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CROSSTALK BETWEEN CDK5 AND MEK–ERK SIGNALLING UPON OPIOID RECEPTOR STIMULATION LEADS TO UPREGULATION OF ACTIVATOR P25 AND MEK1 INHIBITION IN RAT BRAIN

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Abstract—Cyclin-dependent kinase 5 (cdk5) participates in opioid receptor signalling through complex molecular mechanisms. The acute effects of selective μ-(fentanyl) and δ-(SNC-80) opioid receptor agonists, as well as the chronic effects of morphine (the prototypic opiate agonist mainly acting at μ-receptors), modulating cdk5 and activators p35/p25 and their interactions with neurotoxic/apoptotic factors, dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) and extracellular signal-regulated kinase (ERK) were quantified (Western Blot analyses) in the rat corpus striatum and/or cerebral cortex. To assess the involved mechanisms, MDL28170 was used to inhibit calpain activity and SL327 to disrupt MEK (ERK kinase)-ERK activation. Acute fentanyl (0.1 mg/kg) and SNC-80 (10 mg/kg) induced rapid (7–60 min) 2- to 4-fold increases of p25 content, without induction of cdk5/p25 pro-apoptotic c-Jun NH_2-terminal protein kinase or aberrant cleavage of poly(ADP-ribose)-polymerase-1, a hallmark of apoptosis. In contrast, fentanyl and SNC-80 stimulated cdk5-mediated p-Thr75 DARPP-32 (+116–166%; PKA inhibition) and p-Thr286 MEK1 (+21–82%; MEK inactivation), and this latter effect resulted in uncoupling of MEK to ERK signals. Calpain inhibition with MDL28170 (cleavage of p35 to p25) attenuated fentanyl-induced p25 accumulation (−57%), but not the stimulation of p-Thr286 MEK1 or p-Thr75 DARPP-32. MEK–ERK inhibition with SL327 fully prevented fentanyl-induced p25 upregulation. Notably, chronic morphine treatment (10–100 mg/kg for 6 days) also increased p25 content and p25/p35 ratio (and activated/inactivated MEK1) in rat brain cortex, which indicated that p25 upregulation persisted under the sustained stimulation of μ-opioid receptors. The results demonstrate that the acute stimulation of opioid receptors leads to upregulation of p25 activator through a MEK–ERK and calpain-dependent pathway, and to disruption of MEK–ERK signalling by a cdk5/p35-induced MEK1 inhibition. Moreover, the effects induced by the sustained stimulation of μ-receptors with morphine suggest the participation of cdk5/p25 complex in opiate-induced long-term neuroplasticity.

Key words: opioid receptors, cdk5, p25/p35, MEK–ERK, SL327, MDL28170.

INTRODUCTION

Protein kinases play a central role in the short- and long-lasting effects induced by opiates and other abused drugs, participating in the acquisition of tolerance, sensitization, and other behavioural hallmarks of drug addiction (Christie, 2008; Lee and Messing, 2008). The serine/threonine cyclin-dependent kinase 5 (cdk5), an atypical cdk member, has gained especial interest for its ability to mediate different forms of structural and behavioural plasticity (Benavides and Bibb, 2004; Angelo et al., 2006; Lai and Ip, 2009; Barnett and Bibb, 2011), including those induced by opiate drugs (Ferrer-Alcón et al., 2003; Narita et al., 2005; Xie et al., 2009). Activation of cdk5 requires its association with p35 cofactor, a non-cyclin regulatory protein mainly expressed in neurons (Tsai et al., 1994; Lew et al., 1994). In turn, calpain activity targets p35 to yield p25 (Kusakawa et al., 2000; Nath et al., 2000), a more potent and long-lasting cdk5 activator (Patrick et al., 1999). Aberrant cdk5/p25 activation was initially associated with neurotoxicity and neurodegeneration (Patrick et al., 1999; Lee et al., 2000; but also see Taniguchi et al., 2001). Recent findings, however, indicate that p25-mediated neurotoxicity is not a key feature of Alzheimer's disease (Engmann et al., 2011). The stimulation of cdk5/p35/p25 by morphine in the rodent brain *in vivo* is also controversial (Ferrer-Alcón et al., 2003; Contet et al., 2008). Recently, cocaine and *d*-amphetamine were shown to stimulate rodent striatal cdk5 through the induction of p25, which may underlie some of the neuroplastic and/or neurotoxic effects of psychostimulants (Meyer et al., 2008; Mlewski et al., 2008).

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Abbreviations: BCA, bicinchoninic acid; cAMP, cyclic AMP; cdk5, cyclin-dependent kinase 5; ctx, cerebral cortex; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DMSO, dimethyl sulphoxide; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; i.p., intraperitoneal; JNK, c-Jun NH_2-terminal kinase or stress-activated protein kinase (SAPK); MAPK, mitogen-activated protein kinase; MEK, ERK kinase; MKP-1, MAPK phosphatase-1; NMDA, *N*-methyl-D-aspartate; p-, phosphorylated; PAC, phosphatase of activated cells; PARP, poly(ADP-ribose)-polymerase-1; PKA, protein kinase A; PP-1, protein phosphatase-1; s.c., subcutaneous; SDS–PAGE, dodecyl sulphate polyacrylamide gel electrophoresis; SEM, standard error of the mean; str, corpus striatum; SW, spontaneous withdrawal; t-DARPP, truncated DARPP-32.

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Beyond the possible neurotoxic effects of p25 (Lee et al., 2000), cdk5/p35/p25 activation may have other important outcomes. Cdk5 phosphorylates Thr75 of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), which results in protein kinase A (PKA) inhibition (Bibb et al., 1999; Yger and Girault, 2011). This cdk5 activity has been shown to regulate dopaminergic and glutamatergic signals, both of which are important in the molecular mechanisms of drugs of abuse including opiates (Yger and Girault, 2011). On the other hand, cdk5 is linked to extracellular signal-regulated kinase (ERK), which is involved in opiate-induced neuroplasticity (Girault et al., 2007; Zhai et al., 2008; Brami-Cherrier et al., 2009). Thus, the cdk5 activator p35 is under the control of ERK-mediated transcription (Harada et al., 2001). In addition, cdk5 negatively regulates ERK activity by phosphorylating the ERK kinase MEK1 at Thr286, which results in MEK inactivation (Sharma et al., 2002; Takahashi et al., 2005). It is well established that μ- and δ-opioid receptors can inhibit cAMP production and induce p-ERK1/2, simultaneously, in cell culture systems (Audet et al., 2008). Although opioid receptors also transduce signals through the activation of the ERK pathway *in vivo*, substantial discrepancies have been reported concerning the acute effects of opiate drugs in the brain. Thus, acute morphine (10 mg/kg, 20–30 min) has been shown to either downregulate or upregulate ERK activation in the mouse nucleus accumbens (Eitan et al., 2003; Valjent et al., 2004). Paradoxically, acute swim stress in rats was reported to induce a marked stimulation of MEK without the concomitant activation of ERK in various brain regions (Shen et al., 2004).

The present study was designed to unravel the existence of functional interactions between cdk5/p35/p25, neurotoxic/apoptotic signalling, DARPP-32/truncated (t)-DARPP, and/or MEK–ERK pathways modulating the acute effects of opiate agonists that selectively stimulate μ- or δ-opioid receptors (i.e. fentanyl and SNC-80, respectively) in the rat brain. The chronic effect of morphine, the prototypic opiate agonist acting at μ-receptors, was also investigated to assess the possible involvement of cdk5/p35/p25 and MEK in opiate dependence and withdrawal. The major findings indicate that opiate agonists rapidly promote the crosstalk of cdk5 and MEK–ERK at two different points of their signalling cascades: first, p25 content is markedly upregulated by a MEK–ERK and calpain-dependent mechanism; second, MEK to ERK signal is attenuated by cdk5/p35-induced MEK1 inhibition. Moreover, chronic morphine also upregulated p25 and activated/inactivated MEK in rat brain cortex, indicating lack of tachyphylaxia to these targets upon the sustained stimulation of μ-receptors. These functional interactions may have important roles in the neuroplasticity induced by opiate drugs.

EXPERIMENTAL PROCEDURES

Animals

A total number of 174 adult male Sprague–Dawley rats (200–240 g) were used (Charles River, Barcelona, Spain). The rats were housed in the animal facilities in methacrylate cages (Panlab S.L., Barcelona) with wood shavings for nesting material (Ultrasorb, Panlab) under controlled environmental conditions of temperature (20 ± 2 °C), humidity (70%) and light/dark cycle (light period: 8:00–20:00), and they had free access to a standard diet (Panlab A04) and tap water. The animals were handled daily for at least 2 days before the neurochemical experiments to reduce stress during drug administrations. All animal care and experimental procedures were conducted according to standard ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the Local Bioethical Committee (UIB). All efforts were made to minimise the number of animals used and their suffering.

Drug treatments

Groups of randomly allocated rats were acutely treated (subcutaneous, s.c.) with the potent μ-opioid receptor agonist fentanyl (0.1 mg/kg) for 7, 15, 30 and 60 min. Other rats were injected (intraperitoneal, i.p.) with the δ-agonist SNC-80 (10 mg/kg) for 15, 30, and 60 min. Control rats received saline (1 ml/kg, s.c., 15–30 min) or dimethyl sulfoxide (DMSO; 1 ml/kg, i.p., 15–30 min), respectively. To assess the specificity of the opioid receptor involved, groups of rats were injected with the non-selective μ-antagonist naloxone (10 mg/kg, i.p.) 60 min before fentanyl (0.1 mg/kg, s.c., 15 min), or with the selective δ-antagonist naltrindole (5 mg/kg, i.p.) 30 min before SNC-80 (10 mg/kg, i.p., 30 min). The acute dose regimens of fentanyl and SNC-80 in rats were similar to those used in previous neurochemical studies (Asensio et al., 2006; García-Fuster et al., 2008a). Other doses of fentanyl (0.01 and 0.03 mg/kg, s.c., 30 min) on brain cdk5/p35/p25 contents were also tested to assess dose–response effects.

To investigate the role of calpain-mediated effects (cleavage of p35 to p25) upon μ-opioid receptor activation, the calpain inhibitor MDL28170 (50 mg/kg, i.p.) was administered alone (60 min) or 45 min before fentanyl (0.1 mg/kg, s.c., 15 min). Other rats received vehicle (DMSO) followed by fentanyl as indicated. Control animals received DMSO plus saline injections. Similar doses of systemically injected MDL28170 (20–50 mg/kg) have been shown to inhibit (40–50%) calpain activity in rat brain (Markgraf et al., 1998; Wang et al., 2008; Thompson et al., 2010). To assess the involvement of ERK in the regulation of cdk5/p35/p25 signalling by fentanyl, the sequential activation of MEK–ERK was disrupted *in vivo* with the compound SL327 (Ramos-Miguel et al., 2010), a brain penetrating and selective inhibitor of MEK1/2 (Selcher et al., 1999). To this end, groups of rats were treated with SL327 (20 mg/kg, i.p.) alone (90 min) or 75 min before fentanyl (0.1 mg/kg, s.c., 15 min). For comparison, other rats received DMSO and, 75 min later, the same dose of fentanyl. Control rats received DMSO plus saline. The dose of SL327 was chosen from previous studies that reported effective brain ERK inhibition in rat striatum and cortex (Selcher et al., 1999; Ramos-Miguel et al., 2010).

Finally, groups of rats were chronically treated with morphine (sustained stimulation of μ-opioid receptors) to assess the modulation of cdk5/p35/p25 and activated/inactivated MEK in opiate dependence and during spontaneous withdrawal (SW). To this end, the rats were injected (i.p.) with morphine three times daily (at 09:00, 15:00 and 21:00 h), during six consecutive days, with escalating doses of the opiate (day 1: 10, 10 and 10 mg/kg; day 2: 10, 20 and 20 mg/kg; day 3: 20, 20 and 40 mg/kg; day 4: 40, 40 and 80 mg/kg; day 5: 80, 80 and 100 mg/kg; day 6: 100 mg/kg). Rats under this treatment protocol developed a strong morphine physical dependence, which resulted in a severe abstinence syndrome following SW (maximum behavioural scores at 24 h) (Ramos-Miguel et al., 2011). Control rats received parallel injections of saline (1 ml/kg, i.p.). After the chronic morphine treatment, the rats were left undisturbed in their home cages for 2 h (basal chronic effect) and 12 h, 24 h, 48 h, 72 h, and 96 h (SW effects). These groups of rats had been used