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Comparative studies of the neuro-excitatory behavioural effects of morphine-3-glucuronide and dynorphin A(2-17) following spinal and supraspinal routes of administration

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ABSTRACT

Morphine-3-glucuronide (M3G) administered centrally produces dose-dependent neuro-excitatory behaviours in rodents via a predominantly non-opioid mechanism. The endogenous opioid peptide, dynorphin A (Dyn A) (1-17), is rapidly cleaved *in vivo* to the relatively more stable fragment Dyn A(2-17) which also produces excitatory behaviours in rodents via a non-opioid mechanism. This study investigated the possible contribution of Dyn A(2-17) to the neuro-excitatory behaviours evoked by supraspinally and spinally administered M3G in male Sprague–Dawley (SD) rats. Marked qualitative differences in behaviours were apparent following administration of M3G and Dyn A(2-17). Administration of 11 nmol i.c.v. doses of M3G produced intermittent myoclonic jerks, tonic–clonic convulsions, and ataxia, as well as postural changes, whereas i.c.v. Dyn A(2-17) at 15 nmol produced effects on body posture alone. Administration of 11 nmol i.t. doses of M3G produced intermittent explosive motor activity, and touch-evoked agitation, as well as postural changes, whereas i.t. Dyn A(2-17) at 15 nmol produced postural changes, touch-evoked agitation, and paralysis. Pre-treatment with Dyn A antiserum (200 μg) markedly attenuated total behavioural excitation following i.c.v. and i.t. administration of Dyn A(2-17) by ~94% and 78%, respectively. However, total behavioural excitation following i.c.v. and i.t. administration of M3G was less markedly attenuated (both ~27%) by pre-treatment with Dyn A antiserum, with reductions in tonic–clonic convulsions (~43%), explosive motor behaviour (~28%), and touch-evoked agitation (~22%). The present findings discount a major role for Dyn A in mediating the neuro-excitatory effects of M3G, although it may contribute to maintaining some individual neuro-excitatory behaviours.

1. Introduction

The recommendation in 1986 by the World Health Organization for the use of morphine as the drug of choice in the management of moderate to severe cancer-related pain had led to a large increase in morphine prescribing, as well as an increase in the magnitude of the doses of morphine that are prescribed. However, the reported incidence of neuro-excitatory side effects such as allodynia, myoclonus, and seizures has also increased as the number of patients receiving large doses of morphine has grown (Smith, 2000).

Morphine is eliminated in humans mainly by glucuronidation to form morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), with M3G being the major metabolite (Sawe, 1986). The ratios for area under the plasma concentration versus time curves (AUC) of M3G and M6G to morphine were reported to be ~56 and 10, respectively, following oral morphine dosing in humans (Osborne et al., 1990). However, in the rat the glucuronidation of morphine results in formation of only M3G (Kuo et al., 1991). While M6G was shown to act as a potent μ-opioid receptor agonist in the rat, M3G was without antinociceptive activity (Pasternak et al., 1987).

In fact, administration of M3G produces dose-dependent neuro-excitatory behavioural effects that include myoclonus, seizures, explosive motor activity, touch-evoked agitation, and hyperalgesia, following central routes of administration in the rat (Bartlett et al., 1994a; Halliday et al., 1999; Woolf, 1981; Wright et al., 2001; Yaksh and Harty, 1988). Studies of the neuro-excitatory effects of centrally administered M3G found that pre-treatment with naloxone suppressed the behavioural excitation produced by low (1.1 nmol) but not high (11 nmol) doses of M3G (Halliday et al., 1999). In earlier studies, however, i.c.v. pre-treatment with the competitive N-methyl-D-aspartate (NMDA) receptor antagonist, LY274614, prior to high doses (15 nmol) of i.c.v. M3G dose-dependently suppressed the neuro-excitatory effects produced by M3G in rats (Bartlett et al., 1994a). Subsequent *in vitro* studies in cultured rat embryonic hippocampal neurones demonstrated that M3G mediates its neuro-excitatory effects via a predominantly non-opiodergic

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mechanism involving indirect activation of the NMDA receptor (Hemstapat et al., 2003).

The endogenous opioid peptide, dynorphin A (Dyn A) (1-17), binds selectively and with high affinity ($K_i = 0.05 \pm 0.01$ nM) to κ-opioid receptors, and also has a high affinity for μ- ($K_i = 1.6 \pm 0.2$ nM) and δ- ($K_i = 1.25 \pm 0.1$ nM) opioid receptors (Zhang et al. 1998). However, Dyn A(1-17) also has a moderate affinity ($K_i = 0.41 \pm 0.01$ μM) for the non-competitive binding site on the NMDA receptor (Shukla et al., 1997). By contrast, Dyn A(2-17) does not bind to opioid receptors (Walker et al., 1982a), but appears to bind with high affinity ($K_d = 9.4 \pm 1.6$ nM) to the NMDA receptor complex in its 'closed/desensitised' state (Tang et al., 1999). Additionally, Dyn A(1-17) and Dyn A(2-17) potentiate responses to NMDA via a non-opioid mechanism (Lai et al., 1998). *In vivo*, Dyn A(1-17) is rapidly cleaved to its relatively more stable des-tyrosine fragment peptide, Dyn A(2-17) (Young et al., 1987), which produces excitatory electrophysiological and motor effects analogous to those of Dyn A(1-17) via an opioid receptor-independent mechanism (Walker et al., 1982a,b).

A number of studies have shown that following administration of Dyn A(1-17) or its fragment peptides, excitatory behaviours and effects on motor function are produced in a manner similar to those evoked by centrally administered M3G. For instance, Dyn A(1-17) and a series of related opioid and non-opioid fragment peptides produced excitatory motor effects in mice following i.c.v. administration (Shukla et al., 1997). Other earlier studies also reported seizure activity following i.c.v. administration of Dyn A(1-17) and Dyn A(2-17) in rats (Walker et al., 1982b). Similarly, i.c.v. administration of Dyn Ia (Dyn A (1-13)-Tyr-Leu-Phe-Asn-Gly-Pro), a peptide analogue derived from the structure of adrenal Dyn I, produced wild running, 'pop-corn' jumping, hind limb jerking, and barrel rolling in mice that were prevented by pre-treatment with the NMDA receptor antagonists, ketamine and dextromethorphan, in addition to a dose-dependent suppression of acetic acid-induced writhing that was reversed by the κ-opioid antagonist, norbinaltorphimine (Shukla et al., 1992).

In other studies, single i.t. bolus doses of Dyn A(1-17), Dyn A(1-13), Dyn A(2-17), and Dyn A(2-13) evoked long-lasting tactile allodynia in rats (Vanderah et al., 1996). Dyn A(1-17)-evoked allodynia was blocked by pre- but not post-treatment with the NMDA receptor antagonist, MK-801, whereas pre-treatment with naloxone was without effect (Vanderah et al., 1996). In related studies, single i.t. doses of Dyn A(1-17) and Dyn A(2-17) also evoked long-lasting allodynia in mice, and pre-treatment with the NMDA receptor antagonists, MK-801 and LY235959, but not naloxone blocked the allodynic response to Dyn A(1-17) (Laughlin et al., 1997).

Taken together, the foregoing studies raise the possibility that the neuro-excitatory behaviours evoked by centrally administered M3G may be mediated, at least in part, by the release of Dyn A(1-17), with rapid N-terminal cleavage in the CNS to form the relatively more stable des-tyrosine fragment, Dyn A(2-17) (Young et al., 1987), and subsequent activation of the NMDA receptor. Hence, the aim of the present study was to assess the efficacy of pre-treatment with Dyn A antiserum to suppress the neuro-excitatory behaviours evoked by single bolus doses of i.t. and i.c.v. M3G in rats. Additionally, as a positive control, the efficacy of Dyn A antiserum was confirmed by showing that it completely suppressed the opioid receptor-independent excitatory behaviours evoked by Dyn A(2-17).

2. Materials and methods

2.1. Experimental animals

Ethical approval for the study was obtained from the Animal Experimentation Ethics Committee of The University of Queensland. Adult male Sprague–Dawley (SD) rats (210 ± 10 g) were purchased from the Herston Medical Research Centre, The University of Queensland (Brisbane, Australia). Rats were housed in a temperature controlled room (21 °C ± 2 °C) on a 12/12 hour light/dark cycle, with food and water available *ad libitum*. Rats were given an acclimatization period of at least 48 h prior to the commencement of the experimental procedures.

2.2. Drugs and materials

Morphine-3-glucuronide (M3G) was purchased from Sigma (Sydney, NSW, Australia). Preservative free rabbit Dyn A(1-17) antiserum with 100% cross-reactivity for Dyn A(1-13) and Dyn A(1-8) was purchased from Peninsula Laboratories, Inc. (San Carlos, CA, USA). Dyn A(2-17) was purchased from Auspep Pty Ltd (Melbourne, Vic, Australia). Zoletil 100® (100 mg/ml) was purchased from Virbac, Pty Ltd (Sydney, NSW, Australia) and xylazine hydrochloride (Ilium Xylazil-20®) vials (20 mg/ml) were purchased from Troy Laboratories, Pty Ltd (Sydney, NSW, Australia). Sodium benzylpenicillin vials containing 600 mg of powder were purchased from CSL Ltd (Melbourne, Vic, Australia). Normal saline and lignocaine (lidocaine) vials were purchased from Delta West Pty Ltd (Perth, WA, Australia). Dental cement (Metropair® X-linked cold cure acrylic powder and liquid) was purchased from Henry Schein Halas (Brisbane, Qld, Australia). Silk sutures (Dysilk Black Braided Siliconised Silk) were purchased from Dynek Pty Ltd (Adelaide, SA, Australia). Malachite green dye (BDH Chemicals) was purchased from Lab Supply (Brisbane, Qld, Australia). Topical antibiotic powder was purchased from Apex Laboratories Pty Ltd (Sydney, NSW, Australia). Betadine™ antiseptic solution was purchased from Faulding Pharmaceuticals (Adelaide, SA, Australia). Enrofloxacin vials (Baytril™ 50 mg/ml) were purchased from Bayer Ltd (Sydney, NSW, Australia). Polyethylene tubing (I.D. 0.28 mm and O.D. 0.61 mm) was purchased from Dural Plastics and Engineering (Sydney, NSW, Australia).

2.3. Intracerebroventicular cannula implantation

Guide cannulae were prepared from 21 gauge stainless steel needles cut to a length of 8 mm and bevelled at 45°. Cannula plugs were cut from 25 gauge needles such that the tip extended 0.5 mm beyond the tip of the guide cannula. Guide cannulae and plugs were stored in 70% ethanol until use. Rats were deeply anesthetized with a mixture of xylazine (10 mg/kg) and Zoletil 100® (100 mg/kg) administered by a single i.p. injection, while lightly anesthetized with oxygen:carbon dioxide (1:1 v/v). Immediately following loss of the foot withdrawal reflex, the rat's head was shaved and the skin cleaned with Betadine®. A midline incision was made along the length of the skull and lignocaine (lidocaine) solution was applied topically, before the connective tissue was gently removed from the top of the skull to expose the Bregma. Guide cannulae were positioned stereotaxically above the left lateral ventricle (0.8 mm posteria, 1.5 mm lateral, and 3.3 mm ventral to Bregma) using co-ordinates taken from the rat Brain Atlas of Paxinos and Watson (1986), and fixed in position with dental cement. The scalp was sutured and 60 mg of benzylpenicillin was administered via s.c. injection. Rats were allowed 5–7 days to recover prior to drug administration. The cannula plugs remained in place except during the i.c.v. injection procedure.

Cannula placement was assessed at the conclusion of the i.c.v. behavioural studies by injection of malachite green dye. Rats were lightly anesthetized with oxygen:carbon dioxide (1:1 v/v) to facilitate i.c.v. injection of 1 μl of a 100 mg/ml solution of malachite green dye, then immediately euthanized, and the brain was removed for gross dissection. Results from rats were excluded from the study when the dye was not distributed throughout the ventricles and/or clumping of dye in the periventricular tissue was apparent.

2.4. Intrathecal cannula implantation

Rats were anesthetized as described above, then the lumbar spinal region and the back of the neck were shaved, and the skin cleaned

with Betadine®. The L6 lumbar vertebra was located by palpation and an ~5 cm incision was made along the back at the midline. The lumbar muscles surrounding L5 and L6 were subsequently cut and removed to expose the L5/L6 spinous processes. The L6 spinous processes were then removed and the dura was exposed by removing the soft tissue beneath the L5 iliac arch. The dural membrane was subsequently pierced using a 23 gauge needle, allowing leakage of CSF to be observed. A fine polyethylene cannula (I.D. 0.28 mm and O.D. 0.61; length = 20 cm) pre-filled with saline was carefully advanced ~1 cm through the pierced dural membrane into the lumbar subarachnoid space. A small volume of saline was injected via the cannula. If backflow of saline was observed around the cannula, the cannula was removed and re-inserted, and the saline 'leak test' repeated. If saline leakage still persisted, the rat was excluded from further experimentation. For rats with correct cannula insertion, the cannula was sutured onto muscle tissue surrounding L5/L6, and further secured into position using dental cement. The cannula was then exteriorized via a subcutaneous tunnel through a small incision at the back of the neck, and the wound sutured. An s.c. injection of 60 mg of benzylpenicillin and 5 mg/kg of enrofloxacin was given postoperatively, and topical antibiotic powder was applied.

On the day after i.t. cannula implantation, rats were administered 20 µl of a 2% (v/v) lignocaine (lidocaine) solution to verify correct i.t. cannula placement. If reversible bilateral hind limb paralysis was observed, behavioural testing commenced after a recovery period of at least 2 days. Rats not exhibiting temporary bilateral hind limb paralysis or showing motor deficits following i.t. cannula insertion were euthanized.

Cannula placement was assessed again at the conclusion of the i.t. behavioural studies by injection of 30 µl of 100 mg/ml malachite green dye solution. Rats were euthanized and decapitated ~30 s after injection of dye, and the spinal column was surgically exposed. Results from rats were excluded from the study when leakage of the dye was apparent at the site where the cannula entered the back muscles above L6, or when the dye did not distribute along the spinal cord for at least 3–4 cm.

2.5. Drug dosing regimens

In the present study, M3G was administered i.c.v. and i.t. at a dose of 11 nmol. This dose produced pronounced neuro-excitatory behaviours following i.c.v. administration in earlier studies from our laboratory (Halliday et al., 1999), and was similar to the estimated mean (±S.D.) ED_{50} value of 13.2 (±1.3) nmol for the total behavioural excitation evoked by M3G was reported in other studies from our laboratory (Wright et al., 2001). Dyn A(2-17) was administered i.c.v. and i.t. at a dose of 15 nmol based on earlier studies showing an ED_{50} of 14.3 nmol for producing motor effects following i.c.v. administration of Dyn A(1-17) to laboratory rodents (Shukla et al., 1997), and that 15 nmol i.t. doses of Dyn A(1-17) or Dyn A(2-17) produced long-lasting allodynia (Vanderah et al., 1996). Furthermore, as preliminary investigation in the present study showed that doses of Dyn A(2-17) larger than 15 nmol produced severe motor dysfunction in agreement with previous reports from other laboratories (Faden and Jacobs, 1983, 1984; Stevens and Yaksh, 1986), the dose of Dyn A(2-17) was capped at 15 nmol.

2.5.1. Intracerebroventicular studies

Drugs were dissolved in 0.9% saline and delivered in a 1 µl injection volume using a 5 µl SGE® syringe, except for Dyn A antiserum where a 10 µl volume was used due to its limited solubility. Groups of rats were administered one of the following dosing regimens: (i), 0.9% saline followed by 0.9% saline ($n=3$); (ii), 0.9% saline followed by 11 nmol of M3G ($n=11$); (iii), 0.9% saline followed by 15 nmol of Dyn A(2-17) ($n=8$); (iv), 200 µg of Dyn A antiserum followed by 0.9% saline ($n=3$); (v), 200 µg of Dyn A antiserum followed by 11 nmol of M3G ($n=4$); (vi), 200 µg of Dyn A antiserum followed by 15 nmol of Dyn A (2-17) ($n=4$). Pre-treatments were administered 10 min prior to the second treatment.

2.5.2. Intrathecal studies

Drugs were dissolved in 0.9% saline and delivered using a 25 µl SGE® syringe. Groups of rats were administered one of the following dosing regimens: (i), 10 µl of 0.9% saline followed by 5 µl of 0.9% saline ($n=3$); (ii), 200 µg of Dyn A antiserum in 10 µl followed by 10 µl of 0.9% saline ($n=3$); (iii), 10 µl of 0.9% saline followed by 11 nmol of M3G in 5 µl ($n=8$); (iv), 200 µg of Dyn A antiserum in 10 µl followed by 11 nmol of M3G in 5 µl ($n=4$); (v), 10 µl of 0.9% saline followed by 15 nmol of Dyn A(2-17) in 10 µl ($n=9$); (vi), a mixture of 200 µg of Dyn A antiserum in 10 µl combined with 15 nmol of Dyn A(2-17) in 10 µl ($n=6$). Injections were followed by a 20 µl flush with 0.9% saline to ensure complete delivery of the injection volume, with pre-treatments administered 20 min prior to the second treatment.

2.6. Quantification of neuro-excitatory behaviours

Neuro-excitatory behaviours were assessed using a modification of the behavioural paradigm outlined in other studies from our laboratory (Bartlett et al., 1994a; Halliday et al., 1999; Wright et al., 2001), which was based on a scoring paradigm used in earlier studies for assessing opiate withdrawal in rats (Rasmussen et al., 1991). The studies were carried out by a single observer between 10 am and 1 pm. Initially, each rat was placed in a plexiglass observation chamber (45×25×25 cm) and allowed to acclimatize to the testing environment for at least 30 min. Neuro-excitatory behaviours were subsequently scored at 5, 15, 25, 35, 50, 60, and 80 min after administration of each i.c.v. or i.t. treatment. Eight behaviours were scored at the times specified above over a 30 s interval.

Intermittent myoclonic jerks and chewing were scored on a 5 point scale on the basis of absolute frequency (0 = no incidents, 1 = 1–5 incidents, 2 = 6–10 incidents, 3 = 10–15 incidents, and 4 = >15 incidents). Intermittent tonic–clonic convulsions and explosive motor activity were scored on a 5 point scale by severity (0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = extreme). The following 4 behaviours were scored individually: ataxia (1 = mild, 2 = marked); body posture (0 = normal, 1 = abnormal, 2 = extreme); paralysis (0 = none, 2 = partial hind limb paralysis, 4 = bilateral hind limb paralysis); lastly, touch-evoked agitation after brushing of the flank with a blunt probe (0 = none, 1 = avoidance of probe, 2 = biting probe or mild vocalization, 3 = extreme vocalization).

2.7. Data analysis

The scores for each behaviour were plotted against time over the 80 min post-dosing interval, and the area under the curve (AUC) for each behaviour was determined by trapezoidal integration. The AUC values for each of the 8 individual behaviours were then summed to give the total excitation score for each rat ($\sum$AUC values). Between-group statistical comparisons were performed using the Mann–Whitney test, as implemented by GraphPad Prism® software (San Diago, USA). The statistical criterion for significant difference was $p<0.05$.

3. Results

3.1. Neuro-excitatory behaviours following i.c.v. or i.t. administration of M3G or Dyn A(2-17)

Mean scores for individual neuro-excitatory behaviours following i.c.v. and i.t. administration of 11 and 15 nmol doses of M3G and Dyn A (2-17) respectively, are shown in Figs. 1 and 2. There were marked qualitative differences in the neuro-excitatory behaviours evoked by

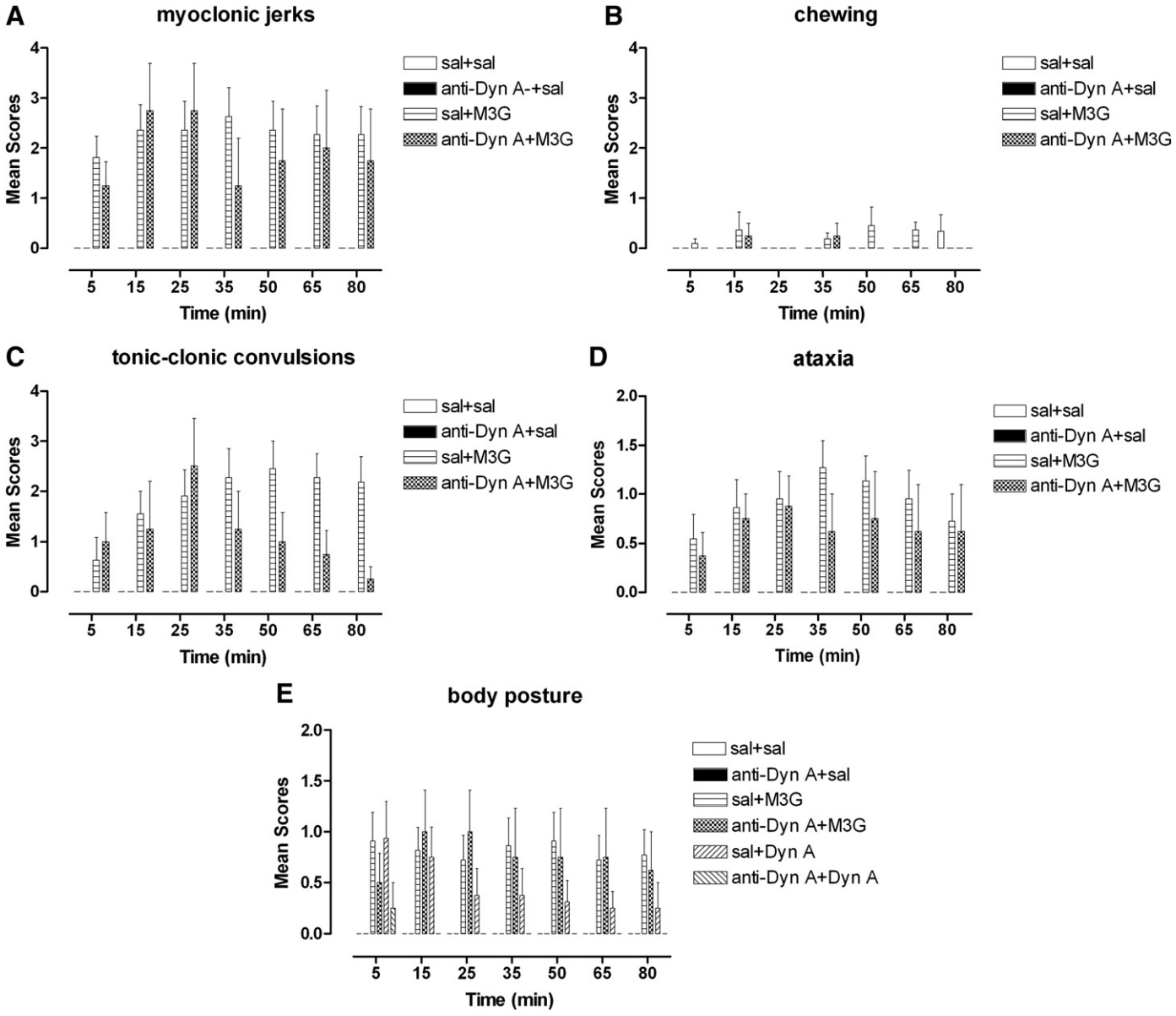

Fig. 1. Mean (±S.E.M.) behavioural excitation scores for rats following i.c.v. administration of morphine-3-glucuronide (M3G) at 11 nmol or the dynorphin A (Dyn A) fragment peptide Dyn A(2-17) at 15 nmol, 10 min after i.c.v. pre-treatment with 0.9% saline (sal) or 200 μg of Dyn A antiserum (anti-Dyn A).

i.c.v. and i.t. bolus doses of M3G and Dyn A(2-17) in rats. The behavioural effects produced by i.c.v. M3G included intermittent bouts of myoclonic jerks, chewing, tonic–clonic convulsions, ataxia, as well as changes in body posture, whereas i.t. M3G produced intermittent episodes of explosive motor activity, and touch-evoked agitation as well as changes in body posture. By contrast, i.c.v. Dyn A(2-17) produced only changes in body posture, whereas i.t. Dyn A(2-17) produced both touch-evoked agitation and hind limb paralysis, as well as changes in body posture.

3.1.1. Intracerebroventicular administration

Intermittent myoclonic jerks of both the head and limbs were observed in rats over the 80 min observation period, when i.c.v. M3G followed pre-treatment with i.c.v. saline. As expected, myoclonic jerks were not seen in rats administered i.c.v. saline or when i.c.v. Dyn A antiserum was followed by i.c.v. saline (Fig. 1A). The mean (±S.E.M.) AUC values for the extent and duration of myoclonic jerks in rats pre-treated with i.c.v. saline or Dyn A antiserum prior to i.c.v. M3G were not significantly different ($p>0.05$, Table 1).

Mild intermittent chewing was observed in rats over the 80 min observation period, when i.c.v. M3G followed pre-treatment with i.c.v. saline (Fig. 1B). The mean (±S.E.M.) AUC value for the extent and duration of chewing was lower (~68%) in rats pre-treated with i.c.v. Dyn A antiserum prior to i.c.v. M3G, compared with that from rats pre-treated with i.c.v. saline prior to i.c.v. M3G, but this difference did not reach statistical significance ($p>0.05$, Table 1).

Intermittent tonic–clonic convulsions were observed in rats over the 80 min observation period, when i.c.v. M3G followed pre-treatment with i.c.v. saline. Consistent with expectations, tonic–clonic convulsions were not seen in rats administered only i.c.v. saline, or when i.c.v. Dyn A antiserum was followed by i.c.v. saline (Fig. 1C). The mean (±S.E.M.) AUC value for the extent and duration of tonic–clonic convulsions was lower (~43%) in rats pre-treated with i.c.v. Dyn A antiserum prior to i.c.v. M3G, compared with that from rats pre-treated with i.c.v. saline prior to i.c.v. M3G, but this difference did not reach statistical significance ($p>0.05$, Table 1).

Ataxia and abnormal body posture comprising of an arched back were observed in rats over the 80 min observation period, when i.c.v.

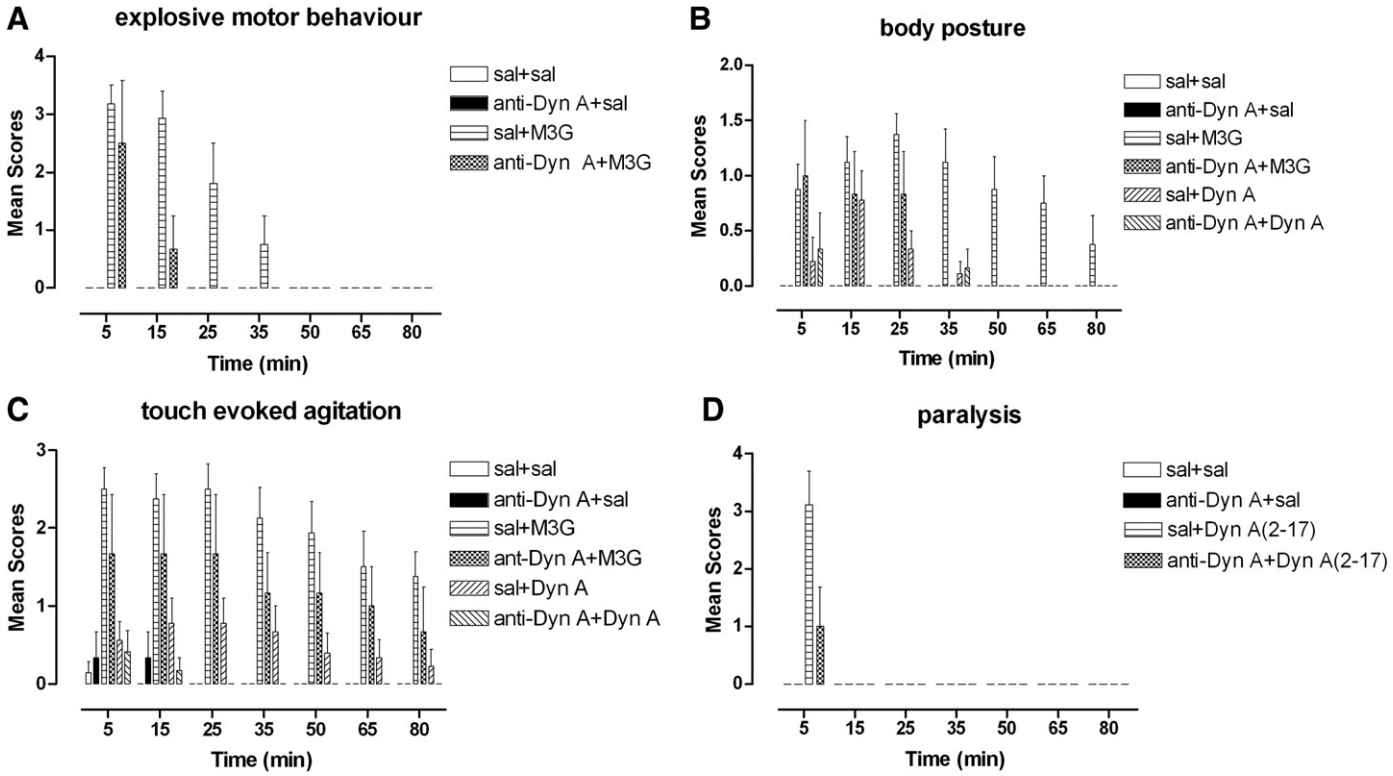

Fig. 2. Mean (±S.E.M.) behavioural excitation scores for rats following i.t. administration of morphine-3-glucuronide (M3G) at 11 nmol or the dynorphin A (Dyn A) fragment peptide Dyn A(2-17) at 15 nmol, 10 min after i.t. pre-treatment with 0.9% saline (sal) or 200 μg of Dyn A antiserum (anti-Dyn A).

M3G followed pre-treatment with i.c.v. saline. As expected, neither ataxia nor an abnormal body posture was seen in rats administered only i.c.v. saline, or when i.c.v. Dyn A antiserum was followed by i.c.v. saline (Fig. 1D). The mean (±S.E.M.) AUC values for the extent and duration of each of ataxia and abnormal body posture in rats pre-treated with i.c.v. saline or Dyn A antiserum prior to i.c.v. M3G were not significantly different ($p>0.05$, Table 1).

When i.c.v. Dyn A(2-17) at 15 nmol followed pre-treatment with i.c.v. saline, abnormal body posture consisting of flattened torso was observed in rats over the 80 min observation period (Fig. 1E). This behaviour was abolished by pre-treatment with dynorphin A antiserum (Fig. 1E).

The mean (±S.E.M.) total behavioural excitation score (∑AUC) was lower (~28%) in rats pre-treated with i.c.v. Dyn A antiserum prior to i.c.v. M3G, compared with that from rats pre-treated with i.c.v. saline prior to i.c.v. M3G, but this difference did not reach statistical significance ($p>0.05$) possibly due to the small sample size in the group pre-treated with Dyn A antiserum (Table 1). The mean (±S.E.M.) ∑AUC was significantly lower (~93%, $p<0.05$) in rats administered i.c.v. Dyn A (2-17) following pre-treatment with i.c.v. saline, compared with that from rats administered i.c.v. M3G following pre-treatment with i.c.v. saline (Table 1).

3.1.2. Intrathecal administration

Intermittent explosive motor behaviour was observed until ~50 min post-dosing, when i.t. M3G followed pre-treatment with i.t. saline. As expected, explosive motor behaviour was not seen in rats administered only i.t. saline, or when i.t. Dyn A antiserum was followed by i.t. saline (Fig. 2A). The mean (±S.E.M.) AUC value for the extent and duration of explosive motor behaviour was lower (~28%) in rats pre-treated with i.t. Dyn A antiserum prior to i.t. M3G, compared with i.t. saline pre-treatment, but this difference did not reach statistical significance ($p>0.05$, Table 2).

Abnormal body posture consisting of an arched back was observed in rats over the 80 min observation period, when i.t. M3G followed pre-treatment with i.t. saline. As expected, abnormal body posture was not seen in rats administered only i.t. saline, or when i.t. Dyn A antiserum was followed by i.t. saline (Fig. 2B). Pre-treatment with i.t.

Table 1
Mean (±S.E.M.) AUC values for the extent and duration of neuro-excitatory behaviours in rats following i.c.v. administration of morphine-3-glucuronide (M3G) at 11 nmol or the dynorphin A (Dyn A) fragment peptide Dyn A(2-17) at 15 nmol, 10 min after i.c.v. pre-treatment with 0.9% saline (sal) or 200 μg of Dyn A antiserum (anti-Dyn A).

Treatment	sal + sal ($n=3$)	anti-Dyn A + sal ($n=3$)	sal + M3G ($n=11$)	anti-Dyn A + M3G ($n=4$)	sal + Dyn A(2-17) ($n=8$)	anti-Dyn A + Dyn A(2-17) ($n=4$)
Excitatory behaviour						
Myoclonic jerks	0	0	181 ± 38[a]	150 ± 62	0	0
Chewing	3 ± 3	0	19 ± 8	6 ± 3	0	0
Tonic–clonic convulsions	0	0	155 ± 34[a]	89 ± 36	0	0
Ataxia	0	0	75 ± 18	52 ± 21	0	0
Body posture	0	0	63 ± 18	60 ± 26	33 ± 17	2 ± 2
Total behavioural excitation						
∑AUC	3 ± 3	0	493 ± 99[a]	357 ± 140	33 ± 17[b]	2 ± 2

[a] $p<0.05$: sal + M3G versus sal + sal.
[b] $p<0.05$: sal + Dyn A versus sal + M3G.

Table 2
Mean (±S.E.M.) AUC values for the extent and duration of neuro-excitatory behaviours in rats following i.t. administration of morphine-3-glucuronide (M3G) at 11 nmol or the dynorphin A (Dyn A) fragment peptide Dyn A(2-17) at 15 nmol, 10 min after i.t. pre-treatment with 0.9% saline (sal) or 200 μg of Dyn A antiserum (anti-Dyn A).

Treatment	sal + sal ($n=3$)	anti-Dyn A + sal ($n=3$)	sal + M3G ($n=8$)	anti-Dyn A + M3G ($n=4$)	sal + Dyn A(2-17) ($n=9$)	anti-Dyn A + Dyn A(2-17) ($n=6$)
Excitatory behaviour						
Explosive motor activity	0	0	81 ± 16[a]	58 ± 34	0	0
Body posture	0	0	73 ± 16[a]	46 ± 23	14 ± 4[b,c]	5 ± 3
Touch-evoked agitation	1 ± 1	6 ± 6	156 ± 25[a]	122 ± 45	41 ± 18[b,c]	5 ± 4
Hind limb paralysis	0	0	0	0	23 ± 4	8 ± 5
Total behavioural excitation						
∑AUC	1 ± 1	6 ± 6	310 ± 50[a]	227 ± 96	78 ± 22[b,c,d]	17 ± 7

[a] $p<0.05$: sal + M3G versus sal + sal.
[b] $p<0.05$: sal + Dyn A(2-17) versus sal + sal.
[c] $p<0.05$: sal + Dyn A(2-17) versus anti-Dyn A + Dyn A(2-17).
[d] $p<0.05$: sal + Dyn A(2-17) versus sal + M3G.

Dyn A antiserum abolished the abnormal body posture produced by i.t. M3G, by 35 min post-dosing (Fig. 2B). Although the mean (±S.E.M.) AUC value for the extent and duration of abnormal body posture in rats pre-treated with i.t. Dyn A antiserum prior to i.t. M3G was lower (~37%) compared with that from rats pre-treated with i.t. saline prior to i.t. M3G, this difference did not reach statistical significance ($p>0.05$, Table 2).

When i.t. Dyn A(2-17) followed pre-treatment with i.t. saline, abnormal body posture consisting of a flattened torso was observed until ~50 min post-dosing (Fig. 2B). This behaviour was abolished by pre-treatment with dynorphin A antiserum, by ~15 min post-dosing (Fig. 2B). The mean (±S.E.M.) AUC value for the extent and duration of abnormal body posture in rats administered i.t. Dyn A following pre-treatment with i.t. saline was significantly lower (~81%, $p<0.05$), compared with that from rats administered i.t. M3G following pre-treatment with i.t. saline (Table 2).

Touch-evoked agitation was observed in rats over the 80 min observation period, when i.t. M3G followed pre-treatment with i.t. saline. As expected, touch-evoked agitation was not seen in rats administered only i.t. saline, or when i.t. Dyn A antiserum was followed by i.t. saline (Fig. 2C). The mean (±S.E.M.) AUC value for the extent and duration of touch-evoked agitation was lower (~22%) in rats pre-treated with i.t. Dyn A antiserum prior to i.t. M3G, compared with that from rats pre-treated with i.t. saline prior to i.t. M3G, but this difference did not reach statistical significance ($p>0.05$, Table 2).

When i.t. Dyn A(2-17) followed pre-treatment with i.t. saline, touch-evoked agitation was observed over the 80 min observation period (Fig. 2C). This behaviour was abolished by pre-treatment with Dyn A antiserum, by ~25 min post-dosing (Fig. 2C). The mean (±S.E.M.) AUC value for touch-evoked agitation in rats administered i.t. Dyn A(2-17) following pre-treatment with i.t. saline was significantly lower (~74%, $p<0.05$), compared with that from rats administered i.t. M3G following pre-treatment with i.t. saline (Table 2).

Administration of i.t. Dyn A(2-17) to rats following pre-treatment with saline resulted in bilateral hind limb paralysis until ~15 min post-dosing. The mean (±S.E.M.) AUC value for the extent and duration of paralysis was lower (~65%) in rats pre-treated with i.t. Dyn A antiserum prior to i.t. Dyn A(2-17), compared with that from rats pre-treated with i.t. saline prior to Dyn A(2-17), but this difference did not reach significance ($p>0.05$, Table 2).

The mean (±S.E.M.) ∑AUC in rats pre-treated with i.t. Dyn A antiserum prior to i.t. M3G was lower (~27%), compared with that from rats pre-treated with i.t. saline prior to i.t. M3G, but this difference did not reach significance ($p>0.05$) possibly due to the small sample size in the group pre-treated with Dyn A antiserum. By contrast, the mean (±S.E.M.) ∑AUC in rats pre-treated with i.t. Dyn A antiserum prior to i.t. Dyn A(2-17) was significantly lower (~78%, $p<0.05$), compared with that from rats pre-treated with i.t. saline prior to i.t. Dyn A (Table 2). The mean (±S.E.M.) ∑AUC was significantly lower (~75%, $p<0.05$) in rats administered i.t. Dyn A(2-17) following pre-treatment with i.t. saline, compared with that from rats administered i.t. M3G following pre-treatment with i.t. saline (Table 2).

4. Discussion

The present study found that i.c.v. bolus doses of M3G at 11 nmol produced neuro-excitatory behavioural effects in rats that included intermittent myoclonus of the face and limbs, chewing, tonic–clonic convulsions, ataxia, as well as an abnormal body posture characterised by an arched back. These findings are consistent with those from previous studies from our laboratory (Bartlett et al., 1994a; Halliday et al., 1999; Wright et al., 2001). Explosive motor activity was also observed when larger doses of M3G (15 nmol) were administered via the i.c.v. route, in an earlier study from our laboratory (Bartlett et al., 1994a).

In the present study, i.t. bolus doses of M3G at 11 nmol produced intermittent explosive motor behaviour, changes in body posture characterised by an arched back, and touch-evoked agitation. In earlier studies, i.t. administration of M3G at 15 μg (32.5 nmol) resulted in severe spontaneous agitation characterised by biting and scratching of the skin near the cannula tip, and touch-evoked agitation (Yaksh and Harty, 1988). Strong spontaneous agitation was also reported in other studies following i.t. administration of M3G at 25 μg (54 nmol) (Suzuki et al., 1993).

Previous studies reported that i.c.v. bolus doses of Dyn A(1-17) or Dyn A(2-17) at 10 nmol in rats produced altered body posture, seizure activity and EEG changes by a non-opioid mechanism (Walker et al., 1982a,b). In other studies, i.c.v. bolus doses of Dyn A(1-17) produced opioid receptor-independent excitatory motor effects in mice that included wild running, 'pop-corn' jumping, hind limb jerks and barrel rolling, for a period of 1–2 min, with an ED_{50} of 14.3 nmol (Shukla et al., 1997).

In other studies, administration of 10–50 nmol i.t. doses of Dyn A (1-17) or Dyn A(2-17) produced hind limb paralysis in rats that was still present 48 h later in some animals administered 50 nmol doses (Faden and Jacobs, 1983, 1984). Pre-treatment with competitive or non-competitive NMDA receptor antagonists (CPP or dextrophan, respectively) significantly attenuated the hind limb paralysis induced by Dyn A(1-17) and Dyn A(2-17) in rats (Bakshi and Faden, 1990). Similarly, mechanical allodynia that developed over a 14 day period from 24 h after i.t. administration of 15 nmol doses of Dyn A(1-17) in rats was blocked by the non-competitive NMDA receptor antagonist, MK-801 (Vanderah et al., 1996).

In the present study, administration of 15 nmol i.t. doses of Dyn A (2-17) produced bilateral hind limb paralysis that commenced ~5 min post-dosing, and lasted for a period of ~5–10 min. Additionally, i.t. bolus doses of Dyn A(2-17) produced changes in body posture

characterised by a flattened torso lasting until ~50 min post-dosing, and touch-evoked agitation over the 80 min observation period. However, i.c.v. administration of Dyn A(2-17) at 15 nmol produced only changes in body posture that were also characterised by a flattened torso over the 80 min observation period.

By contrast, Shukla et al. (1997) reported dose-dependent excitatory motor effects lasting for 1–2 min following i.c.v. administration of Dyn A(1-17) and a number of related opioid and non-opioid fragment peptides to mice, but Dyn A(2-17) was not included in the study by Shukla et al. (1997). The lack of initial excitatory motor effects observed in the present study following i.c.v. administration of Dyn A(2-17) may possibly be due to the use of Dyn A(2-17) in the present study or it may reflect a species difference, as the present study was undertaken in rats, whereas the study by Shukla et al. (1997) used mice.

Importantly, there were marked qualitative differences in the nature of the excitatory behaviours evoked by single bolus doses of centrally administered M3G and Dyn A(2-17) in the present study. Following i.c.v. administration, M3G evoked intermittent myoclonic jerks, chewing, tonic–clonic convulsions, as well as ataxia and alterations in body posture, whereas Dyn A(2-17) resulted only in an altered body posture. Although both M3G and Dyn A(2-17) both produced changes in body posture different effects were observed, such that M3G produced an arched back whereas Dyn A(2-17) produced a flattened torso. Additionally, following i.t. administration, Dyn A(2-17) induced hind limb paralysis, whereas M3G did not.

Pre-treatment with Dyn A antiserum markedly attenuated the total neuro-excitatory behavioural score following i.c.v. and i.t. administration of bolus doses of Dyn A(2-17), thereby confirming the efficacy of Dyn A antiserum for blocking the neuro-excitatory actions of Dyn(2-17). This observation is similar to that of other studies, whereby i.t. administration of Dyn A antiserum (200 μg) blocked thermal hyperalgesia in rats that occurred secondary to increases in spinal Dyn A content produced by spinal nerve injury (Malan et al., 2000). By contrast, i.t. administration of Dyn A antiserum (200 μg) did not significantly attenuate the neuro-excitatory behaviours evoked by spinally or supraspinally administered M3G. When taken together with the marked qualitative differences in behaviours evoked by M3G and Dyn A(2-17) in the present study, our findings indicate that Dyn A does not appear to be directly involved in mediating the neuro-excitatory behaviours evoked by M3G following central routes of administration.

M3G has a very low affinity for binding sites on the NMDA receptor (Bartlett et al., 1994b). However, *in vitro* studies from our laboratory have demonstrated that M3G activates the NMDA receptor indirectly (Hemstapat et al., 2003), although it does not alter potassium-evoked release of glutamate from synaptosomes (Bartlett and Smith, 1996). More recently, electrophysiological studies in spinal cord slices have found that M3G produces a naloxone-insensitive concentration-dependent suppression of inhibitory neurotransmission, via a pre-synaptic reduction in evoked γ-amino butyric acid (GABA)ergic and glycinergic postsynaptic currents (Moran and Smith, 2002).

Our present findings together with those of earlier studies (Bartlett et al., 1994a; Halliday et al., 1999; Woolf, 1981; Wright et al., 2001; Yaksh and Harty, 1988), suggest that the neuro-excitatory behavioural effects evoked by M3G result from the indirect activation of NMDA receptors, following suppression of inhibitory neurotransmission in the central nervous system by M3G. However, the mechanism whereby M3G acts to suppress inhibitory neurotransmission in the central nervous system does not appear to directly involve Dyn A, and hence remains to be determined.

In conclusion, the present study found marked qualitative differences in the behaviours evoked by M3G compared with those evoked by Dyn A(2-17) following supraspinal and spinal routes of administration. Consistent with expectations, i.t. administration of Dyn A antiserum markedly attenuated the effects of Dyn A(2-17) on body posture, touch-evoked agitation and hind limb paralysis. As Dyn A antiserum did not significantly attenuate the neuro-excitatory behaviours evoked by centrally administered M3G, our findings discount a major role for Dyn A in mediating the neuro-excitatory effects of M3G, although it may contribute to maintaining some individual neuro-excitatory behaviours.

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