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Imbalance of HCN1 and HCN2 expression in hippocampal CA1 area impairs spatial learning and memory in rats with chronic morphine exposure

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ABSTRACT

The hyperpolarization-activated cyclic-nucleotide-gated non-selective cation (HCN) channels play a vital role in the neurological basis underlying nervous system diseases. However, the role of HCN channels in drug addiction is not fully understood. In the present study, we investigated the expression of HCN1 and HCN2 subunits in hippocampal CA1 and the potential molecular mechanisms underlying the modulation of HCN channels in rats with chronic morphine exposure with approaches of electrophysiology, water maze, and Western blotting. We found that chronic morphine exposure (5 mg/kg, sc, for 7 days) caused an inhibition of long-term potentiation (LTP) and impairment of spatial learning and memory, which is associated with a decrease in HCN1, and an increase in HCN2 on cell membrane of hippocampal CA1 area. Additional experiments showed that the imbalance of cell membrane HCN1 and HCN2 expression under chronic morphine exposure was related to an increase in expression of TPR containing Rab8b interacting protein (TRIP8b) (1a-4) and TRIP8b (1b-2), and phosphorylation of protein kinase A (PKA) and adaptor protein 2 μ2 (AP2 μ2). Our results demonstrate the novel information that drug addiction-induced impairment of learning and memory is involved in the imbalance of HCN1 and HCN2 subunits, which is mediated by activation of TRIP8b (1a-4), TRIP8b (1b-2), PKA and AP2 μ2.

1. Introduction

Drug addiction is a complex neuropsychiatric disorder, which has been considered as a result of pathological changes in neuronal plasticity in brain circuitry responsible for learning and memory (Bell et al., 2000; Kandel, 1997; Nestler, 2001; Robinson and Kolb, 1997; Scannevin and Huganir, 2000). Several studies have reported that acute and chronic administration of opioids produce impaired effects on memory processes. Administration of opioids impaired acquisition or retention of memory in passive-avoidance (Izquierdo, 1979), Y-maze (Ma et al., 2007), or water maze paradigms (Farahmandfar et al., 2010; Li et al., 2001). However, the cellular mechanisms that underlie memory deficits caused by drug addiction are not fully understood.

Changes in the electrophysiological properties of neurons involved in the reward circuit seem to be of utmost importance in addiction. The hyperpolarization-activated cation current, I_h, exhibited fundamental physiological functions such as determining the neuronal resting membrane potential (Doan and Kunze, 1999), integration of synaptic input to neurons (Nolan et al., 2004), synaptic plasticity (Huang and Hsu, 2003), neurotransmitter release (Mellor et al., 2002), and the rhythmicity of various brain regions (Magee, 1999). Studies about the electrophysiological properties of I_h suggest its potential role in the neurological basis underlying addiction diseases, especially in areas of the reward system (Chu and Zhen, 2010; Hopf et al., 2007; McDaid et al., 2008; Migliore et al., 2008; Okamoto et al., 2006). Arencibia-Albite and colleagues reported a 40% decrease in I_h amplitude and 45% reduction in the number HCN channels in the VTA after cocaine sensitization (Arencibia-Albite et al., 2012).

HCN channels underlying the I_h current were tetramers made of four distinct channel subunits: HCN1–4. The expression pattern of HCN1–4 subunits has been shown to strongly determine the

Abbreviations: HCN channels, Hyperpolarization-activated cyclic-nucleotide-gated non-selective cation channels; CA1, Cornu ammonis 1; LTP, Long-term potentiation; TRIP8b, TPR containing Rab8b interacting protein; PKA, Protein kinase A; AP2, Adaptor protein 2; CNS, Central nervous system; fEPSP, Field excitatory postsynaptic potential; I/O curves, Input/output curves; VTA, Venta tegmental area; DA, Dopamine; MWM, Morris water maze; AAK1, Adaptor-associated kinase 1.

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biophysical properties of the I_h and its potential modulatory role in cell excitability (Benndorf et al., 2012; Berrera et al., 2006; DiFrancesco and Tortora, 1991; Wainger et al., 2001). Previous reports have been shown that the dysfunction of HCN channels are involved in several brain disorders, including Parkinson's disease, epilepsy, inflammatory and neuropathic pain (Chu and Zhen, 2010; Emery et al., 2011; Ludwig et al., 2003). However, little is known about the role of HCN channels in drug addiction. HCN channels were widely expressed in the central nervous system, including those areas critical to drug addiction: nucleus accumbens (NAc), prefrontal cortex (PFC), ventral tegmental area (VTA) and hippocampus (Notomi and Shigemoto, 2004; Santoro et al., 2000). The hippocampus was known to be one of the major areas associated with spatial learning and memory, which also played an important role in opioid dependence (Chauvet et al., 2011; Everitt and Robbins, 1997; Lu et al., 2000; Muller et al., 1996; Rezayof et al., 2003). There was a consensus that HCN1/HCN2 subunits were the most abundant subunits in hippocampal CA1 (Brewster et al., 2005; Franz et al., 2000; Notomi and Shigemoto, 2004). Although one study has indicated that cocaine sensitization induce a significant increase in glycosylated and non-glycosylated HCN2 proteins in the mesocorticolimbic system (Santos-Vera et al., 2013), the intrinsic mechanism was still unclear. Based on these findings, we hypothesized that changes of HCN channels in hippocampus might be a potential mechanism underlying the effects of chronic morphine exposure on cognitive function. Thus, the present study was undertaken to determine whether and how the expression of HCN1/HCN2 subunits in hippocampal CA1 regions was altered in rats with chronic morphine exposure and investigate molecular mechanisms underlying the modulation of HCN channels.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (200–250 g) were used in the present study. The animals were housed five per cage with free access to water and food, a 12 h light/dark cycle, and a temperature-controlled environment. Animals were adapted to these conditions for at least 3 days before experiments. The experimental protocol was approved by the Ethic Committee of Animal Use for Teaching and Research, Tongji Medical College, Huazhong University of Science and Technology (Approval number: SCXK(E)2010-0009, No. 4200900000283).

2.2. Animals treatment

The experimental procedure was detailed in Fig. 1a. After removing unqualified animal with morris water maze (MWM), animals were randomly divided into two groups: saline group and chronic morphine exposure group. In chronic morphine exposure group, rats were injected with morphine (5 mg/kg, sc, Shenyang, China) once per day for 7 consecutive days. Rats in saline group were injected with isovolumetric saline. All experiments were performed 2 h after the injection to avoid the effect of acute morphine treatment on locomotion of rats On days 2–7, morris water maze (MWM) was performed 2 h after the injection of morphine or saline to evaluate the spatial learning and memory performances of rats. Two hours after the last morphine or

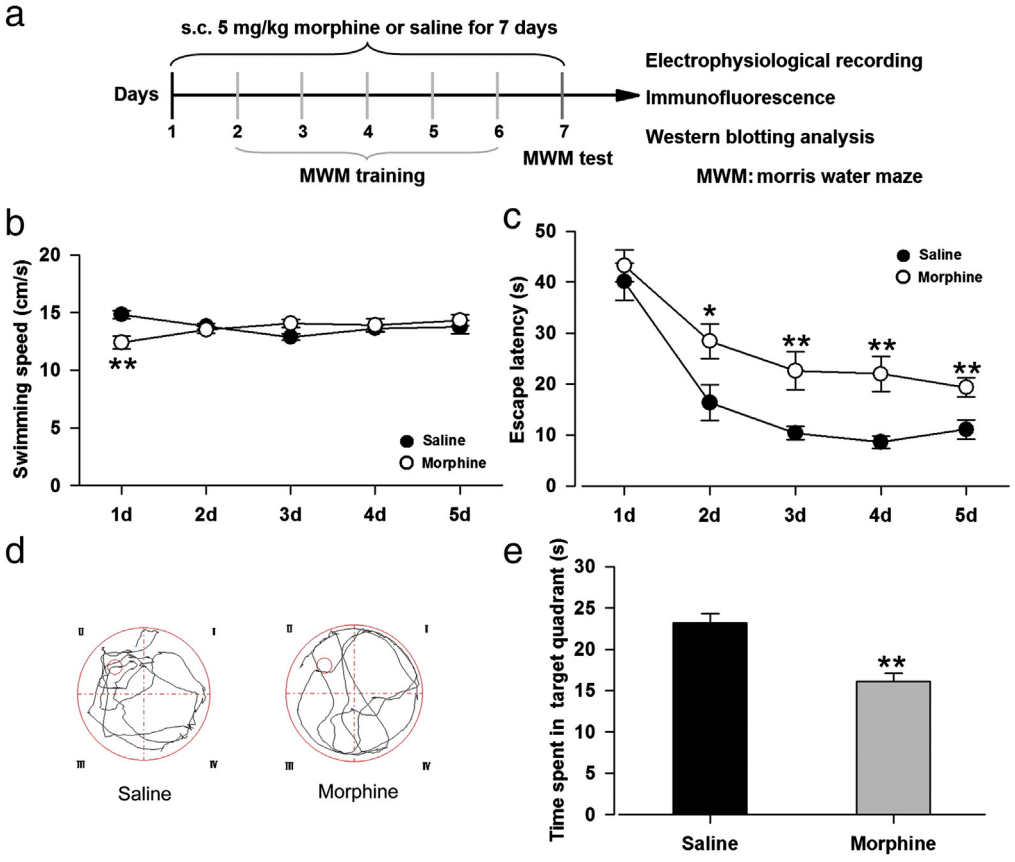

Fig. 1. Effects of chronic morphine exposure on spatial learning and memory processes in rats. a. Experimental procedure. b. The average speed of rats from the first day to the fifth day. On day 1, the swimming speed in chronic morphine exposed rats was faster than that in saline group rats. From day 2 to day 5, no difference was showed between saline with chronic morphine exposure group. c. The escape latencies to find the hidden platform from the first day to the fifth day. Chronic morphine exposure at 5 mg/kg significantly prolonged the escape latency of days 2–5 during training procedure. d. Swimming trajectories on the test day. e. The time spent in the target quadrant on the test day. Rats exposed with morphine resulted in a significant decrease of the time spent in the target quadrant. n = 11 for per group, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline group.

saline injection on day 7, the field excitatory postsynaptic potential (fEPSP) and molecular biological studies in hippocampal CA1 area were carried out as described below.

2.3. Morris water maze task

Morris water maze (MWM) task was carried out as previously described by Yang (Morris, 1984; Yang et al., 2013) with slight modification. Briefly, a circular pool (150 cm in diameter and 50 cm in deep) was filled with 23 ± 2 °C water to a depth of 21 cm. A hidden circular platform (6 cm in diameter) was located in the center of the target quadrant (quadrantII), submerged 1.5 cm beneath the surface of the water. The animal escape latency, swimming speed and the amount of time spent in the target quadrant were measured automatically using a computer-based image analyzer morris water-maze tracking system MT-200 (ChengDu Technology & Market Co., LTD, Chengdu, Sichuan province, China).

For MWM training, rats were subjected to a session of four trials per day with four different starting positions for 5 consecutive days (days 2–7). In each trial, animals were given a maximum of 60 s to find the platform. After mounting the platform, the rats were allowed to remain there for 30 s. If the rat failed to find the platform in 60 s, it was placed on the platform and allowed to rest for 30 s. On day 7, MWM test probe consisting of a 60 s free swim period without the platform was performed to test spatial memory. After the last trial, the rat was towel dried and placed in a holding cage under a heating lamp before it was returned to the home cage.

2.4. Electrophysiological recordings in vivo

Two hours after the last morphine or saline injection on day 7, rats in each group were anesthetized with urethane (1.5 g/kg, ip) and core temperature was maintained at 37 °C. Recordings of field excitatory postsynaptic potentials (fEPSPs) were carried out from CA1 stratum radiatum of the hippocampus in response to stimulation of the Schaffer collateral/commissural pathway using techniques described previously (Li et al., 2013). At 30 min after electrodes insertion, input/output (I/O) curves were generated by systematic variation of the stimulus intensity (10–28 V) to evaluate synaptic potency. Stimulus pulses were delivered at 0.3 Hz and three responses at each current level were averaged. Test fEPSP were evoked at a frequency of 0.3 Hz and at a stimulus intensity adjusted to give a fEPSP amplitude of 50–60% maximum response. LTP was induced by high frequency stimulation (HFS, 100 pulses at 100 Hz, 4 trains, 5 min stimulus intervals) at a stimulus intensity that evoked a fEPSP of approximately 80% of maximum response. After HFS, the amplitude of fEPSP was recorded for at least 60 min. fEPSP amplitude was expressed as mean ± S.E.M. % of the baseline fEPSP amplitude recorded over at least a 20-min baseline period. It was defined as a successful induction of LTP if the amplitude of fEPSP change exceeded 20% (Bliss and Collingridge, 1993). After the experiment, the sites of stimulating and recording electrodes were routinely verified by histology.

2.5. Immunohistochemistry

Two hours after the last morphine or saline injection on day 7, rats in each group were deeply anesthetized with urethane (1.5 g/kg, ip) and transcardially perfused with 0.9% saline solution, followed by a fixative containing 4% paraformaldehyde in 0.1 m phosphate buffer (pH 7.4). After the transcardial fixation, the brain was removed, postfixed in the same fixative overnight, and sectioned coronally at 5 μm on a Vibratome. Sections of the hippocampus were incubated with xylene, and 100%, 90%, 80%, 70% ethanol solutions. Slides were then boiled in sodium citrate buffer for 20 min, incubated sequentially with 3% H_2O_2 for 30 min and 5% bovine serum albumin (BSA) for 1 h. The sections were incubated in the primary antibody solutions rabbit anti-mouse NeuN (1:400, MAB377, Millipore) overnight at 4 °C and were transferred into the appropriate secondary antibodies DyLight 488 Affinipure Goat Anti-Mouse IgG (H + L) (E032210, EarthOx, LLC) for 2 h. Sections were analyzed with Olympus FluoView 1200 confocal microscope system (Olympus Corporation, Japan).

2.6. Western blotting analysis

Rats in each group were killed by decapitation under anesthesia 2 h after the last treatment injection. Brains were quickly removed. Coronal sections of 400 μm at the level of the hippocampus were made using a Leica® Vibratome VT1000S (Leica, Germany), and subdissected into CA1, CA3, and dentate gyrus (DG) regions under a dissecting microscope (as shown in Fig. 3c) (Powell et al., 2008). The samples were stored at -80 °C until required -80 °C amended as below zero 80 °C. Membrane protein extracts were prepared using a ProteoExtract Native Membrane Protein Extraction kit (71772-3, Calbiochem/Merck Biosciences). Protein concentrations were measured using a BCA Protein Assay kit (Pierce) (Smith et al., 1985). Proteins were fractionated on 10% SDS-PAGE gels and transferred to PVDF membranes (03010040001, Roche). After being blocked in 5% milk for 1 h at room temperature, membranes were incubated overnight at 4 °C with the following primary antibodies: anti-NeuN (1:2000, MAB377, Millipore), anti-HCN1 (1:800, NBP1-20250, Novus), anti-HCN2 (1:200, APC-030, Alomone labs), anti-TRIP8b (1a-4) (1:500, NeuroMab clone N212/3), Anti-TRIP8b (1b-2) (1:200, NeuroMab clone N212A/34), anti-AAK1 (H-140) (1:200, sc-134662, Santa Cruz), anti-Adaptin 2 (M-16) (1:400, sc-6422, Santa Cruz) and p-AP2 μ2 (pT156) (1:4000, 3312-1, Epitomics), PKA α/β/γ (H-56) (1:100, sc-98951, Santa Cruz), anti-phospho-PKA (Thr197) (1:500, 4781, Cell Signaling), or GAPDH (1:5000, cw0100, Cwbiotech). The antigen–antibody complexes were visualized with horseradish peroxidase (HRP) conjugated secondary antibodies (1:5000; Proteintech Group Inc., China) by using immobilon western chemiluminescent HRP substrate (WBKLS0500, Millipore). A monoclonal antibody directed against GAPDH was used as a control to normalize protein expression levels. Immunoreactive protein bands were then quantified by densitometric scanning method using NIH ImageJ software.

2.7. Statistical analysis

All analyses were performed using SPSS 16.0 software (SPSS Inc., USA) and data were presented as mean ± SEM. Differences between mean values were evaluated using Independent-Samples Student's *t* test. Some data in swimming speed and the latency in MWM test and I/O curves were tested by one ANOVA with repeated measures. *P* values less than $\alpha = 0.05$ were considered statistically significant.

3. Results

3.1. Spatial learning and memory processes in rats with chronic morphine exposure

During five training days (days 2–6), the escape latencies and swimming speeds of each group were recorded and analyzed. As shown in Fig. 1b, on day 2, the swimming speed in chronic morphine exposure rats was faster than that in saline group rats ($n = 11$, $F(1, 20) = 13.67$, $P < 0.01$ vs. saline group). From days 3 to 6, the swimming speed in two groups showed no significant differences, which suggests that chronic morphine exposure does not affect the locomotion of rats (Fig. 1b, $n = 11$, $P > 0.05$ compared with saline group rats). However, the latency was significantly longer in rats with chronic morphine than in saline group from days 3 to 6 (Fig. 1c, $n = 11$, day 3: $F(1, 20) = 6.08$, $P < 0.05$; day 4: $F(1, 20) = 9.40$, $P < 0.01$; day 5: $F(1, 20) = 13.51$, $P < 0.01$; day 6: $F(1, 20) = 9.68$, $P < 0.01$ vs. saline group), which indicates that

chronic morphine exposure impairs spatial learning ability. In MWM test (day 7), the time spent in the target quadrant was significantly shorter in rats with chronic morphine exposure than in saline group (Fig. 1e, n = 11, t(20) = 4.703, $P < 0.01$ vs. saline group). These findings indicate that spatial learning and memory processes are impaired in rats with chronic morphine exposure.

3.2. Hippocampal LTP and I/O functions at the schaffer collateral-CA1 synapses in rats with chronic morphine exposure

To determine the electrophysiological basis for the changes of spatial learning and memory processes, we examined the effect of chronic morphine exposure on hippocampal CA1 LTP and I/O functions, which is a neurological basis of learning and memory (Bliss and Collingridge, 1993; Martin and Morris, 2002). Chronic morphine exposure did not affect the I/O curves in the CA1 area measured by the fEPSP amplitude (Fig. 2b, 10 V: F(1, 10) = 0.07, 13 V: F(1, 10) = 0.51, 16 V: F(1, 10) = 0.55, 19 V: F(1, 10) = 0.82, 22 V: F(1, 10) = 0.65, 25 V: F(1, 10) = 0.21, 28 V: F(1, 10) = 0.12, n = 6, $P > 0.05$). This result suggested that chronic morphine exposure does not affect the basal synaptic transmission at the schaffer collateral-CA1 synapses in rats. In rats treated with saline, HFS of the Schaffer collateral inputs to CA1 pyramidal cells induced a stable LTP in the amplitude of fEPSP (Fig. 2c–d, 20–50 min after HFS: 210.34 ± 8.17% of baseline values; 50–80 min: 196.14 ± 6.66% of baseline values, n = 6, $P < 0.01$). In rats with chronic morphine exposure, LTP induced by HFS was significantly reduced (20–50 min after HFS: 170.97 ± 7.45% of baseline values, n = 6, t(10) = 3.560, $P < 0.01$; 50–80 min: 155.56 ± 5.02% of baseline values, t(10) = 4.862, $P < 0.01$ vs. saline group).

3.3. NeuN expression in hippocampal CA1 area of rats with chronic morphine exposure

Changes of neuronal cell bodies and synaptic contacts in CA1 region of the hippocampus have been implicated in memory function, which may be responsible for spatial memory impairment. In the present study, the neuron specific marker protein NeuN in hippocampus CA1 was evaluated using an anti-NeuN antibody in order to explore the cellular mechanism for morphine-induced impairment of spatial learning and memory processes. No significant difference in protein level of NeuN was observed in hippocampal CA1 area of rats with saline or chronic morphine exposure (Fig. 3d, 0.85 ± 0.12, n = 4, $P > 0.05$, vs. saline group). Correspondingly, we stained the cross section of hippocampal CA1 area with the anti-NeuN antibody to count neuron number of the cross section to correlate the Western blot results. Results showed that no C of cross section of hippocampal CA1 area was observed in rats with chronic morphine exposure (Fig. 3a–b, CA1: 200×, n = 4). These results suggest that the impairment of spatial learning and memory is not related to alteration of hippocampal CA1 neurons in rats with chronic morphine exposure.

3.4. HCN1/HCN2 expression in hippocampal CA1 area of rats with chronic morphine exposure

It is believed that change in HCN channel expression and function plays a potential role in the neurological basis underlying many nervous system diseases (Emery et al., 2011; Li et al., 2010; Nolan et al., 2004; Postea and Biel, 2011). In the present study, we determined the total proteins, cell membrane proteins, and intracellular proteins of HCN1 and HCN2 subunits in hippocampal CA1 area with Western blot analyses. As illustrated in Fig. 4a–b, no change was observed in total protein of HCN1 subunit in hippocampal CA1 area of rats with chronic morphine exposure (Fig. 4a, 1.06 ± 0.08, n = 4, $P > 0.05$ vs. saline group), but total protein of HCN2 was increased (Fig. 4b, 1.85 ± 0.22, n = 4, t(3) = −3.909, $P < 0.05$ vs. saline group). However, the cell membrane HCN1 subunit in hippocampal CA1 area was decreased in rats with chronic morphine exposure (Fig. 4c, 0.51 ± 0.05, n = 4, t(6) = 7.917, $P < 0.01$ vs. saline group), while the intracellular HCN1 protein was significantly increased (Fig. 4c, 1.68 ± 0.13, n = 4, t(3) = −4.352, $P < 0.05$ vs. saline group). Interestingly, both cell membrane and

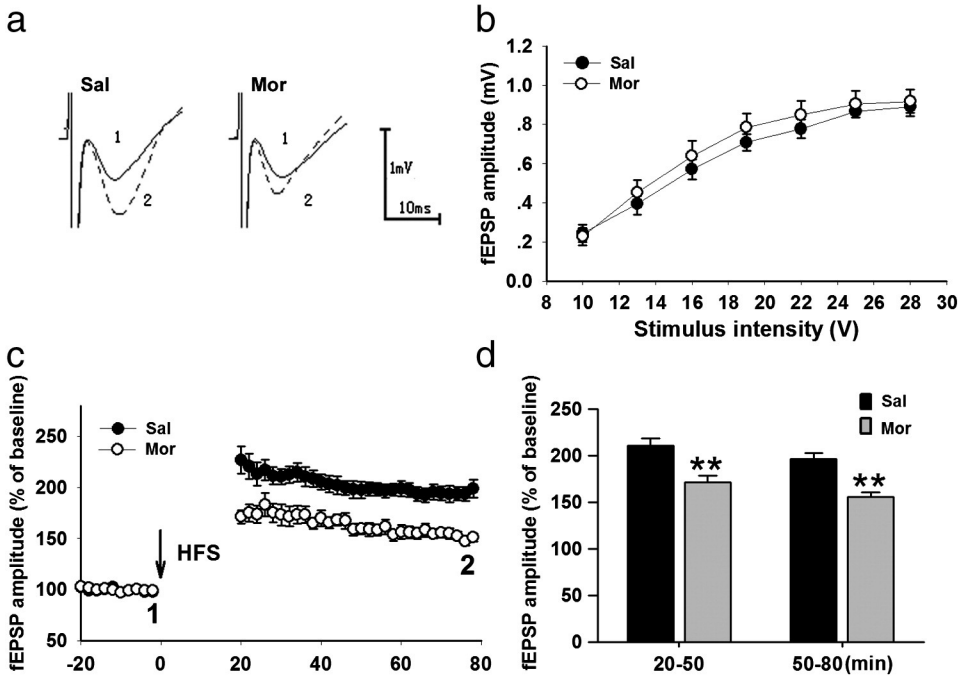

Fig. 2. Effects of chronic morphine exposure on the hippocampal I/O functions and LTP at the schaffer collateral-CA1 synapses in rats in vivo. a. Representative fEPSP recorded before (solid line) and after HFS (dashed line). b. I/O curves of the fEPSP amplitude in the CA1 area as a function of stimulus intensity before induction of LTP. Chronic morphine exposure did not affect the I/O curves in the CA1 area measured by the fEPSP amplitude. n = 6, $P > 0.05$ vs. saline group. c. The linear graph of normalized fEPSP amplitude at the schaffer collateral-CA1 synapses in rats in vivo. The normalized fEPSP amplitude of chronic morphine exposured rats was obviously reduced than that of saline group rats. The downward filled arrow indicates HFS. d. The histogram of average fEPSP amplitude at 20–50 min and 50–80 min after HFS in two groups. n = 6, $^{**}P < 0.01$ vs. saline group.

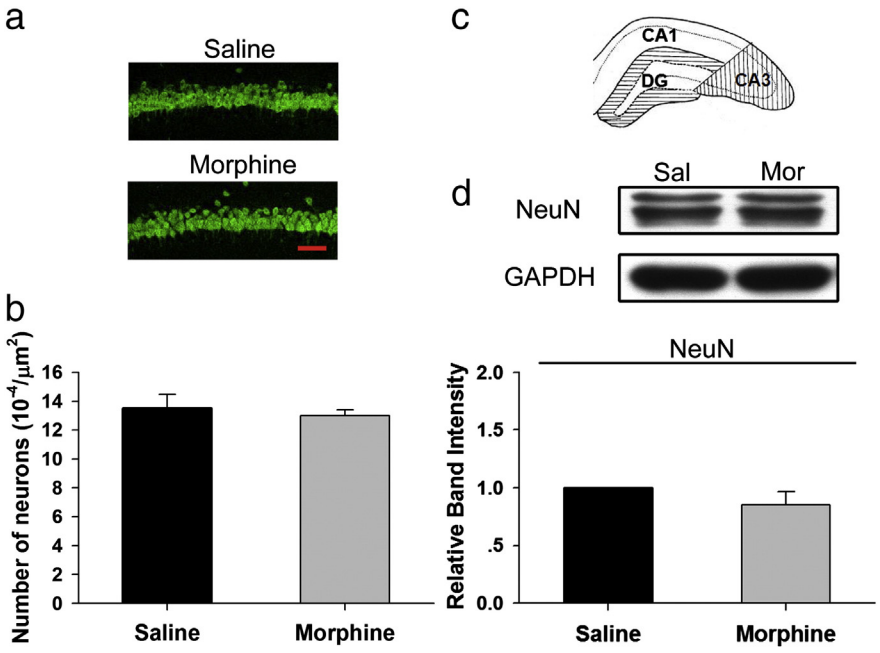

Fig. 3. Effects of chronic morphine exposure on the neuronal survival of neurons in rat hippocampal CA1 area. a. Representative photomicrographs of immunohistochemical staining with an anti-NeuN antibody in hippocampal CA1 (200×, scale bar, 100 μm) areas. b. Neuron number of the cross section of hippocampal CA1 area. No significant change in neuron cell number of cross section of hippocampal CA1 area was observed in rats with chronic morphine exposure (n = 4, $P > 0.05$ vs. saline group). c. Schematic illustration of sectioning of hippocampus technique for regional analysis. d. NeuN protein levels by Western blotting analysis. The protein levels of NeuN in two groups showed no significant difference in hippocampal CA1 area. n = 4, $P > 0.05$ vs. saline group.

intracellular HCN2 proteins were increased in hippocampal CA1 area in rats with chronic morphine exposure (Fig. 4d, n = 4, surface expression: 1.43 ± 0.05, $t(3) = -7.920$, $P < 0.01$; intracellular expression: 1.64 ± 0.20, $t(6) = -2.836$, $P < 0.05$ vs. saline group). These results suggest that chronic morphine exposure induces an imbalance of HCN1/HCN2 expression.

3.5. Expression of TRIP8b (1a-4) and TRIP8b (1b-2), p-AP2 μ2 and p-PKA in hippocampal CA1 area of rats with chronic morphine exposure

TRIP8b plays an important role in HCN channel trafficking in rat hippocampus (Lewis et al., 2009; Santoro et al., 2009). We found that TRIP8b (1a-4) and TRIP8b (1b-2) protein levels were significantly increased in hippocampal CA1 region in rats with chronic morphine exposure (Fig. 5a–b, n = 4, 1a-4: 1.37 ± 0.08, $t(6) = -4.802$, $P < 0.01$; 1b-2: 1.63 ± 0.10, $t(6) = -5.297$, $P < 0.01$ vs. saline group).

Accumulating evidence has demonstrated that subunits of adaptor proteins AP2 complex is a major TRIP8b-interacting proteins (Kirchhausen, 1999; Popova et al., 2008; Santoro et al., 2011). In the present study, no change was observed in AP2 α1 protein (Fig. 5e, 0.94 ± 0.03, n = 4, $P > 0.05$ vs. saline group), but p-AP2 μ2 was increased in rats with chronic morphine exposure (Fig. 5f, 2.03 ± 0.20, n = 4, $t(3) = -4.521$, $P < 0.05$ vs. saline group).

Additionally, AAK1, a member of the Prk/Ark family of serine/threonine kinases, could decrease AP2-mediated internalization of HCN channels via combined with AP2 and facilitating the phosphorylation of AP2 μ2 subunit (Conner and Schmid, 2002; Popova et al., 2008, Santoro et al., 2011). We found that no alteration of AAK1 protein was observed in rats with chronic morphine exposure (Fig. 5g, 0.98 ± 0.06, n = 4, $P > 0.05$ vs. saline group), suggesting that AAK1 is not involved in morphine-induced imbalance of HCN1/HCN2 expression. Phosphorylated PKA was found to promote phosphorylation of AP2 μ2 subunit and therefore regulate HCN cell membrane expression (Ricotta et al., 2002). In this observation, p-PKA level was increased in rats chronic morphine exposure (Fig. 5c, 1.78 ± 0.23, n = 4, $t(3) = -3.243$, $P < 0.05$ vs. saline group), suggesting that p-PKA may be involved in the imbalance of HCN1/HCN2 expression in rats with chronic morphine exposure.

4. Discussion

In the present study, we demonstrated the new finding that chronic morphine exposure impaired spatial learning and memory in rats and reduced LTP in hippocampal CA1 area. Western blot analysis revealed that expression of membrane HCN1 protein was decreased, while HCN2 was increased. The imbalance of membrane HCN1 and HCN2 expression was related to up-regulation of TRIP8b (1a-4) and TRIP8b (1b-2), and increased phosphorylation of adaptor protein 2 μ2 (AP2 μ2) and PKA.

It is generally recognized that the hippocampus is one of the major areas associated with spatial learning and memory, which also plays an important role in opioid dependence (Chauvet et al., 2011; Everitt and Robbins, 1997; Lu et al., 2000; Muller et al., 1996; Rezayof et al., 2003). Drug addiction and learning and memory were modulated by the same neurotrophic factors, shared certain intracellular signaling cascades, and associated with similar adaptations in neuronal morphology, such as the formation or loss of dendritic spines (Hyman, 2005; Hyman and Malenka, 2001; Nestler, 2001). Long-term potentiation (LTP), a form of synaptic plasticity at hippocampus, is a cellular mechanism that is believed to reflect the synaptic mechanisms of learning and memory (Bliss and Collingridge, 1993; De Roo et al., 2008; Pastalkova et al., 2006). Several studies have shown that hippocampal LTP induced by high frequency stimulation is greatly reduced by chronic exposure to morphine or heroin (Bao et al., 2007; Lu et al., 2010; Portugal et al., 2014; Pu et al., 2002; Salmanzadeh et al., 2003; Xia et al., 2011). A recent study demonstrated that chronic morphine exposure impaired hippocampal LTP, and accompanied an increase of SK2 (small conductance Ca^{2+}-activated potassium type 2) activity. Inhibition of the SK2 channel function can restore LTP impairment that occurs following context-dependent sensitization to morphine (Fakira et al., 2014). Consistent with these studies, we found that chronic morphine exposure at 5 mg/kg for 7 days diminished hippocampal LTP at the schaffer

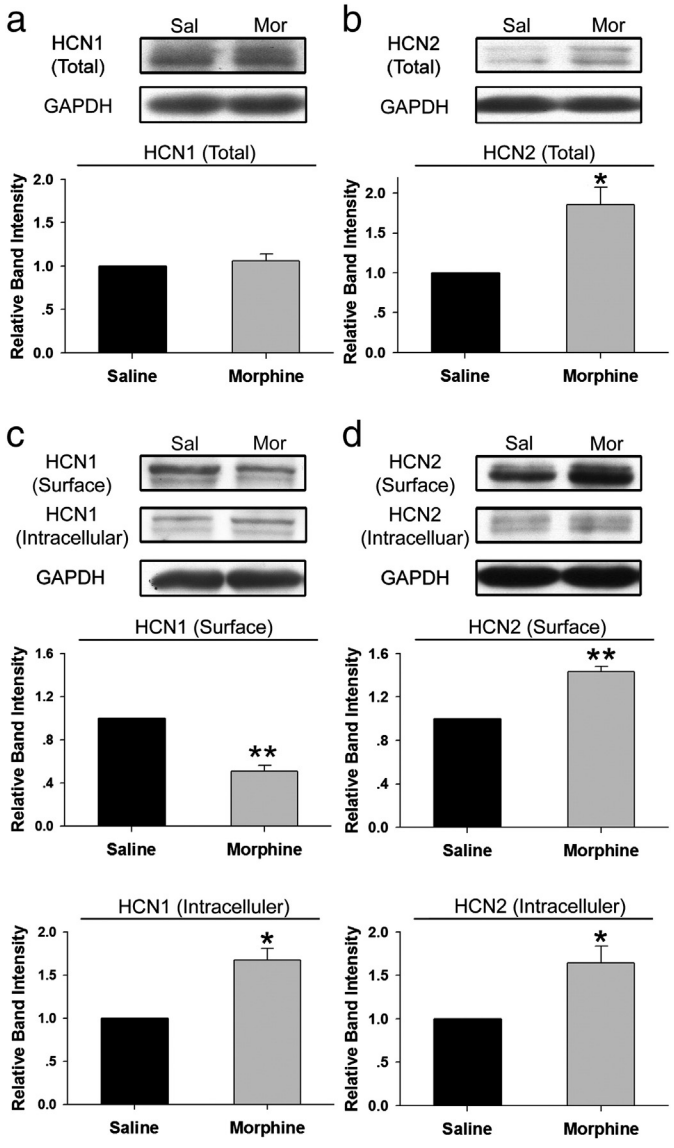

Fig. 4. Chronic morphine exposure resulted in an imbalance of HCN1/HCN2 cell membrane expression in hippocampal CA1 area. a. The total protein levels of HCN1 channel subunit in all groups showed no significant differences in hippocampal CA1 area. b. The total protein levels of HCN2 channel subunit was markedly increased in hippocampal CA1 area of chronic morphine exposed rats. c. The cell membrane expression of HCN1 channel subunit in hippocampal CA1 area of chronic morphine exposed rats was significantly decreased, while the intracellular level of HCN1 was significantly increased. d. Both the cell membrane expression and the intracellular expression of HCN2 channel subunit in hippocampal CA1 area of chronic morphine exposed rats were significantly evaluated. n = 4 for per group, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline group.

collateral-CA1 synapses, which might be associated with memory deficiencies induced by morphine.

Changes of neuronal cell bodies and synaptic contacts in CA1 region of the hippocampus have been implicated in memory function, which may be the structural plasticity mechanism responsible for spatial memory impairment (Ishikawa et al., 2014; Kitamura and Inokuchi, 2014). Our study showed that the no significant change of NeuN protein in hippocampal CA1 area was observed in rats with chronic morphine exposure, suggesting the inhibition of the hippocampal CA1 LTP and impairment of spatial learning and memory processes are related to dysfunction of neurons under chronic morphine addiction, such as the alteration of electrophysiological properties of neurons (Miladi-Gorji et al., 2014; Robinson et al., 2002; Yang et al., 2013).

The hyperpolarization-activated cyclic-nucleotide-gated nonselective cation (HCN) channels exhibit fundamental physiological

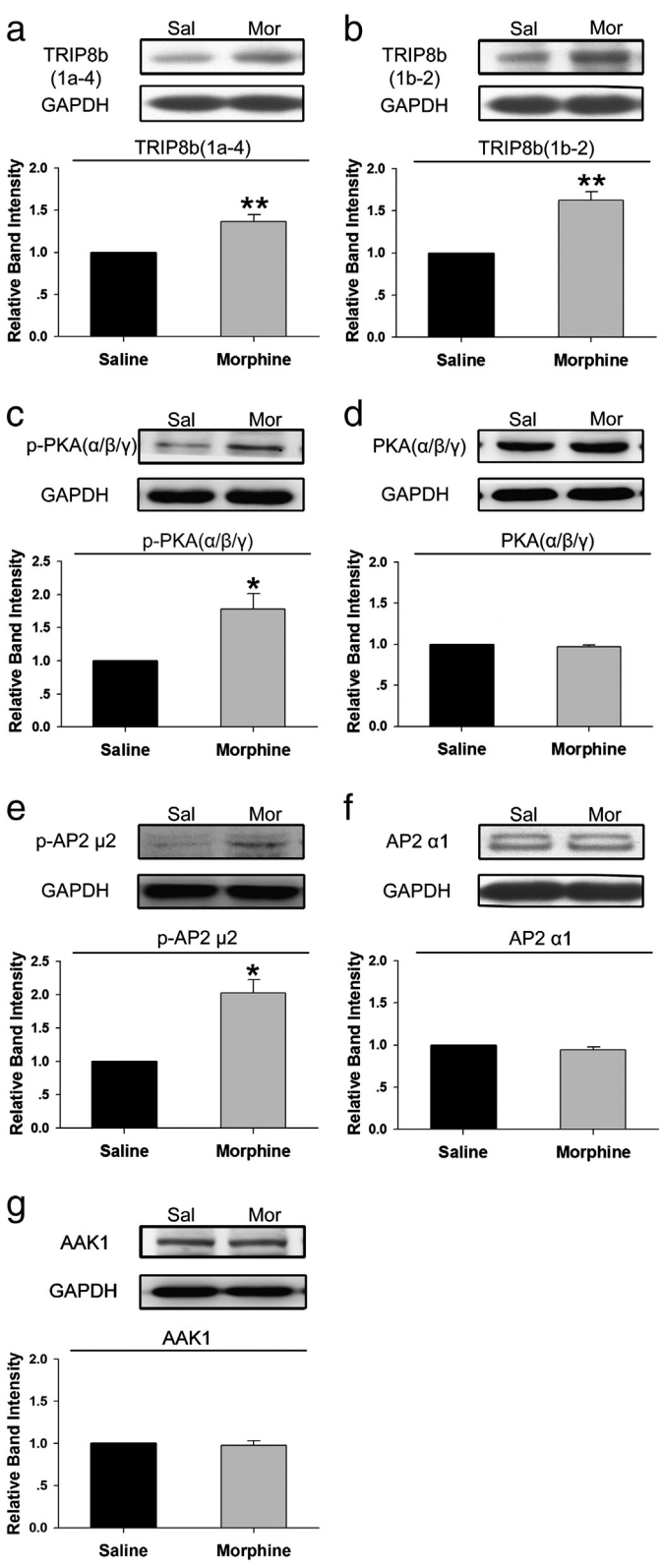

Fig. 5. Chronic morphine exposure could not only increase the expression of TRIP8b (1a-4) and TRIP8b (1b-2), but also regulate the function of TRIP8b via p-AP2 μ2 and p-PKA. a. TRIP8b (1a-4) protein level was significantly increased in hippocampal CA1 region in chronic morphine exposed rats. b. TRIP8b (1b-2) protein levels were also markedly evaluated in hippocampal CA1 region in chronic morphine exposed rats. c. p-PKA α/β/γ levels in chronic morphine exposed rats showed a notable increase. d. PKA α/β/γ levels remained unchanged among all groups. e. The expression of p-AP2 μ2 was significantly increased in chronic morphine exposed rats. f. AP2 α1 levels in all groups showed no significant differences. g. AAK1 levels remained unchanged among all groups. n = 4 for per group, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. saline group.

functions and play a vital role in the neurological basis underlying nervous system diseases (Emery et al., 2011; Nolan et al., 2004; Postea and Biel, 2011). HCN1 and HCN2 are the most abundant subunits of HCN channels in the hippocampus (Brewster et al., 2005; Franz et al., 2000; Notomi and Shigemoto, 2004). Our results showed that chronic morphine exposure (5 mg/kg, sc, for 7 days) caused a decrease in HCN1 cell membrane expression, and an increase in HCN2 cell membrane expression. However, increased intracellular HCN1 and HCN2 proteins were observed in hippocampal CA1 area in rats with chronic morphine exposure. Our observation for the up-regulation of HCN2 expression is consistent with the observation from Santos-Vera and colleagues, in which chronic cocaine exposure caused a significant increase in glycosylated and non-glycosylated proteins of HCN2 channels in the mesocorticolimbic system of rats (Santos-Vera et al., 2013). In rat hippocampus, amounts of HCN1 protein was over eight-fold higher than that of HCN2, so that HCN1 subunit was likely a major molecule to form homomeric channels (Brewster et al., 2005). Glycosylation of the HCN channels stabilize cell membrane expression of heteromeric HCN1/HCN2 constructs in heterologous neuron systems (Zha et al., 2008). Therefore, an imbalance of HCN1/HCN2 expression may implicate disorders of neurophysiological function (Lewis and Chetkovich, 2011).

Ample evidences have shown that changes in HCN channels alter synaptic plasticity, which is important for behavior such as learning and memory. Nolan and colleagues found that HCN1 knockout mice demonstrated deficits in their ability to learn to swim to a visible platform (Nolan et al., 2003). They further found that deletion of forebrain HCN1 would significantly enhance LTP amplitude at perforant path synapses but not at Schaffer collateral synapses, which caused increases in both short-term and long-term forms of spatial memory (Nolan et al., 2004). LTP in the direct perforant path to CA1 pyramidal cells was enhanced in mice carrying a global deletion of HCN2 (Matt et al., 2011). Moreover, HCN channel blocker ZD7288 could produce a dose-dependent inhibition of the induction of LTP at the Schaffer collateral-CA1 synapse of hippocampus by reducing the amount of glutamate release (He et al., 2010). These studies suggest that alteration of HCN channels plays a critical role in the neurological basis underlying memory deficiencies. Based on the above findings, we hypothesized that the decreased ratio of cell membrane HCN1 and HCN2 caused by chronic morphine exposure might be related to the formation of functional heteromeric channels via increasing the combinatorial interaction of the more abundant HCN1 molecules with HCN2 isoforms, which may alter the neuronal electrophysiological activity, i.e. LTP in the hippocampus and be responsible for the impairment of synaptic plasticity and memory deficits underlying chronic morphine exposure.

It has been documented that the brain-specific HCN channel auxiliary protein TRIP8b plays an important role in the trafficking of HCN channels through interacting with a conserved tripeptide sequence in the COOH terminus of HCN channels (Santoro et al., 2011; Zerial and McBride, 2001). Therefore, functional combinatorial interaction of TRIP8b isoforms with HCN protein resulted in a strong up- or down-regulation of HCN channels in the cell membrane. TRIP8b (1b-2) strongly decreases the cell membrane expression of HCN1 and HCN2 (Santoro et al., 2004), while TRIP8b (1a-4) promotes HCN1 surface expression, but reduces HCN2 surface membrane expression (Lewis et al., 2009; Santoro et al., 2009; Zolles et al., 2009).

In the present study, TRIP8b (1a-4) and TRIP8b (1b-2) protein levels were significantly increased in hippocampal CA1 region, and accompanied with a reduction of membrane HCN1 expression in rats with chronic morphine exposure, which is consistent with the previous report (Santoro et al., 2004). However, the up-regulation of HCN2 surface membrane expression could not be interpreted by the increase of TRIP8b (1a-4) and TRIP8b (1b-2). This suggests that other regulatory molecules, such as AP2, AAK1 or PKA, are involved in the action of morphine (Conner and Schmid, 2002; Popova et al., 2008; Santoro et al., 2011). Our results demonstrated a significant increase of p-AP μ2 and p-PKA in rats with chronic morphine exposure. The phosphorylation of PKA induced by chronic morphine exposure enhanced the effect of AAK1 on the phosphorylation of AP μ2, which decreases HCN2 internalization (Conner and Schmid, 2002; Popova et al., 2008; Ricotta et al., 2002; Santoro et al., 2011). This is likely related to the up-regulation of cell membrane HCN2 expression observed in rats with chronic morphine exposure. Collectively, the combined effect of the related regulatory molecules ultimately participated in the imbalance of cell membrane HCN1/HCN2 expression induced by chronic morphine exposure.

5. Conclusion

Drug addiction places an enormous burden on society through its repercussions on crime rate and healthcare, which is increasingly seen as a result of pathological changes in neuronal plasticity in brain circuitry responsible for learning and memory. Studies on the electrophysiological properties of HCN channels suggest its potential role in the neurological basis underlying nervous system diseases. In the present study, we found that the phosphorylation of PKA in hippocampal CA1 region caused by chronic morphine exposure enhanced the effect of AAK1 on the phosphorylation of AP μ2, accompanied by the increase in TRIP8b (1a-4) and TRIP8b (1b-2), and consequently induced an imbalance of HCN1/HCN2 surface expression, which might account for the impairment effect of chronic morphine exposure on spatial memory. Taken together, these findings suggest that changes in cell membrane HCN1/HCN2 subunits are important neurobiological basis in CNS adaptations that occur in drugs addiction and are likely relevant in the development of new strategy for the treatment of drugs addiction.

Conflict of interest

Authors declare that they have no conflict of interest.

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