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Enhanced Activities of δ Subunit-containing $GABA_A$ Receptors Blocked Spinal Long-term Potentiation and Attenuated Formalin-induced Spontaneous Pain

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Abstract—The δ subunit-containing γ-Aminobutyric acid type A receptors (δ-$GABA_A$Rs) are located at extrasynaptic sites and persistently active in the control of neuronal excitability. Here we recorded primary afferent C fiber-evoked field potentials in the superficial dorsal horn of rat spinal cords *in vivo* and investigated the possible influence of δ-$GABA_A$Rs activities on nociceptive synaptic transmission. We found that δ-$GABA_A$Rs-preferring agonist 4,5,6,7-tetrahydroisoxazolol [4,5-c] pyridine-3-ol (THIP), when topically applied onto spinal cord dorsum, inhibited the basal synaptic responses in a dose-dependent manner. Low-frequency stimulation (LFS) of sciatic nerves elicited long-term potentiation (LTP) of C fiber transmission, a synaptic correlate of central sensitization. Pretreatment with THIP before LFS delivery blocked the induction of LTP. When applied at 30 min and 180 min post-LFS, THIP reduced the magnitudes of established LTP. Intraplantar injection of formalin naturally evoked LTP in anesthetized rats. Spinal administration of THIP not only reversed formalin-induced LTP, but alleviated the spontaneous painful behaviors and mechanical hyperalgesia. Biochemical analysis demonstrated that δ-$GABA_A$Rs activation by THIP decreased the synaptic expression and phosphorylation of AMPA receptor GluA1 subunit in formalin-injected rats, and meanwhile, increased synaptic GluA2 content, allowing the switch of GluA2-lacking AMPA receptors to GluA2-containing ones at synapses. THIP also suppressed the synaptic accumulation and phosphorylation of NMDA receptor GluN1 subunit in formalin-injected rats. Our data suggested that enhanced δ-$GABA_A$Rs activities blunted the initiation and maintenance of spinal LTP, which correlated with the amelioration of central sensitization of nociceptive behaviors.

Key words: tonic inhibition, γ-aminobutyric acid type A receptors, THIP, long-term potentiation, AMPA receptor.

INTRODUCTION

Ionotropic γ-Aminobutyric acid type A receptors ($GABA_A$Rs) are pentameric hetero-oligomers assembled from several subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3). The subunit composition not only determines the biological and pharmacological properties of $GABA_A$Rs, but dictates their localization on plasma membrane (Jacob et al., 2008). $GABA_A$Rs with α1-3, β and γ subunits are typically clustered at postsynaptic membrane (Jacob et al., 2008), where they mediate fast inhibitory synaptic transmission and generate the phasic inhibition. A small fraction of synaptic $GABA_A$Rs is also composed of α5, β and γ subunits. The functional loss of synaptic GABAergic inhibition represents one of the key contributors to central sensitization after peripheral tissue or nerve injuries (Zeilhofer and Zeilhofer, 2008). Benzodiazepines specifically target γ subunit-containing $GABA_A$Rs and generate great alleviation of pathological pain (Knabl et al., 2008; Ralvenius et al., 2015).

Evidence has been accumulating that a large fraction of $GABA_A$Rs is distributed at extrasynaptic or perisynaptic sites. These receptors are putatively assembled into α4βδ, α6βδ and α5βγ heteropentamers (Jacob et al., 2008). Distinct from those with γ subunits, the δ subunit-containing $GABA_A$Rs (δ-$GABA_A$Rs) show less sensitivity to benzodiazepines but bind with a higher affinity to GABA transmitter and neurosteroids (Belelli et al., 2009). Extrasynaptic $GABA_A$Rs persistently produce the tonic inhibition. Neuroactive steroids that preferentially enhance the tonic inhibitory conductance mediated by δ-$GABA_A$Rs regulate glutamatergic neurotransmission

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Abbreviations: ACSF, artificial cerebrospinal fluid; AMPARs, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype of glutamate receptors; EDTA, ethylenediaminetetraacetic acid; $GABA_A$Rs, γ-Aminobutyric acid type A receptors; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LFS, low-frequency stimulation; LTP, long-term potentiation; NMDARs, N-methyl-D-aspartate subtype of glutamate receptors; PVDF, polyvinylidene difluoride; THIP, 4,5,6,7-tetrahydroisoxazolol [4,5-c] pyridine-3-ol; δ-$GABA_A$Rs, δ subunit-containing γ-Aminobutyric acid type A receptors.

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and plasticity as well as pain sensitization (Stell et al., 2003; Shen et al., 2010; Vashchinkina et al., 2012; Whissell et al., 2013; Patte-Mensah et al., 2014).

Previous studies have illustrated that δ-$GABA_A$Rs are functionally expressed in spinal cord dorsal horn neurons (Bonin et al., 2011). Following peripheral nerve injury, there is a selective reduction in the protein expression of spinal δ subunit (Iura et al., 2016), implicating a diminished GABAergic tonic inhibition during pathological pain. The loss of δ-$GABA_A$Rs might contribute to the sensitization of nociceptive behaviors. In formalin tests, δ-deficient mice display increased pain hypersensitivity during the second inflammatory phase (Bonin et al., 2011). 4,5,6,7-Tetrahydroisoxazolol [4,5-c] pyridine-3-ol (THIP) (also known as gaboxadol) is a δ-$GABA_A$Rs-specific agonist at low concentrations (Adkins et al., 2001; Brown et al., 2002). A potent antinociceptive action is observed in several animal models of pain when δ-$GABA_A$Rs activities are enhanced by systematic or intrathecal application of THIP (Krogsgaard-Larsen et al., 2004; Rode et al., 2005, 2007; Asiedu et al., 2012). The mechanisms for THIP to relieve pain might involve the inhibition of action potential discharge in spinal dorsal horn neurons (Bonin et al., 2011). However, it remains uncharacterized as yet whether extrasynaptic δ-$GABA_A$Rs regulate the nociceptive transmission from periphery onto superficial dorsal horn neurons. The current study recorded primary afferent C fiber-evoked field potential in rats and tested the possible role of δ-$GABA_A$R in the modification of nociceptive transmission and plasticity.

EXPERIMENTAL PROCEDURES

Animals, intrathecal administration and behavioral tests

Animal experiments were conducted in accordance to the guidelines of the Animal Care and Use Committee of Lanzhou University. We purchased total 214 male adult Sprague-Dawley rats and 24 Kunming mice (8–12 weeks of age) from the Experimental Animal Center of Lanzhou University. Two to three animals were housed per cage with food and water available *ad libitum*. To administrate drugs intrathecally (Hylden and Wilcox, 1980), the animals were restrained by a pelvic girdle and a 30-gauge needle linked to a 25-μl microsyringe was inserted between L5 and L6 vertebra. The drugs in a volume of 10 μl (for rats) or 5 μl (for mice) were then injected slowly. For behavioral tests, the animals were adapted to the testing environment for at least 1 h before formalin (for rats: 2.5% in 50 μl; for mice: 1.5% in 20 μl) was subcutaneously injected into the plantar surface of left hindpaw (Liu et al., 2008). Immediately after injection, the animal was returned to the chamber and spontaneous painful behaviors were observed for 1 h. The mechanical hyperalgesia was examined before and after formalin injection into the dorsal surface of right hindpaw (Bravo-Hernandez et al., 2016). The calibrated Von Frey monofilament 250 mN (Stoelting, Wood Dale, IL, USA) was applied perpendicularly to the base of the third toe on the plantar surface for 10 times to determine the mechanical withdrawal frequency. We averaged the withdrawal frequencies after three trials. The reflexes for righting, placing and grasping were tested at 60 min after intrathecal drug application (Tao et al., 2008; Suo et al., 2013). In the righting reflex test, the rat was made to lie on the back on a table surface. We recorded if the animal could resume the normal upright position immediately. In the grasping reflex test, we put the rat on a wire grid inclined at 90° to see if the rat could grasp the wire for 30 s. The placing reflex was examined by drawing the dorsal surface of one hind paw to touch the edge of a table. The animal should reflexively place the hind paw onto the table surface. Each reflex was tested for 6 times, and the number of normal reflexes was recorded as the reflex score. All the experiments were conducted blindly by the researchers.

Electrophysiological recordings

The rats were anesthetized deeply by intraperitoneal injection of urethane (1.5 g kg^{-1}). The trachea was cannulated to allow autonomous respiration. The right femoral artery and vein were cannulated for blood pressure monitoring and saline infusion (0.8–1 ml h^{-1}), respectively. After a laminectomy, the animal was fixed in a stereotaxic instrument. The left sciatic nerve was exposed and covered with warmed paraffin oil. We formed an agarose pool around the exposed spinal segment in order to focally administrate drugs. When the dura mater was longitudinally incised, the artificial cerebrospinal fluid (ACSF; in mM: 119.0 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.0 NaH_2PO_4, 26.0 $NaHCO_3$, 11.0 D-glucose, pH 7.4) was added into the agarose pool. THIP (Gaboxadol hydrochloride; Sigma, St. Louis, MO) was dissolved in ACSF before use. The glass recording pipettes were filled with the internal fluid containing (mM) 135.0 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$ and 10.0 HEPES (pH 7.4), and had the resistance of 2–3 MΩ (Ikeda et al., 2006; Drdla-Schutting et al., 2012). A silver hook electrode was used to deliver testing stimuli (monophasic square pulses, 25 V, 0.5-ms duration) onto sciatic nerves at a 60-s interval, and the C fiber-evoked field potentials were recorded at the depth of 100–300 μm from the surface of spinal cord (Drdla-Schutting et al., 2012). Low frequency stimulation (60 V, 0.5-ms duration, 2 Hz, 2 min) was used to induce long-term potentiation (LTP) of C fiber transmission (Drdla-Schutting et al., 2012). The synaptic responses were amplified by a DAM50 extracellular amplifier (WPI, Sarasota, FL, USA), filtered by using a bandwidth of 0.1–1000 Hz and sampled at 5 kHz. A feedback-controlled heating pad was used to keep the colorectal temperature at 37–38 °C during the entire experiment.

Subcellular fractionation and Western blot

The rats were anesthetized deeply by intraperitoneal injection of sodium pentobarbital (60–90 mg kg^{-1}). After a laminectomy, the spinal cord was quickly removed into ice-cold ACSF bubbled with 95% O_2/5% CO_2. The dorsal quadrant of spinal cord was dissected out, divided into the left and right side, and homogenized in ice-cold Lysis buffer (in mM: 10.0 Tris·HCl, pH 7.6,

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