Abstract—The partial opioid agonist thienorphine is currently in Phase II clinical trials in China as a candidate drug for the treatment of opioid dependence. However, its effect on synaptic plasticity in the NAc (nucleus accumbens) remains unclear. In the present study, we measured structural parameters of the synaptic interface to investigate the effect of thienorphine, morphine or a combination of both on synaptic morphology in the NAc of rats. Expression of synaptophysin was also examined. Ultrastructural observation showed that synaptic alterations were less pronounced after chronic thienorphine administration than after chronic morphine administration. Animals that received thienorphine had thinner postsynaptic densities and shorter active zones in the NAc compared with those in the saline group, but the active zone was larger, and the cleft narrower, than those in the morphine group. Furthermore, synaptophysin expression in the NAc was significantly greater after chronic administration of thienorphine, morphine, or both, than after saline. These results identified interesting differences between thienorphine and morphine in their effects on synaptic structure and synaptophysin expression in the rat NAc. Further study is deserved to investigate thienorphine as a new treatment for opioid dependence. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: thienorphine, nucleus accumbens, synaptic structure, synaptophysin, synaptic plasticity.

INTRODUCTION

Drug abuse is a global social and medical problem. The partial opioid agonist buprenorphine has proved effective in long-term detoxification, alleviating the protracted withdrawal syndrome, and preventing drug craving (Cheskin et al., 1994). However, its poor oral absorption and potential for dependence hinder its suitability as a desirable agent to treat opioid dependence (Heel et al., 1979). A strategy in the development of new treatments for opioid addiction is to find other partial opioid agonists with a long duration of action but with high oral bioavailability. In a search for such compounds, thienorphine, a novel analog of buprenorphine, was synthesized by our institute (Liu et al., 2005). Thienorphine is a partial opioid agonist with high oral bioavailability, both antinociceptive activity and morphine antinociception-blocking activity, and persistently inhibits morphine-induced dependence (Zhao et al., 2004; Yu et al., 2006; Kong et al., 2007). It is being developed as a candidate to treat opioid dependence and is now in a Phase II clinical trial in China.

Long-lasting synaptic plasticity in the brain, especially in the nucleus accumbens (NAc), is thought to play a crucial role in the persistence of drug addiction (Lüscher and Malenka, 2011). Synaptic plasticity includes not only changes in efficacy of synaptic transmission but also changes in synaptic morphology. Structural plasticity and morphological changes are the basis for functional plasticity (Wilbrecht et al., 2010). Structural parameters of the synaptic interface, such as postsynaptic density (PSD) thickness, length of the active zone, width of the synaptic cleft and curvature of the synaptic interface, are reliable parameters for quantitative analysis of synaptic plasticity (Jing et al., 2004). Morphological changes in PSD and the active zone reflect receptor and ion channel alterations in the postsynaptic membrane, as well as changes in synaptic transmission efficacy. Morphological changes in the synaptic cleft also play an important role in the magnitude and kinetics of synaptic activity. To some extent, curvatures of changes in the synaptic interface represent the functional and active state of neurons (Kennedy, 2000; Takagi et al., 2000). Structural plasticity involves synapse formation, maturation, elimination, and maintenance, as well as the expression of synapse-associated proteins. Synaptophysin, a major synaptic vesicle protein, is a marker for synaptic activity and synapse formation during development (Thiele et al., 2000). Some findings strongly suggest that synaptophysin plays an important role in the regulation of mu-opioid receptor trafficking and signaling (Liang et al., 2007).

The effect of thienorphine on synaptic plasticity in the NAc remains unknown. Therefore, in the present study, we used electron microscopy to measure the structural parameters of the synaptic interface, in order to investigate the effects of thienorphine, morphine, or a combination of both, on the morphology and ultrastructure of the rat NAc. Finally, we also examined synaptophysin expression using Western blotting.

EFFECTS OF THIENORPHINE ON SYNAPTIC STRUCTURE AND SYNAPTOPHYSIN EXPRESSION IN THE RAT NUCLEUS ACCUMBENS

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Abbreviations: DMSO, dimethyl sulfoxide; NAc, nucleus accumbens; PSD, postsynaptic density; RER, rough endoplasmic reticulum; TEM, transmission electron microscopy.

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EXPERIMENTAL PROCEDURES

Animals

The experiments were carried out in male Wistar rats (weighing 180–200 g) supplied by the Beijing Animal Center (Beijing, China). The rats were acclimated to a colony room with ambient temperature (22 ± 1 °C), humidity (50 ± 10%), and a 12-h light/dark cycle with food and water available ad libitum for at least 3 days before the start of the experiment. All animal experiments were performed in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). The experimental procedures were approved by the local Committee on Animal Care and Use.

Laboratory reagents

Thienorphine hydrochloride was synthesized in our institute (Liu et al., 2005). Morphine hydrochloride was produced by Qinghai Pharmaceutical Factory (Xining, China). Rabbit anti-synaptophysin antibody was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Zhongshan Biotechnology (Beijing, China). All other reagents were of analytical grade and purchased from commercial resources. Thienorphine was dissolved in dimethyl sulfoxide (DMSO), then diluted to desired concentrations in distilled water containing <2% DMSO just before use. Morphine was dissolved in saline (0.9% NaCl). All drugs were injected in a volume of 2 ml/kg.

Drug treatment

For the assessment of NAc synaptic structures, rats received thienorphine (1 mg/kg, s.c.), morphine ( escalating dose: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 50, 50, 50 mg/kg), thienorphine + morphine, or saline, three times daily for 2 weeks. For measurement of synaptophysin expression, rats received thienorphine (1 mg/kg, s.c.), morphine (10, 20, 20, 30, 40, 40, 50 mg/kg, s.c.), thienorphine + morphine, or saline, three times daily for 8 days. Thienorphine was injected 30 min before morphine injection on each day in the thienorphine + morphine group.

Electron microscopy for the assessment of synaptic structure

Six hours after the last injection on days 3, 5 and 8, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and sacrificed. The brain was rapidly removed from the cranium, placed on an ice-cold plate and the NAc dissected out, quickly frozen in liquid nitrogen, and transferred to a −80 °C freezer where it was stored until Western blot analysis. For analysis, samples were thawed, weighed, and homogenized in 10 volumes of extraction buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mM sodium vanadate and 1 mM EDTA pH 8.0). Samples were then centrifuged at 16,000 g for 30 min at 16,000g and the supernatant removed. Protein content was determined using the method of Bradford (1976). The NAc samples were diluted in extraction buffer and proteins denatured in boiling water for 5 min. Equal amounts of each NAc protein were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred electrophoretically to nitrocellulose membranes. Nitrocellulose membranes in duplicate were then blocked with 5% non-fat milk in phosphate-buffered saline. The membranes were subsequently incubated with primary rabbit anti-synaptophysin antibody. Immunoreactivity was then detected by incubation with horseradish peroxidase-conjugated secondary antibody. Specific complexes were detected using an enhanced chemiluminescence kit (Millipore, MA, USA) according to the manufacturer’s instructions. Western blots were scanned and analyzed with Image J software (U.S. National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data are expressed as mean ± S.E.M. for four animals per group unless otherwise stated. Statistical analyses were performed by a one-way or two-way analysis of variance followed by Dunnett’s t test. Student’s t test was used
when two independent groups were compared. Statistical significance was accepted at $P < 0.05$.

**RESULTS**

**Effect of chronic thienorphine treatment on synaptic ultrastructure**

Ultrastructural changes in the NAc tissue of rats were observed by TEM (Fig. 1). Karyotheca, rough endoplasmic reticulum (RER), ribosomes and other organelles were clearly discernible in the saline control group. Normal nuclei and normal chromatin were apparent. Both presynaptic and postsynaptic membranes were clearly observed. In the three drug groups, degenerative changes occurred. However, neuronal ultrastructure in the thienorphine group appeared closer to that in the saline group than the morphine or morphine + thienorphine groups, with a greater degree of RER and ribosomal structure retained; some neurons were atrophied, with obvious borders with the surrounding tissue. Chromatin was condensed and some vacuoles were observed in mitochondria.

**Measurement and analysis of synaptic interface structure parameters**

Significant differences were observed between the drug treatment groups in the structural parameters of the synaptic interface. Morphine treatment for 2 weeks significantly reduced the length of the active zone and thickness of the PSD and increased the width of the cleft compared to the saline group (Table 1).

The length of the active zone and thickness of the PSD in rats that had received morphine were, respectively, 60.1% ($P < 0.05$) and 70.4% ($P < 0.05$) of that in the saline group, and cleft width was increased to 132.2% ($P < 0.05$). In the thienorphine group rats, the length of the active zone and thickness of the PSD were also decreased compared to the saline group, but compared with the morphine group, the active zones were 16.3% longer ($P < 0.05$) and cleft widths were 83.9% ($P < 0.05$) of those in the morphine group. The length of the active zone, thickness of the PSD and width of the cleft in rats that received morphine + thienorphine were similar to those in the thienorphine group. No differences in

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*Fig. 1.* Synaptic ultrastructure of the NAc in rats in the four treatment groups. The right side is an enlarged image of box in the left side. (A-1,2) saline, clear synaptic structures can be observed; (B-1,2) morphine, presynaptic and postsynaptic membranes are obscured and mitochondria are swollen; (C-1,2) thienorphine, relatively clear and intact presynaptic and postsynaptic components; (D-1,2) thienorphine + morphine, presynaptic and postsynaptic membranes are obscured, and mitochondria are swollen.
synaptic interface curvature were observed between the four groups.

Effect of chronic thienorphine treatment on synaptophysin expression

The expression of synaptophysin was determined by western blotting. Six hours after the last injection on days 3 or 5, no differences were observed in the expression of synaptophysin among the four groups. However, on day 8, synaptophysin expression in the NAc of rats that received morphine, thienorphine, and morphine + thienorphine was significantly greater than that in the saline control group \((P < 0.05)\), with no differences observed among the three drug groups (Fig. 2).

DISCUSSION

In the present study we performed a morphological observation and a quantitative ultrastructural analysis of NAc synapses in rats treated with thienorphine, and also determined synaptophysin expression. Interestingly, thienorphine administration decreased the length of the active zone and thickness of the PSD and increased synaptophysin expression. The present results provide ultrastructural evidence of thienorphine-induced synaptic plasticity in the NAc of rats and demonstrate changes in synaptophysin expression after thienorphine. In contrast, we also found a reduction in active zone length and PSD thickness, as well as an increase in synaptophysin expression, in rats that received morphine, while the results of the morphine + thienorphine group were comparable to those in the thienorphine group.

Abuse of drugs produces widespread effects on the synaptic connections and function of neurons throughout the brain’s reward circuitry (Russo et al., 2009), which synaptically connects a wide variety of nuclei. Of these nuclei, the NAc plays a vital role in the cognitive, hedonic and motor effects of drugs of abuse, and in the transition to addiction (De Biasi and Dani, 2011). Chronic morphine treatment has previously been reported to induce changes in morphology of NAc neurons in behaviorally sensitized animals (Alcantara et al., 2011). Consistent with these and other findings (Robinson and Kolb, 1999), the present results provide direct evidence for the effect of morphine on synaptic structural plasticity. Changes in neuron morphology resulting from repeated morphine treatment suggest that changes in synaptic connections are also occurring. As a partial agonist of opioid receptors, thienorphine potently binds to mu, delta and kappa opioid receptors and partially stimulates mu and kappa opioid receptors (Yu et al., 2006). In the present study, chronic thienorphine administration led to pathological ultrastructural changes in NAc synapses, suggesting that the changes in the NAc core were associated with thienorphine’s high affinity for the opioid receptor.

Thienorphine affected the function of those regions and led to the remodeling of synaptic membranes, including changes in PSD thickness and active zone length. The PSD is a specialization of the cytoskeleton at the synaptic junction and is made up of a mixture of proteins, including cytoskeletal and scaffold proteins, glutamate receptors, calmodulin binding protein, ion channels, and signaling molecules (Trinidad et al., 2005). The active zone is a specialized membrane domain in the presynaptic neuron and contains the molecular machinery required for calcium-dependent synaptic vesicle fusion and recycling (Scheiffele, 2003; Sudhof, 2004). The PSD and the active zone are essential components of a complex synaptic signaling assemblage and play an important role in synaptic regulation and plasticity (Klauck and Scott, 1995). Chronic morphine exposure induces changes in proteins of the PSD (Prokai et al., 2005; Li et al., 2006) and PSD thickness. The reduction in active zone length and PSD thickness induced by morphine and thienorphine in the NAc of rats could impair synaptic transmission efficiency. Thienorphine affected these factors less than morphine did, possibly owing to the strong binding of thienorphine to opioid receptors, in contrast to morphine’s easy dissociation from them. In addition, we also observed that the effect of morphine + thienorphine on synaptic structure was similar to that of thienorphine, implicating that thienorphine inhibited the effect of morphine, providing further evidence in support of the drug as a new treatment for opioid dependence.

Abuse of drugs is a compulsive behavioral abnormality in which pathological changes in synapse structure occur, as well as changes in expression of synapse-associated proteins such as synaptophysin. Synaptophysin is an abundant integral membrane protein of synaptic vesicles. A number of studies have shown that it plays an important role in the regulation of neurotransmitter release and synaptic plasticity (Kwon and Chapman,

### Table 1. Effects of thienorphine on synaptic interface structure parameters in the NAc of rats

<table>
<thead>
<tr>
<th>Synaptic interface structure parameters</th>
<th>Group</th>
<th>Saline</th>
<th>Morphine</th>
<th>Thienorphine</th>
<th>Thienorphine + Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curvature of interface</td>
<td>1.0 ± 0.05</td>
<td>1.0 ± 0.03</td>
<td>1.1 ± 0.04</td>
<td>1.1 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Length of active zone (nm)</td>
<td>352.5 ± 91.75</td>
<td>211.9 ± 50.00(^*)</td>
<td>246.4 ± 29.34(^*)</td>
<td>240.8 ± 40.07(^*)</td>
<td></td>
</tr>
<tr>
<td>Thickness of PSD (nm)</td>
<td>41.2 ± 5.18</td>
<td>29.0 ± 6.45</td>
<td>31.2 ± 3.40</td>
<td>32.4 ± 3.55</td>
<td></td>
</tr>
<tr>
<td>Width of cleft (nm)</td>
<td>14.8 ± 1.94</td>
<td>19.6 ± 3.95</td>
<td>16.4 ± 3.15(^*)</td>
<td>16.7 ± 2.30(^*)</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Data shown are the mean of all synapses examined in each group \((n = 55–65 \text{ synapses per group})\); Student’s \(t\) test was used when two independent groups were compared.

\(^*\) \(P < 0.01\) compared with the saline group.

\(^*\) \(P < 0.01\) compared with the morphine group.
Interestingly, previous work (Liang et al., 2007) and our current observations show that synaptophysin is constitutively associated with the mu-opioid receptor and may be involved in receptor trafficking and signaling. In the present study, we showed that morphine, thienorphine, or both, significantly increased synaptophysin expression after 8 days of treatment. One explanation for the observed effects is that the action of morphine or thienorphine is mediated by the mu-opioid receptor, which could be modulated by synaptophysin. Overexpression of synaptophysin enhances the endocytosis of mu-opioid receptors activated by morphine or thienorphine, suggesting that abnormal synaptophysin expression may contribute to the development of morphine or thienorphine tolerance and to the neuronal adaptation with altered functions of neuronal circuits, including changes in neuronal plasticity (Liang et al., 2007).

CONCLUSIONS

Taken together, we have identified the differences between thienorphine and morphine in their effects on synaptic structure and synaptophysin expression in the rat NAc, and demonstrated that thienorphine alters synaptic ultrastructure and increases the expression of synaptophysin. These results provide the first evidence that thienorphine regulates neuronal plasticity in the NAc, and opens an avenue of research into thienorphine as a new treatment for opioid dependence.

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