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RACK1, a potential target to decrease morphine reward in mice

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ABSTRACT

Morphine reexposure induces the decrease of receptor for activated C-kinase 1 protein (RACK1) levels in frontal cortex, and the increase of p-ERK (extracellular signal-regulated kinase) levels in mouse frontal cortex, striatum, hippocampus and nucleus accumbens (NAcc). Moreover, RACK1 is associated with the core kinases of the ERK pathway, Raf, MEK, and ERK. The purpose of this study is to investigate the effect of overexpression of RACK1 on the conditioned place preference (CPP) and the level of p-ERK in morphine reexposure mice. Mice were subcutaneously injected with morphine on the 2nd, 4th, 6th, and the 8th day, saline was delivered the next day. After mice showed place preference, RACK1 was administered by intraventricular injection 20 minutes after injection of morphine on the 11th, 13th, 15th, and 17th day. CPP was measured on the 18th day. It was found that morphine reexposured mice showed a decreased RACK1 level in the frontal cortex, striatum and an increased RACK1 level in hippocampus and NAcc, but this effect was reversed after administration of RACK1. In this study we demonstrated that RACK1 decreased p-ERK and erased CPP during reexposure of morphine and there was no an effect in reexposure saline mice. It strongly suggests that RACK1 may play a crucial role in morphine reexposured mice and the RACK1 has the potential to be a remedy to the morphine reward.

Key words
Addiction • Morphine • RACK1 • ERK • CPP (Conditioned place preference)

Abbreviations
RACK1: receptor for activated C-kinase 1 protein • CPP: Conditioned place preference
ERK: extracellular signal-regulated kinase

Introduction

Drug addiction is a devastating disease that affects 200 million people in globe. 100,000 people died due to drug abuse and 10 million people have lost the ability to work every year. Recently the role of RACK1 in morphine addiction has attracted much attention. The development of morphine addiction depends on a complex interplay of genetics, other biological factors, and social factors (Leshner, 1997; Robbins et al., 1999). Mechanistically, addiction is thought to depend on neurochemical systems within specific neural circuits (Nestler, 2001) and endogenous systems (Mcgough et al., 2004).

Drug addiction results partially from the distortion of dopamine controlled plasticity, and extracellular signal-regulated kinase (ERK) also plays an important role in the underlying molecular mechanisms

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of this process. Activation of ERK1/2 in response to addictive drugs was first reported to occur in the ventral tegmental area (VTA) after a five-day exposure to morphine or cocaine in rats (Berhow et al., 1996). Subsequently, acute treatment with cocaine in mice led a rapid transient increase of ERK phosphorylation in the NAcc (Valjent et al., 2000), lateral bed nucleus of stria terminalis, central amygdala and deep layers of the prefrontal cortex (Valjent et al., 2001). This effect can also be observed in other types of drugs abuse such as D9-tetrahydrocannabinol (THC), D-amphetamine, 3, 4-methylene-dioxy-methamphetamine, morphine and nicotine (Valjent et al., 2001; Choe et al., 2002; Salzmann et al., 2003; Valjent et al., 2004). Blockade of ERK activation prevents long-lasting behavioral changes, including psychomotor sensitization and conditioned place preference. It also interferes with drug craving and drug associated memory reconsolidation.

RACK1, a 36 kDa homologue of the β subunit of G proteins, is a member of the WD-40 family proteins characterized by the highly conserved internal WD-40 repeats (Trp-Asp) (Hu et al., 2006). Due to its association with a large number of signaling proteins such as protein kinase C(PKC) (Besson et al., 2002), Src and integrin β subunit (Chang et al., 2001), phosphodiesterase PDE4D5 (Steele et al., 2001), STAT (Kubota et al., 2002), IGF-I receptor (Hermanto et al., 2002), Epstein-Barr virus BZLF1 protein and HIV-1 Nef protein (Baumann et al., 2000; Gallina et al., 2001), RACK1 has been identified as an anchoring or adaptor protein in multiple intracellular signal transduction pathways. *In vivo* injection of RACK1 increases striatal BDNF expression, RACK1 reduces ethanol consumption and sensitization (Mcgough et al., 2004). The cortical slices from aged rats have reduced levels of RACK1 (roughly 50%) in comparison with those observed in adult and middle-aged animals (Pascale et al., 1996b). When bound to the IP3 receptor, RACK1 regulates intracellular Ca^{2+} level, potentially contributing to processes such as learning, memory and synaptic plasticity. By binding to the NMDA receptor, it dictates neuronal excitation and sensitivity to ethanol.

Here we test the possibility that ERK is part of a pathway that is involved in morphine reexposure. We also assess the interactions between RACK1, ERK, and morphine reexposure, and determine whether the RACK1-ERK pathway regulates the behavioral actions of morphine reexposure.

Methods

Animals

Male C57BL/6J mice initially weighing were 25-30 g. The room temperature was kept at 23 ± 1°C. Food and water were available *ad libitum*. The experimental protocol was conducted according to the Guidelines of the Ethics Review Committee for Animal Experimentation of Sichuan University.

pcDNA 3.1-RACK1 plasmid construction

Total RNA of mouse brain was isolated with TriZol reagent (Invitrogen) according to the protocol provided by the manufacturer. The cDNA was synthesized from the total RNA using the first strand cDNA synthesis kit (Fermentas). The full-length cDNA of mouse RACK1 (Genbank Accession No. NM_008143.3) was amplified by PCR. The oligonucleotide primers were as follows: Sense (5'-TAAAGCTTATGACCGAGCAGATGACCC-3'), Antisense (5'-CCTCTAGATTGACGGTACCAATAGTTAC-3'). The cloned RACK1 cDNA fragment was digested by Hind III and Xba I and then inserted into the mammalian expression vector pcDNA3.1(+) (Invitrogen). The pcDNA 3.1 - RACK1 vector was transformed into *E. Coli* DH5α and subjected to DNA sequencing.

Conditioned place preference (CPP)

The conditioned place preference (CPP) apparatus consisted of two visually distinct conditioning compartments (one with white-colored walls and wire mesh flooring and the other with black-colored walls and steel rod flooring) connected by a smaller center compartment. The time spent in morphine-paired compartment during a 15 minutes session was measured automatically.

Western blot

Protein concentrations were measured by the method of Bradford (Bradford, 1976), using BSA as a standard. Samples (50 μg) were resolved on a 12% SDS-PAGE and transferred to PVDF membrane. Membranes were probed with the appropriate anti-

bodies, PVDF membrane were chromogenic by diaminobenzidine. Bands were then scanned by EPSON PERFECTION 1260 instrument and analyzed by Image-pro plus 4.5 software.

Immunohistochemistry

The brains prepared from sacrificed mice were fixed in 10% paraformaldehyde. Subsequently, they were dehydrated and embed in paraffin. Serial sections of 4 um were heated to 60°C and dehydrated in xylene and graded alcohols. They were then put in dilution of 3% H_2O_2 for 10 min. Antigen retrieval was performed with 0.01 M citrate buffer at pH 6.0 at 95°C. Sections were blocked with 5% BSA, then incubated with primary antibody diluted in 0.01 M PBS (pH 7.6) for 12 h, followed by incubations with biotinylated IgG peroxidase secondary antibody for 30 min. Slides were chromogenic by diaminobenzidine. Slides were counterstained with haematoxylin and mounted. Brain slices were then scanned by Nikon ECLIPSE 55i instrument.

Experiment protocol

Seventy-two mice were randomly divided into six groups, including saline group (Group I), morphine group (Group II), morphine/RACK1 treat group (Group III), morphine/vehicle control plasmid group (Group IV), saline/RACK1 treat group (Group V), saline/vehicle control plasmid group (Group VI). On day 1 (pretest), mice were placed into the center compartment and allowed access to both conditioning compartments for 30 min. Mice (Group II to Group IV) were conditioned by pairing a 20 min exposure of the white compartment with morphine (10 mg/kg, S.C.) injection, and of the other compartment with saline injection on alternating days until subjects received four pairings each of morphine and saline with their respective conditioning chambers. On day 10 (Test 1), animals were tested for CPP by placing them in the center compartment and allowing access to both conditioning compartments for 15 min. On day 11, a hole (diameter 1 mm) was drilled by dentistry drill in the right lateral ventricle (anteroposterior, -1.5; mediolateral, -1.4; dorsoventral, -3.0, relative to bregma) of subjects anesthetized with Chloral Hydrate. On day 11, 13, 15, 17, the mice received i.c.v. RACK1 or vehicle control plasmid (5 μl; flow rate, 5 μl/5 min) 20 minutes after injection of morphine. On day 14 and 18, CPP was tested. The nuclei of the sacrificed mice were prepared for later analysis with western blot and immunohistochemistry. RACK1 was transfected with *in vivo* Cationic polymer transfection reagent (polyplus-transfection) according to the protocol provided by the manufacturer.

Statistical analysis

Data were depicted in figures as means ± SEM and were analyzed by one-way ANOVA (Post Hoc Multiple Comparisons) for group differences (SPSS 13.0).

Results

1. RACK1 plasmid erases previously acquired CPP

Mice were conditioned to morphine in a 9-day unbiased CPP protocol and mice in Group I, V and VI received only saline injections. On day 11, 13, 15 and 17, mice of Group III and IV were administered with morphine and placed in the morphine-paired compartment. 20 minutes later, mice were injected with either RACK1 (Group III) or vehicle (Group IV). When these animals were tested for CPP on the 18th day (Test 3), CPP can be observed in the morphine/vehicle-treated Group IV, but not in the moephine/RACK1 treated Group III (Fig. 1B). These results show that the administration of RACK1 erases previously acquired CPP with reexposure to morphine.

2. Effects of reexposure to morphine and RACK1 plasmid on RACK1 levels in brain

Reexposure to morphine could change the expression of RACK1 in the nuclei on Test 3. Compared with saline administration, morphine reexposure mice induced a significant decrease in RACK1 levels (68 ± 10%) in the frontal cortex and striatum (38 ± 11%) (Group II, Fig. 2A, B). In contrast, the RACK1 level was found increased in the hippocampus (22 ± 13%) and NAcc (25 ± 17%) (Group II, Fig. 2A, B).

Endo-free RACK1 plasmid or vehicle control plasmid was administered on the 11th, 13th, 15th, and 17th day. Mice were sacrificed and brains protein extracts were prepared to subject western blot on the 18th day. Compared with morphine/vehicle control plasmid treated mice (Group IV), the RACK1 level

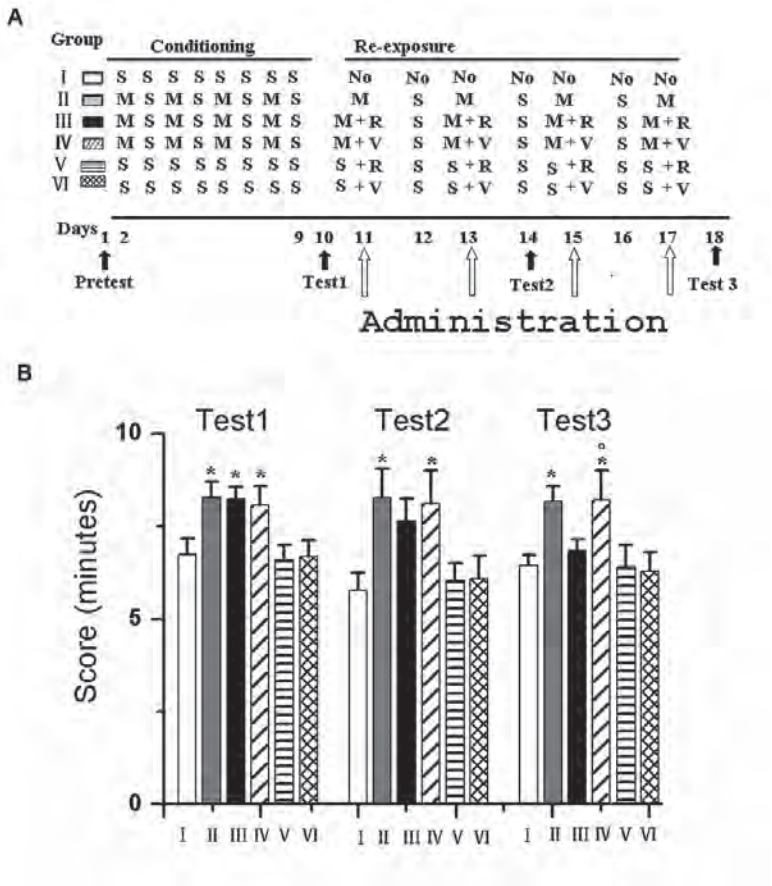

Fig. 1. - Reexposure to morphine in the presence of RACK1 erase CPP. Experimental design: S, saline; M, morphine 10 mg/kg; R, RACK1, 2.5 ug, 5 ul; V, vehicle control plasmid pcDNA 3.1, 2.5 ug, 5 ul. (Fig. 1A). All morphine-conditioned groups (Group II to IV) had a significant CPP at Test1 ($P < 0.05$). The administration of RACK1 20 minutes after morphine reexposure suppressed the place preference when animals were tested 24 h later. At Test 2, CPP was slightly diminished in morphine/RACK1-treat Group IV ($P > 0.05$). At Test 3, CPP was erased in morphine/RACK1-treat Group IV ($P < 0.05$). Data are means ± SEM (eight mice per group). Post Hoc Multiple Comparisons, Group I *vs.* Group II to IV: * $P < 0.05$; Group III *vs.* Group IV: ° $P < 0.05$ (Fig. 1B). At Test 2 and Test 3, CPP has no significant change in saline/RACK1-treat mice (Group V) and saline/vehicle control plasmid mice (Group VI).

was significantly increased in the prefrontal cortex and striatum, but decreased in the hippocampus and NAcc (Group III) (Fig. 2A, B).

3. Influence of RACK1 on ERK in morphine reexposure mice

To determine whether the overexpression of RACK1 reduces p-ERK levels in morphine reexposure mice, mice (Group III) were administered with RACK1 as in Fig. 1. Importantly, it was found that RACK1 lowered the protein levels of p-ERK in the cortex, striatum, hippocampus and NAcc (Fig. 3). Hence, RACK1 suppressed the expression of p-ERK in morphine reexposure mice.

4. Influence of RACK1 on ERK in saline reexposure mice

We then tested whether RACK1 play an identical part in saline reexposure mice. To test this hypothesis, mice received injection of RACK1 (Group V) or vehicle control plasmid (Group VI) 20 minutes after compared with saline treated as in Fig. 1A. Compared with saline treated mice, the RACK1 level was significantly increased in the prefrontal cortex and striatum ($p < 0.001$), but reduced in the hippocampus and NAcc ($p < 0.001$) (Group V) (Fig. 4A, E). Importantly, in mice that received RACK1 in the absence of morphine (reexposure saline), ERK phosphorylation was not suppressed (Fig. 4C,

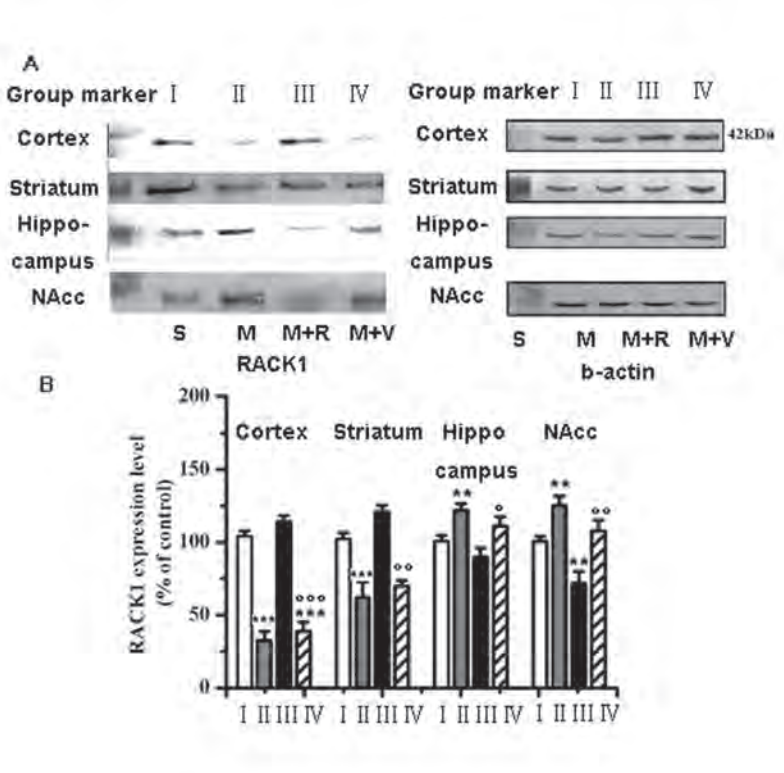

Fig. 2. - Effects of reexposure to morphine and RACK1 plasmid on RACK1 level. To assess RACK1 expression level, RACK1 levels were analyzed at the end of Test 3, by immunoblotting in the prefrontal cortex, striatum, hippocampus and NAcc, and results were quantified (Fig. 2A). Compared with saline-treated mice, a significant decrease of RACK1 level in the cortex and striatum and significant increase in hippocampus and NAcc after reexposure to morphine(Group II). When morphine reexposure mice were administration RACK1, RACK1 expressed increaser in cortex and striatum, decreased in hippocampus and NAcc. Histogram depicts the mean percentage of control (saline only) of the ratio of *RACK1: β-actin* ± SEM; $n = 8$. ** $p < 0.01$, *** $p < 0.001$ compared with control (Fig. 2B). Post Hoc Multiple Comparisons test was as follows: Group I *vs.* Group II to IV, ** $P < 0.01$, *** $P < 0.001$; Group III *vs.* Group IV, °° $P < 0.01$, °°° $P < 0.001$).

F). This finding shows that RACK1 does not play a part in reexposure saline mice, only play a role in reexposure morphine mice.

5. RACK1 is transduced into the cortex and striatum

RACK1 expressed increaser in cortex 24 hour after mice reexposure to morphine was administration RACK1 plasmid, which was reduced in hippocampus and NAcc.

Discussion

We revealed that reexposure of morphine in mice caused up regulation of the p-ERK levels in the prefrontal cortex (123%, $p < 0.05$), striatum (108%), hippocampus (118%, $p < 0.05$) and NAcc (115%, $p < 0.05$). This concurs with a prior report that single or repeated morphine injections activate ERK in many brain regions, including NAcc (Valjent et al., 2000, 2004). In animals trained for CPP with morphine, cocaine or methamphetamine, exposure to the test apparatus is sufficient to activate ERK1/2 in the prefrontal cortex, NAcc and caudate-putamen, lateral bed nucleus of *stria terminalis* and hippocampus (Mizoguchi et al., 2004; Miller and Marshall, 2005; Valjent et al., 2001; Valjent et al., 2005, 2006). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) appears to be required for ERK1/2 activation in striatal slices (Vanhoutte et al., 1999) and *in vivo* (Choe and Wang, 2002). Activation of this pathway is also necessary for long-lasting effects of drugs.
Blockade of ERK before each conditioning to mor-

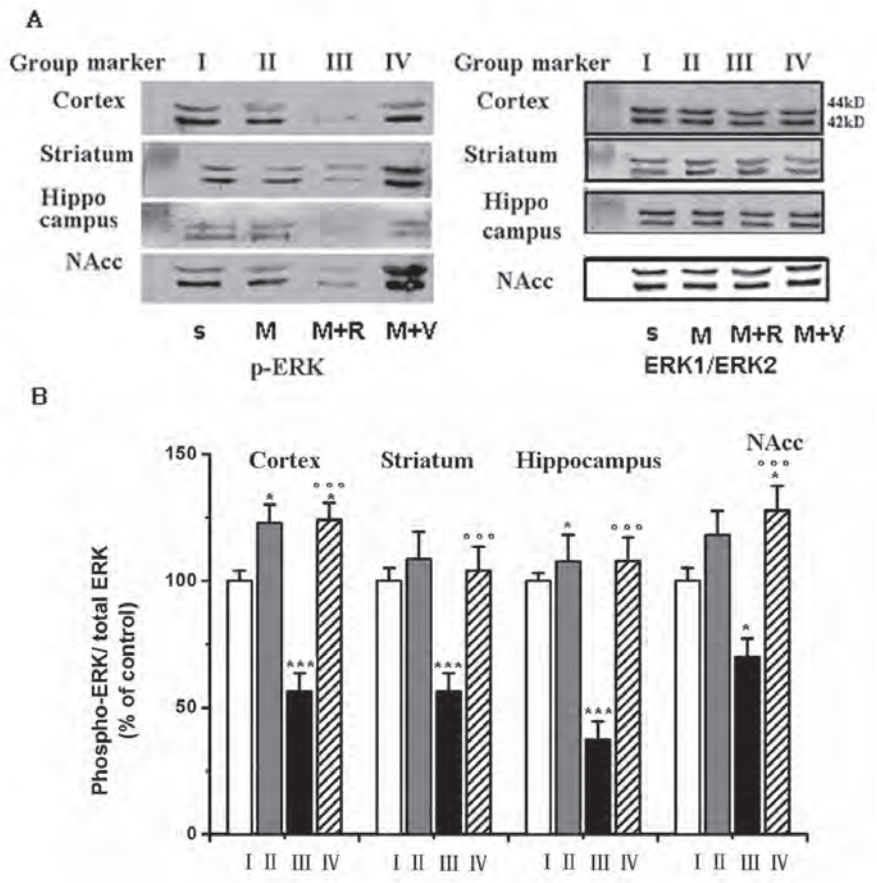

Fig. 3. - Re-exposure to morphine in the presence of RACK1 erases its biochemical correlates. To assess protein phosphorylation during the CPP test, p-ERK and total ERK immunoreactivity were analyzed at the end of Test 3, by immunoblotting in nuclei. Histogram depicts the mean percentage of control (saline only) of the ratio of *p-ERK: total protein* ± SEM; $n = 8$. * $p < 0.05$, *** $P < 0.001$ compared with control (Fig. 3B). Post Hoc Multiple Comparisons test was as follows: Group I *vs.* Group II to IV, * $P < 0.05$; *** $P < 0.001$, Group III *vs.* Group IV, °°° $P < 0.001$.

phine prevents the induction of CPP (Valjent et al., 2000). We examined whether blockade of the ERK pathway was able to modify the behavioral response even if it had been learned. With the RACK1 administration (Group III), we found the p-ERK level was decreased in the prefrontal cortex (56%, $P < 0.001$), striatum (50%, $P < 0.001$), hippocampus (37%, $P < 0.001$) and NAcc (69%, $P < 0.05$). At the same time, we found that mice ERK phosphorylation was inhibited and CPP was also abolished in the Group III mice but not in the vehicle-treat Group IV. Conditioned place preference score (minutes) was 8.26 in Group IV and 6.88 in Group III respectively ($p < 0.05$). RACK1 did not play a role in p-ERK in saline administration mice Group V. The present results demonstrate that RACK1 only plays an important role in suppressing the effects of reexposure of morphine.

Our present study was designed to test the hypothesis that the RACK1 is key regulator in morphine reexposure and its mechanism is to regulate the expression of p-ERK during reexposure to morphine.

In this study we have revealed that morphine reexposure can cause a down regulation of RACK1 levels in the prefrontal cortex, the finding is consistent with the report that RACK1 expression is reduced after chronic treatment (Escribá and García-Sevilla, 1999). We have also found morphine reexposure can increase RACK1 in the hippocampus. Ethanol treatment results in the release of RACK1 from the NMDAR complex through a

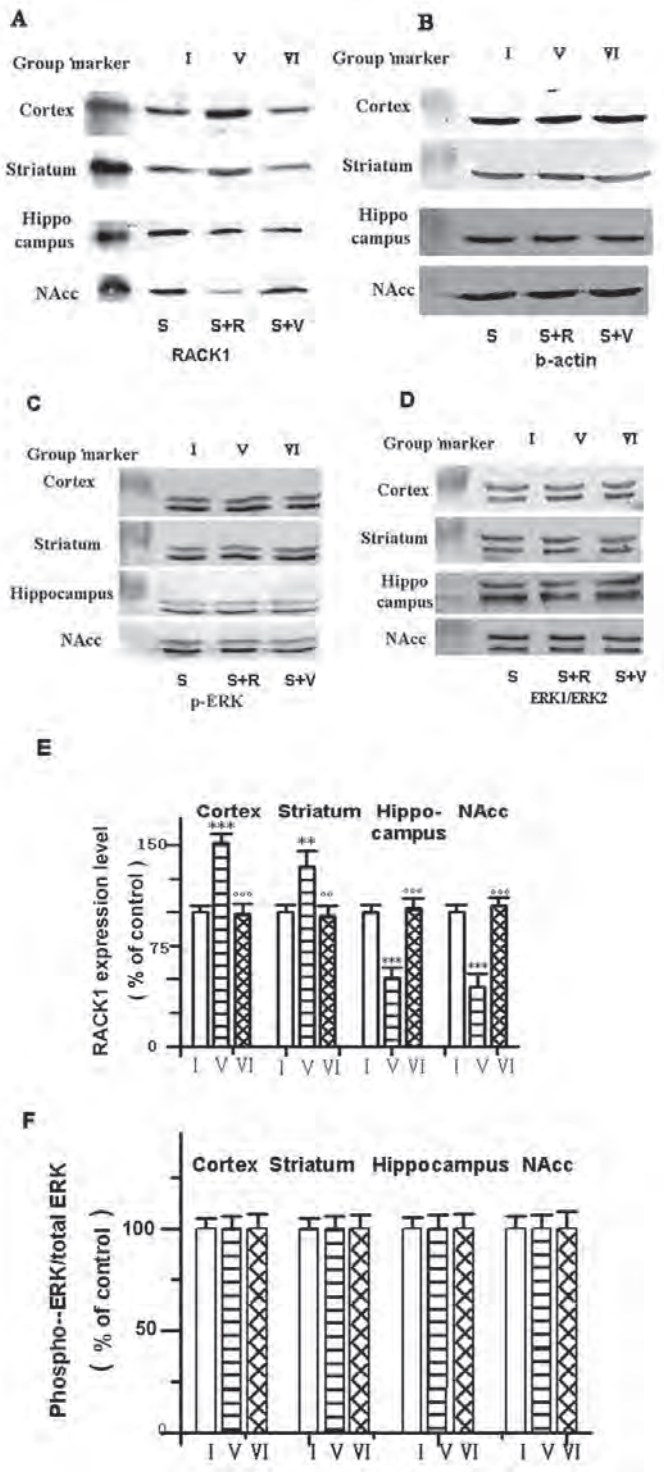

Fig. 4. - Influence of RACK1 plasmid on RACK1 expression in saline reexposure mice. (A, B, E) In addition to Groups I-IV, treated as in Fig. 1, Groups V and VI received a saline injection, and 20 minutes later RACK1 or vehicle injection respectively. RACK1 were analyzed at the end of Test 3, by immunoblotting in nuclei. The results were quantified. Histogram depicts the mean percentage of control (saline only) of the ratio of *RACK1: β-actin* ± SEM; $n = 8$. ** $P < 0.01$; *** $P < 0.001$ compared with control (Fig. 4E). Post Hoc Multiple Comparisons test was as follows: Group I *vs.* Group V to VI, ** $P < 0.01$; *** $P < 0.001$, Group III *vs.* Group IV, °°° $P < 0.001$. (C, D, F) Re-exposure to saline in the presence of RACK1 and its biochemical correlates. p-ERK and total ERK immunoreactivity were analyzed at the end of Test 3 by immunoblotting in nuclei. The results were quantified. Histogram depicts the mean percentage of control (saline only) of the ratio of *p-ERK: total ERK* ± SEM; $n = 8$. * $p > 0.05$ compared with control (Fig. 4F).

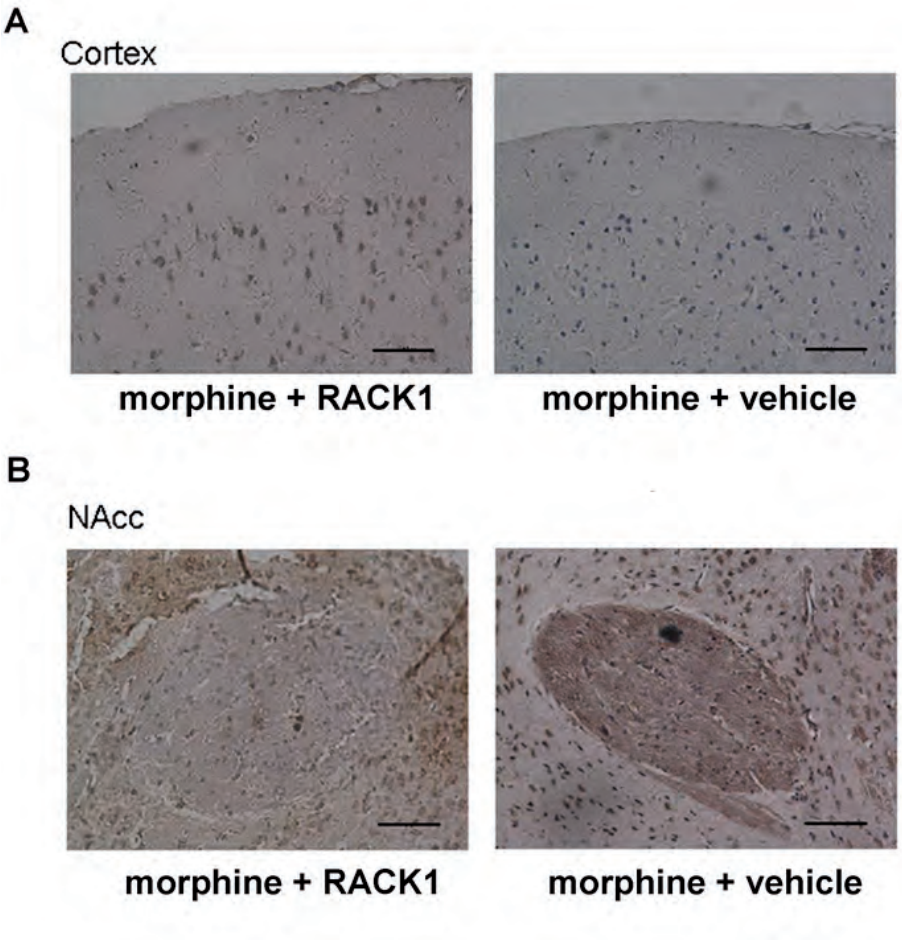

Fig. 5. - Immunohistochemistry images showed RACK1 was transfected into the cortex 24 hours after intraventricular injection. However this expression effect was reversed in NAcc. Reexposure morphine Mice were i.c.v. with vehicle or RACK1 5 ul. RACK1 in the cortex was detected by using anti-RACK1 antibodies. Scale bar = 50 um.

mechanism that depends on the activation of the cAMP/PKA signaling cascade in the hippocampus (Yaka et al., 2003). Opiate drugs modulate the PKC levels in brain (Ventayol et al., 1997), and there is a parallel modulation of brain RACK1 and its ligands PKC-α and -β after the various morphine treatments and withdrawal states (Escribá and García-Sevilla, 1999).

In the present study we have revealed morphine reexposure can cause a down regulation of the RACK1 levels in the prefrontal cortex and striatum but up-regulation in the hippocampus and NAcc. But the effect was reversed after the administration of the Endo-free RACK1 plasmid. Compared with vehicle-treated mice (Group IV), the RACK1 level increased significantly in the prefrontal cortex and striatum in RACK1-treated mice (Group III); the RACK1 level was 32.5% (Group IV) and 112% (Group III) respectively in the prefrontal cortex, 42% (Group IV) and 107% (Group III) respectively in the striatum. RACK1 expression was analyzed by Western blotting and immunohistochemistry.

First of all, Group III showed RACK1 reduced expression of p-ERK and CPP was erased, this certified our model of elimination addiction is successed. Secondly, morphine addiction is not decided by a nucleus, but by the team play of a number of nuclei. The level of RACK1 is not the same in different nuclei, RACK1 is reduced in the cortex and striatum but increased in the hippocampus and *nucleus accumbens*. The combined effect of RACK1 in different nuclei leads to addiction. We can not simply

say that increased RACK1 level correlates with addiction or reduced RACK1 level correlates with non-addiction, because the RACK1 level in different nuclei is inconsistent. It was very interesting that the effect of RACK1 was reversed after the addicted mice were injected exogenous RACK1, at the same time that place preference was erased. Therefore, RACK1 plays an important role in the morphine reexposure. From the physiological point of view, the cortex and striatum show positive feedback whereas the hippocampus and NAcc show negative feedback. Even more interesting was the fact that p-ERK level showed the same trend in different nuclei, p-ERK express was reduced in different nuclei after administration of RACK1.

In conclusion, the results of the current study indicate that (i) opiate addiction could be linked to alterations in RACK1 level, which differs among different brain regions. (ii) the exogenous RACK1 can decrease p-ERK in different nuclei during mice exposed to morphine.

Acknowledgments

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