8.042$,

D: Test time: 30 min



E: Test time: 120 min



F: Test time: 360 min

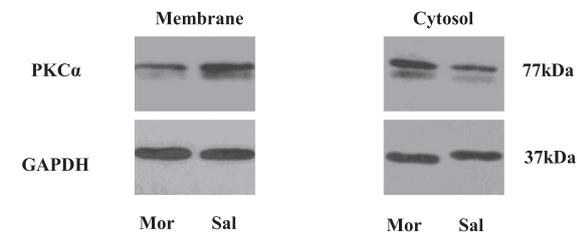


Fig. 2. (continued)

$P=0.001$; 120 min: $t_{10}=-3.596$, $P=0.005$; as seen in Fig. 3A and B).

4. Discussion

The present study used Semi-quantitative Western Blotting to investigate the effects on expressions of PKC α and PKC δ in the left IMM of prenatally morphine-exposed, one-day old chicks which showed memory impairments in PAL tasks. The main results included: (1) ITM and LTM of the PAL paradigm were significantly disrupted in chicks which were injected with morphine during E5-8, which were fully in line with our previous studies (He et al., 2010). (2) The reduced PKC α activity in left IMM was observed in prenatally morphine-exposed chicks with impaired LTM. (3) The translocation of PKC δ from cytosol to membrane in left IMM was involved in impairment of LTM in chicks prenatally exposed to morphine. (4) Neither PKC α nor PKC δ in left IMM were associated with the impaired ITM induced by prenatal morphine exposure.

The signaling pathways underlying the neurobehavioral deficits induced by prenatal opiate exposure, cover a large number of molecules, including the protein kinase C (Huleihel and Yanai, 2006; Katz et al., 2008; Shahak et al., 2003; Steingart et al., 2000; Yaniv et al., 2004). For example, Yaniv et al. (2004) reported that prenatal exposure of mice to heroin abolished the translocation and activation of PKC γ and PKC β II induced by cholinergic receptor, which seemed to be responsible for the deficits in hippocampus-related learning and memory behavior. Furthermore, they also conducted parallel experiments using chick embryos, which had the similarity of effects on PKC isoforms and imprinting behavior to those seen in the mouse (Yanai et al., 2004). Those findings based on the cross-species investigations clearly suggested that the postnatal neurobehavioral teratogenicity associated with prenatal opiate exposure might be attributable to the abnormalities in the responsiveness of PKC isoforms at the relevant brain region. To our knowledge no work involving the learning capacity

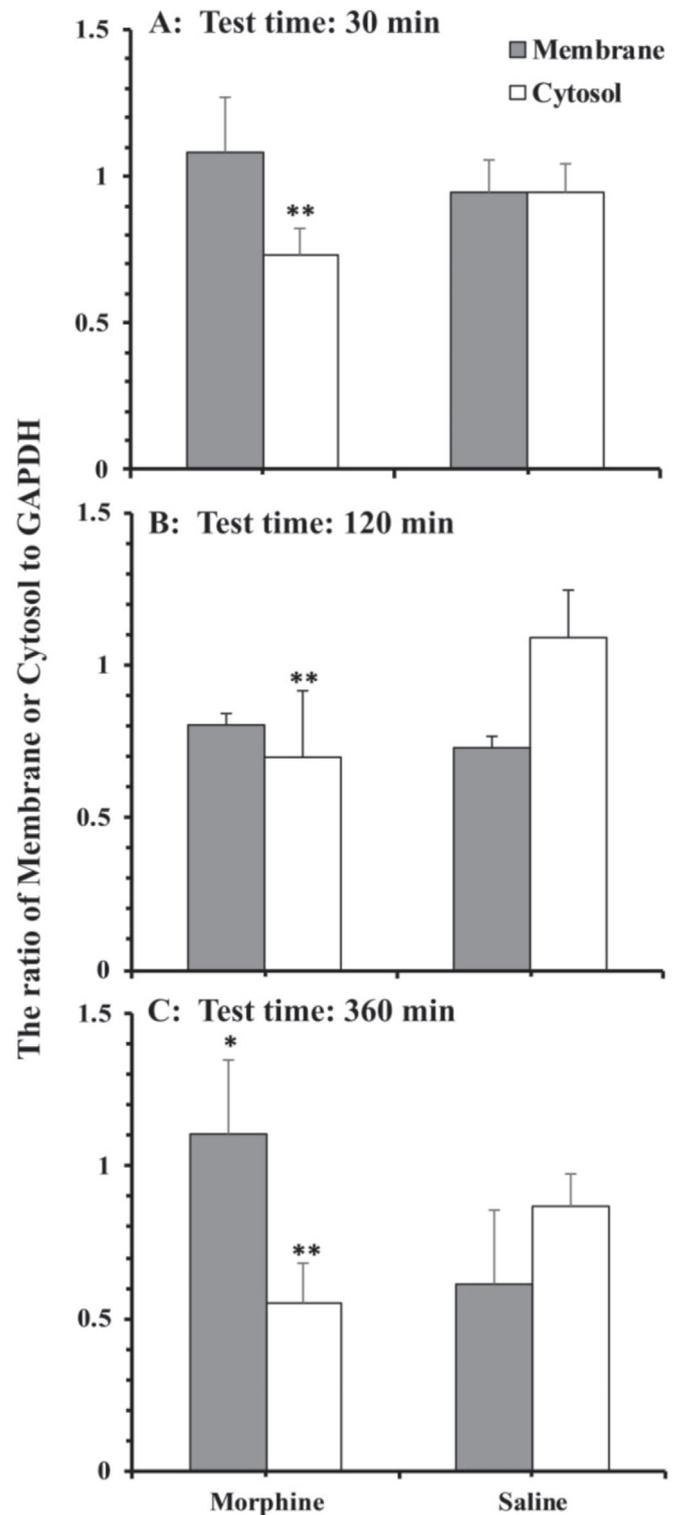
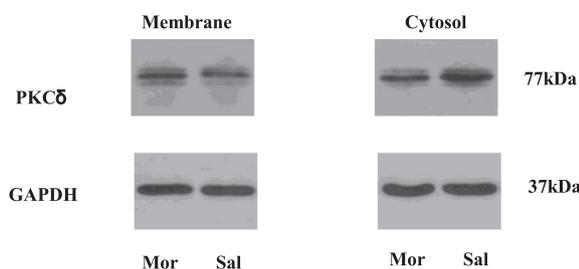


Fig. 3. Effects of prenatal morphine exposure on the protein levels of PKC δ of the left IMM in the passive avoidance learning tasks. A–C. The expression of PKC δ in the left IMM membrane and cytosol fractions at 30 min, 120 min and 360 min respectively after training. Data, obtained from 12 to 18 chicks in each group, are expressed as mean \pm S.E.M. *, $P < 0.05$ or **, $P < 0.01$ versus prenatal saline group. D–F. Representative bands from Western blots. Mor=morphine; Sal=saline.

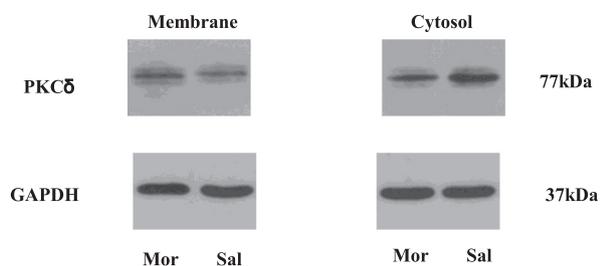
for avian species prenatally exposed to morphine and the associative alterations in PKC expression has been attempted.

It is well known that the protein kinase C (PKC) isoforms are thought to be a family of “memory kinases”, which have the vital impact in the memory acquisition and maintenance (Sun and Alkon, 2014).

D: Test time: 30 min



E: Test time: 120 min



F: Test time: 360 min

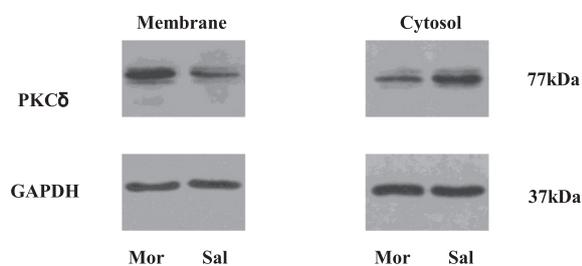


Fig. 3. (continued)

For more than two decades, PKC activation and its phosphorylated substrates have been found related to learning and memory in a variety of species, such as *Lymnaea stagnalis*, chick, rodents, and rabbits (Bank et al., 1988; Bowers et al., 1995; Sheu et al., 1993; Takigami et al., 2014). More recently, increasing evidence demonstrates that the conventional PKC isoform PKC α has been implicated in the formation of aversive and high-impact memories (Lucke-Wold et al., 2015). For example, it has been shown that PKC α is genetically linked to the traumatic memory and to the risk for posttraumatic stress disorder (PTSD) in heavily traumatized genocide survivors (de Quervain et al., 2012). PKC α also plays an important role in strong memory formation mediated by α -adrenergic receptors (Dong et al., 2009). Furthermore, it has been shown that the activation of the PKC α is crucially involved in the formation of LTM of taste avoidance conditioning in pond snails (Takigami et al., 2014). In addition, studies on mammals have indicated that chronic exposure to morphine significantly reduces the levels of PKC- α/β in the hippocampus and results in spatial memory impairments in the Morris water maze tasks (Lu et al., 2010). These findings may support evidence for our present data showing that the level of PKC α in the membrane fractions in left IMM was decreased but that in the cytosol showed increased in prenatally morphine-exposed chicks with impaired LTM (120 and 360 min; as seen from Fig. 2B and C), when compared with prenatally saline-exposed chicks. It is well-known that the translocation of PKC isozymes from cytosol to membrane means PKC's activation. So, this could suggest that prenatal morphine exposure-induced LTM impairments have been associated

with concomitant marked down-regulation of the activity of PKC α in left IMM. However, unlike the activities of PKC α , there was an obvious translocation of PKC δ from cytosol to membrane in left IMM in prenatal morphine group compared with prenatal saline group, while test of memory was conducted at 360 min after training (Fig. 3C). The current results indicated that the activation of PKC δ in left IMM may be responsible for the impairment of LTM induced by prenatal morphine exposure.

PKC δ belongs to the novel subclass of PKC isoforms and plays important roles in neuronal survival, differentiation, proliferation and apoptosis regulation (Ferri et al., 2006; Kim et al., 2013; Sparatore et al., 1993), and also has been involved in learning and memory (Apostolatos et al., 2012). Gallagher et al. (2000) have demonstrated that in vitro the inhibition of PKC δ to be associated with the increased polysialyltransferase (PST) activity and with the increased expression in polysialylation of neural cell adhesion molecule (NCAM PSA) which is required for memory consolidation. Similarly, they have also discovered the decreased expression of PKC δ in the hippocampus of rats reared in the complex environment, accompanied by the significant increase in frequency of hippocampal polysialylated neurons (Gallagher et al., 2001). Moreover, pharmaco-behavioral experiments on Wistar rats have indicated that chronic injection of curcumin, which is known to promote the degradation of PKC δ , could increase the frequency of polysialylated cells in the dentate infragranular zone in vivo and noticeably improve the acquisition and consolidation of spatial learning in the water maze paradigm (Conboy et al., 2009). In consideration of the occurrence of the synthesis of neuron/glia cell adhesion molecule during the formation of LTM of the PAL tasks (Scholey et al., 1995), it is reasonable to speculate that the translocation of PKC δ may become one of signals for suppression of the synthesis of NCAM in the left IMM of prenatally morphine-exposed chicks with impaired LTM, although the present study could not provide direct evidence for the effects of PKC δ on synthesis of NCAM following prenatal morphine exposure. However, it merits further investigation.

Moreover, according to the part of the present results, we did indeed not see the significant decreases in the level of PKC α in membrane fraction of prenatal morphine group compared with prenatal saline group when detected at 30 min after training, although the level of PKC α in the cytosol fraction of prenatal morphine group was higher than that of prenatal saline group (Fig. 2A). Similarly, compared with prenatal saline group, the big increases in the expression of PKC δ in membrane fraction of prenatal morphine group did not happen when detected at 30 and 120 min after training (Fig. 3A-B), although the level of PKC δ in the cytosol fraction of prenatal morphine group was significantly lower than that of prenatal saline group (Fig. 3A and B). The present results indicate that little differences are observed between the two groups regarding the expressions of PKC α and PKC δ in the membrane fraction, implying that activities of PKC α or PKC δ in left IMM of prenatal morphine exposure may not be different from that of prenatal saline exposure. Therefore, we cannot conclude that PKC α and PKC δ in left IMM are required for ITM impairment and/or for the breakdown of early phase of LTM of chicks prenatally exposed to morphine. It has been suggested that agents acted on protein kinases (such as PKA, PKC, and PKG) could successfully prevent ITM formation with strong MeA training, which do not produce amnesia with weak training (Rosenzweig et al., 1993), demonstrating that the difference in concentration of MeA may result in qualitatively different forms of memory arising as a consequence of the different induction regimes (Crowe and Hamalainen, 2001). Therefore, it is possible that prenatal morphine-induced the impairments of ITM and/or the early phase of LTM might not be attributed to one or more of the altered expression of PKC isoforms in the left IMM, based on the present PAL paradigm of the weak training (20% MeA), which need in-depth investigations.

Because the changes of PKC α and PKC δ activity during memory

consolidation in the chick resemble similar phenomena in the mammal, it seems likely that there are common underlying mechanisms for the effects of prenatal opiates exposure on PKC modulation in long-term synaptic plasticity (LTP), functions of the acetylcholine system and the GABAergic transmissions (Steingart et al., 1998; Yanai et al., 2000). Our previous experiments (unpublished) suggested an obvious loss of GABA-containing neurons in IMM of chicks exposed to morphine during E5–8, which may be related to the impaired IMM GABAergic inhibitory function, known to be involved in the memory formation and consolidation (Godinho et al., 2016). Moreover, it has been reported that bryostatin, a potent agonist of PKC, can significantly enhance GABAergic neurotransmission in pyramidal neurons by activating the PKC α - and PKC ϵ -dependent pathway, which may contribute to the improvement of learning and memory in rats (Xu et al., 2014). We hypothesized that dysfunction of GABAergic-PKC signaling would be one of the neural mechanisms underlying cognitive deficits in offspring treated with morphine during pregnancy.

5. Conclusion

The present study for the first time demonstrates that PKC α and PKC δ are differentially involved in the IMM-related cognitive deficits induced by prenatal morphine exposure, in a manner identical to the previous findings in mammals. Regulation in activations of PKC α and PKC δ in the relevant brain locus-based learning may be useful for the improvement of learning and memory in prenatally morphine-exposed offspring.

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