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Morphine-6β-glucuronide has a higher efficacy than morphine as a mu-opioid receptor agonist in the rat locus coeruleus

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1 The pharmacological properties of the active morphine metabolite, morphine-6β-D-glucuronide (M6G), and the parent compound were compared in rat locus coeruleus neurons by electrophysiological recording in brain slices.

2 M6G and morphine activated potassium currents in voltage clamped neurons, which were blocked by the opioid receptor antagonist naloxone.

3 Both M6G and morphine behaved as partial agonists that produced maximal responses smaller than the system maximum, which was measured using [Met^5]-enkephalin. M6G produced a larger maximal response (78%) than morphine (62%), which we estimated was due to a 2–4 fold difference in the relative efficacy of the agonists.

4 3-O-methoxynaltrexone, which has been reported to behave as a selective antagonist of a M6G preferring receptor, was equally effective at blocking currents produced by M6G and the selective mu-opioid receptor agonist DAMGO.

5 M6G currents were occluded by a prior application of morphine, and were reduced when mu-opioid receptors were desensitized by using [Met^5]-enkephalin.

6 Morphine-3β-D-glucuronide did not affect action potential firing or membrane currents in locus coeruleus neurons and had no effect on currents produced by M6G.

7 These results show that the relative efficacy of M6G is higher than morphine in locus coeruleus neurons, contrary to what has been shown using mu-opioid receptors expressed in cell clones.
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Keywords: Mu-opioid receptor; morphine; glucuronide; relative efficacy; 3-O-methoxynaltrexone; locus coeruleus; electrophysiology; analgesia

Abbreviations: DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin; M3G, morphine-3β-D-glucuronide; M6G, morphine-6β-D-glucuronide

Introduction

Glucuronidation, the principle route of morphine metabolism in humans, forms two biologically active metabolites: morphine-6β-D-glucuronide (M6G) and morphine-3β-D-glucuronide (M3G) (Yoshimura *et al*., 1973). M6G binds preferentially to the mu-opioid receptor and has a pharmacological profile that is similar to morphine but M3G binds with around a 300 fold lower affinity to opioid receptors (Löser *et al*. 1996) and causes behavioural effects that are mediated by unknown pharmacological mechanisms.

When administered centrally, M6G can be over 100 fold more potent than morphine at producing antinociception (Pasternak *et al*., 1987; Sullivan *et al*., 1989; Frances *et al*., 1990; 1992; Krzanowska *et al*., 1998). M6G is also more potent than morphine (146 : 1) at producing rewarding effects measured with place preference testing following intracerebral administration (Abbott & Franklin, 1991). Binding studies show that M6G has an equal or lower affinity for the mu-opioid receptor than morphine (Abbott & Palmour, 1988; Chen *et al*., 1991; Hucks *et al*., 1992; Lambert *et al*., 1993; Mignat *et al*., 1995; Löser *et al*. 1996; Brown *et al*., 1997b; Pan *et al*., 1999). In order to explain these differences in the potencies of M6G and morphine, it has recently been hypothesized that M6G has selective antinociceptive and other behavioural agonist effects at a receptor that is not activated by morphine (reviewed by Pasternak & Standifer, 1995). Evidence that this putative receptor is a splice variant of the MOR-1 gene encoding the mu-opioid receptor has come from 'knockdown' studies that have reduced receptor expression by injecting antisense oligonucleotides into the cerebral ventricles. Antisense probes targeted against exons 1 or 4 of the MOR-1 gene selectively reduce antinociception produced by morphine, but not M6G. Conversely, probes targeted against exons 2 or 3 affect antinociception produced by M6G, but not morphine (Rossi *et al*., 1995a; 1996; 1997). More recently the analgesic effects of morphine (but not M6G) were reduced by antisense probes targeted against exons 6, 7, 8 or 9 of the MOR-1 gene (Pan *et al*., 1999). Dissociation of the analgesic effects of M6G and morphine has also been reported in knockout mice that lack either the first or second exon of the MOR-1 gene (although we cannot confirm this result in our laboratory, see Connor *et al*., 1999). It was found that M6G produced analgesia in mice homozygous for the exon 1 but not the exon 2 mutation, whereas morphine did not produce analgesia in mice homozygous for either mutation (Schuller *et al*., 1999). Binding studies using [^{3}H]-M6G have identified two sites in calf and mouse brain: a low affinity site that corresponds to the classical mu-opioid receptor and a high affinity site that has a low affinity for morphine (Brown *et al*., 1997a,b). It has been reported that 3-O-methylnaltrexone can discriminate the high affinity site in competition studies using [^{3}H]-M6G, and when used at low doses blocks analgesia produced by M6G, but not morphine (Brown *et al*., 1997a).

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Interpretation of the evidence supporting a unique receptor that mediates M6G but not morphine analgesia has been influenced by the assumption that there is no difference in the relative efficacy of the two compounds. Although this is supported by functional *in vitro* pharmacological studies that have used clonal cell lines or guinea-pig ileum (Lambert *et al*., 1993; Schmidt *et al*., 1994; Brown *et al*., 1997b), similar studies have yet to be performed on central nervous system neurons. In mice there is a 10 fold difference in the fraction of opioid receptors occupied by doses of M6G and morphine that are equipotent in producing analgesia, which is evidence against the efficacy of these drugs being the same (Frances *et al*., 1992).

Rat locus coeruleus neurons are arguably the most extensively characterized *in vitro* bioassay system used to investigate mu-opioid receptor function in the brain (reviewed by Christie, 1991; Christie *et al*., 1997). Our aim in this study was to make a pharmacological comparison of M6G and morphine by using brain slice recording from locus coeruleus neurons.

Methods

Male Sprague-Dawley rats were used in all of the experiments. For intracellular microelectrode recording experiments we used rats that weighed 150–200 g and were more than 6 weeks old. For patch clamp recording experiments we used rats that were 12–21 days old.

All rats were killed under halothane anaesthesia after which their brains were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.4; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 11; $NaHCO_3$, 25; and equilibrated with 95% O_2 : 5% CO_2. Up to three 300 μm thick slices that included the locus coeruleus were cut in the horizontal plane. These were kept in a holding chamber filled with oxygenated ACSF maintained at 35°C before being used for experiments.

Electrophysiological recordings were made from locus coeruleus neurons using both intracellular microelectrode and whole cell patch clamp techniques. For intracellular microelectrode recording, slices were placed in a chamber (1.5 ml vol^{-1}) mounted under a stereo-dissecting microscope and were continuously superfused (1.5 ml min^{-1}) with ACSF (35°C). The locus coeruleus was visualized using transillumination. Recordings were made with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA, U.S.A.) using microelectrodes filled with 2 M KCl that had resistances of 28–40 MΩ. Membrane currents were recorded in discontinuous voltage-clamp mode, during which the switching frequency (4–5.2 kHz) and capacitance compensation were adjusted by observing the headstage voltage response. This was monitored on a separate oscilloscope to ensure that the voltage transients across the microelectrode decayed fully before voltage sampling.

For whole-cell patch clamp recording, slices were placed in chamber mounted on the stage of an upright microscope and viewed using a water-immersion objective (Zeiss × 40) and infrared-differential interference contrast optics. Recordings were made using an Axopatch 200 A amplifier (Axon Instruments) and patch pipettes (4–6 MΩ) containing (mM): K^+ gluconate, 125; NaCl, 15; $MgCl_2$, 2; ethylene glycol-bis(b-amino-ethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 11; MgATP, 2; guanosine 5′-triphosphate (GTP), 0.25; and N′-2-ethanesulphonic acid (HEPES), 10; adjusted to a pH of 7.3 with KOH and an osmolarity of 280–285 mosmol l^{-1}. Data were used only if the recordings were made when the series resistance was below 25 MΩ.

Recordings from electrophysiological experiments were digitized, acquired and stored on a computer using PClamp or Axotape software (Axon Instruments).

Analysis of data

Concentration-effect curves for opioid agonists were constructed by measuring the steady-state amplitude of agonist currents, which were standardized against the maximal response to [Met^5]-enkephalin. We have shown previously that [Met^5]-enkephalin behaves as a high efficacy full agonist in the locus coeruleus (Osborne & Williams, 1995).

Concentration-effect data from each neuron was fitted to a logistic function of the form:

$$E = M\frac{[A]^n}{[A]^n + k^n} \quad (1)$$

which E and [A] are the pharmacological effect and concentration of agonist respectively, M is the maximum response, k is the EC_{50} and n is the slope parameter.

The operational model of agonism (Black & Leff, 1983; Leff, 1988) was fitted in a modified form (Trzeciakowski, 1999b) to the average concentration-effect curve data sets obtained for M6G and morphine where:

$$E = \frac{E_M\ \tau_{app}\ [A]}{K_{app} + (1 + \tau_{app})[A]} \quad (2)$$

in which E_M is the operational maximum response of the tissue, τ_{app} is the apparent operational efficacy and K_{app} is the apparent dissociation constant for the agonist. As both M6G and morphine behaved as partial agonists relative to [Met^5]-enkephalin, to obtain estimates of K_{app} and τ, concentration-response curves for M6G and morphine were fitted simultaneously with the operational model (equation 2) with E_M constrained to 100% (the maximum response to [Met^5]-enkephalin) (see Leff & Giles, 1992).

Curve fitting was performed using a simplex optimization algorithm implemented using Kaleidagraph or Axograph software. Data means are given with s.e.means.

Drugs used

Stock solutions of [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO, Sigma), [Met^5]-enkephalin (Sigma-Aldrich), 3-O-methoxynaltrexone hydrochloride (NIDA), morphine hydrochloride (Glaxo), morphine-3β-D-glucuronide (NIDA), morphine-6β-D-glucuronide (NIDA), and naloxone (Sigma-Aldrich) were made in distilled water and stored at 4°C. All drugs were applied by superfusion.

Results

Measurements of concentration-effect relationships

Mu-opioid receptor agonists produce outward currents in rat locus coeruleus neurons by opening G protein-coupled inward rectifying (GIRK) potassium channels (Miyake *et al*., 1989; Grigg *et al*., 1996). Figure 1a shows outward currents (V_{hold}: −60 mV) produced in rat locus coeruleus neurons by different concentrations of M6G and a maximally effective concentration of [Met^5]-enkephalin (30 μM), which we have previously shown to behave as a full agonist in this system (Christie *et al*., 1987; Osborne & Williams, 1995).

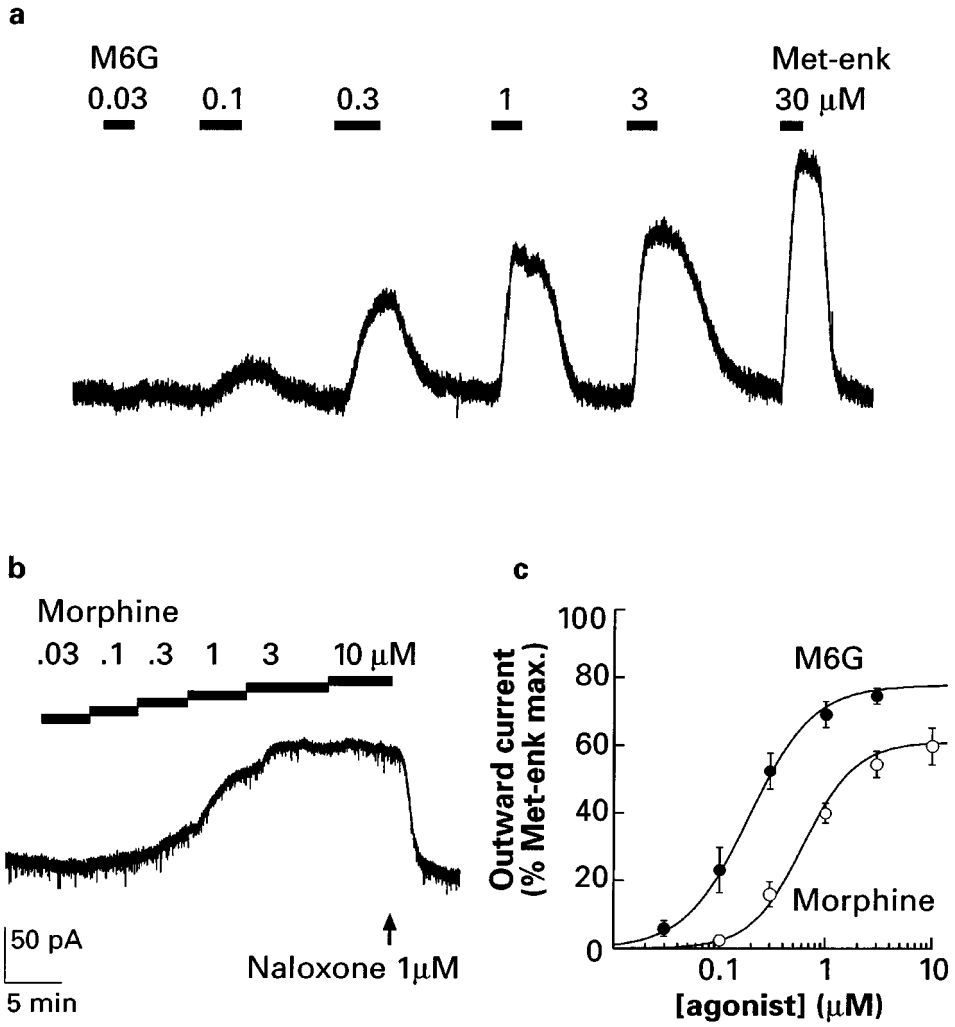

Figure 1 M6G and morphine behave as partial agonists in locus coeruleus neurons. In (a) is shown a continuous voltage clamp recording (holding potential: −60 mV) in which outward currents were produced by different increasing concentrations of M6G and a single maximally effective concentration of [Met^5]-enkephalin. In (b) is shown currents produced by cumulative applications of morphine. Note that the current does not desensitize during a 5 min application of 10 μM morphine and that the current was completely blocked by 1 μM naloxone. In (c) concentration-effect curves for M6G and morphine are shown using data expressed as a percentage of the amplitude of the [Met^5]-enkephalin maximum. In both cases the maxima for both curves are smaller than the system maximum measured with [Met^5]-enkephalin. The curves are also vertically and horizontally displaced from one another. The lines through the data are the average logistic fit. Vertical bars show s.e.mean.

Currents produced by M6G were always smaller than the maximum [Met^5]-enkephalin current. This is illustrated by the concentration-effect relationship for M6G shown in Figure 1c that was obtained by combining experiments that used randomized (one cell), sequential (two cells) and cumulative (two cells) agonist concentration applications (which produced comparable results). The average of the estimates of the maximum response obtained from individual fits of a logistic function (equation 1) to data from five neurons was 77.7±2.19% (of the maximum [Met^5]-enkephalin current). The corresponding estimates of the pEC_{50} and Hill slope were −6.72±0.095 and 1.45±0.168 respectively.

Also plotted in Figure 1c is the average of the concentration-effect relationships that were measured for morphine in five neurons. Due to the prolonged period required for the effects of morphine to reverse with washout all of these experiments were performed using cumulative agonist concentration applications (Figure 1b). From these data the average estimates of the maximum, pEC_{50} and Hill slope parameters were 61±5.6%, −6.21±0.095 and 1.77±0.297 respectively. In comparison to M6G, morphine produced a smaller maximum response (*t*-test: $P=0.024$, 8 d.f.) and was 3.2 fold less potent ($P=0.005$) but showed no difference in the Hill slope ($P=0.367$).

Relative efficacies of M6G and morphine

The fractional responses of M6G and morphine relative to [Met^5]-enkephalin indicated that both are partial agonists. The operational model (Black & Leff, 1983) has been used to estimate the dissociation constants for partial agonists in the case where a full agonist can be used to estimate E_M, the operational maximum response of the tissue (Leff *et al*., 1992). Concentration-response data were therefore fitted simultaneously using a modified form of the operational model (equation 2) with E_M constrained to 100% of the [Met^5]-enkephalin current. From this analysis we estimated pK values of −5.9 and −6.1 for M6G and morphine respectively, and apparent efficacy values (τ_{app}) of 2 and 4.6, which provided an efficacy ratio equal to 2.3.

The relative efficacy ratio can be also estimated using:

$$\frac{\varepsilon_{M6G}}{\varepsilon_{morphine}} = \frac{(E_{mM6Gx})_{M6G}}{(E_{mM6Gx})_{morphine}} \frac{(EC_{50})_{morphine}}{(EC_{50})_{M6G}} \frac{K_{M6G}}{K_{morphine}} \quad (3)$$

in which ε is the efficacy E_{max} agonist maximum response, EC_{50} effective concentration 50% and K is the true equilibrium dissociation constant of the agonist (Trzeciakowski 1999a,b). The dissociation constants of M6G and morphine have not been measured in locus coeruleus neurons but binding and competition studies indicate that the affinity of M6G for the mu-opioid receptor is equal or no more than 5–10 fold lower than that of morphine. If $K_{M6G} = K_{morphine}$ then the efficacy ratio of M6G to morphine estimated from our data is equal to 4.1. Figure 2 illustrates the effect on the relative efficacy of the two agonists when the ratio $K_{M6G} : K_{morphine}$ is varied.

Antagonist effects of 3-O-methoxynaltrexone

M6G has been reported to bind with high affinity to a novel receptor that is selectively antagonized by 3-O-methoxynaltrexone (Brown *et al*., 1997a). The antagonist effect of 3-O-methoxynaltrexone on M6G currents measured in juvenile brain slices with patch clamp recording is shown in Figure 3. Concentrations of 3-O-methoxynaltrexone between 30 nM and 3 μM competitively antagonized currents produced by M6G ($n=4$). However the figure also illustrates the similar effect 3-O-methoxynaltrexone had on currents produced by the selective mu-opioid receptor agonist DAMGO. A low concentration of 3-O-methoxynaltrexone (30 nM) caused shifts in the concentration-effect curves for M6G and DAMGO of 10 ± 3.1 and 14 ± 5.7 fold respectively ($n=3$), that were not significantly different (paired t-test: $P=0.57$, 2 d.f.).

Shown in Figure 4 are currents produced by maximally effective concentrations of M6G and morphine in the same neuron. The recording illustrates the difference in current amplitudes and also the slower reversal of the morphine current with washout (although this was not quantified).

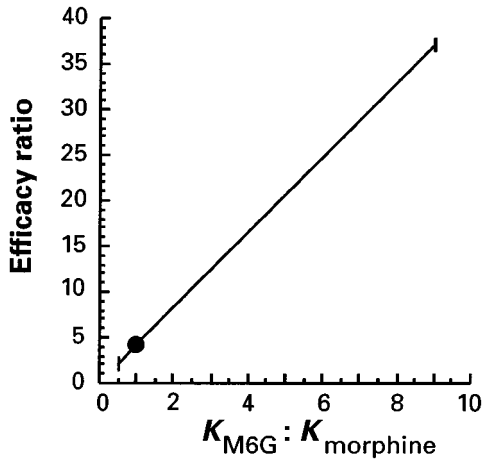

Figure 2 Efficacy ratios (M6G:morphine) predicted using dissociation constants obtained from binding studies. Shown is a plot of the efficacy ratio calculated by the equation method of Trzeciakowski (1999a) expressed as a function of the ratio of the affinity constants for M6G and morphine, which were obtained from published binding studies (Table 1). The values assigned to the following variables were estimated from the concentration-effect curves for each agonist: $(E_m)_{M6G} = 77.7\%$, $(E_m)_{morphine} = 61\%$, $(EC_{50})_{M6G} = 19$ μM, and $(EC_{50})_{M6G} = 6.2$ μM. The filled circle indicates an efficacy ratio of 4.1 calculated when the dissociation constants of M6G and morphine are equal.

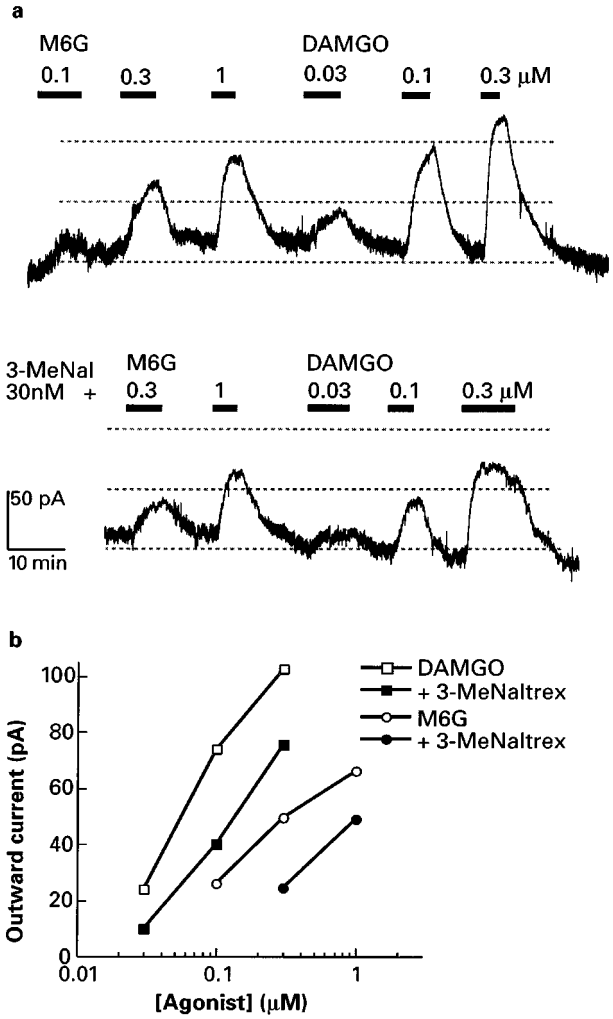

Figure 3 M6G and DAMGO currents are antagonized equally by 3-O-methoxynaltrexone. In (a) is shown recording sequences made from a single neuron. In the upper panel are control responses produced by three concentrations of M6G and the selective mu-opioid agonist DAMGO. In the lower panels 3-O-methoxynaltrexone caused a similar reduction in the currents produced by both agonists. In (b) the data for this experiment has been plotted.

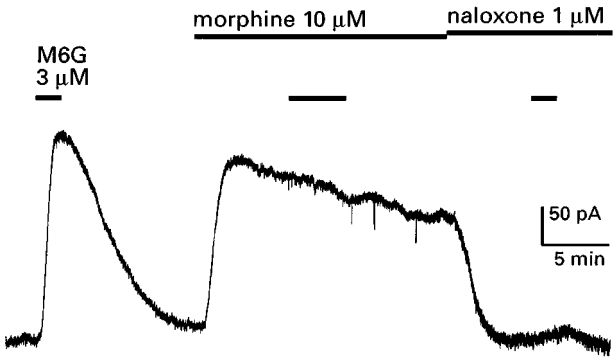

Figure 4 M6G currents are occluded by morphine. The effect of M6G is shown on a locus coeruleus neuron voltage clamped at −60 mV. M6G produced an outward current when superfused alone but had no effect when superfused during an application of morphine. Normally the current produced by morphine would reverse slowly during washout but superfusion with naloxone caused an immediate return to the original baseline and also blocked the current response to a third application of M6G.

Combined applications of morphine (10 μM) and M6G (3 μM) did not produce any further increase in current amplitude when compared to the effect of a single agonist application ($n=5$). In contrast, following prior application of the full receptor agonist [Met^5]-enkephalin, simultaneous application of M6G caused a decrease in current amplitude ($n=3$). Naloxone (300 nM – 1 μM), an opioid receptor antagonist with poor subtype selectivity, completely blocked currents produced by M6G ($n=5$) or morphine ($n=2$) (Figure 4).

Extended applications of opioid agonists at supermaximally effective concentrations can transiently desensitize mu-opioid receptors in locus coeruleus neurons (Harris & Williams, 1991; Osborne & Williams, 1995). In Figure 5 a desensitizing application of [Met^5]-enkephalin (30 μM applied for 5 min) reduced the current produced by M6G (3 μM) by 38%.

Morphine-3β-D-glucuronide

The major morphine metabolite in humans, M3G, has been reported to show biological activity in some *in vivo* bioassays. We were unable to show pharmacological activity of this metabolite in the rat locus coeruleus. M3G (10 μM, $n=3$) had no effect on the membrane current of locus neurons voltage clamped at −60 mV (Figure 6a) or on spontaneous action potentials measured by bridge recording (Figure 6b). The current produced by M6G (1 μM) was not antagonized by M3G (Figure 6a). Finally, currents produced by superfusing slices with NMDA (30 μM) were not affected by M3G ($n=2$) (data not shown).

Discussion

In this study the agonist properties of morphine were compared to those of the active metabolite morphine-6β-glucuronide (M6G). This was done by using electrophysiological techniques to record from locus coeruleus neurons in rat brain slices. Previous studies have shown that locus coeruleus neurons express mu-opioid receptors that use pertussis toxin sensitive G proteins to open G protein-gated inward rectifier potassium (GIRK) channels (Miyake *et al*., 1989; Grigg *et al*., 1996). Neither delta nor kappa opioid receptors couple to ion channels in these neurons. In the present study M6G acted as an opioid agonist that produced an outward potassium current which was completely blocked

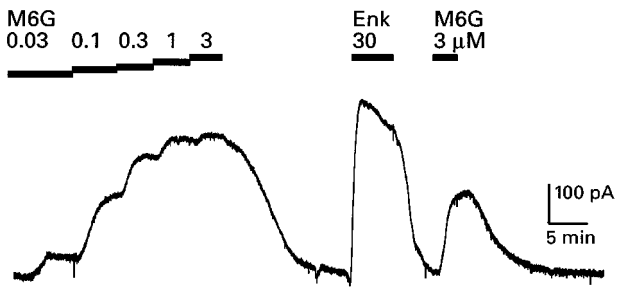

Figure 5 M6G currents are reduced following desensitization of mu-opioid receptors with [Met^5]-enkephalin. In the experiment shown following a cumulative application of three concentrations of M6G, a 30 μM concentration of [Met^5]-enkephalin was applied for 5 min, which causes transient homologous desensitization of mu-opioid receptors. This treatment also caused desensitization of the M6G (3 μM) current.

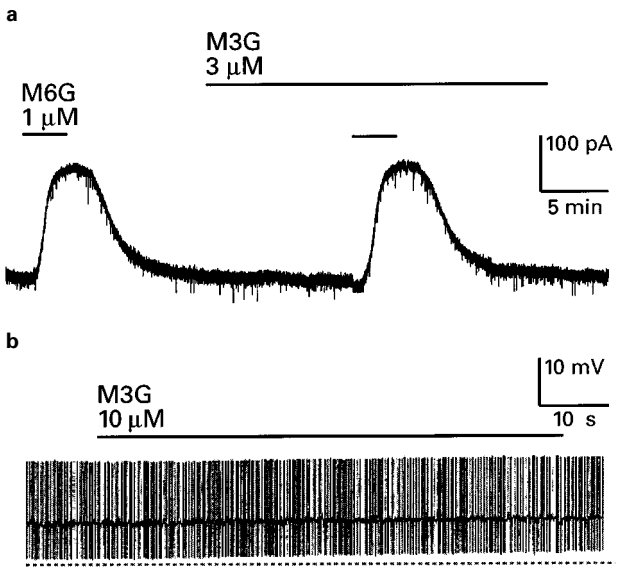

Figure 6 M3G which does not bind to opioid receptors had no electrophysiological effects in locus coeruleus neurons. In (a) M3G produced no change in current in a neuron voltage clamped at −60 mV and did not antagonize currents produced by M6G. In (b) M3G had no effect on the rate or pattern of firing in a recording made from a spontaneously active locus coeruleus neuron.

by the opioid receptor antagonist naloxone. M6G and morphine both behaved as partial agonists but M6G had a higher relative efficacy than morphine. Although 3-O-methoxynaltrexone has been reported to discriminate a receptor that is selective for M6G and not morphine (Brown *et al*., 1997b), in the locus coeruleus even a low concentration of 3-O-methoxynaltrexone produced equal shifts in the concentration-effect curves of M6G and the mu-opioid receptor agonist DAMGO.

We found that M6G and morphine behaved as partial agonists because both produced maximal responses that were smaller than system maximum measured with [Met^5]-enkephalin. We have previously shown [Met^5]-enkephalin and the mu selective agonist DAMGO behave as full agonists in locus coeruleus neurons, by reducing the receptor reserve with the irreversible antagonist β-chlornaltrexamine or by desensitizing mu-opioid receptors (Christie *et al*., 1987; Osborne & Williams, 1995). In the present study where M6G and morphine both behaved as partial agonists the difference in their maximal responses indicated that M6G had a higher efficacy than morphine (see Kenakin, 1999). A 2 to 4 fold difference in efficacy was estimated by fitting the data to the operational model using the method described by Leff *et al*. (1992) and by using an equation method (Trzecianowski, 1999a) where it was assumed that the dissociation constants of M6G and morphine were the same. As shown in Table 1 M6G has consistently been found in binding studies to have a similar or slightly lower affinity for mu-opioid receptors than morphine. In locus coeruleus neurons the efficacy of morphine is also lower than normorphine, which is another active metabolite that has a maximal response nearly equal to the system maximum (Christie *et al*., 1987; Osborne & Williams, 1995).

We found no evidence to show that a receptor activated by M6G and distinct from the receptor activated by morphine was present in locus coeruleus neurons. It has been proposed that such a receptor is responsible for the greater analgesic potency shown by M6G *in vivo* and may be a splice variant of the MOR-1 clone (Rossi *et al*., 1995a,b; 1996; 1997; Pan *et*

Table 1 Binding affinities of M6G and morphine for mu-opioid receptors

	Tissue	*M6G*	*Morphine*	*Ratio (M6G : M)*	*Labelled ligand*
K_D *values (nM)*					
Brown *et al*., 1997b	MOR-1 CHO	3.3			
Pan *et al*., 1999	MOR-1 CHO	4.1 – 5.6	1.5 – 5.3	0.98 – 3.2	
K_i *values (nM)*					
Abbot & Palmour, 1988	Rat brain	25	2.8	8.9	[^{3}H]-dihydromorphine
		29	3.5	8.3	[^{3}H]-etorphine
		88	12.5	7	[^{3}H]-naloxone
Chen *et al*., 1991	Rat brain	0.6	1.2	0.5	[^{3}H]-DAMGO
Christensen & Reiff, 1991	Bovine caudate n.	2.5	2.1	1.2	[^{3}H]–morphine
Frances *et al*., 1992	Rabbit brain	20.3	5.6	3.6	[^{3}H]-diprenorphine
Hucks *et al*., 1992	Rat brain	94	13	7.2	[^{3}H]-DAMGO
Lambert *et al*., 1993	SH-SY5Y	393	96	4.1	[^{3}H]-diprenorphine
Mignat *et al*., 1995	Guinea-pig brain	3.5	1.8	1.9	[^{3}H]-DAMGO
Löser *et al*., 1996	Rat brain (1 site)	11.1	5.9	1.9	[^{3}H]-DAMGO
	(2 site)	8.3/8.4	2.1/6.7	4/1.3	[^{3}H]–DAMGO
Brown *et al*., 1997b	MOR-1 CHO	14.2	18.1	0.8	[^{3}H]-M6G
		24.4	9.53	2.6	[^{3}H]-morphine
	Mouse brain	19.2	10.2	1.9	[^{3}H]-M6G
		19.7	6.5	3.0	[^{3}H]-morphine
Pan *et al*., 1999	MOR-1 CHO	4.1 – 5.6	1.5 – 5.3	0.98 – 3.2	[^{3}H]-DAMGO

al., 1999; reviewed by Pasternak & Standifer, 1995). A binding site has been identified in calf and mouse brain homogenates that has a higher affinity for [^{3}H]-M6G than the traditional mu-opioid receptor (K_D 60 pM c/f 1.9 nM) and a low affinity for morphine (Brown *et al*., 1997b). It has been reported that these two sites can be discriminated using the opioid antagonist 3-O-methoxynaltrexone, which has IC_{50} values of 12 nM and 400 nM measured using [^{3}H]-M6G in mouse brain (Brown *et al*., 1997a). However we found that concentrations of 3-O-methoxynaltrexone as low as 30 nM caused equal rightward shifts in the concentration-effect curves for both M6G and the mu-opioid selective agonist DAMGO in locus coeruleus. As competition studies show that DAMGO does not bind to the high-affinity M6G site in brain (Löser *et al*., 1996; Brown *et al*., 1997b), this result indicated that M6G was as an agonist, and 3-O-methoxynaltrexone was an antagonist, of the classical mu-opioid receptor in locus coeruleus neurons. This conclusion was further supported by the failure of M6G and morphine currents to summate when maximally effective concentrations of agonists were applied together. This 'occlusion' was consistent with M6G and morphine having a common action at the same receptor, but does not rule out the possibility that a M6G specific receptor could be coupled to the same G proteins or same GIRK channels as the mu-opioid receptor. We also showed in this study that the current produced by M6G could be desensitized by [Met^5]-enkephalin. In locus coeruleus neurons desensitization produced by [Met^5]-enkephalin is homologous for mu-opioid receptors and produces minimal cross-desensitization with other receptors, such as alpha$_2$-adrenoceptors, that couple to GIRK channels (Harris & Williams, 1991). Taken together, these experiments indicated that if a functional receptor activated by M6G and not morphine were expressed in locus coeruleus neurons, then it does not couple to GIRK channels. Our results also question whether functional responses of a putative M6G preferring receptor can be discriminated using 3-O-methoxynaltrexone as responses to mu-opioid receptor agonists in locus coeruleus neurons were antagonized by a concentration of 3-O-methoxynaltrexone only 3 fold higher than the IC_{50} reported for inhibition of the high affinity binding site for [^{3}H]-M6G (Brown *et al*, 1997a,b).

Our findings are consistent with the main conclusion drawn by Frances *et al*. (1992) that the relative efficacy of M6G and morphine are different. This was demonstrated in mice by determining the fractional receptor occupancy (measured using [^{3}H]-diprenorphine) achieved by doses of M6G or morphine that were equally effective in producing antinociception. M6G occupied less than 1% of the available opioid receptors whereas morphine occupied more than 50%. It has been widely assumed in the literature that differences in the relative efficacy of M6G and morphine do not contribute to the markedly different potencies these drugs have in producing antinociception when administered centrally. This argument has been supported by measurements of agonist inhibition of adenylyl cyclase activity made in SH-SY5Y cells and in Chinese hamster ovary cells transfected with the MOR-1 clone (Lambert *et al*., 1993; Brown *et al*., 1997b). In these cells M6G and morphine behave as partial agonists and show no difference in maximal response. However, the relative efficacies of agonists can only be compared across different natural and recombinant systems when the active state of receptor, the concentration and species of G proteins, and the post-stimulus signalling pathways that are coupled to the receptor are the same (see Kenakin, 1999; Trzeciakowski, 1999a,b).

In conclusion, our findings and those of Frances *et al*. (1992) question the notion that the relative efficacies of M6G and morphine are the same. Opioid agonists act on receptors distributed throughout a complex network of neural circuits to produce analgesia. These receptors utilize several different signalling pathways and multiple effector mechanisms. Clearly there is ample capacity in this system for even small differences in the relative efficacy of MG6 and morphine to be amplified in order to produce large differences in analgesic potency. Further experimental work is required to quantify the extent to which the efficacy and biodistribution of M6G and morphine determines the relative potency of these drugs when injected into the central nervous system. The contribution made by these factors to the results of receptor knockdown and knockout studies should also be carefully assessed.

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