NeuroToxicology 51 (2015) 51–57

Contents lists available at ScienceDirect

NeuroToxicology

The effect of different durations of morphine exposure on mesencephalic dopaminergic neurons in morphine dependent rats

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ARTICLE INFO

Article history:
Received 24 February 2015
Received in revised form 4 September 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
Morphine dependence
Ventral tegmental area
Substantia nigra
TH-positive cell
Fluoro-Jade B

ABSTRACT

Mesencephalic dopaminergic neurons are heavily involved in the development of drug dependence. Thyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, plays an important role in the survival of dopaminergic neurons. Therefore, this study investigated TH changes in dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra (SN), as well as the morphine effects on dopaminergic neurons induced by different durations of morphine dependence. Models of morphine dependence were established in rats, and paraffin-embedded sections, immunohistochemistry and western blotting were used to observe the changes in the expression of TH protein. Fluoro-Jade B staining was used to detect degeneration and necrosis, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) detected the apoptosis of mesencephalic dopaminergic nerve cells. Immunohistochemistry and western blotting showed that the number of TH positive cells and the protein levels in the VTA and SN were significantly decreased in the rats with a long period of morphine dependency. With prolonged morphine exposure, the dopaminergic nerve cells in the VTA and SN showed degeneration and necrosis, while apoptotic cells were not observed. The number of VTA and SN dopaminergic nerve cells decreased with increasing periods of morphine dependence, which was most likely attributable to the degeneration and necrosis of nerve cells induced by morphine toxicity.

1. Introduction

It is well-known that morphine has an obvious analgesic effect, but this drug is also highly addictive. The abuse of morphine has increased greatly in recent years (Compton and Volkow, 2006; Volkow and Skolnick, 2012), which has led to a series of undesirable effects, and has become a significant medical and public health problem. The mesencephalic dopamine system, one of the key brain regions involved in drug addiction, participates in almost all of the rewarding effects of dependency drugs and is thought to be the final pathway for the reward system (Masserano et al., 1996; Schmidt et al., 2001). Previous studies have shown that mesencephalic dopaminergic neurons are critically involved with the behavioral sensitization caused by morphine (Bai et al., 2012) and are associated with stress-induced opioid seeking (Graziane et al., 2013). Most of the studies on morphine have investigated functioning and metabolic functions, while pathological studies are lacking. Among the transcription factors involved in regulation of mesencephalic dopaminergic neurons, tyrosine hydroxylase (TH, the rate-limiting enzyme in dopamine synthesis (Jankovic et al., 2005; Reddy et al., 2011)) plays a critical role in the survival of dopamine neurons. However, the changes of TH expression in mesencephalic dopaminergic neurons during chronic morphine dependence have not been described. A recent study presented the changes of TH mRNA and protein in the VTA after one week of morphine dependence (García-Pérez et al., 2014), but the changes associated with longer morphine treatment remain unclear. The morphine dependent rats in our study were treated with morphine for longer durations, with the longest being 6 weeks, for the purpose of mimicking the characteristics of humans with long periods of morphine exposure and to investigate the changing patterns of TH in mesencephalic dopaminergic neurons, as well as neuronal degeneration, necrosis and apoptosis. The findings provide morphological evidence for studying the mechanism of nerve injuries induced by morphine dependence.

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http://dx.doi.org/10.1016/j.neuro.2015.09.005
0161-813X/

2. Materials and methods

2.1. Reagents

Rabbit monoclonal anti-TH antibody was purchased from Abcam (UK, CN: MAB318), the immunohistochemical kit was purchased from Zhongshan Goldenbridge Biotech (China, CN: SP9001), Fluoro-Jade B was purchased from Millipore (USA, CN: AG310), the TUNEL kit was purchased from Promega (USA, CN:#G7132), and the morphine hydrochloride injection was produced in the first pharmaceutical factory of Shenyang (China).

2.2. Animals

Adult male Wistar rats (Experimental Animal Center, Hebei Medical University, China) weighing 300 ± 20 g were adapted to a 12/12 h light/dark cycle, with free access to food and water. This study was approved by the Institutional Review Board for Animal Experiments at Hebei Medical University. The rats were randomly divided into the following groups: the control, 1 week, 3 week and 6 week morphine dependent groups (n = 12).

2.3. Model of morphine dependence

The model of morphine dependence was established by increasing subcutaneous injections of morphine hydrochloride. The three groups of morphine dependent animals were injected subcutaneously in the back with morphine hydrochloride twice daily (8:00, 20:00) for 5 days. The initial dose administered was 10 mg/kg and was increased by 10 mg/kg every other day until the 5th day of treatment. The control rats received equal volumes of saline. The animals were thoroughly disinfected with alcohol (75%) prior to every injection with a disposable needle/syringe. The rats were then confirmed to be dependent on morphine after 5 days of morphine administration. This process was described by (Maldonade et al., 1992), and involved observing signs of opiate withdrawal such as wet dog shakes, stretching, cleaning fur, swallowing, teeth chattering, ptosis, and diarrhea. Following this assessment, 30 mg/kg morphine were administered twice daily (8:00, 20:00) until 1, 3 or 6 weeks post-establishment of dependence.

2.4. Tissue preparation

Tissue used for staining was harvested and fixed immediately in 10% formalin. Brain slices beginning at −5.30 mm from the bregma were obtained using a stereotaxic atlas (Paxinos and Watson, 2007). The tissue was subsequently dehydrated in a graded ethanol series and embedded in paraffin. Sections (4 mm) were prepared for immunohistochemical staining. The brain samples for western blot were harvested at 0 °C and frozen immediately in liquid nitrogen. Tissues of the VTA and SN were dissected using a punching device with a 1-mm internal diameter. The anatomical locations and boundaries of each region were determined using the rat brain Atlas of Paxinos and Watson (2007). Tissues of interest were homogenized in ice-cold homogenization medium consisting of 20 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.1 mM PMSF, 1 mM dithiothreitol (DTT) and proteinase inhibitor cocktail tablets. The homogenates were centrifuged at 15,000 × g at 4 °C for 30 min, and the supernatants were collected as protein samples.

2.5. Immunohistochemistry

Deparaffinized sections were pretreated with microwave antigen retrieval, 3% H_2O_2 in cold methanol for 30 min, and goat serum for 30 min. Next, the tissues were incubated with a monoclonal antibody against rabbit TH (1:200) overnight at 4 °C. The tissues were then incubated for 1 h with biotinylated secondary antibody and subsequently with alkaline phosphatase (AP)-conjugated biotin for 30 min. Finally, AP-red was used for visualization. The tissues were counterstained with hematoxylin to visualize the location of immunostaining.

2.6. Fluoro-Jade B staining

Sections were dewaxed with an alcohol series and then rinsed in distilled water for 2 min. Slides were then transferred for 10 min to a 0.0001% solution of Fluoro-Jade B dissolved in a 0.1% acetic acid vehicle. The slides were then rinsed through three changes of distilled water for 1 min per change. Excess water was drained onto a paper towel, and the slides were then air dried on a slide warmer at 50 °C for at least 5 min. The air dried slides were then cleared in xylene for at least 1 min and coverslipped with neutral balata.

2.7. TUNEL assay

The TUNEL assay was used to assess the extent of apoptosis in the treated cells. The slides were washed with PBS and fixed using 4% paraformaldehyde. The slides were stained by adding fluorescein 12-dUTP to nicked ends of DNA and then visualized with a fluorescent light microscope. Green fluorescence was correlated with DNA fragmentation.

2.8. Cell counting

Eight rats from each group were used for morphological observation. Using the serial section technique, we took one out of every three sections. Following a comparison of the sections after TH immunohistochemical staining, the sections with the largest area of the VTA and SN were selected, and the numbers of TH positive cells in the two regions were counted in a 100× field view. Two independent observers who were blinded to the experimental conditions performed the counts and calculated the average number of TH positive cells. The protocol for counting the numbers of degenerating and necrotic neurons in Fluoro-Jade B staining was the same as described above.

2.9. Western blot analysis

Four rats from each group were used for the western blot analysis. Tissue extracts (50 μg of protein/lane) were loaded, separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with anti-TH (1:200) and β-actin (Abcam, UK) antibodies. Then, incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG was performed. For the X-ray film exposure, the enhanced chemiluminescence system was employed. The optical densities of the bands were measured using LabWorks 4.5 software. TH expression was quantified with respect to the signals of the corresponding β-actin band.

2.10. Statistical methods

Statistical analysis was performed with one-way ANOVA. The results are presented as the mean ± SEM. The threshold for statistical significance was defined as $P < 0.05$.

3. Results

3.1. TH expression in the VTA

The VTA region was easily identified from the surrounding tissue when labeled with the TH antibody. The cytoplasm and