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MAP kinase activation by mu opioid receptor involves phosphatidylinositol 3-kinase but not the cAMP/PKA pathway

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Abstract The involvement of protein kinases was studied in mu opioid receptor activation of mitogen-activated protein (MAP) kinase using cells transfected with the receptor clone. The cAMP/protein kinase A (PKA) pathway is known to be the major biochemical pathway for mu opioid receptor signaling. However, our data showed that stimulating adenylyl cyclase or activating PKA had no effect on mu receptor enhancement of MAP kinase activity, suggesting that the cAMP/PKA pathway is not involved in mediating the mu receptor activation of MAP kinase. Inhibition of phosphatidylinositol (PI) 3-kinase reduced mu receptor enhancement of MAP kinase activity, suggesting PI 3-kinase involvement. Together, these results show that cross-talk between the mu opioid receptor and the MAP kinase cascade is not mediated by the cAMP/PKA pathway, but involves PI 3-kinase.

Key words: Mu opioid receptor; Mitogen-activated protein kinase; Protein kinase A; Phosphatidylinositol 3-kinase

1. Introduction

Mitogen-activated protein (MAP) kinases are serine/threonine kinases that play an important role in the regulation of cell growth and differentiation. They are activated by growth factor receptors and other tyrosine kinase receptors and they in turn activate various regulatory molecules in the cytoplasm and in the nucleus [1–3]. They have also been shown to be activated by G protein-coupled receptors. A number of protein kinases, including protein kinase A (PKA), protein kinase C (PKC) and protein tyrosine kinase (PTK), are known to be involved in the mechanism by which G protein-coupled receptors stimulate MAP kinase activity [1,4–6]. More recently, phosphatidylinositol (PI) 3-kinase has been reported to participate in G protein-coupled receptor activation of MAP kinase [7,8], further implicating the complexity of protein kinase involvement in mediating the cross-talk between G protein-coupled receptors and the MAP kinase cascade.

Opioids are some of the most effective pain-relieving drugs used in the clinical management of pain [9–11]. In addition to their analgesic effect, opioid peptides and alkaloids also affect a number of physiological functions including hormone secretion, neurotransmitter release, feeding, gastrointestinal motility and respiratory activity [12]. Opioids achieve their effects by activating three types of membrane-bound receptors, mu, delta and kappa [10,13], of which the mu opioid receptor is the major site for commonly used opioid narcotica, including both naturally existing drugs such as morphine and codeine as well as synthetic compounds such as fentanyl and methadone.

The mu opioid receptor is a member of the G protein-coupled receptor family [14,15]. At the cellular level, it exerts two types of effects: a decrease in the intracellular level of cAMP by inhibiting adenylyl cyclase (AC) activity [16,17] and reduction of the neuronal excitability by inhibiting Ca^{2+} channels and activating K^+ channels [18]. These cellular effects induced by mu opioid receptor have been shown to be modulated by protein kinases, including PKA, PKC and Ca^{2+}/calmodulin-dependent protein kinase II [19].

The mu opioid receptor has recently been shown to activate MAP kinase [20,21], involving the activity of both PTK and PKC [21]. However, the pathway by which the mu opioid receptor cross-talks with the MAP kinase is not clear. In particular, it is of interest to determine whether the cAMP/PKA pathway is involved, since it is the major second messenger pathway for the mu opioid receptor [17], and the cloned mu opioid receptor has been shown to couple to the cAMP/PKA pathway either in transiently transfected COS cells [14] or in stably transfected Chinese hamster ovary (CHO) cells [22]. We sought to address this question in this study.

2. Materials and methods

2.1. Cell culture

CHO-K1 cells were stably transfected with the rat mu opioid receptor as previously described [22]. The stably transfected cells (designated as CHO-K1 μ18) were cultured in Ham's F-12 medium (Gibco BRL, Baltimore, MD, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA) in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

2.2. MAP kinase activity assay

Cells were grown to subconfluence in 100 mm diameter dishes, serum-free starved overnight (~12 h) and incubated with the indicated compounds for the indicated time at 37°C. Cells were then washed three times with ice-cold PBS, scraped into 0.5 ml of ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 15 mM NaF, 5 mM EDTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin and 10 μg/ml aprotinin) and homogenized with a Dounce homogenizer (20–30 strokes). The homogenate was centrifuged at $50\,000 \times g$ for 20 min and the supernatant was assayed for MAP kinase activity, using the p42/p44 MAP kinase enzyme assay system (Amersham, Buckinghamshire, UK). Protein concentrations of cell lysate were determined by the method of Bradford [23]. All data are shown as mean ± S.E.M.

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Abbreviations: AC, adenylyl cyclase; CHO, Chinese hamster ovary; MAP, mitogen-activated protein; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; 8-CPT-cAMP, 8-chlorophenylthio-cAMP; PI, phosphatidylinositol

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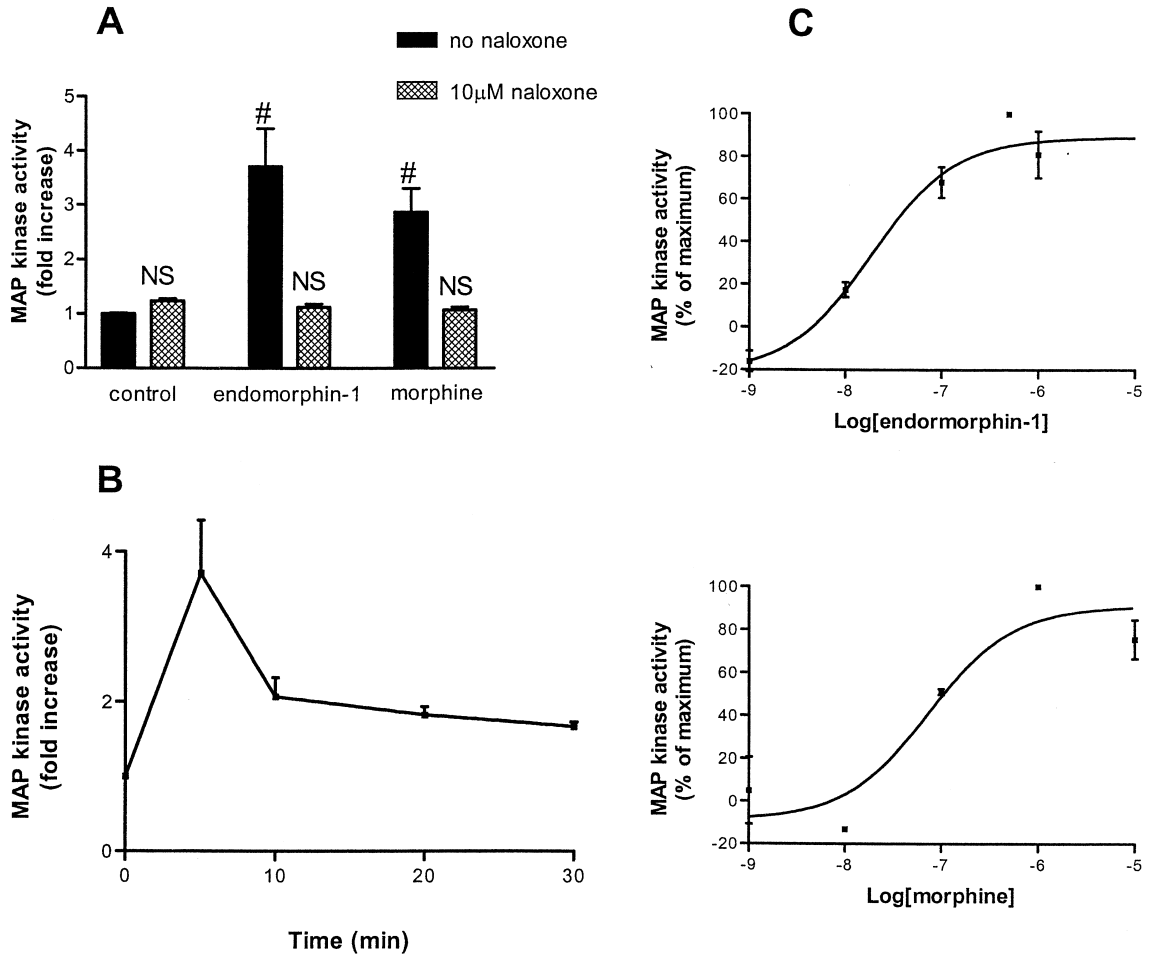

Fig. 1. MAP kinase activation by the mu opioid receptor. (A) Cells were incubated with 500 nM endomorphine-1 or 1 μM morphine for 5 min, with or without 10 μM naloxone. #: Significantly different from control. NS: not significantly different from control. (B) Transient activation of MAP kinase by the mu receptor. Cells were treated with 500 nM endomorphine-1 for the indicated time intervals. (C) Dose-response relations of mu opioid receptor-mediated MAP kinase activation. Cells were stimulated for 5 min by the mu opioid receptor agonists morphine (top panel) or endomorphine-1 (bottom panel) at the indicated concentrations (in molar). EC_{50} values are 80 nM for morphine and 20 nM for endomorphine-1.

3. Results

3.1. Mu opioid receptor-mediated MAP kinase activation

To examine mu opioid receptor-mediated activation of MAP kinase activity, we used CHO-K1 μ18 cells that have been stably transfected with a cloned mu opioid receptor cDNA [22]. For agonists, we utilized both morphine, the plant alkaloid agonist from opium that displays relative selectivity for the mu opioid receptor over other types of opioid receptors [10,24], and endomorphine-1, a recently discovered endogenous peptide that displays high affinity and selectivity for the mu opioid receptor [25] with full agonist activity [26]. Both agonists increased MAP kinase activity over the non-stimulated control level (Fig. 1A) and the stimulatory effects by both agonists were completely blocked by the opioid antagonist naloxone (Fig. 1A). In CHO cells not transfected with the mu opioid receptor, these opioid agonists had no effect on the level of MAP kinase activity (data not shown). MAP kinase activation was rapid and transient, with the increase of MAP kinase activity peaking at about 5 min after the agonist addition before declining toward the baseline level (Fig. 1B). Both endomorphine-1 and morphine showed concentration-dependent stimulation of MAP kinase activation as indicated by their dose-response curves (Fig. 1C). By comparison, endomorphine-1 appeared more potent in stimulating MAP kinase than morphine, with a lower EC_{50} value (20 nM) than that of morphine (80 nM).

3.2. Mu opioid receptor activation of MAP kinase cascade involves a pathway distinct from the cAMP/PKA pathway

In the MAP kinase cascade, MEK, a MAP kinase kinase, is the major activator of MAP kinase activity by tyrosine and threonine phosphorylation of MAP kinases [5]. To verify that the mu opioid receptor activation of MAP kinase activity is mediated by MEK, we used a MEK inhibitor, PD98059 [27], and examined mu receptor-induced MAP kinase activation. As shown in Fig. 2A, the MEK inhibitor PD98059 completely blocked the MAP kinase activation by endomorphine-1, confirming that MEK indeed mediates the mu opioid receptor activation of MAP kinase.

At the cellular level, opioid receptors decrease AC activity, resulting in reduced cAMP levels [17]. The cloned mu opioid receptor has also been shown to negatively couple to the cAMP/AC pathway either in transiently transfected COS cells

[14] and in stably transfected CHO cells [22]. Thus, it is of interest to determine whether the cAMP/PKA pathway is involved in mu receptor-induced MAP kinase activation. As shown in Fig. 2B, AC activator forskolin had little effect on the basal level of MAP kinase activity (forskolin data not significantly different from the control) and it did not appear to affect mu receptor activation of MAP kinase (endomorphine-1+forskolin results not significantly different from the endomorphine-1 alone results). Furthermore, 8-chlorophenylthio-cAMP (8-CPT-cAMP), a membrane-permeable cAMP analogue that can activate PKA, also had no influence either on the basal MAP kinase activity (8-CPT-cAMP data not significantly different from the control) or on mu receptor-induced MAP kinase activity (endomorphine-1+8-CPT-cAMP results not significantly different from the endomorphine-1 alone results). These results indicate that activating the cAMP/PKA pathway neither alters basal MAP kinase activity nor interferes with mu receptor enhancement of MAP kinase activity. These results therefore suggest that the cAMP/PKA pathway is not involved in mediating the mu opioid receptor activation of MAP kinase. In other words, mu receptor activation of MAP kinase involves a signaling pathway distinct from receptor inhibition of the cAMP/PKA pathway.

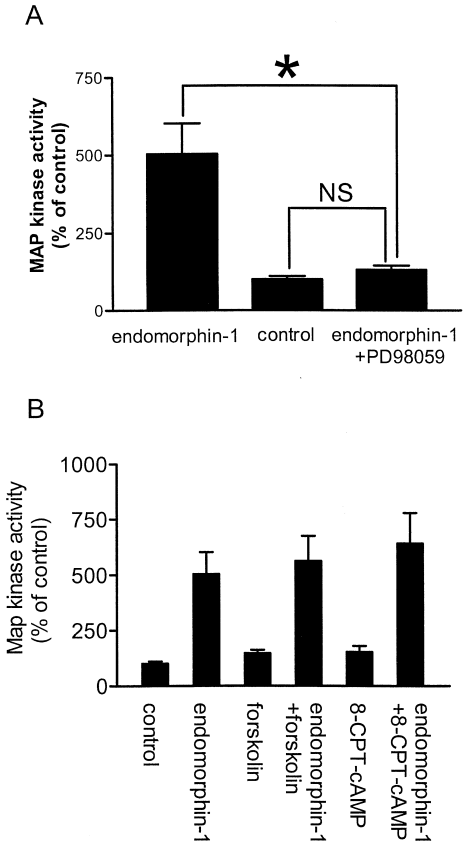

Fig. 2. Mu opioid receptor stimulation of MAP kinase activity involves a pathway distinct from the cAMP/PKA pathway. (A) MEK involvement. Cells were incubated for 5 min with 500 nM endomorphine-1 with or without 15 min pretreatment of the MEK inhibitor PD98059 (100 μM). *: Significantly different ($P < 0.05$), NS: not significantly different. (B) The cAMP/PKA pathway is not involved. Cells were stimulated for 5 min with 500 nM endomorphine-1 with or without 10 min pretreatment of either the AC activator forskolin (30 μM) or the cAMP analogue 8-CPT-cAMP (1 mM).

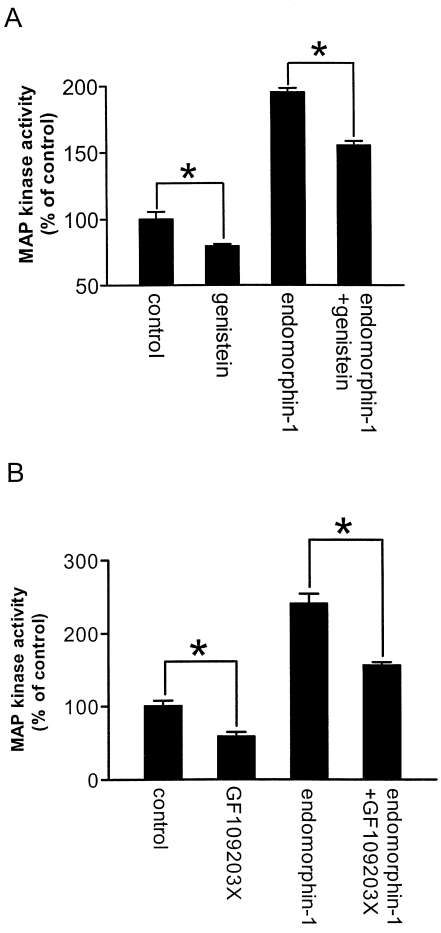

Fig. 3. Both basal- and receptor-mediated MAP kinase activation are modulated by PTK and PKC. (A) PTK effect. Cells were treated with PTK inhibitor genistein (20 μM, 2 h pretreatment) alone or with genistein plus endomorphine-1 (500 nM, 5 min). (B) PKC effect. Cells were incubated with PKC inhibitor GF109203X (1 μM, 4 h pretreatment) alone or with GF109203X plus endomorphine-1 (500 nM, 5 min). *: Significantly different ($P < 0.05$).

3.3. PTK and PKC modulate both basal and receptor-activated MAP kinase activity

Fukuda et al. reported that both genistein, a PTK inhibitor, and GF109203X, a PKC inhibitor, were able to reduce opioid receptor-mediated MAP kinase activation [21]. Using these two protein kinase inhibitors, we confirmed the results of Fukuda et al. in our CHO-K1 μ18 cells (Fig. 3A, right-hand two bars: endomorphine-1+genistein results significantly different from endomorphine-1 alone results; Fig. 3B, right-hand two bars: endomorphine-1+GF109203X results significantly different from endomorphine-1 alone results). Moreover, we observed that without activating the mu opioid receptor, the basal activity of MAP kinase (control) was also significantly reduced by genistein (Fig. 3A, left-hand two bars) or GF109203X (Fig. 3B, left-hand two bars). These results suggest that there is a basal level of endogenous PTK and PKC activity in these cells to stimulate MAP kinase activity, which can be blocked by their respective inhibitors.

3.4. PI 3-kinase is involved in mu opioid receptor activation of MAP kinase

It was recently reported that PI 3-kinase can mediate cer-

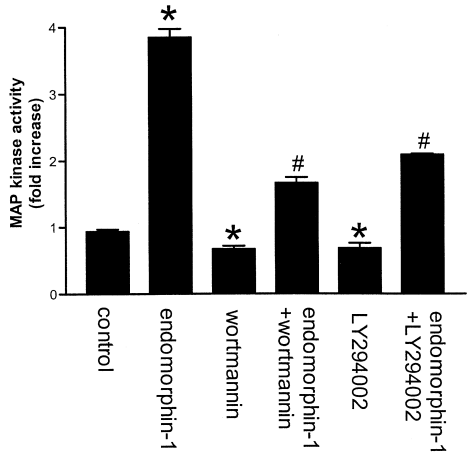

Fig. 4. PI 3-kinase is involved in mu opioid receptor enhancement of MAP kinase activity. Cells were incubated either with a PI 3-kinase inhibitor (100 nM wortmannin or 50 μM LY294002) for 30 min or with 500 nM endomorphine-1 for 5 min with or without 30 min pretreatment of the inhibitors. #: Significantly different from endomorphine-1 treatment cells ($P < 0.05$), *: significantly different from untreated (control) cells ($P < 0.05$).

tain G protein-coupled receptor activation of the MAP kinase signaling pathway [7,8] and that such linkage is provided by $G_{\beta\gamma}$ subunits [8]. To examine whether PI 3-kinase is involved in mediating the mu receptor effect, we employed two structurally distinct inhibitors of PI 3-kinase, wortmannin [28] and LY294002 [29]. We observed that both wortmannin and LY294002 reduced mu opioid receptor-induced MAP kinase activation (Fig. 4). Compared with endomorphine-1 alone treatment, MAP kinase activity for either endomorphine-1+wortmannin treatment or endomorphine-1+LY294002 treatment was significantly decreased. These results indicate that PI 3-kinase is involved in mu opioid receptor activation of MAP kinase. Moreover, either wortmannin or LY294002 alone inhibited the endogenous MAP kinase activity (Fig. 4, data for wortmannin alone or LY294002 alone significantly different from untreated control), suggesting that PI 3-kinase may exert a basal level activation on the MAP kinase.

Table 1
Multiple kinase modulation of MAP kinase activity

Treatment	MAP kinase activity (% of control)
Untreated control	100 ± 9.7
End	209 ± 18
Ge+GF	60.7 ± 9.0
End+Ge+GF	85.0 ± 8.5
Ge+Wor	49.3 ± 4.7
End+Ge+Wor	76.7 ± 7.5
GF+Wor	55.7 ± 7.4
End+GF+Wor	71.7 ± 9.1
Ge+GF+Wor	47.4 ± 1.0
End+Ge+GF+Wor	88.6 ± 16.1

Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 5 min with 500 nM endomorphine-1 with or without pretreatment of two different kinase inhibitors together: 20 μM genistein (2 h), 1 μM GF109203X (4 h) and 100 nM wortmannin (30 min). The respective controls of double-kinase inhibitor treatment alone were also included. MAP kinase activity is expressed as percentage of control. All results are significantly different from control ($P < 0.05$). End: endomorphine-1, Ge: genistein, GF: GF109203X, Wor: wortmannin.

3.5. Multiple kinase modulation in a parallel fashion

Since MAP kinase activation by mu opioid receptor was not completely blocked by the inhibition of any one of the three kinases PTK (Fig. 3A), PKC (Fig. 3B) or PI 3-kinase (Fig. 4), we tested combined inhibition of two kinases. The results are summarized in Table 1. Upon mu receptor activation of MAP kinase, combined inhibition of PTK and PKC with genistein plus GF109203X (End+Ge+GF) brought the MAP kinase level to below the untreated control (85% of control), combined inhibition of PTK and PI 3-kinase with genistein plus wortmannin (End+Ge+Wor) resulted in a reduction to 77% of control and combined inhibition of PKC and PI 3-kinase (End+GF+Wor) yielded 72% of control. In addition, inhibition of any two kinases alone, without endomorphine stimulation of the mu opioid receptor, resulted in a significant reduction of the basal level of MAP kinase activity (Ge+GF at 61% of control, Ge+Wor at 49% of control and GF+Wor at 56% of control). Furthermore, inhibition of all three kinases also resulted in significant reduction of both basal (Ge+GF+Wor at 47% of control) and endomorphine-stimulated (End+Ge+GF+Wor at 88% compared to End alone at 209%) MAP kinase activity (Table 1). Taken together, these results suggest that the three kinases, PI 3-kinase, PTK and PKC, all participate in modulating the MAP kinase activity in these cells.

4. Discussion

The mu opioid receptor is the major cellular target of potent opioid analgesics [10]. It mediates agonist effects via G protein coupling to cellular effectors and cAMP/PKA is the major biochemical pathway for mu opioid receptor signaling [16,17]. It has been reported that stimulation of the mu opioid receptor resulted in an increase in MAP kinase activity [20,21]. However, the signal transduction pathway that leads from stimulation of mu opioid receptor to MAP kinase activation is not clear. Since there is considerable evidence that cAMP plays a role in the regulation of MAP kinase activity, either inhibitory [30,31] or stimulatory [32,33], it would be of interest to determine whether the cAMP/PKA pathway provides the link for mu receptor activation of MAP kinase activity. Our results demonstrated that mu receptor stimulation of MAP kinase activity was not influenced either by activating AC with forskolin or by stimulation of protein kinase A with a cAMP analogue (Fig. 2B). Thus, it appears that the cAMP/PKA pathway is not involved in mu opioid receptor-mediated MAP kinase activation in these cells.

For cross-talk from G protein-coupled receptors to the MAP kinase signaling cascade, there has been evidence for the involvement of a number of protein kinase pathways. For instance, reports have suggested pathways both dependent on and independent of the stimulation of PKC and elevation of cytosolic Ca^{2+} [34,35]. Also, PTKs mediating growth factor effects have been shown to be modulated by heterotrimeric G protein subunits [1,36]. More recently, PI 3-kinase involvement in mediating the signaling from G protein-coupled receptors to the MAP kinase cascade has been shown [7,8], suggesting additional complexity in the cross-talk between the G protein pathway and the MAP kinase pathway. Our results suggest that PI 3-kinase is involved in mediating the mu opioid receptor activation of MAP kinase (Fig. 4), similar to the involvement of PKC and PTK [21]. More-

over, we observed that inhibitors for these three kinases, when applied alone, could not completely block the MAP kinase activation by mu opioid receptor (Figs. 3 and 4), suggesting that these kinases may act in an additive manner to modulate mu receptor enhancement of MAP kinase activity.

In summary, our results indicate that the mu opioid receptor activates MAP kinase by a different pathway from its coupling to the cAMP/PKA pathway. This raises the question as to what extent, if any, the two pathways of the mu receptor action may interact. In this regard, it is interesting to note that because neurons are terminally differentiated cells, 'long-term' mechanisms involved in cell growth and proliferation such as the MAP kinase pathway are not thought to play a major role in the neuronal function. Traditionally, neurotransmitter receptors, including the mu opioid receptor, have been viewed to exert 'short-term' actions on neurons, i.e. modulating neuronal excitability by changing ion channel conductances and by regulating the protein phosphorylation status via kinases such as PKA. With the increasing evidence that many neurotransmitter receptors do activate the MAP kinase pathway, this view may need to be expanded. It is plausible that activation of MAP kinase by these receptors may result in altered cellular processes in the brain, either in glia cells that do proliferate or in neurons to cause 'set point' changes in the cell physiological status.

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