Neuroscience 153 (2008) 823–831

AMITRIPTYLINE INDUCES NUCLEAR TRANSCRIPTION FACTOR-κB–DEPENDENT GLUTAMATE TRANSPORTER UPREGULATION IN CHRONIC MORPHINE-INFUSED RATS

Y.-H. TAI,[a] R.-Y. TSAI,[b] Y.-H. WANG,[a] C.-H. CHERNG,[a] P.-L. TAO,[c] T.-M. LIU[a] AND C.-S. WONG[a]*

[a]*Department of Anesthesiology, Tri-service General Hospital and National Defense Medical Center, #325, Chenggung Road, Section 2, Neihu 114, Taipei, Taiwan*

[b]*Graduate Institute of Medical Science, National Defense Medical Center, No. 161, Section 6, Min-Chuan East Road, Neihu 114, Taipei, Taiwan*

[c]*Department of Pharmacology, National Defense Medical Center, Taipei, Taiwan*

Abstract—We previously showed that intrathecal co-administration of amitriptyline with morphine upregulates the expression of the glial glutamate transporters glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) and restores neuronal glutamate transporter excitatory amino acid carrier 1 (EAAC1) expression in chronically morphine-infused rats. The present study examined the role of nuclear transcription factor-κB (NF-κB) in the regulation of the expression of GLAST, GLT-1, and EAAC1 following long-term amitriptyline/morphine co-infusion. Male Wistar rats were implanted with two intrathecal catheters with or without a microdialysis probe; one of the catheters was used for continuous infusion of saline (control), morphine (15 μg/h), or morphine plus amitriptyline (both 15 μg/h) for 5 days, while the other was used for a single daily intrathecal injection of the NF-κB inhibitor Ro106–9920 (10 μl of 10 μM) for 5 days. We found that amitriptyline co-infusion restored the antinociceptive effect of morphine (4.5-fold right-shift in the morphine dose-response curve compared with a 65-fold right-shift in its absence) and this effect was inhibited by Ro106–9920 administration (48-fold right-shift). Moreover, amitriptyline/morphine co-infusion increased IκBα phosphorylation and the translocation of NF-κB p65 from the cytosol to the nucleus. Daily intrathecal injection of Ro106–9920 prevented the amitriptyline/morphine-induced NF-κB p65 translocation and reversed the amitriptyline/morphine-induced GLAST and GLT-1 upregulation and inhibited the restoration of EAAC1 expression. The Ro106–9920 injections abolished the inhibitory effect of amitriptyline on the morphine-evoked release of excitatory amino acids into the spinal cerebrospinal fluid (CSF) dialysates. In conclusion, amitriptyline/morphine co-infusion restores the antinociceptive effect of morphine and upregulates GLAST and GLT-1 expression and restores EAAC1 expression to baseline levels, thus reducing excitatory amino acid levels in the spinal CSF dialysates. The mechanism involves activation of the NF-κB pathway, but may also involve other pathways.

Key words: amitriptyline, nuclear transcription factor-kB, glutamate transporters, excitatory amino acids, microdialysis, morphine tolerance.

Glutamate, the main excitatory neurotransmitter in the mammalian CNS, is critical for spinal excitatory synaptic transmission (Danbolt, 2001). It is important to maintain the extracellular glutamate concentration at the physiological level. Excessive activation of glutamate receptors is critical in chronic opioid-induced neuronal adaptation, such as opioid tolerance, dependence, withdrawal (Trujillo and Akil, 1991, 1995; Nestler, 1996), and neuropathic pain-associated hyperalgesia (Urban and Gebhart, 1998; Coderre et al., 1993; Porreca et al., 2002). The termination of glutamatergic transmission and the clearance of excess glutamate, which is neurotoxic, are regulated by high capacity Na^+-dependent glutamate transporters, including the astrocytic transporters glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) and the neuronal transporter excitatory amino acid carrier 1 (EAAC1). Glutamate transporters are known to play a critical role in maintaining glutamate homeostasis and controlling excitatory signaling (Robinson and Dowd, 1997; Mennerick et al., 1999; Sims and Robinson, 1999).

Expression of glutamate transporters is regulated at the transcriptional, translational, and post-translational levels (Gegelashvili and Schousboe, 1997). Recently, nuclear transcription factor-κB (NF-κB) –dependent transcription was proposed as a target for regulation of glutamate transporter expression (Zelenaia et al., 2000; Rodriguez-Kern et al., 2003; Sitcheran et al., 2005; Wang et al., 2006). Previous studies have shown that treatment with dibutyryl-cAMP, epidermal growth factor, or other growth factors increases GLT-1 mRNA levels and GLT-1 promoter-controlled reporter gene expression (Zelenaia et al., 2000; Su et al., 2003; Sitcheran et al., 2005), these effects depend on the phosphatidylinositol 3-kinase (PI-3K) and NF-κB pathways (Zelenaia et al., 2000). NF-κB has been shown to directly regulate the GLT-1 promoter (Sitcheran et al., 2005). A previous study demonstrated that spinal glucocorticoid receptors, acting through the NF-κB pathway, regulate EAAC1 expression after peripheral nerve injury

*Corresponding author. Tel: +886-2-87927008; fax: +886-2-87927009.
E-mail address: w82556@ndmctsgh.edu.tw (C.-S. Wong).

Abbreviations: CSF, cerebrospinal fluid; EAAC1, excitatory amino acid carrier 1; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter-1; IL-1β, interlukin-1β; IL-6, interlukin-6; MPE, maximal possible antinociceptive effect; NF-κB, nuclear transcription factor-κB; PBS, phosphate-buffered saline; PI-3K, phosphatidylinositol 3-kinase; TNFα, tumor necrosis factor α; TTBS, Tris-buffered saline containing 0.01% Tween 20.

0306-4522/08$32.00+0.00 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2008.02.055

and contribute to the development of neuropathic pain (Wang et al., 2006).

Tricyclic antidepressants are widely used to treat neuropathic and inflammatory pain (Sawynok et al., 1999; McCarson et al., 2005). In our previous study (Tai et al., 2006), we demonstrated that intrathecal co-administration of amitriptyline (at picomolar concentrations) with morphine attenuates morphine tolerance and maintains the antinociceptive effect of morphine. These effects of amitriptyline were shown to be mediated by inhibition of the expression of the pro-inflammatory cytokines tumor necrosis factor α (TNFα), interlukin-1β (IL-1β), and interlukin-6 (IL-6) in glial cells and prevention of the downregulation of glutamate transporters (even increasing GLAST and GLT-1 expression) in chronically morphine-infused rats, thus enhancing synaptic excitatory amino acid uptake and reducing excitatory amino acid levels in the spinal cerebrospinal fluid (CSF) dialysate (Tai et al., 2006). In the present study, we therefore examined whether NF-κB activation plays a role in glutamate transporter expression in rats chronically co-infused with morphine and picomolar concentrations of amitriptyline.

EXPERIMENTAL PROCEDURES

Animal preparation

A total of 150 rats were used. After a basal tail-flick test, the rats were randomly divided into: (1) a saline-infused group, (2) an amitriptyline-infused group, (3) a saline-infused/Ro106–9920 injected group, (4) a morphine-infused group, (5) a morphine-/amitriptyline-infused group, and (6) a morphine-/amitriptyline-infused and Ro106–9920 injected group. All rats were implanted with two intrathecal catheters as in our previous study (Tai et al., 2006). Briefly, male Wistar rats (350–400 g) were anesthetized with phenobarbital (60 mg/kg, intraperitoneally), then all rats were implanted with two intrathecal catheters inserted via the atlantooccipital membrane into the intrathecal space at the level of the lumbar enlargement of the spinal cord (L1–L2), and 25% were also implanted in the same region with a microdialysis probe. One intrathecal catheter was connected to a mini-osmotic pump (model 2001; Alzet, Palo Alto, CA, USA) for infusion of saline (1 μl/h), morphine (15 μg/h), or morphine/amitriptyline (both 15 μg/h) for 5 days, while the other was used for a single daily injection of the NF-κB inhibitor Ro106–9920 (10 μl; 1–10 μM as indicated) for 5 days. Ro106–9920 inhibits NF-κB activation via selective inhibition of IκBα ubiquitination (Swinney et al., 2002). After surgery, the rats were housed individually and maintained on a 12-h light/dark cycle with food and water freely available. Rats with any neurological deficits were excluded from the study ($<$10%). All experiments conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and were approved by the National Defense Medical Center Animal Care and Use Committee. Efforts were made to minimize their suffering and the number of animals used.

Construction of the intrathecal catheter and microdialysis probe

Intrathecal catheter was constructed using an 8 cm polyethylene tube (0.008 inch inner diameter, 0.014 inch outer diameter; Spectranetics, Colorado Springs, CO, USA) and a 3.5 cm Silastic tube (Dow Corning, Midland, MI, USA). The Silastic tube was inserted into the polyethylene tube and the joint sealed with epoxy resin and silicon rubber. The construction of the spinal microdialysis probe was modified from that in previous studies (Marsala et al., 1995), as described previously (Wen et al., 2003; Sung et al., 2004; Tai et al., 2006). The probe was constructed using two 7 cm polyethylene tubes and a 4.2 cm cuprophane hollow fiber (Hospal, M100 Pre Set, France). A nichrome-formvar wire (0.0026 inch diameter; A-M System, Everret Inc., WA, USA) was passed through a polycarbonate tube (194 μm outer diameter, 102 μm inner diameter; 0.7 cm in length) and the cuprophane hollow fiber (active dialysis region), then connected to a polyethylene catheter with epoxy glue. The middle portion of the cuprophane hollow fiber was bent to form a U-shaped loop, and both ends of the dialysis loop, which consisted of Silastic tubes, were sealed with silicone. The dead space of the dialysis probe was 8 μl. Using this technique, it is possible to measure CSF amino acids for up to 12 days after implantation.

Behavioral tests

Rats were placed in plastic restrainers for the antinociception assay. The tail-flick latency was measured using the hot water immersion test (52$\pm$0.5 °C) before drug infusion, then daily after the start of infusion for 5 days; at this temperature, the average baseline latency was 2$\pm$0.5 s and the cutoff time 10 s. The percentage of the maximal possible antinociceptive effect (% MPE) was calculated as (maximum latency$-$baseline latency)/(cut off latency$-$baseline latency)$\times$100. Morphine antinociceptive dose-response curves were constructed using a computer-assisted linear regression program. Values for the analgesic dose of 50% of the MPE (AD_{50}) were calculated from linear regression analysis of the morphine dose-response curves.

Preparation of spinal cord cytosolic and nuclear fractions

After drug treatment as described above, rats were killed by exsanguination under isoflurane anesthesia, then laminectomy was performed at the lower edge of the twelfth thoracic vertebra, and the lumbar enlargement (L1–L2) was removed and immediately separated into ventral and dorsal parts, which were stored at $-$80 °C until used. A part of the frozen dorsal part of the lumbosacral spinal cord segments was fractionated into cytosolic, membrane, and nuclear fractions using a cytoplasmic, nuclear, and membrane compartment protein extraction kit as recommended by the manufacturer (Biochain Institute, Inc., Hayward, CA, USA). The cytosolic and nuclear fractions were checked by Western blotting with anti-rat α-tubulin and anti-rat histone 1 antibodies, respectively, and the proteins were then separated by SDS-10% PAGE, and immunoblotted with mouse anti-rat IκBα (1:1000), anti-rat *phospho*-IκBα (1:500), or anti-rat NF-κB p65 (1:500) antibodies (all from Santa Cruz, CA, USA).

Spinal cord preparation and Western blotting

Some of the frozen dorsal parts of the lumbar spinal cord enlargement were homogenized in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2% Triton X-100, 100 μg/ml of phenylmethylsulfonyl fluoride, 1 μg/ml of aprotinin), then the homogenate was centrifuged at 100,000$\times g$ for 30 min at 4 °C and the supernatant used for Western blotting for glutamate transporters. The protein concentrations of the samples were determined by the BCA method (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard. Equal amounts of protein (20 μg) were adjusted to a similar volume with loading buffer (2% SDS, 25% glycerol, 62.5 mM Tris, 350 mM EDTA, 0.002% Bromophenol Blue, 10% β-mercaptoethanol, pH 6.8), denatured by heating at 95 °C for 5 min, and separated on 10% SDS–polyacrylamide gels, then transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon™-p; Millipore, Bedford, MA, USA). The membranes were blocked with 5% milk in Tris-buffered saline containing 0.01% Tween 20 (TTBS) and incubated overnight