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Research report

Mu-opioid receptor knockout mice are more sensitive to chlordiazepoxide-induced anxiolytic behavior

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ABSTRACT

We have previously demonstrated benzodiazepine binding in the cortex and hippocampus of mu-opioid receptor knockout (KO) mice. It is known that benzodiazepine receptors are involved in regulating anxiety-like behaviors. Thus, the present study was designed to examine whether there are changes in anxiety-like behavior in mice lacking mu-opioid receptors. To produce anxiolytic activity (less anxiety), the prototype benzodiazepine receptor agonist chlordiazepoxide (CDP, 5 mg/kg) was intraperitoneally administered in wild type (WT) and mu-opioid receptor KO mice. We found that compared to WT mice, mu-opioid receptor KO mice showed enhanced anxiolytic activity to CDP, including increased number of entries into open arm, increased percentage of the time spent in open arms, and decreased percentage of the time spent in enclosed arms in the elevated plus-maze test. We also assessed protein expression of the gamma-aminobutyric acid (GABA) synthetic enzyme (glutamic acid decarboxylase; GAD). Western blotting data indicated that neither the lack of mu-opioid receptors nor CDP treatment altered cortical or hippocampal GAD_{65} or GAD_{67} protein expression. These data indicate that compared with WT, mu-opioid receptor KO mice experienced less anxiety and exhibited enhanced anxiolytic activity to CDP treatment, and these effects were not dependent on GAD_{65} or GAD_{67} protein expression. Our previous and present data suggest that the anxiolytic activity displayed in mu-opioid receptor KO mice is associated with upregulation of the benzodiazepine receptor system.

1. Introduction

Opioids modulate neural processes that are essential to anxiety-related behaviors (Asakawa et al., 1998; Good and Westbrook, 1995). It was previously reported that morphine induces anxiolytic effects after both peripheral (Shin et al., 2003) and central (Good and Westbrook, 1995) administration. In addition, intracerebroventricular administration of endomorphine 1, a mu-opioid receptor agonist, increases anxiolytic action in mice as assessed by the elevated plus-maze test (Asakawa et al., 1998). Systemic administration of the non-specific opioid antagonist naltrexone increases anxiety in rats (Zhang et al., 1996). However, intrahippocampal administration of naloxone, another non-selective opioid receptor antagonist, increases anxiolytic-like behavior (Solati et al., 2010). Although it is known that the mu-opioid receptor system is involved in regulating anxiety-like behavior, their exact role remains unclear.

Gamma-aminobutyric acid (GABA)-mediated systems are involved in anxiety management (Trincavelli et al., 2012). Intrahippocampal injections of $GABA_A$ receptor agonists, such as muscimol, have an anxiolytic-like effect (Ashabi et al., 2011). Blockade of $GABA_A$ receptors using antagonists like bicuculline reduces anti-anxiety in rats (Kataoka et al., 1991). The available evidence indicates that mu-opioid receptors are expressed in GABAergic neurons in many regions, such as the cortex (Liao et al., 2005), hippocampus (Liao et al., 2005; Zieglgansberger et al., 1979), amygdala (Sugita and North, 1993), and throughout the central nervous system, and opioids exert numerous effects by affecting GABA release (Kalyuzhny and Wessendorf, 1997). Activation of

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mu-opioid receptors by morphine decreases presynaptic GABA release, which inhibits adenylate cyclase in hippocampal slices (Uchida et al., 1995). Thus, mu-opioid receptors may regulate anxiolytic behavior via GABA-mediated synaptic transmission.

$GABA_A$ receptors contain 3 types of binding sites for GABA (muscimol), benzodiazepine, and chloride ions (Miller and Lonstein, 2011). We previously demonstrated compensatory upregulation of [^{3}H]-flunitrazepam (native type 1 benzodiazepine) binding in the hippocampus of mu-opioid receptor knockout (KO) mice (Tien et al., 2007). GABAergic receptor systems modulate anxiety-like behavior, especially through benzodiazepine binding sites (Robertson, 1980; Trincavelli et al., 2012). Chlordiazepoxide (CDP) was the first benzodiazepine to be synthesized in the mid-1950s (Lopez-Munoz et al., 2011), and many others have followed. Here, we used CDP treatment to determine if upregulated $GABA_A$ receptors in mu-opioid receptor KO mice were involved in regulating anxiolytic-like behavior.

GABA is synthesized from the amino acid glutamate by the enzyme glutamic acid decarboxylase (GAD) (Fong et al., 2005). There are 2 isoforms of GAD with molecular weights of 65,000 and 67,000, referred to as GAD_{65} and GAD_{67}, respectively (Erlander et al., 1991). GAD_{65} is associated with anxiety-like behavior in mice; GAD_{65}-deficient mice exhibit increased anxiety-like behavior in open field and elevated zero-maze tests (Kash et al., 1999). Although there is no direct evidence for a role of GAD_{67} in anxiety-like behavior, this isoform is found in the cell body and is responsible for maintaining basal GABA levels (Kash et al., 1999). We investigated anxiety-like behavior and cortical and hippocampal GAD_{65} and GAD_{67} levels in mu-opioid receptor KO and wild type (WT) mice.

2. Materials and methods

2.1. Chemicals

CDP hydrochloride was purchased from Sigma (St. Louis, MO, USA) and dissolved in saline immediately before use. Thirty minutes before performing the behavioral test, mice in the experimental groups received a single intraperitoneal (i.p.) injection of 5 mg/kg CDP. Our preliminary study indicated that for WT and mu-opioid receptor KO mice, mice treated with 5 or 10 mg/kg CDP spent more time in the open arm during the elevated plus-maze test than those treated with 2.5 mg/kg of CDP. Control animals received an equivalent volume of saline.

2.2. Animals

Mu-opioid receptor KO mice were provided by Prof. John Pintar (Robert Wood Johnson Medical School, Piscataway, NJ, USA) and were bred in Dr. P.L. Tao's laboratory. These mice were developed by disrupting exon-1 of the mu-opioid receptor-1 gene through homologous recombination as described previously (Schuller et al., 1999). WT mice were of the parental C57BL/6J strain. All mice used in this study were male and 12–16 weeks old. The mice were randomly divided into groups of 8 mice each and maintained in a laboratory on a 12-h light/dark cycle at a constant temperature of 22 ± 2 °C. All procedures for animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Fu Jen Catholic University, Taiwan, ROC.

2.3. Elevated plus-maze test

The elevated plus-maze test was used to assess anxiety behavior (Agmo and Belzung, 1998; Ku et al., 2011). The elevated plus-maze was a modified form of that used by Lister (1987) and consisted of 2 open arms (30 cm × 5 cm × 0.5 cm; $L \times W \times H$) and 2 closed arms (30 cm × 5 cm × 15 cm; $L \times W \times H$) with an open roof and arranged so that 2 pairs of identical arms were opposite each other. Arms emerged from a central platform (5 cm × 5 cm), and the entire apparatus was raised to a height of 40 cm above the floor. Mice received the test compound and were placed individually in the center of the maze, facing one of the open arms. Each test lasted 5 min, and each mouse was tested only once (Lister, 1987). We recorded the numbers of open and enclosed arm entries (arm entry is defined as all 4 paws into an arm), the total time each animal spent in various sections of the maze (open arms, center, and enclosed arms), and the total distance traveled in 5 min. The results were expressed as the number of open or enclosed arm entries and the percentage of time spent in open or enclosed arms (time spent in open or enclosed arms divided by the sum of time spent in both arm types). The drugs were administered i.p. before performing the tests. Room darkness was adjusted at 80–100 lux using a luminescent meter. All tests were conducted between 9:00 AM and 4:00 PM.

2.4. Brain sample preparation

The mice were euthanized by rapid decapitation after i.p. injection of tribromoethanol (0.2 ml/g body weight) (Sigma, St. Louis, MO, USA). Brain samples were obtained after decapitating the mice. The brains were removed from the skulls, and the cortex and hippocampus were immediately separated by gross dissection using mouse atlases (Paxinos and Franklin, 2001). All tissue was stored at −30 °C until use.

2.5. Western blot analysis

Immunoblotting assays were performed according to our previously described method (Ku et al., 2011) with modifications. Brain samples were homogenized in buffer (10 mM Tris–HCl), pH 7.4, 1 mM ethylenediaminetetraacetic acid, 300 mM sucrose, 0.5 mM dithiothreitol, 1 mM benzamidine, 0.3 mM phenylmethylsulfonyl fluoride, and a protease inhibitor (Roche, Mannheim, Germany), and protein levels were determined with the Bradford method (Bio-Rad, Hercules, CA, USA). Protein samples (6–12 μg/μl) were heated for 5 min at 95 °C after 1:2 (v/v) dilution in Laemmli Sample buffer (Bio-Rad) containing 5% (w/v) β-mercaptoethanol. Equal amount of protein (10–20 μg/10 μl) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 1 h at 120 V. Proteins were transferred to nitrocellulose membranes (Bio-Rad) for 1 h at 100 V. Nonspecific binding was blocked with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Primary antibodies were diluted to 1:1000 for GAD_{65} (sc-5601; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 1:2000 for GAD_{67} (ab-52249; Abcam, Cambridge, UK) in blocking buffer overnight at room temperature. An antibody against β-actin antibody (1:10,000; Sigma) was used as a reference to ensure equal loading. Blots were washed and incubated in goat anti-rabbit IgG linked to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:1500 in PBS containing 0.05% Tween 20 for 1 h at room temperature. The immunoreaction product was visualized by chemiluminescence on Hyperfilm (Amersham Pharmacia, Piscataway, NJ, USA).

2.6. Data quantification and statistical analysis

The behavioral data were analyzed by two-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls multiple comparison tests. Alterations in CDP-stimulated anxiolytic behaviors (less anxiety) in WT or mu-opioid receptor KO mice are presented as the mean ± SEM and were analyzed by Student's t tests. Quantified Western blot data were analyzed by two-way ANOVA followed by post hoc Student–Newman–Keuls multiple comparison tests. For all data, the results are presented as mean ± standard error of the mean (SEM), and differences were considered significant at $p < 0.05$.

3. Results

3.1. Mu-opioid receptor KO mice exhibit enhanced anxiolytic activity following CDP stimulation

The elevated plus-maze test was used to assess anxiety behavior (Agmo and Belzung, 1998; Schmitt et al., 2002); it is based on rodents' natural tendencies to avoid open spaces, and it does not contain any experimenter-controlled aversive element. To investigate potential consequences of lack of mu-opioid receptors on GABAergic functions, CDP was administered to either WT or mu-opioid receptor KO mice. CDP is a GABAergic drug and induces anxiolytic activity (less anxiety). Similar to previous reports (Cryan et al., 2004; Garcia et al., 2011), we found that CDP treatment increased plus-maze open arm exploration without modifying locomotor activity in the plus-maze enclosed arm (Fig. 1A). Two-way ANOVA of the elevated plus-maze test data following CDP injections showed that absence of the mu-opioid receptor gene, CDP treatment, and the interaction of absence of the mu-opioid receptor gene × CDP treatment had significant effects on the number of open arm entries [$F(1, 28) = 33.181$, $p < 0.001$; $F(1, 28) = 27.603$, $p < 0.001$; $F(1, 28) = 4.363$, $p = 0.046$, Fig. 1A], the percentage of total time spent in open arms [$F(1, 28) = 35.201$, $p < 0.001$; $F(1, 28) = 30.978$, $p < 0.001$; $F(1, 28) = 5.302$, $p = 0.029$, Fig. 1B], and the percentage of total time spent in enclosed arms [$F(1, 28) = 35.201$, $p < 0.001$; $F(1, 28) = 30.978$, $p < 0.001$; $F(1, 28) = 5.302$, $p = 0.029$, Fig. 1B].