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Involvement of glycine receptor α1 subunits in cannabinoid-induced analgesia

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ABSTRACT

Some cannabinoids have been shown to suppress chronic pain by targeting glycine receptors (GlyRs). Although cannabinoid potentiation of α3 GlyRs is thought to contribute to cannabinoid-induced analgesia, the role of cannabinoid potentiation of α1 GlyRs in cannabinoid suppression of chronic pain remains unclear. Here we report that dehydroxylcannabidiol (DH-CBD), a nonpsychoactive cannabinoid, significantly suppresses chronic inflammatory pain caused by noxious heat stimulation. This effect may involve spinal α1 GlyRs since the expression level of α1 subunits in the spinal cord is positively correlated with CFA-induced inflammatory pain and the GlyRs antagonist strychnine blocks the DH-CBD-induced analgesia. A point-mutation of S296A in TM3 of α1 GlyRs significantly inhibits DH-CBD potentiation of glycine currents (I_{Gly}) in HEK-293 cells and neurons in lamina I-II of spinal cord slices. To explore the *in vivo* consequence of DH-CBD potentiation of α1 GlyRs, we generated a GlyRα1^{S296A} knock-in mouse line. We observed that DH-CBD-induced potentiation of I_{Gly} and analgesia for inflammatory pain was absent in GlyRα1^{S296A} knock-in mice. These findings suggest that spinal α1 GlyR is a potential target for cannabinoid analgesia in chronic inflammatory pain.

1. Introduction

Cannabis has been used to relieve chronic pain in human for centuries (Johnson et al., 2010; Martin-Sanchez et al., 2009; Murray et al., 2007). Both Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), the major psychoactive and nonpsychoactive components of cannabis (Wang et al., 2008), are found to produce analgesic effects in chronic pain (Costa et al., 2007; Johnson et al., 2013). While the primary action site of THC in brain is cannabinoid receptor type 1 (CB1) (Kawamura et al., 2006), emerging evidence suggests that some of THC-induced analgesic effects are independent of CB1 receptors (LedentOV et al., 1999; Costa et al., 2005). For instance, the analgesic effect of THC remained intact in the tail-flick reflex (TFR) test in CB1 receptor knock-out mice (Wilson and Nicoll, 2002), and this THC-induced effect was not affected by intrathecal injection of a selective CB1 receptor antagonist either (Hohmann et al., 2005). In addition, CBD could also induce analgesic effects without activating CB1 receptors (Costa et al., 2007; Izzo et al., 2009; Long et al., 2006). These observations suggest that there are additional targets other than CB1 receptors contributing to cannabis analgesia.

Emerging evidence has suggested that some cannabinoids can directly or indirectly act on the glycine receptor (GlyR), an inhibitory ion channel, through a CB receptor-independent mechanism in the central nervous system (Anderson et al., 2009; Hejazi et al., 2006; Lozovaya et al., 2005; Xiong et al., 2011). Molecular cloning

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has identified four isoforms of the GlyR α subunit (α1-4) and a single isoform of the β subunit (Betz and Laube, 2006). In general, these GlyRs are functional as pentameric assemblies containing homomeric α subunits or heteromeric α/β subunits (Lynch, 2009). In adults, α1 and α3 are the most abundant GlyR subunits in the spinal cord (Shiang et al., 1995; Robert Harvey et al., 2004). The α1 GlyR is considered to be critical for neuromotor activity since several naturally occurring point-mutations that cause functional deficiency of α1 GlyRs are closely associated with hyperekplexia, an exaggerated startle disease, in human (Zhou and ChillagNigro, 2002). Meanwhile, the α3 GlyR, which is abundantly expressed in the superficial laminae of spinal cord dorsal horn, plays an important role in the suppression of inflammatory-induced hypersensitivity to pain (Robert Harvey et al., 2004).

Previous studies have demonstrated that cannabinoid potentiation of GlyR α3 subunits contributes to cannabinoid-induced analgesia. For instance, Dehydroxylcannabidiol (DH-CBD), a chemically modified cannabinoid, can alleviate acute pain and chronic inflammatory pain by targeting spinal α3 GlyRs (Xiong et al., 2012a). Additionally, cannabinoids can also act on GlyR α1 subunits (Foadi et al., 2010; Xiong et al., 2012b), which are localized in brain areas participating in pain transmission such as brainstem and spinal cord (Baer et al., 2009; McDearmid et al., 2006; Piechotta et al., 2001), suggesting that GlyR α1 subunits may also be involved in cannabinoid analgesia. However, it's very challenging to directly explore the role of α1 GlyR in the sensation of pain because of the lack of subunit-specific agonists and antagonists. Genetically engineered animal models have been used and shown to be valuable for identifying the physiological roles of targeted genes *in vivo*. The previous studies revealed that a serine at 296 (S296) in the third transmembrane domain of the α1 GlyR is critical for cannabinoid potentiation (Xiong et al., 2011). To investigate the role of cannabinoid-α1 GlyR interaction in chronic pain, we have generated an GlyRα1^{S296A} mutation knock-in mouse line. Using electrophysiological recordings and behavioral measurements, we examined the efficacy of DH-CBD potentiation of I_{Gly} in spinal slices of these mutant mice and their sensitivity to DH-CBD-induced analgesic effect in chronic pain.

2. Methods

2.1. Animals

C57BL/6 female mice used in the experiments were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) at the age of 8 weeks. A Strain 129SvJ mouse genomic DNA based gene targeting construct (see Fig. 5) was used to convert serine at position 296 to alanine and to introduce a silent mutation that adds a NdeI restriction site for genotyping. This construct was linearized and transfected into R1 mouse embryonic stem cells (Nagy et al., 1993) as previously described (Homanics et al., 1997). G418 resistant embryonic stem cell clones were analyzed for gene targeting by Southern blot analysis (details available from G.E.H.). Correctly targeted clones were microinjected into C57BL/6J (Jackson Laboratory, Bar Harbor, ME, USA) blastocysts to produce germline competent chimeric founder mice. Chimeric males were bred to C57BL/6J females to produce mice that harbored the Aneo allele (see Fig. 5B). Aneo animals were subsequently crossed to C57BL/6J FLPe expressing transgenic mice (Rodriguez et al., 2002) to remove the drug selectable neomycin cassette. The FLPe transgene was removed from the pedigree by backcrossing to C57BL/6J. Heterozygous (S/A) breeders were intercrossed to produce wild type (S/S), heterozygotes, and homozygous knock-in (A/A) animals. Genotyping was performed on tail snips using following primers: forward:5′-GAATCTTCCAGGCAACATTTCAG-3′, reverse:5′-AGTATCCCACCAAGCCAGTCTTT-3'. After weaning, mice were housed in an animal room with ad libitum access to rodent chow and water with 12-h light/dark cycles (lights on at 8:00 a.m.). All mice were between 8 and 12 weeks of age for behavioral experiments. Each mouse was used for only one experiment, and all mice were DH-CBD-naïve at the start of each experiment. Only female mice were used for experiments. Male mice are not applicable in hot-plate test because that the skin of scrotum of male mice is more sensitive than the paw to thermal pain (Bölcskei et al., 2010). All experiments were approved by the Institutional Animal Care and Use Committee at each university and conducted in accordance with the University of Science and Technology of China guidelines with regard to the use of animals in research.

2.2. Hot-plate test

Experiments were conducted during the light cycle (8 a.m.–8 p.m.) in a separated and air conditioned (22 °C) testing room. The hot-plate test was performed by using an electronically controlled 25 * 37 cm hotplate analgesia meter (Hot/Cold Plate; Ugo Basile, Italy) heated to 52 °C (±0.1 °C). The female mice were individually placed on the hot plate apparatus, and the latency of hind paw-flutter, -lift or -lick was taken as the index of nociceptive threshold. This was simultaneously registered by two observers. The animals were taken off immediately after the first reaction or after a maximum time of 30 s. After each test the arena was cleaned with 75% ethanol.

2.3. CFA-induced inflammation

First, animals were tested for nociceptive threshold in the hot-plate test. Following the establishment of baseline latencies all mice received a subplantar injection of 20 μL CFA (1:4 in saline) or vehicle controls (saline). Mice were re-tested for changes in nociceptive threshold before and 4 h, 24 h and 72 h after subplantar injection.

2.4. Startle reflex test

The mice were placed in the chamber of the Med Associates Startle Reflex System (Med Associates Inc.) and allowed to habituate for 5 min. Then the mice were given a test to measure their startle responses using 30 pseudorandom white noise startle stimuli (10 per intensity, 85 dB, 90 dB or 95 dB) with a variable 58–63s inter-trial interval (ITI).

2.5. Rotarod test

To test motor coordination and equilibrium, animals were placed on the rotating rod of the rotarod apparatus (XR1514, Xinruan Inc., Shanghai, China) on two successive days. Before the first training sessions, the mice were habituated staying on the stationary rod for 2 min. A total six trials of the rotarod test were carried out using an accelerating protocol from 4 to 60 rpm in 300s with 20 min ITI. Three training sessions every day were performed for 2 days. The latency (sec) to slip off the rod was automatically recorded (maximal trial duration: 300 s). After falling, the mice were immediately placed back into the home cage. When finished, the apparatus and testing area were cleaned with 75% ethanol.

2.6. Western blotting

The spinal cord tissues of lumbar level L3 to L5 from mice with CFA inflammation or vehicle controls were homogenized and sonicated followed by centrifugation at 10000g, 4 °C for 30 min. The