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SEX DIFFERENCES IN U50,488H-INDUCED PHOSPHORYLATION OF P44/42 MITOGEN-ACTIVATED PROTEIN KINASE IN THE GUINEA PIG BRAIN

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Abstract—Recently there has been a widespread interest in the development of kappa opioid receptor (KOPR) ligands for treatment of pain, depression and anxiety, and prevention of stress-induced drug relapse. However, most of these preclinical studies have been conducted using male experimental animals. In the present study we examined if sex differences exist in neural activity induced by the KOPR agonist trans-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide methanesulfonate (U50, 488H). Here, we used immunohistochemistry to detect activation (phosphorylation) of p44/42 mitogen-activated protein kinase (MAPK) as an indicator of neural activity. Following habituation to injection for 3 days, adult guinea pigs received a single injection of U50,488H (5 mg/kg, s.c.) and perfused 30–45 min later. U50,488H-induced an increase in the number of cells immuno-positive for phosphorylated p44/42 MAPK in subregions of the amygdala, thalamus, paraventricular nucleus of the hypothalamus, periaqueductal gray, and dorsal raphe nuclei. In contrast, U50,488H-induced a decrease in immuno-positive cells in the ventrolateral and lateral orbital cortex. Pretreatment with the KOPR antagonist norbinaltorphimine (10 mg/kg, i.p.) 18 h prior to U50,488H significantly reversed the effects of U50,488H in most regions. In addition, we observed a notable sex difference in the basolateral amygdala; in males, U50,488H induced an increase in immuno-positive cell numbers but a decrease in females. However, across other brain regions males were generally more sensitive to U50,488H-induced alterations than females. These results suggest the need to include female subjects in studies examining emotional responses to KOPR ligands.

Key words: kappa opioid receptor, sex difference, guinea pig, p44/42 MAP kinase, neural activity.

INTRODUCTION

The kappa opioid receptor (KOPR) is one of three major types of opioid receptors, which include μ, δ and κ or mu opioid receptor (MOPR), delta opioid receptor (DORP), and KOPR, respectively. Activation of the KOPR produces many effects including analgesia, dysphoria, water diuresis, antiprurisis and attenuates cocaine craving in addicts (reviewed in Liu-Chen, 2004). KOPRs are localized throughout the rostral–caudal axis of the mammalian brain. Radioligand binding studies in rodents reveal that KOPR is enriched in the claustrum (CLA), hypothalamus, endopiriform nucleus, caudate putamen, nucleus accumbens (NAc) and neocortex (Quirion et al., 1987; Mansour et al., 1988; Quirion and Pilapil, 1991; Wang et al., 2011). Of interest, studies indicate that KOPR distribution in the human brain is more similar to the guinea pig brain than to the rat or mouse brain. For example, in guinea pig and human brains, but not the rat or mouse brain, KOPR is abundant in the cerebellum, in deep layers (layers V and VI) of the cortex and is found in striosomes (having patchy distribution) within the striatum (Quirion et al., 1987; Mansour et al., 1988; Quirion and Pilapil, 1991; Wang et al., 2011). Therefore, we consider the guinea pig to be a more appropriate small animal model for KOPR studies.

Early studies by Levine and colleagues (Gear et al., 1996a,b) found that in patients undergoing molar teeth extraction partial KOPR agonists, such as pentazocine, butorphanol and nalbuphine, produced greater analgesic efficacy in women than men. In contrast, studies in rodents and non-human primates have revealed that selective full KOPR agonists produced greater antinociceptive effects in males than females (Craft, 2003; Rasakham and Liu-Chen, 2011 and references therein).

Recently, we demonstrated endpoint-dependent sex differences in behavioral pharmacology of KOPR in

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Abbreviations: ACTH, adrenocorticotrophic hormone; ANOVA, analysis of variance; BLA, basolateral amygdala; BSA, bovine serum albumin; CeA, central amygdala; CLA, claustrum; DAB, 3,3′-diaminobenzidine; DPX, distrene, plasticiser, xylene; DRN, dorsal raphe nuclei; HPA, hypothalamic-pituitaryadrenal; KOPR, kappa opioid receptor; LO, lateral orbital cortex; MAPK, mitogen activated protein kinase; MeA, medial amygdala; MOPR, mu opioid receptor; NAc, nucleus accumbens; NGS, normal goat serum; norBNI, norbinaltorphimine; PAG, periaqueductal gray; PB, phosphate buffer; PFA, paraformaldehyde; PVN, paraventricular nucleus of the hypothalamus; ROI, region of interest; THA, thalamus; U50,488H (U50), trans-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide methanesulfonate; U69,593, (5a,7a,8b)-(−)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)1-oxaspiro (4,5) dec-8-yl) benzeneacetamide; VLO, ventrolateral orbital cortex; VMH, ventromedial hypothalamus.

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guinea pigs (Wang et al., 2011). Males showed greater analgesia/antinociception and abnormal postural changes in response to the KOPR agonist trans-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide methanesulfonate (U50,488H) than females. In contrast, U50,488H inhibited cocaine-induced hyperactivity to greater extents in females than in males. Moreover, anatomical KOPR distribution and receptor-G protein coupling supported the behavioral differences observed between the sexes (Wang et al., 2011). [^{3}H]U69,593, binding studies showed that males had higher levels of KOPR in the periaqueductal gray (PAG), a critical brain region involved in pain. In addition, males showed higher KOPR levels and U50,488H-stimulated [^{35}S]GTPγS binding in several areas related to pain, sedation, and motor control.

Although KOPR distribution and *in vitro* receptor functionality provided support for an anatomical basis for the observed behavioral differences, the downstream effect of KOPR agonist on neural activity has not been examined.

In the present study we aimed to investigate potential sex differences in neural activity induced by the KOPR agonist U50,488H. We use phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) (also known as extracellular regulated kinases 1 and 2, ERK1/2) as an indicator of neural activity *in vivo*. Activation of p44/42 MAP kinase via phosphorylation (P-p44/42 MAPK) has been used as a marker of altered neural activity since the administration of many drugs, including morphine, psychostimulants, and antipsychotic drugs, produce distinct and specific patterns of changes in P-p44/42 MAPK in select brain regions (for example, see Eitan et al., 2003; Valjent et al., 2004). In addition, p44/42 MAPK activity has been shown to underlie many forms of synaptic plasticity, such as learning and memory (Roberson et al., 1999), drug addiction (Valjent et al., 2004) and mu opioid analgesic tolerance (Eitan et al., 2003). It is important to note that enhancement in P-p44/42 MAPK in brain regions after U50,488H is distinctly different from increased P-p44/42 MAPK in cells expressing the KOPR *in vitro*, which typically has an initial peak at 5–10 min (Li et al., 1999; Bohn et al., 2000).

In the present study we use P-p44/42 MAPK as an indicator of neuronal activity, similar to the use of c-Fos expression (Chang et al., 1988; Liu et al., 1994; Bot and Chahl, 1996). Importantly, P-p44/42 MAPK is an ideal target for our studies since, unlike c-Fos, P-p44/42 MAPK activity occurs rapidly and is upstream to the induction of c-Fos expression. Induction of c-Fos expression commonly occurs 2–3 h after drug administration and is more related to long-term adaptive changes.

EXPERIMENTAL PROCEDURES

Animals

Age-matched male and female Dunkin–Hartley guinea pigs (450–600 g) were used for these studies. Animals were housed at the Temple University School of Medicine animal facility and used in compliance with IACUC regulations. Guinea pigs were housed in same sex groups of three per cage (54 × 38 × 28 cm). Animals were allowed to acclimate to animal facilities for at least 1 week prior to any handling and experiments. Animals were placed in a standard rat cage (40 × 20 × 20 cm), two (same sex) per cage, for transport to and from the animal facility on a covered animal transport cart. Animals were allowed to acclimate to the laboratory for at least 90 min prior to handling and remained in the laboratory for at least 1 h prior to returning to the central animal facilities.

Chemicals

The KORP agonist U50,488H and KOPR antagonist norbinaltorphimine (norBNI) was kindly provided by the National Institute on Drug Abuse (NIDA) (Bethesda, MD, USA). Sodium pentobarbital was purchased from Ovation Pharmaceuticals, Inc. (Deerfield, IL, USA) through the Central Animal Facilities at Temple University. Rabbit phosphorylation specific p44/42 (Thr202/Tyr204) MAP kinase antibody was from Cell Signaling (Beverly, MA, USA). Phosphorylation specific p44/42 MAP kinase recognizes only the phosphorylated (activated) form of p44/42 MAPK. Biotinylated goat anti-rabbit IgG, and avidin biotin-peroxidase complex (ABC) were from Vector Labs (Burlingham, CA, USA). 3,3′-Diaminobenzidine (DAB) was from Sigma Chemical Co. (St. Louis, MO, USA). All other reagent-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Drug treatment and perfusion

Guinea pigs were habituated to daily s.c. injections of saline (1 ml/kg) for 3 days (2× per day, between 9:00–12:00 and 16:00–18:00). On the day of perfusion, animals were injected with U50,488H at 5 mg/kg in a volume of 1 ml/kg or 0.9% saline (1 ml/kg). We examined the effect at the dose of 5 mg/kg as this dose produced strong behavioral effects in guinea pigs (Wang et al., 2011). A separate group of guinea pigs were treated with norBNI approximately 18 h prior to U50,488H or saline treatment. Twenty-seven minutes following drug administration, guinea pigs were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) then transcardially perfused approximately 30–45 min after U50,488H injection with 800 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) (pH 7.4). We chose this time point given that peak pharmacological effects at this dose were observed previously in the guinea pig (Wang et al., 2011). Brains were removed and post-fixed in 4% PFA in 0.1 M PB overnight at 4 °C and then incubated at 4 °C in 30% sucrose for 2 days for cryoprotection.

Immunohistochemistry

Coronal sections of the tissue were cut at 50 μm. Free-floating sections were processed by immunohistochemistry immediately after sectioning. Sections were washed 3 × 5 min in 0.01 M PBS. Sections were then treated for 30 min with a solution of 10% methanol and 0.3% H_2O_2 in 0.01 M PBS to inactivate endogenous peroxidases followed by washing in 0.01 M PBS 3 × 5 min and blocking with 5% normal goat serum (NGS)/1% bovine serum albumin (BSA)/0.2%Triton X-100 solution for 1 h at room temperature. Sections were then incubated with rabbit phospho-specific 44/42 primary antibody (1:2000 in 1% NGS/1%BSA/0.2%Triton X-100) overnight on a shaker at 4 °C. The sections were washed 3 × 5 min followed by incubation in secondary antibody, biotinylated goat anti-rabbit IgG (1:100) in 1%NGS/1%BSA/0.2%Triton X-100 for 90 min. Sections were washed and then incubated in ABC (1:100) in 0.01 M PBS for 1 h. Sections were then washed and stained using DAB (0.02%) and 0.0072% H_2O_2 prepared in distilled water. Following staining, tissue sections were rinsed once in distilled water for 5 min and then washed 3 × 5 min in 0.01 M PB. The sections were mounted on subbed slides with 0.01 M PB and air-dried