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Morphine-induced trafficking of a mu-opioid receptor interacting protein in rat locus coeruleus neurons

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ABSTRACT

Opiate addiction is a devastating health problem, with approximately 2 million people currently addicted to heroin or non-medical prescription opiates in the United States alone. In neurons, adaptations in cell signaling cascades develop following opioid actions at the mu opioid receptor (MOR). A novel putative target for intervention involves interacting proteins that may regulate trafficking of MOR. Morphine has been shown to induce a re-distribution of a MOR-interacting protein Wntless (WLS, a transport molecule necessary for secretion of neurotrophic Wnt proteins), from cytoplasmic to membrane compartments in rat striatal neurons. Given its opiate-sensitivity and its well-characterized molecular and cellular adaptations to morphine exposure, we investigated the anatomical distribution of WLS and MOR in the rat locus coeruleus (LC)–norepinephrine (NE) system. Dual immunofluorescence microscopy was used to test the hypothesis that WLS is localized to noradrenergic neurons of the LC and that WLS and MOR co-exist in common LC somatodendritic processes, providing an anatomical substrate for their putative interactions. We also hypothesized that morphine would influence WLS distribution in the LC. Rats received saline, morphine or the opiate agonist [D-Ala2, N-Me-Phe4, Gly-ol5]-enkephalin (DAMGO), and tissue sections through the LC were processed for immunogold–silver detection of WLS and MOR. Statistical analysis showed a significant re-distribution of WLS to the plasma membrane following morphine treatment in addition to an increase in the proximity of gold–silver labels for MOR and WLS. Following DAMGO treatment, MOR and WLS were predominantly localized within the cytoplasmic compartment when compared to morphine and control. In a separate cohort of rats, brains were obtained from saline-treated or heroin self-administering male rats for pulldown co-immunoprecipitation studies. Results showed an increased association of WLS and MOR following heroin exposure. As the LC–NE system is important for cognition as well as decisions underlying substance abuse, adaptations in WLS trafficking and expression may play a role in modulating MOR function in the LC and contribute to the negative sequelae of opiate exposure on executive function.

1. Introduction

Opioids are a class of drugs that are the most effective analgesics known for many types of pain. However, their clinical utility is limited by tolerance and the propensity for addiction. Opioid addiction afflicts approximately 2 million people in the United States (SAMHSA, 2011). Heroin and non-medical use of prescription opioids rank among the top drugs of dependence that contribute significantly to the 193 billion dollars/year cost of addiction (United States Department of Justice, 2011). Worldwide the estimated annual prevalence of illicit opioid use is a staggering 26–36 million or roughly 0.6–0.8% of the total population (UNODC, 2012). Opioid detoxification, substitution, and maintenance paradigms are the current mainstay of treatment but despite these efforts, opioid abuse and related overdose has continued to escalate in the past decade (CDC, 2012). Novel targets for opioid abuse and elucidating the neuronal pathways that perpetuate substance abuse are needed.

Previous studies have reported conflicting results concerning the effect of chronic morphine on MOR density (Castelli et al., 1997; Pert

Abbreviations: WLS, Wntless; MOR, mu-opioid receptor; LC, locus coeruleus; NE, norepinephrine; DAMGO, [D-Ala2, N-Me-Phe4, Gly-ol5]-enkephalin; TH, tyrosine hydroxylase; GPCR, G-protein coupled receptor; GIP, G-protein interacting protein; MORIP, mu-opioid receptor interacting protein; i.c.v, intracerebroventricular; PKC, protein kinase C; GRK, G-protein receptor kinase; GIRK, G-protein inwardly rectifying potassium channel; CREB, cAMP response element-binding protein.

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et al., 1975; Petruzzi et al., 1997). The magnitude of change and inconsistency between reports has led to the hypothesis that tolerance and dependence are not readily explained by changes in receptor density (Fleming and Taylor, 1995). Despite a number of putative mechanisms that have been put forth, current studies have failed to identify any single regulatory mechanism underlying tolerance to opioids (Christie, 2008) suggesting that opioid tolerance is a complex, multifaceted process involving the interplay of multiple regulatory mechanisms occurring both at the cellular and circuit level (Williams et al., 2013). Using fluorescence recovery after photobleaching, the movement of MORs on the plasma membrane was found to be agonist-dependent (Sauliere-Nzeh Ndong et al., 2013). Morphine-bound receptors were more restricted to the membrane, whereas [D-Ala^2, N-MePhe^4,Gly-ol5] enkephalin (DAMGO)-bound receptors either moved freely or were restricted, possibly to clathrin-coated pits. As a partial agonist, morphine does not show significant internalization (Keith et al., 1996; Kovoor et al., 1998; Van Bockstaele and Commons, 2001) compared to etorphine and DAMGO that show robust desensitization and internalization in response to agonist treatment (Blanchet et al., 2003; Bohm et al., 1997; Johnson et al., 2006; Van Bockstaele and Commons, 2001; Virk and Williams, 2008; Wang et al., 2008). The dynamic regulation of MORs at the plasma membrane following acute and chronic administration of opioids may therefore direct downstream signaling (for review see, Williams et al., 2013).

A novel target potentially regulating MOR involves G-protein coupled receptor interacting proteins (GIP) (Bockaert et al., 2004, 2010; Milligan, 2005; Ritter and Hall, 2009). Recently, a MOR interacting protein (MORIP) has been identified, the mammalian ortholog of Drosophila Wntless (WLS)/Evi/Sprinter or GPR177 (Jin et al., 2010a,b) WLS may possibly serve as a substrate underlying alterations in neuronal structure, synaptic organization and molecular adaptations characteristic of opioid dependence (Jin et al., 2010a,b; Reyes et al., 2010a, 2011). WLS contains four (Goodman et al., 2006), seven (Banziger et al., 2006) or eight (Bartscherer et al., 2006) membrane spanning domains and is essential in mediating the secretion of Wnt signaling proteins (Banziger et al., 2006; Bartscherer et al., 2006). We have previously demonstrated with immunoelectron microscopy that WLS and MOR differentially interact after opioid agonist exposure in rat striatal neurons (Reyes et al., 2011). In those studies, morphine caused a marked shift in WLS from the cytoplasm to the plasma membrane, where it co-localized with MOR.

Morphine-induced cellular and molecular adaptations in noradrenergic neurons of the locus coeruleus (LC) have long been recognized and robustly investigated (Williams et al., 2013). The LC is a compact, homogeneous norepinephrine (NE)-containing nucleus that innervates the entire neuraxis through a divergent efferent system. It is the sole source of NE in many forebrain regions that have been implicated in cognition (*e.g.*, cortex and hippocampus (Foote et al., 1983) and its rate of discharge is positively correlated to behavioral and electroencephalographic indices of arousal (Aston-Jones and Bloom, 1981a,b; Berridge and Waterhouse, 2003; Foote et al., 1980; Page and Valentino, 1994). Given the opiate-sensitivity of the LC and the well-defined molecular and cellular adaptations of LC noradrenergic neurons following opiate exposure, we sought to investigate the distribution of WLS with respect to MOR in this brain region. In addition, we also examined whether morphine causes a re-distribution of WLS in LC neurons when compared to saline or DAMGO using high resolution immunoelectron microscopic analysis. Finally, we investigated whether heroin exposure caused an increase in MOR/WLS protein interactions using co-immunoprecipitation analysis.

2. Methods

2.1. Subjects

Twelve adult male Sprague–Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN, USA; 250–270 g) housed two to three to a cage (20 °C, 12-h light, 12-h dark cycle lights on 0700) were used in this study. They were allowed *ad libitum* access to standard chow and water. All procedures were approved by The Institutional Animal Care and Use Committee at Thomas Jefferson University and Penn State College according to the revised *Guide for the Care and Use of Laboratory Animals* (1996), The Health Research Extension Act (1985) and the PHS Policy on Humane Care and Use of Laboratory Animals (1986). All efforts were made to utilize only the minimum number of animals necessary to produce reliable scientific data, and experiments were designed to minimize any animal distress.

2.2. Drug treatment

Adult male rats received intracerebroventricular (i.c.v.) injections of morphine (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 0.9% saline to a concentration of 10 mg/ml and administered at 1.0 μg/kg (n = 5), 0.9% saline in a volume of 25 μl/kg (n = 5) or DAMGO (Tocris Bioscience, Ellsville, MO, USA) at 5 μg/kg body weight (n = 5). Rats were anesthetized with isoflurane (Abbott Laboratories, North Chicago, USA, IL; 0.5–1.0%, in air) *via* a specialized nose cone affixed to the stereotaxic frame (Stoelting Corp., Wood Dale, IL, USA) and placed in a stereotaxic apparatus for surgery. Micropipettes (Kwik-Fil, 1.2 mm outer diameter; World Precision Instruments, Inc., Sarasota, FL, USA) with tip diameters of 20–25 μm were filled with saline, morphine or DAMGO. The tips of the micropipettes were placed at the following coordinates, 3.5 mm posterior from bregma, 1.4 mm medial/lateral, 3.7 mm ventral from the top of the skull. The stereotaxic coordinates of the injection sites were based on the rat brain atlas of Paxinos and Watson (1997). Saline, morphine or DAMGO was injected using a Picospritzer (General Valve Corporation, Fairfield, NJ, USA) at 24–26 psi and over a 10 min period. Pipettes were left at the site of injection for 5 min following drug or vehicle administration.

Thirty minutes following i.c.v. injections of saline, morphine or DAMGO, rats were euthanized. The time of euthanasia post-treatment was selected based on previous studies from our group (Jin et al., 2010a; Reyes et al., 2011; Van Bockstaele and Commons, 2001). Using an *in vitro* technique in MOR expressing human embryonic kidney 293 cells a MOR/WLS complex was detected 1 h following DAMGO treatment (Jin et al., 2010a). Moreover, another *in vivo* study using high resolution electron microscopy in rat brain showed significant MOR internalization 30 min following treatment with the opiate agonist, etorphine (Van Bockstaele and Commons, 2001), as well as with changes in MOR and WLS localization in rat striatal neurons (Reyes et al., 2011). Finally, it is well known that agonist-induced trafficking and up-regulation may occur rapidly from seconds to minutes (Norgauer et al., 1991; Zigmond et al., 1982) and the time point of 30 min was considered to be optimal for detecting changes in trafficking patterns.

2.3. Immunoblotting

Tissues were harvested from adult Sprague–Dawley rats, frozen on dry ice, and thawed prior to homogenization in lysis buffer (Hannan et al., 2008). Brain regions were dissected and lysates prepared immediately after animals were sacrificed. Crude membrane fractions from rat brain lysates were prepared as previously described (Karpa et al., 1999). Membrane fractions were separated on SDS-containing10% polyacrylamide gels, then transferred to polyvinylidine difluoride (PVDF) membranes. Filters were blocked for 2 h in Tris-buffered saline with Tween-20 (TBS-T; 20 mM Tris,pH 7.4; 275 mM NaCl, 3 mM KCl, 1% Tween-20) containing 10% dry milk. Blots were incubated with chicken anti-WLS antibodies (Gene-Tel Laboratories, Madison, WI, USA; 1:5000–1:2500 dilution) for 1 h, then horseradish-peroxidase (HRP)-conjugated donkey-anti-chicken secondary antibodies (1:15,000) for 1 h. Immunoreactivity was detected using an Enhanced Chemiluminescence (ECL) Plus kit (GE Healthcare, Piscataway, NJ).