Research Report

RACK1 affects morphine reward via BDNF

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ABSTRACT

Chronic morphine addiction may trigger functional changes in the mesolimbic dopamine system, which is believed to be the neurobiological substrate of opiate addiction. Brain derived neurotrophic factor (BDNF) has been implicated in addiction-related pathology in animal studies. Our previous studies have shown that RACK1 is involved in morphine reward in mice. The recent research indicates nuclear RACK1 by localizing at the promoter IV region of the BDNF gene and the subsequent chromatin modifications leads to the activation of the promoter and transcription of BDNF. The present study was designed to investigate if shRACK1 (a short hairpin RNA of RACK1) could reverse the mice’s behavioral responses to morphine and BDNF expression in hippocampus and prefrontal cortex. No significant changes were observed in vehicle-infused mice which received no morphine treatment (CONC) and shRACK1-infused mice which received no morphine treatment (CONR), whereas vehicle-infused mice preceded the morphine injection (MIC) showed increased BDNF expression in hippocampus and prefrontal cortex, as compared to vehicle-infused mice which received no morphine treatment (CONC). Intracerebroventricular shRACK1 treatment reversed these, and in fact, ShRACK1-infused mice preceded the morphine injection (MIR) showed reduced BDNF expression in hippocampus and prefrontal cortex, as compared to vehicle-infused mice which received no morphine treatment (CONC). Intracerebroventricular shRACK1 treatment reversed these, and in fact, ShRACK1-infused mice preceded the morphine injection (MIR) showed reduced BDNF expression in hippocampus and prefrontal cortex, as compared to MIC. In the conditioned place preference (CPP) test, inactivating RACK1 markedly reduces morphine-induced conditioned place preference. Non-specific changes in CPP could not account for these effects since general CPP of shRACK1- and vehicle-infused animals was not different. Combined behavioral and molecular approaches have support the possibility that the RACK1-BDNF system plays an important role in the response to morphine-induced reward.

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Abbreviations: RACK1, protein receptor for activated C kinase 1; BDNF, brain-derived neurotrophic factor; shRACK1, a short hairpin RNA of RACK1; PFC, prefrontal cortex; HC, hippocampus; DG, dentate gyrus; NAC, nucleus accumbens; VTA, ventral tegmental area.
1. Introduction

Drug addiction is regarded as the chronic brain disease, which is likely to share much in common in neural adaptations, synaptic plasticity, and related molecular mechanisms with memory. The mesolimbic dopamine projections, originating from VTA and terminating in the ventral striatum, prefrontal cortex and hippocampus, are linked to spatial memory processes and are important for shaping drug-taking behaviors and reward (Girou et al., 1996; Kesner et al., 2004; Swanson, 2000).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of nerve growth factor related proteins (Theozen, 2000). Several recent studies have demonstrated neuromodulatory effects of BDNF (Poo, 2001) on learning and memory, depression (Altar et al., 1992; Heaton et al., 2003; Siuciak et al., 1997) and drug addiction (Filip et al., 2006; Grimm et al., 2003; McCough et al., 2004; Miller and Mooney, 2004).

Previous results have shown that the expression of RACK1 mRNA and protein was markedly increased in prefrontal cortex and hippocampus of chronic morphine exposed mice and shRACK1 (a short hairpin RNA of RACK1) can block chronic morphine-induced conditioned place preference (CPP) in mice (Wan et al., 2009). Thus, RACK1 has been shown to be involved in the process of morphine reward. The recent research indicates nuclear RACK1 by localizing at the promoter IV region of the BDNF gene and the subsequent chromatin modifications leads to the activation of the promoter and transcription of BDNF (He et al., 2010).

In this study, we set out to demonstrate that chronic morphine increases the expression of BDNF in the prefrontal cortex and hippocampus of mice and conversely, that both reward and expression of BDNF are suppressed by shRACK1. Thus, we investigated whether (1) chronic morphine increases the expression of BDNF in the prefrontal cortex and hippocampus of mice; (2) shRACK1-treated mice shows reduced preference for morphine reward; and (3) RNA and protein expressions of BDNF induced by chronic morphine reward are suppressed in shRACK1-treated mice.

2. Results

2.1. Interference effects

To verify the role of shRACK1 (a short hairpin RNA of RACK1) in chronic morphine reward in mice again (Wan et al., 2009), we treated the mice with shRACK1 and evaluated the interference efficiency of shRACK1 in hippocampus and prefrontal cortex by RT-PCR.

A two-way ANOVA showed significant effects for drug (F1,16=5.67, p=0.031), plasmid (F1,16=31.71, p=0.001) and an interaction (F1,16=5.18, p=0.037) in hippocampus; and showed significant effects for drug (F1,16=5.92, p=0.027), plasmid (F1,16=24.96, p=0.001) and a significant interaction (F1,16=4.64, p=0.047) in prefrontal cortex.

Pairwise comparison using the Bonferroni test showed that RACK1 mRNA levels in the hippocampus (p=0.0006) and prefrontal cortex (p=0.0024) were significantly lower in the CONR group vs CONC group. And that RACK1 mRNA levels were significantly greater in the MIC group vs CONC group. shRACK1 reduced the morphine-induced high expression of RACK1 mRNA to levels similar to those of CONR mice (Fig. 1A). RACK1 mRNA levels were up-regulated in hippocampus (p=0.027) and prefrontal cortex (p=0.025), relative to CONC mice. The expressions of RACK1 mRNA did not differ between CONR and MIR groups, neither in hippocampus (p=1.000) nor in prefrontal cortex (p=1.000). Moreover, RACK1 mRNA levels in hippocampus (p=0.000) and prefrontal cortex (p=0.002) were inhibited significantly, as compared to MIC mice. The expressions of RACK1 protein were also decreased markedly in the PFC and granular cells of the hippocampus (Fig. 1C, B), as compared to MIC mice.

2.2. Rewarding effects

Preferences for the places paired with 10 mg·kg−1 morphine s.c. were analyzed in CONC, CONR, MIC and MIR mice. Two-way ANOVA revealed a significant differences of drug (F1, 16=49.616, p=0.000), plasmid (F1,16=43.396, p=0.000) and a significant interaction (F1,16=12.572, p=0.003). Bonferroni post hoc comparison revealed that repeated morphine administration induced a significant increase in CPP score for the MIC mice, compared to CONC and CONR mice, as anticipated (p=0.000*, n=10). Moreover, compared to MIC mice the CPP score of MIR mice was significantly decreased (p=0.000#, n=10). However, there was no significant difference in the place preference between CONC and CONR (p=0.28, n=10); and between MIR and CONR (p=0.15, n=10) (Fig. 2). Thus, pretreatment with shRACK1 plasmid conferred tendencies toward lower morphine place preference.

2.3. Effect of shRACK1 on BDNF mRNA expression in different condition

Repeated morphine administration significantly increased the levels of BDNF mRNA in hippocampus and prefrontal cortex as compared to saline treated group and shRACK1 treatment significantly decreased the levels of BDNF mRNA in hippocampus and prefrontal cortex as compared to MIC mice.

A two-way ANOVA (drug and plasmid) revealed a significant effect of drug (F1,16=14.21, p=0.002), plasmid (F1,16=22.70, p=0.001) and a significant interaction (F1,16=21.62, p=0.001) in hippocampus; and a significant effect of drug (F1, 16=5.47, p=0.033), plasmid (F1,16=20.16, p=0.001) and a significant interaction (F1,16=14.40, p=0.002) in prefrontal cortex.

Bonferroni post hoc tests showed that mice subjected to morphine exhibited a significant increase of BDNF mRNA levels in hippocampus and prefrontal cortex by Bonferroni post hoc test). Relative to saline control, shRACK1 reduced the morphine-induced high expression of BDNF mRNA in the hippocampus and prefrontal cortex relative to saline control. shRACK1 reduced the morphine-induced high expression of BDNF mRNA in the hippocampus and prefrontal cortex (Fig. 3). BDNF mRNA levels were up-regulated (p=0.000* in hippocampus and 0.003* in prefrontal cortex, n=5, respectively by Bonferroni post hoc test), relative to CONC mice. The expressions of BDNF mRNA did not differ between CONR and MIR groups, neither in hippocampus nor in prefrontal cortex (p=1.000 and 1.000, n=5, respectively by Bonferroni post hoc test). Moreover, BDNF mRNA levels in hippocampus and prefrontal cortex were markedly down-regulated (p=0.0004 and 0.000#, n=5, respectively by Bonferroni post hoc test), as compared to MIC mice.
RACK1 protein expression in Hippocampus by IHC (OD)

RACK1 protein expression in PFC by IHC (OD)

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2.4. Effect of shRACK1 on BDNF protein expression of prefrontal cortex and hippocampus

BDNF protein expression in prefrontal cortex and hippocampus was detected by western blot and IHC, respectively. As compared to saline treated group, repeated morphine administration significantly increased the levels of BDNF protein in prefrontal cortex (Two-way ANOVA: $F_{1,16}=5.465$, $p=0.033$; $p=0.007$ by Bonferroni post hoc test, Fig. 4) and hippocampus (Two-way ANOVA: $F_{1,16}=17.21$, $p=0.001$; $p=0.000$ by Bonferroni post hoc test, Fig. 5B), especially in CA1, CA3 and DG (Fig. 5A), whereas shRACK1 administration remarkably reduced the BDNF protein expression in the prefrontal cortex (Two-way ANOVA: $F_{1,16}=20.16$, $p=0.001$; $p=0.000^*$ by Bonferroni post hoc test, Fig. 4) and hippocampus (Two-way ANOVA: $F_{1,16}=23.71$, $p=0.000$; $p=0.000^*$ by Bonferroni post hoc test, Fig. 5B), compared to MIC group.

3. Discussion

Drug addiction can be considered as a form of neural plasticity, which might involve changes in several brain structures, such as the hippocampus and cerebral cortex, other than the VTA and the NAc (Nestler, 2001). There is growing evidence that up-regulation of BDNF expression was related with several drugs of abuse, such as amphetamine (Meredith et al., 2002), morphine (Takayama and Ueda, 2005) and cocaine (Grimm et al., 200). For example, BDNF expression was shown to be up-regulated in VTA neurons (Bolaños and Nestler, 2004; Butovsky et al., 2005; Grimm et al., 2003; amygda (Grimm et al., 2003; Meredith et al., 2002), striatum (Graham et al., 2007; Zhang et al., 2002), mPFC (Butovsky et al., 2005; Le, Foll et al., 2005) and noradrenergic neurons (Akbarian et al., 2002; Hatami et al., 2007). Therefore, BDNF is considered to have a key role in the response to drug addiction (Angelucci et al., 2007; Horger et al., 1999; Joe et al., 2007; Kim et al., 2005; Lu et al., 2004; Tsai, 2007) by modulating synaptic transmission and plasticity. Especially, increased levels of BDNF can induce a change to an opiate-dependent-like reward state when expressed in the ventral tegmental area in rats (Vargas-Perez et al., 2009).

Although changes in BDNF expression following exposure to drugs of abuse have been reported, relatively little is known about the mechanism of it.

Fig. 1 – Effect of shRACK1 on RACK1 expression. (a) Effect of shRACK1 on RACK1 mRNA expression. RNA from hippocampus (a) and prefrontal cortex (b) was isolated after chronic (8-days) administration of saline or morphine solutions. RNA abundance was determined by reverse-transcription (RT)-PCR with gene-specific primers described above. Compared with CONC group, RACK1 mRNA expression was markedly decreased in CONR group. However, the RACK1 mRNA in the MIC group was significantly increased than that in CONC group. shRACK1 administrations could reverse morphine induced increases of RACK1 mRNA. The optical density for RACK1 was shown as a proportion of β-actin optical density. *Bonferroni-corrected P-values < 0.05 vs CONC mice. #Bonferroni-corrected P-values < 0.05 vs MIC mice. (b) Effect of shRACK1 on RACK1 protein expression in hippocampus. (a) Film images of IHC for RACK1 on tissue sections taken from mice in different condition. The scale bar equals 0.02 mm. (A) Granular cells of the hippocampus of saline-treated mice with control plasmid are light positive for RACK1; (B) Granular cells of the hippocampus of saline-treated mice with shRACK1 RNA plasmid are light positive for RACK1; (C) Granular cells of the hippocampus of morphine-treated mice with control plasmid are strongly positive for RACK1; (D) Granular cells of the hippocampus of morphine-treated mice with shRACK1 RNA plasmid are light positive for RACK1. *Bonferroni-corrected P-values < 0.05 vs CONC mice. #Bonferroni-corrected P-values < 0.05 vs MIC mice. (b) Optical density measurements of RACK1 labeling in the prefrontal cortex and hippocampus. *Bonferroni-corrected P-values < 0.05 vs CONC mice. #Bonferroni-corrected P-values < 0.05 vs MIC mice.

Fig. 2 – Evaluate the rewarding effects by CPP score. The CPP score was designated as the time spent in the drug-paired compartment on day 12 minus the time spent in the same compartment in the preconditioning phase on day 3. Bars above the zero-line represent conditioned shifts in preference toward the drug-paired side of the apparatus. Control groups produced no reliable shifts in preference or aversion from baseline to test. Mice exhibited conditioned place preferences (CPP) for the side of the apparatus paired with the effects of morphine (MIC). shRACK1 pretreatment was detected by western blot and IHC, respectively. As compared to saline treated group, repeated morphine administration significantly increased the levels of BDNF protein in prefrontal cortex (Two-way ANOVA: $F_{1,16}=5.465$, $p=0.033$; $p=0.007$ by Bonferroni post hoc test, Fig. 4) and hippocampus (Two-way ANOVA: $F_{1,16}=17.21$, $p=0.001$; $p=0.000^*$ by Bonferroni post hoc test, Fig. 5B), especially in CA1, CA3 and DG (Fig. 5A), whereas shRACK1 administration remarkably reduced the BDNF protein expression in the prefrontal cortex (Two-way ANOVA: $F_{1,16}=20.16$, $p=0.001$; $p=0.000^*$ by Bonferroni post hoc test, Fig. 4) and hippocampus (Two-way ANOVA: $F_{1,16}=23.71$, $p=0.000$; $p=0.000^*$ by Bonferroni post hoc test, Fig. 5B), compared to MIC group.

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Drug addiction can be considered as a form of neural plasticity, which might involve changes in several brain structures, such as the hippocampus and cerebral cortex, other than the VTA and the NAc (Nestler, 2001). There is growing evidence that up-regulation of BDNF expression was related with several drugs of abuse, such as amphetamine (Meredith et al., 2002), morphine (Takayama and Ueda, 2005) and cocaine (Grimm et al., 200). For example, BDNF expression was shown to be up-regulated in VTA neurons (Bolaños and Nestler, 2004; Butovsky et al., 2005; Grimm et al., 2003; amygda (Grimm et al., 2003; Meredith et al., 2002), striatum (Graham et al., 2007; Zhang et al., 2002), mPFC (Butovsky et al., 2005; Le, Foll et al., 2005) and noradrenergic neurons (Akbarian et al., 2002; Hatami et al., 2007). Therefore, BDNF is considered to have a key role in the response to drug addiction (Angelucci et al., 2007; Horger et al., 1999; Joe et al., 2007; Kim et al., 2005; Lu et al., 2004; Tsai, 2007) by modulating synaptic transmission and plasticity. Especially, increased levels of BDNF can induce a change to an opiate-dependent-like reward state when expressed in the ventral tegmental area in rats (Vargas-Perez et al., 2009).

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RACK1 is a multifunctional scaffolding protein known to be involved in the regulation of various signaling cascades in the central nervous system (CNS). In the adult mouse, RACK1 is widely expressed in most brain regions, with relatively higher levels in hippocampus and cortex and cerebellum (Ashique et al., 2006). Chronic morphine administration can cause an up-regulation in the levels of RACK1 mRNA and protein in hippocampus and prefrontal cortex (Wan et al., 2009). In this study, we found that expression of BDNF overlapped with the RACK1 in certain regions in mice, including the hippocampus and PFC. Chronic exposure to morphine also led to a significant increase in BDNF mRNA in the hippocampus and PFC. These findings are in agreement with several recent reports showing that acute exposure of neurons to ethanol leads to increased levels of BDNF and RACK1 mRNA (McGough et al., 2004). In this regard, it should also be noted that increased expression of RACK1 may lead to enhanced expression of BDNF, as nuclear RACK1 by localizing at the promoter IV region of the BDNF gene and the subsequent chromatin modifications leads to the activation of the promoter and transcription of BDNF exon IV (He et al., 2010). Moderate increases in BDNF and RACK1 immunostaining following morphine treatment were observed in CA1, CA3 and DG of hippocampus, especially the abundance of granular cells in DG. Inversely, ablation of RACK1 in the present study prevented the chronic morphine-induced increase in BDNF mRNA and protein levels in mouse hippocampus and prefrontal cortex.

The CPP test is the most commonly used to assess addiction potential of drugs in rodent models. Ablation of RACK1 in the present study also attenuated morphine reward, which is different from the result that over-expression of RACK1 attenuated morphine reward in a CPP design (Liu et al., 2009). A plausible reason for this discrepancy could be the differences of the addictive stage. In the study of Liu et al., the first stage was the mice were injected morphine to induce the conditioned place preference and the second stage was the mice were administered pcDNA 3.1-RACK1 plasmid or vehicle i.c.v. 20 min after morphine re-exposed and measured the CPP again. Whereas in our study, the mice just were injected morphine to induce the conditioned place preference. These differences have been verified by some research, for example chronic exposure of rats to morphine markedly reduced the capacity of hippocampal CA1 LTP, which could be restored to the normal level by re-exposure to opiates (Pu et al., 2002).

In conclusion, the results of the present study demonstrate that chronic morphine exposure leads to up-regulation of BDNF mRNA and protein in areas associated with rewarding, such as the hippocampus and PFC. Pretreatment with shRACK1 plasmid by i.c.v. partially reduced the morphine-induced CPP score and completely abolished the effects of morphine on BDNF mRNA and protein expression in mouse hippocampus and prefrontal cortex. In this study, we demonstrated the importance of the central RACK1-BDNF signaling pathway in the prefrontal cortex and hippocampus for chronic morphine reward in mice. Taken
together with our recent observation that the central RACK1-BDNF signaling pathway is required for morphine reward, the emerging hypothesis is that this pathway may be important for increasing the chronic morphine induced conditioned place preference.

4. Experimental procedure

4.1. Ethics statement

All experimental procedures on mice were approved by the Committee on the Ethics of Animal Experiments of Sichuan University (Permit Number: 2003–149). All surgery was performed under ether anesthesia, and all efforts were made to minimize suffering.

4.2. Animals

Male ICR mice, 6–8 weeks old, were used in the experiments. Animals were housed under standard conditions of ambient temperature (22±2 °C), humidity (55±10%), and light (12 L:12D, lights on at 8:00) and were fed food and water ad libitum. 40 mice were randomly divided into 4 groups, including vehicle-infused mice with saline treatment (CONC), shRACK1-infused mice with saline treatment (CONR), vehicle-infused mice preceded the morphine injection (MIC) and shRACK1-infused mice preceded the morphine injection (MIR).

4.3. Conditioned place preference (CPP)

The CPP test was carried out in a two-chamber apparatus (15 cm wide×30 cm long×15 cm high) with a sliding partition that divided the main unit into two equal-sized chambers. The two chambers differed in floor: one was white with a textured floor, and the other was black with a smooth floor. When the sliding partition was raised, mice could freely move from one chamber to the other. When CPP measured, the partition was raised to 7 cm above the floor. Mice were assayed for the time spent in the two chambers of the apparatus in 15 min. The time that mice spent in the drug-paired chamber was used as the CPP score. Each mouse had three daily adaptation sessions followed by CPP training, when it was given a morphine injection paired with restraint in the white-floor chamber for 30 min or a saline injection paired with restraint in the black-floor chamber for 30 min.

4.3.1. Plasmid

The plasmid of shRACK1 and the control plasmid (vehicle) were donated by Dr. Wang YH, which were synthesized by Shanghai Gene Pharma Co., Ltd. The sequences of shRACK1 are 5′-GATCC GCAAGAAGTTATCAGCACCTTCAAGACGGGTGCTGAT- TAACCTTCTGCTTTTTTGTGCACA-3′ (forward) and 5′-AGCTTGTCGACAAAAAAGCAAGAAGTTATCAGCACCGTCCTT-GAAAGTGCTGATACTTCTTGCG-3′ (reverse). The sequences of vehicle are 5′-GATCCGACTTCATAAGGCGCATGCTTCAAGACGGCATGCGCCTTATGAACTTCTTGCGACA-3′ (forward) and 5′-AGCTTGTGGCAAAAAAGACCTCTATAAGGCACATGCCTGCCTT-GAAAGCATGCGCCTTATGAAAGTGCGCACA-3′ (reverse).

4.3.2. Intracerebroventricular injection (i.c.v. injection)

The i.c.v. injection procedure was adapted from the method described earlier (Mistry et al., 1997). Briefly, the i.c.v. injections were given as follows: under light ether anesthesia, bregma was exposed. An injection volume of 20 μl was delivered over a 60-s period, 2 mm lateral and caudal to bregma at a depth of 2 mm by using a syringe. Proper placement was verified in the experiments (n=1) by injection and localization of Methylene Blue dye before the experiments.

4.3.3. Experimental protocol

During the three adaptation sessions (pre-test), the animals were free to explore the entire apparatus for 15 min and the natural preference of the mice (for the white-floor chamber) was scored. During the conditioning phase, from the 4th day on, all mice were engaged in the basic CPP training for 8 days. Mice were given morphine (MIC and MIR, 10 mg·kg⁻¹) or saline (CONC and CONR) subcutaneously at 10:00 and then confined to the white side of the apparatus for 30 min. On the following day, all of them were given saline at 10:00 and then confined to the white side of the apparatus for 30 min. The 2-day procedure was repeated four times (days 4–11). The CPP test was drug-free and performed 24 h after the last conditioning session (days 12). The behavior of the animals

![Image of western blot analysis](image-url)
was observed and time spent in each compartment was determined by visual analysis of the video. All of the mice were sacrificed 24 h after the CPP test (days 13) by decapitation at 10:00 (10 animals per group). The CPP score was designated as the time spent in the drug-paired compartment on day 12 minus the time spent in the same compartment in the preconditioning phase on day 3. The experimenter scoring the videos of the CPP test was blind to the experimental

Fig. 5 - Effect of shRACK1 on expression of BDNF protein in hippocampus. (A) Film images of IHC for BDNF on tissue sections taken from mice in different condition. The scale bar equals 0.2 mm in (a, c, e, g) and 0.05 mm in (b, d, f, h). (a) DG and CA1,CA3 of hippocampus of saline-treated mice with control plasmid, shows that both the DG and CA1,CA3 of the hippocampus are light positive for BDNF; (b) Enlarged, from panel a, shows that granular cells of the hippocampus are light positive for BDNF; (c) DG and CA1,CA3 of hippocampus of saline-treated mice with shRACK1 RNA plasmid, shows that both the DG and CA1,CA3 of the hippocampus are negative for BDNF; (d) Enlarged, from panel c, shows that granular cells of the hippocampus are negative for BDNF; (e) DG and CA1,CA3 of hippocampus of morphine-treated mice with control plasmid, shows that both the DG and CA1, CA3 of the hippocampus are strongly positive for BDNF; (f) Enlarged, from panel e, shows that granular cells of the hippocampus are strongly positive for BDNF; (g) DG and CA1,CA3 of hippocampus of morphine-treated mice with shRACK1 RNA plasmid, shows that both the DG and CA1,CA3 of the hippocampus are light positive for BDNF; (h) Enlarged, from panel a, shows that granular cells of the hippocampus are light positive for BDNF. Abbreviations: HC, hippocampus; DG, dentate gyrus. (B) Optical density measurements of BDNF labeling in the hippocampus of control plasmid and shRACK1 plasmid mice. *Bonferroni-corrected P-values < 0.05 vs CONC mice. #Bonferroni-corrected P-values < 0.05 vs MIC mice.
treatments. The prefrontal cortex and hippocampus of the sacrificed mice were prepared for later analysis with RT-PCR, immunohistochemistry and western blot.

4.4. Reverse transcription polymerase chain reaction (RT-PCR) for RACK1 and BDNF

RT-PCR was performed to verify the expression of RACK1 and BDNF gene in hippocampus and prefrontal cortex. Brains from 5 of the 10 animals in each group were used for RT-PCR. The brain was rapidly removed, and specimens of the hippocampus and prefrontal cortex were dissected and stored at −80 °C. Total RNA (5 microgram), obtained using Trizol (Invitrogen, USA) according to the manufacturer’s instructions, was subjected to reverse transcription with an oligo-dT18 primer, recombinant RNasin, and M-MLV reverse transcriptase (all Fermentas, EU). PCR was performed with a gene amp PCR system thermal cycler (Eppendorf, German), and Taq DNA polymerase (Fermentas, EU). The cDNA-mixture was allowed to react for 30 (β-actin), 30 (RACK1) or 30 (BDNF) cycles. The sequences of primers used for mouse RACK1 are 5′-ACCAACAGGCGATTGCCG-3′ (forward), and 5′-GCAGACACCGAGATTTCCAATA-3′ (reverse). The sequences of primers used for mouse BDNF are 5′-AGCAGCTCATGCAACATxCATC-3′ (forward), and 5′-AGTCCTTACAGGAAGACT-GAATC-3′ (reverse). The sequences of primers for mouse β-actin are 5′-TCATCATTGGCAACGAGCG-3′ (forward) and 5′-AACGTCCGGCTAGACACAC-3′ (reverse). RACK1 and BDNF primers were designed to amplify mouse RACK1 and BDNF specifically. Primers for β-actin were used in control reactions. The RT-PCR products were analyzed with a 1.5% agarose gel electrophoresis, and the expected amplification lengths were 126 bp, 136 bp and 399 bp, respectively.

4.5. Immunohistochemistry analysis for RACK1 and BDNF level

Brains from 5 of the 10 animals in each group were used for immunohistochemistry. The brains prepared from sacrificed mice were fixed in 10% paraformaldehyde. Subsequently, they were dehydrated and blocked in paraffin. Serial sections (n=5) of 4 μm were cut and processed for immunohistochemistry (Brey et al., 2003). Sections were cleared of paraffin, and endogenous peroxidases were blocked by incubation with 3% H2O2 and washed. Sections of the brains were then incubated with rabbit serum for 15 min at ambient temperature. Subsequently, the sections were incubated overnight with a goat polyclonal anti-RACK1 (Santa Cruz Biotechnology, Inc., USA, 1:100) and anti-mature-BDNF antibody (Millipore, Billerica, MA, 1:100) at 4 °C, followed by the addition of biotinylated rabbit anti-goat IgG secondary antibody (Jinshan, BJ, China). To verify the binding specificity for RACK1 and BDNF, some sections were also incubated with primary antibody only (no secondary) or with secondary antibody only (no primary). In these situations, no immunoreactions were positive in all the experiments carried out. Immunohistochemistry staining was processed in accordance with the manufacturer’s instructions and visualized by the use of diaminobenzidine (DAB) staining. Counterstaining was carried out with Harris hematoxylin (Sigma), according to procedure from Shroyer in Genes Dev, 2005 (Shroyer et al., 2005).

5 sections were analyzed for each animal and the four images were acquired from a randomly selected location in each slide. Digital photos were analyzed with Image-pro plus 6.0 (Media Cybernetics, USA) by an observer blind to the treatment groups. Quantification of RACK1 or BDNF density per tissue was accomplished by determining the proportion of the area of tissue on each image that was red (RACK1 or BDNF) and yellow (remaining tissue) using Image Pro Plus software.

4.6. Western blot analysis for BDNF level of PFC

Mouse prefrontal cortex samples were taken by dissection and homogenized in RIPA lysis buffer (Pierce Biotechnology, Rockford, IL). After homogenization, the samples were centrifuged for 20 min at 16,000 × g at 4 °C. Protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of proteins (40 μg) were loaded to 12% SDS-PAGE gels and separated by electrophoresis. Then proteins were electrophoretically transferred to nitrocellulose membranes. Immunoblot analysis, and visualization were performed using proteins, rabbit anti-mature-BDNF antiserum (18 kDa, 1:500) in 1% bovine serum albumin and Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL) for the detection of horseradish peroxidase (Hamabe et al., 2003). Quantitation of the western blots was performed by densitometric scanning of the film using the Kodak IS4400CF image analysis system and the corresponding software (Eastman Kodak, Rochester, NY).

4.7. Statistical analysis

All data are presented as means±SEM of the mean and statistically evaluated by two-way ANOVA followed by the Bonferroni post hoc test for group differences (SPSS 16.0). Differences with P<0.05 were considered significant.

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