BMC Pharmacology

BioMed Central

Research article

The spinal antinociceptive effects of cholinergic drugs in rats: receptor subtype specificity in different nociceptive tests

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Published: 19 November 2002

BMC Pharmacology 2002, **2**:20

Received: 13 September 2002
Accepted: 19 November 2002

This article is available from: http://www.biomedcentral.com/1471-2210/2/20

Abstract

Background: Several studies have shown that muscarinic cholinergic agonists cause antinociception in humans and animals when given by both spinal and non-spinal parenteral routes. It is uncertain which subtype of muscarinic receptor is involved in spinally mediated antinociceptive effects caused by these drugs. The cholinergic receptor agonists McN-A-343 (M_1 selective; 3.89 to 389 nmol) and carbachol (non-selective; 0.029 to 29 nmol) were used in a rat acute pain model to investigate the involvement of M_1 and non-M_1 subtypes in spinally mediated antinociception. The drugs were injected intrathecally and results from experiments in which drug actions were carefully confined to the spinal cord were used to construct agonist dose response curves.

Results: McN-A-343 frequently diffused rostrally to the brain, away from the lumbosacral site of injection. Thus, in spite of its receptor subtype selectivity, McN-A-343 is a poor probe to use in attempting to identify receptor subtypes involved in spinal cord antinociceptive systems. However, in some experiments McN-A-343 caused spinally mediated antinociception assessed by the electrical current threshold test. Antinociception assessed by the tail flick latency test with intrathecal McN-A-343 was observed and found to involve supraspinal mechanisms. Carbachol caused spinally mediated antinociception assessed by both electrical current threshold and tail flick latency.

Conclusions: The results suggest that M_1 receptors are involved in spinally mediated antinociception revealed by electrical current threshold; other cholinergic receptors (non-M_1) are involved in thermal antinociception at the spinal cord. This contrasts with previous work on spinally mediated cholinergic antinociception. These differences are believed to be due to difficulties in restricting the action of these drugs to the spinal cord.

Background

Muscarinic cholinergic agonists as well as cholinesterase inhibitors possess antinociceptive activity in animal tests [1–5]. Harris et al. [4] showed that the nonselective muscarinic agonist oxotremorine and the cholinesterase inhibitor physostigmine were as efficacious as morphine in the mouse tail flick. It is important to note, in the light of the current paper and other reports that acetylcholine, administered intracerebroventricularly, has been shown to cause antinociception assessed with the mouse tail-flick

test [6]. These analgesic effects were antagonized by muscarinic antagonists but not by opioid antagonists [6]. That observation suggests that muscarinic antinociception assessed with noxious heat can be mediated directly through muscarinic receptors and not indirectly through opioid systems and furthermore that the antinociceptive effects of these compounds can be caused by drug action at receptors in the brain.

Several studies have tried to investigate the muscarinic receptor subtypes involved in the antinociception evoked by muscarinic agonists. Bartolini et al. [7] suggested that M1 receptors were involved. By contrast Dawson et al. [8] suggested that antinociception in the mouse tail flick was mediated by M1 or M3 receptors. More recently, Iwamoto and Marion (1993) [9], working with intrathecally injected muscarinic agonists, suggested that M1 and/or M2 receptor subtypes were involved in the spinal cord. Much of this work has been clouded by doubts about the receptor selectivity of agonist and antagonist drugs that were used. That is also true for the novel muscarinic agonist McN-A-343 (M_1-agonist) that was used in the study reported here. However, a recent study confirmed that McN-A-343 is a selective M_1 agonist [10]. One study used that selectivity of McN-A-343, which was injected intrathecally, to determine the receptor subtype involved with cholinergic antinociception at the level of the spinal cord. The effects on nociceptive thresholds to noxious heat were assessed following McN-A-343 (M_1-agonist), carbachol (non-selective muscarinic cholinergic agonist) and neostigmine (cholinesterase inhibitor), given intrathecally alone and in combination with antagonists [11]. Using this approach it was concluded that muscarinic agonists were potent spinal analgesics, especially those specific for M_1 and/or M_3 receptor sub-types [11]. Thus this study seemed to confirm much of the previous work.

However, in all of the above-cited work it is uncertain how much of the antinociceptive effects observed were due to actions of the drugs at the spinal cord level. Given that muscarinic agonists may cause antinociception by interactions with receptors in the brain [6] it is possible that even an intrathecally administered drug can cause antinociceptive effects by actions at a brain site by spreading away from its spinal site of injection towards more rostral structures, including the brain. Unless suitable experimental controls are performed to assure the experimenter that drug action is confined to the spinal cord, erroneous conclusions may be drawn about the site of action of a drug and thus the involvement of spinal cord receptors. This can influence greatly the side effect profile of a drug and also the conclusion on its suitability for spinal administration to humans.

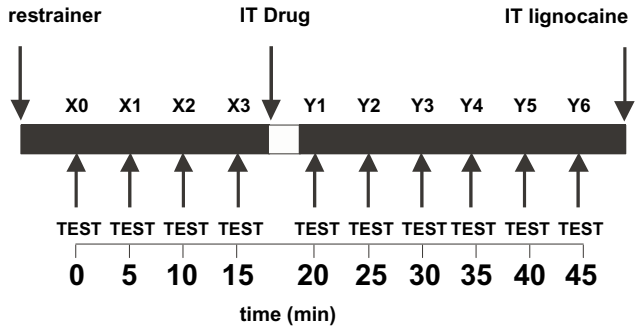

Figure 1
Experimental protocol for nociceptive testing This figure shows the experimental protocol used in all experiments. X values represent ECT (tail and neck) and TFL measurements made before intrathecal (IT) drug administration and Y values represents measurements made after drug administration.

A technique described by Goodchild and Serrao in 1987 enables the site of drug action, brain or spinal cord, responsible for the antinociceptive effects following intrathecal injection of a drug to be determined. This is achieved by measurement of nociceptive threshold using electrical current (ECT) at caudal and rostral skin sites [12]. The ECT is determined at a neck and tail skin site and then an antinociceptive drug is administered intrathecally at the level of the lumbar and sacral segments of the spinal cord responsible for the innervation of caudal dermatomes including the tail. If the drug remains confined to the caudal segments of spinal cord then a rise in tail ECT is noted with no effect on neck ECT. If the drug does spread rostrally to higher levels of spinal cord or brain then a rise in ECT is observed at both tail and neck skin sites. This technique may be combined with concomitant tail flick latency (TFL) measurements to show that a drug affecting this nociceptive modality also produces its effects by a spinal cord action. In the experiments reported in this paper the spinal antinociceptive component of two cholinergic drugs (McN-A-343 and carbachol) was investigated in this acute pain model.

Results

Antinociceptive efficacy of intrathecal McN-A-343

The results of all experiments in this part of the study refer to rats that had a positive result to the lignocaine test both after catheter implantation surgery and after each experiment. Thus in all experiments McN-A-343, was administered intrathecally. This study, as in previous studies using these protocols, revealed that nociceptive thresholds measured each day prior to drug administration remained stable with no significant progressive increase or decrease that might indicate residual drug effects from one day to the next or progressive neurological damage.

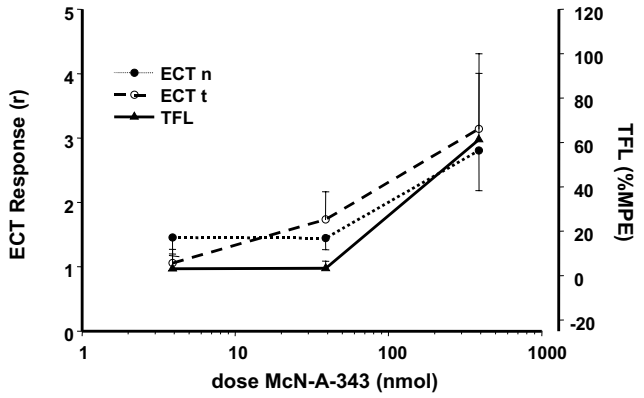

Figure 2
Dose response curves for intrathecal carbachol, all data All ECT (neck and tail) and TFL values recorded in the rats that received intrathecal doses of carbachol that subsequently had positive lignocaine tests. Values for each testing site and modality have been combined at each dose of carbachol and shown as means ± SEM; n = 8–12.

Intrathecal injections of McN-A-343 caused a rapid onset of antinociception when assessed by the ECT test that was maintained for the duration of the experiment. Frequently there was a rise in ECT (n) indicating rostral spread of McN-A-343. Figure 2 shows dose response curves for ECT (t), ECT (n) and TFL including all data. There was no dose of McN-A-343 that caused a rise in ECT (t) without a concomitant rise in ECT (n) in some measurements during the experiment. It was because of this that the data sorting procedure was used to exclude those measurements where there was significant antinociception at the neck skin site (statistically compared with 8% dextrose control) indicative of rostral spread at least as far as the cervical spinal cord and thus possibly to the brain. This allowed construction of dose response relationships in which only spinally mediated effects were included. These dose response curves are shown in figure 3. As the dose of McN-A-343 was increased the number of measurements in which there was no significant rise in ECT (n) decreased compared with the total number of measurements taken for that dose. After the exclusion of those measurements where rostral spread had occurred so that there was no significant antinociceptive effect of McN-A-343 on ECT (n), there was a significant dose dependent increase in ECT (t) ($p < 0.05$, Student's t-test). However there was no significant spinally mediated antinociception observed with the TFL test. The highest dose (389 nmol) of McN-A-343 was found to cause paralysis making impossible the determination of TFL as a measure of antinociception.

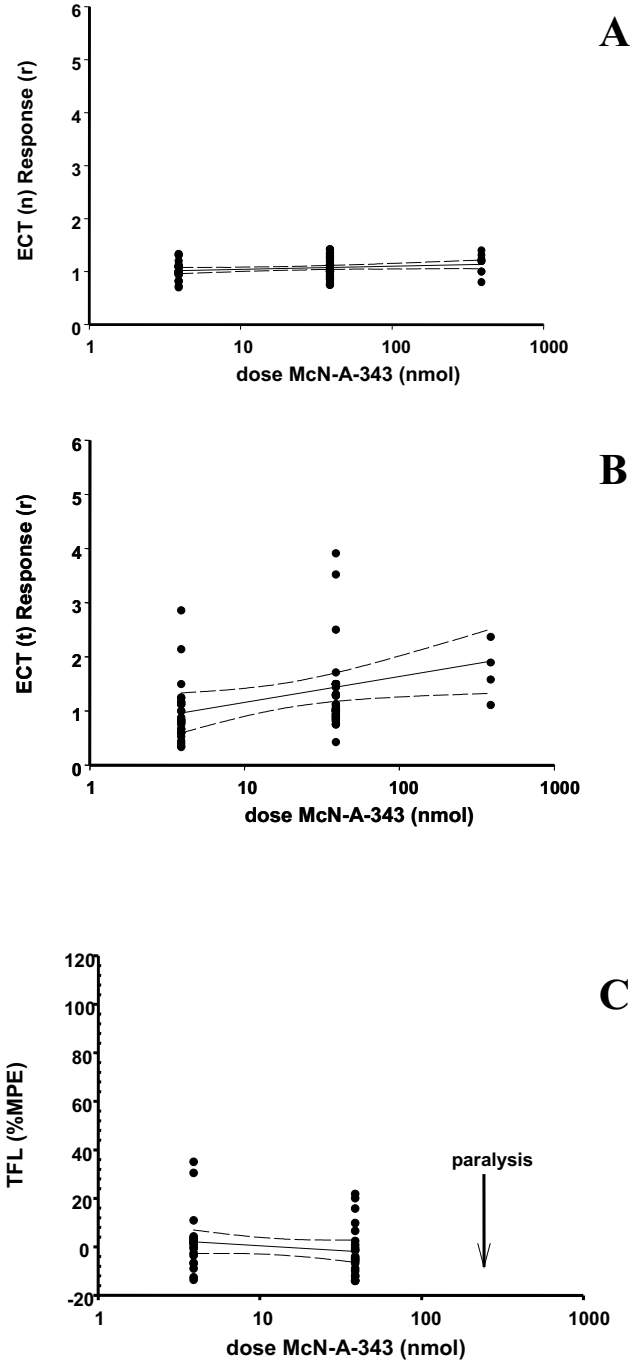

Figure 3
Dose response curves for the spinally mediated antinociceptive effects of intrathecal McN-A-343 The values for ECT (neck, panel A; tail, panel B) and TFL (panel C) are shown on a scatter plot against dose of intrathecal McN-A-343. Only the results from testing times when there was no significant rise in ECT (n) values are shown. These values have been subjected to a logistic regression shown by the continuous line bounded by ± 95% CI shown as broken lines.

Antinociceptive efficacy of intrathecal carbachol

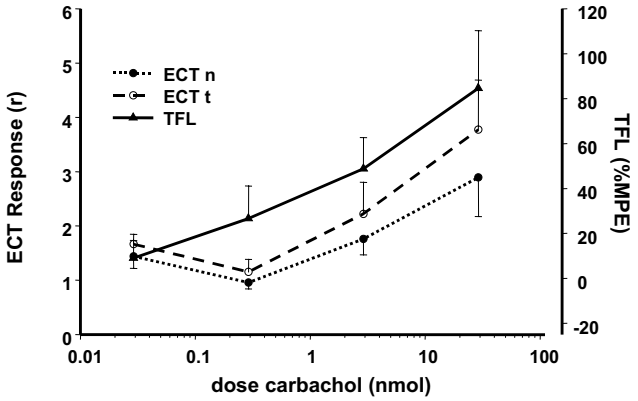

Figure 4
Dose response curves for intrathecal McN-A-343, all data All ECT (neck and tail) and TFL values recorded in the rats that received intrathecal doses of McN-A-343 that subsequently had positive lignocaine tests. Values for each testing site and modality have been combined at each dose of McN-A-343 and shown as means ± SEM; n = 20–25.

As with McN-A-343 IT carbachol caused a rapid rise in both ECT (t) and frequently ECT (n) indicative of rostral spread of the drug in many experiments. Figure 4 shows dose response curves for ECT (t), ECT (n) and TFL including all data. There was no dose of carbachol that caused a rise in ECT (t) without a concomitant rise in ECT (n) for some measurements during the experiment. Therefore, the same data sorting was applied to carbachol that was applied to McN-A-343 to exclude those measurements where rostral spread of the drug had occurred. When spinally confined (i.e. when there was an ECT value in the neck that was not significantly different from the neck ECT values obtained with 8% dextrose controls) there was significant antinociception when assessed by ECT at the tail skin site (figure 5; $p < 0.01$, at the highest dose) although there was a large scatter of results and a shallow dose response regression line. By contrast a clear dose response curve was obtained for TFL. A logistic curve fit allowed calculation of the ED50 for the spinal action of intrathecal carbachol on TFL (1.4 nmol, coefficient of variation = 5.6%; figure 5).

Side effects of intrathecal McN-A-343 and carbachol

A number of significant side effects were noted with the intrathecal administration of the cholinergic agonist drugs. McN-A-343 at doses of 3.89 nmol and 38.9 nmol caused very few side effects, but at the highest dose of 389 nmol where rostral spread was more common many side effects were noted. These ranged from paralysis (28%), death (5%, from respiratory arrest), sedation or tachypnoea (11%), and tail autotomy (11%). In contrast to McN-A-343 the only side effect noted with the use of carbachol was tail autotomy. This autotomy did not appear to be dose dependent.

Discussion

The major obstacle encountered in this study was restricting the action of drugs administered to the lumbosacral segments of the spinal cord. Many drugs given intrathecally do eventually reach the brain via the CSF resulting in drug action at both sites. This is important since many studies have reported that activation of muscarinic cholinergic receptors throughout the CNS causes antinociception [9,13–17].

Previous studies have concluded that M_1 and possibly M_2 and/or M_3 receptors are involved with spinally mediated antinociception [9,11]. These studies used noxious heat as the nociceptive test and the results seem to be at odds with the observations made in gene knockout animals. In M2 gene knockout studies antinociception following muscarinic agonists was markedly reduced implying that M2 receptors are responsible for these effects [18]. Autoradiography studies have shown both M_1 and M_2 muscarinic receptors in laminae II and III of the spinal cord [19]. Functional studies have suggested that the M_1 receptor subtype is responsible for post-synaptic muscarinic antinociception and a recent systematic study by Naguib & Yaksh confirmed this [11,20,21]. These results seem to disagree with an autoradiography study that concluded that there were no M_1 muscarinic receptors in rat spinal cord [22]. However this result relies on the selectivity for the subtypes of muscarinic receptor of the agonist and antagonist drugs used. Thus the background levels of M_1 receptor binding reported by Hoglund and Baghdoyan may actually indicate that there are significant numbers of M_1 receptors in the rat spinal cord [22]. Thus the nature and function of muscarinic receptor subtypes involved with spinally mediated antinociception is still uncertain.

In all of the above-cited in-vivo work it is uncertain how much of the antinociceptive effects observed were due to actions of the drugs at the spinal cord level. Given that muscarinic agonists may cause antinociception by interactions with receptors in the brain [6] it is possible that even an intrathecally administered drug may spread away from its site of injection towards more rostral structures, including the brain and thus cause antinociception. Unless suitable experimental controls are in place to assure the experimenter that drug action is confined to the spinal cord, erroneous conclusions may be drawn about the site of action of a drug and the involvement of specific spinal cord receptors. These have to be performed in every experiment. The model of intrathecal cannulation that is used commonly was shown to deliver drugs selectively to the spinal cord when the method was first described [23].

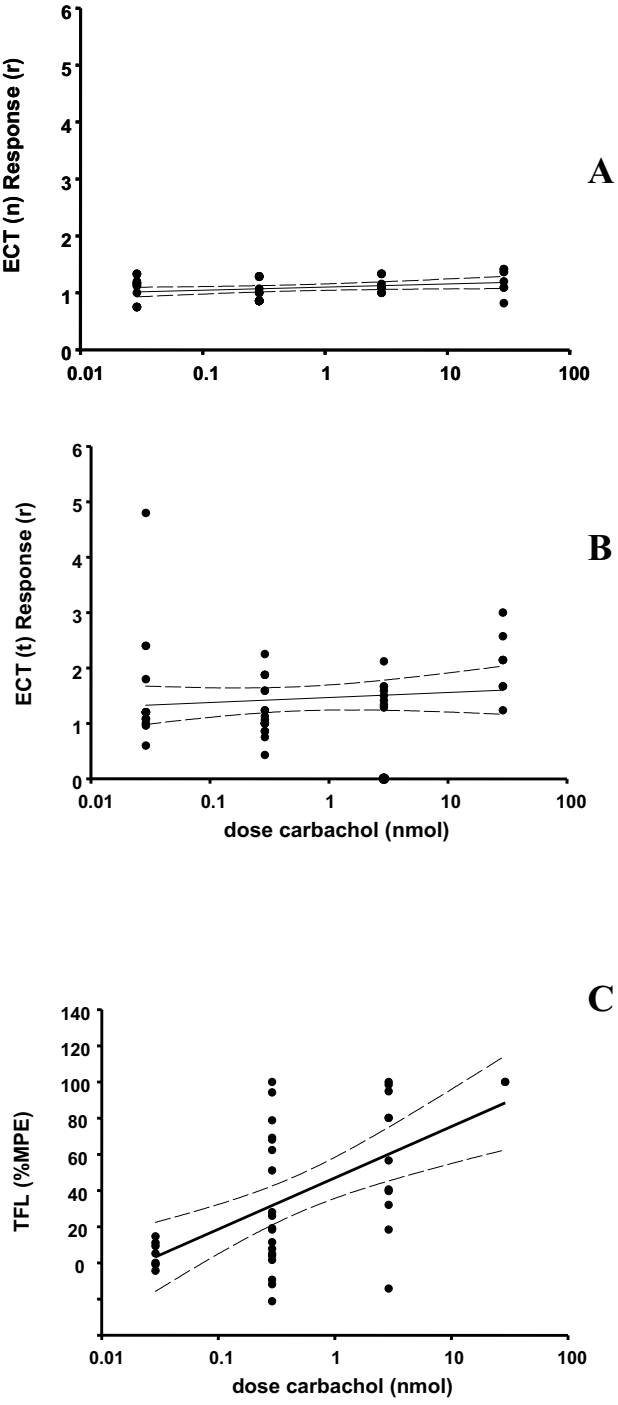

Figure 5
Dose response curves for the spinally mediated antinociceptive effects of intrathecal carbachol The values for ECT (neck, panel A; tail, panel B) and TFL (panel C) are shown on a scatter plot against dose of intrathecal carbachol. Only the results from testing times when there was no significant rise in ECT (n) values are shown. These values have been subjected to a logistic regression shown by the continuous line bounded by ± 95% CI shown as broken lines.

However those checks are rarely applied each time the model is used and it is still possible in individual experiments or in a series of experiments that the catheters could be misplaced or the drugs injected down them might spread rostrally to the brain.

The observations of increases in ECT thresholds in the neck show that drugs injected more caudally at the level of the lumbosacral spinal cord can alter neck ECT measurements and thus they are an indicator of rostral spread of the drug away from lumbosacral segments. In the experiments reported in this paper it must be remembered that even if an antinociceptive effect is observed at the neck skin site this does not positively indicate that the drug has reached the brain because cervical spinal cord segments supply the neck skin site. An increase in ECT (n) does however provide strong evidence that the administered drug has spread rostrally in the spinal cord and thus one cannot be sure that it was confined totally to the spinal cord and that it did not reach the brain to exert a supraspinal effect.

Rostral spread of the cholinergic agonist drugs occurred frequently because of their high water solubility. In order to restrict the movement of drug solution in the CSF the drug was dissolved in an 8% dextrose solution in the experiments reported in this paper. This has a higher specific gravity than CSF and when combined with elevation of the animal's body on an inclined plane head up, it promotes confinement of the drug by gravity to the caudal segments of the spinal cord. In spite of this manoeuvre, rapid rostral spread of the drugs still occurred within five minutes of administration in some animals. Hence the data sorting process using the scattergram was used to extract those measurements where it was confirmed by the ECT (n) measurements that drug effects were confined to the caudal segments of the spinal cord. Values of ECT (tail) and TFL were used for construction of spinally mediated antinociception dose response curves only if their corresponding ECT (n) was not statistically different from ECT (n) values obtained after intrathecal vehicle. The main concern with this approach is the pooling of all post drug measurements over the 30 minutes period instead of the period of peak effect. This is justified given the rapid onset and prolonged effects of these drugs. Pooling of all such measurements at each dose therefore results in an estimate of the average antinociceptive effect over 30 minutes after intrathecal drug.

Antinociceptive efficacy of intrathecal McN-A-343

The first part of the study examined the spinally mediated antinociceptive efficacy of McN-A-343. McN-A-343 has been proposed as a highly selective M_1 agonist [24] and this was confirmed in a recent study designed to settle doubts about the selectivity of McN-A-343 [10]. The re-

sults reported herein suggest that a spinal cord action of McN-A-343 causes significant dose dependent antinociception when determined by ECT (t). Despite the antinociceptive effect of McN-A-343 on ECT there was no antinociceptive effect of McN-A-343 assessed by TFL. At the highest dose (389 nmol) of McN-A-343 TFL was elevated maximally (100% MPE). These animals had suffered hind limb paralysis and so were unable to flick their tail. Thus the 100% MPE measurement may be due to paralysis rather than antinociception although an antinociceptive effect at that dose cannot be ruled out. Paralysis did not affect ECT readings because vocalisation was the response and also the end point. At the 389 nmol dose the rats did vocalise in ECT tests at the tail skin site suggesting that tail anaesthesia had not occurred. This result is different to the findings of Naguib et al [11]. They did not report any side effects at the 389 nmol dose of McN-A-343 suggesting that perhaps they were administering the drug epidurally. This would decrease the amount of drug delivered to the intrathecal space and thus causing an effective rightward shift of the dose response. Many other side effects were observed with the use of McN-A-343 at the 389 nmol dose that were not reported by Naguib et al. The side effects include death, sedation, tachypnoea, tachycardia, tail autotomy as well as hind limb paralysis which are easy to observe and therefore to report. This again suggests that perhaps epidural doses were given in that study [11] and that could explain why their conclusion about M1 receptor involvement in TFL effects differs from the conclusion made after the experiments reported in this paper.

The experiments reported in this paper suggest that spinal cord M1 receptors mediate antinociception assessed by the ECT test. This conclusion is based upon the selectivity of McN-A-343 for that receptor subtype and there is some controversy about this. Although there is the suggestion that it also binds with M_2 and M_3 receptor subtypes this has been refuted by recent studies [10]. However the suggestion for involvement of the M1 subtype in spinal cord by the current study does disagree with binding studies that concluded that there are no M_1 receptors in the rat spinal cord [22]. Thus it is possible if McN-A-343 is not totally selective for the M1 subtype, that the ECT effects of McN-A-343 at the level of the spinal cord are mediated by M_3 receptors.

Even if McN-A-343 were a very selective probe for M1 receptors, it does not seem to be a good probe to investigate the involvement of M1 receptors in spinal cord antinociceptive mechanisms. The high water solubility of this compound led frequently to rostral spread. TFL only rose when McN-A-343 had spread rostrally to elevate ECT(n). Thus TFL antinociception following intrathecal McN-A-343 is a supraspinal effect. Even at the supraspinal level the involvement of M1 receptors in the TFL responses after McN-A-343 is in doubt because it has been shown in M2 gene knockout mice that the antinociceptive effects of muscarinic agonists assessed with the TFL test is markedly reduced [18]. It is possible that neck ECT values rose following increased intraspinal release of acetyl choline caused by intrathecal injection of agonist; this has been shown for carbachol [29]. However this would still indicate spread of the drug away from its site of injection making it impossible to be sure of the site of action. It is also plausible that the negative results in this study for McN-A-343 may be because M1, M2, M3 oligomeric combination receptors may be involved [30].

Antinociceptive efficacy of intrathecal carbachol

The second part of this study examined the antinociceptive efficacy of the non-selective cholinergic agonist, carbachol. It was found that carbachol caused statistically significant antinociception assessed by ECT at the tail skin site when there were no significant changes in ECT (n) thresholds, although there was great scatter of results and a shallow dose response curve. There was a classical and highly significant dose related antinociception for the TFL test. This antinociceptive effect for TFL occurred at doses significantly lower than those observed by Naguib et al. (1997). The ED_{50} for carbachol on paw flick latency (PFL; equivalent to TFL) was 29 nmol in Naguib's study whereas the ED_{50} in this study for carbachol on TFL was 1.4 nmol. One explanation for this is that Naguib et al. may have been giving doses of agonist epidurally.

There are two reasons that point to the fact that previous studies may have given drugs via catheters that had migrated from the intrathecal space epidurally. The first is that others using the same drugs and formulations observed none of the side effects noted in this study. The side effect dose response curve could be shifted rightward if the drug was given into the epidural space and thus lower concentrations diffused from there into the subarachnoid space. The major finding that suggests previous experimenters were giving their doses epidurally is that the observed TFL effects of carbachol (ED_{50} = 1.4 nmol) occurred at doses approximately twenty times lower than those used by prior studies (ED_{50} = 29 nmol) [11]. It is known that if a catheter is placed epidurally a much larger dose than the intrathecal dose of lignocaine will be required to cause the same paralysis.

The ability of intrathecal carbachol to cause thermal antinociception by a spinal cord action contrasts with McN-A-343 that did not cause thermal antinociception when its effects were confined to the spinal cord. This means that a cholinergic receptor other than the M_1 subtype may be responsible for the thermal antinociception caused by carbachol. It has been suggested that cholinergic antinociception may be due to M_1 and M_2 receptor sub-

types so perhaps M_2 is responsible for thermal antinociception [25]. This conclusion fits with the study in M2 gene knockout mice showing that the antinociceptive effects of muscarinic agonists assessed with the TFL test is markedly reduced in these animals [18].

Conclusions

In conclusion this study has shown that cholinergic agonists do produce spinally mediated antinociception affecting both electrical and thermal modalities shown by changes in ECT and TFL. Intrathecal McN-A-343, which binds with M1 and possibly M3 subtypes of muscarinic receptors, produces spinally mediated antinociception when determined by the electrical modality of ECT, but not by the thermal modality of TFL. The observations of spinally mediated antinociception assessed with ECT and TFL following carbachol, when combined with the observations on the activity of McN-A-343, suggest that non-M_1 (probably M_2) muscarinic receptors are involved in *spinally-mediated* thermal antinociception. There are contradictions between studies setting out to define the muscarinic receptor subtypes involved with spinal antinociception. These contradictions probably arise from methodological differences such as not confirming with appropriate controls, correct intrathecal dosing and confinement of drugs and their effects to the spinal cord.

Methods

This work was carried out with the permission from the Monash University Standing Committee On Ethics in Animal Experimentation (SCEAE No. B-97-01). In all experiments attention was paid to ethical guidelines for the investigation of experimental pain in conscious animals [26].

Intrathecal catheter implantation

Male Wistar rats (140–160 g) were anaesthetised with halothane in oxygen-enriched air (FiO_2 = 0.4) and a Portex catheter (I.D. 0.28 mm, O.D. 0.61 mm) was implanted under aseptic conditions into the lumbar subarachnoid space to lie adjacent to the lower lumbar and sacral segments of the spinal cord using a method described previously [12]. After the rats recovered from general anaesthesia they were observed for normal behaviour and movement. If there were any signs of neurological damage, such as paralysis, the animal was killed immediately. Correct placement of the catheter was verified by intrathecal injection of lignocaine (2% 10 μL) into the catheter followed by 15 μL of saline flush. Correct position was confirmed if the animal became paralysed in the hindlimbs within 30 seconds of this local anaesthetic injection [12]. This test was performed immediately after recovery from surgery and also after each experiment. Thus, we were confident that all drugs injected down the catheter were introduced into the lumbar subarachnoid space. A minimum of at least 12 hours elapsed between catheter implantation and nociceptive testing. In previous studies using these protocols it has been shown that nociceptive thresholds measured each day prior to drug administration remain stable with no significant progressive increase or decrease that might indicate residual drug effects or neurological damage [12].

Nociceptive tests

Nociceptive thresholds were measured with two tests: electrical current (ECT) at two skin sites, the neck-ECT (n) and tail – ECT (t); and noxious heat (tail-flick latency, TFL) as described previously [27,28]. A series of experiments using these nociceptive tests were performed on each rat, the first one being on the day after cannulation. One experiment was performed each day on each rat up to a maximum of four. The rats were kept in groups of 5 prior to intrathecal cannulation and then in single boxes in rooms with a 12-h12 h light/dark cycle. They were allowed free access to food and water throughout. Experiments were carried out in a quiet, darkened environment to decrease distraction of the animal by extraneous noises and light.

Electrical current threshold test

Each rat was placed in a plastic restrainer (elevated head up on an inclined plane at 30°) and all the antinociceptive tests were performed sequentially every five minutes as shown in figure 1. Needle electrodes were placed 1 cm apart in the skin at the base of the neck and wire electrodes were applied to the surface of the skin 1 cm and 5 cm from the base of the tail. These pairs of electrodes were connected alternately to a constant current stimulator (50 Hz; 2 ms pulses; 0.5 s train) in order to measure the ECT at each skin site. The ECT was the minimum current necessary to provoke either sharp withdrawal or (more usually) a squeak. Three consecutive stable (pre-drug injection) readings (X1, X2, X3) were obtained and then the intrathecal injection of cholinergic drug (McN-A-343 or carbachol) was given. The tests were repeated every five minutes for a further thirty minutes to obtain six post-injection readings (Y1 – Y6). The responses of individual animals were standardised because the absolute measurement of electrical thresholds in mA varied between animals and even in the same animal at different testing times because of minor differences in electrode position. The standardisation was achieved by each ECT reading obtained after the intrathecal injection by the mean of the three corresponding control (pre-injection) readings for that set of electrodes. This was called the standardised response expressed as a multiple of control (x control).

Tail flick latency (TFL) test

The tip of the tail was painted black after the animal had been placed in the restrainer. The heat from 150 w projec-

tor bulb was focussed on the blackened part of the tail and the time taken for the animal to remove (flick) its tail from the source of the heat was measured. As with the ECT test intrathecal drug was injected after three stable readings had been obtained. The three values taken prior to intrathecal drug were averaged to give a mean latency (TFL pre-drug) and each post drug reading (TFL post-drug) was standardised as a percentage maximum possible effect (%MPE) as follows:

$$\%MPE = \frac{TFL(post\text{-}drug) - TFL(pre\text{-}drug)}{cut\ off\ time - TFL(pre\text{-}drug)}$$

away from the heat source before the lamp was automatically switched off; this was set at 10 s in order to avoid burning the tail.

Tail flick latency was always measured before either electrical threshold and the neck threshold was assessed after the tail measurements. ECT testing of the neck and tail skin sites was performed in every experiment to determine that the antinociceptive drug given was confined to the caudal segments of the spinal cord. For example, a rise in tail thresholds [ECT (t)] after intrathecal drug, but not neck thresholds [ECT (n)], proves that the drug is confined in its actions to the caudal segments of the spinal cord for ECT antinociception and also any concurrent increase in TFL.

Dose response curves for the antinociceptive effects of McN-A-343 (M_1 agonist) and carbachol (non selective cholinergic agonist) were generated using ECT at neck and tail skin sites and TFL. McN-A-343 (Research Biochemicals International) was administered intrathecally to 51 rats in 74 experiments at doses 38.9 – 389 nmol (dissolved in 5 μL 8% glucose solution). Carbachol (Research Biochemicals International) was also administered intrathecally to 22 rats in 41 experiments at doses 0.29 – 29 nmol (dissolved in 5 μL 8% glucose solution). All intrathecal injections were performed via a Hamilton micro syringe that was attached to the catheter. Injections were given in a constant volume of 5 μL of 8% dextrose solution at 15 μL/min. Only one dose of cholinergic drug was injected into each rat per day; the order of doses being random in those rats that received more than one dose of drug.

Controls

Twenty-six rats with intrathecal catheters were given 5 μl intrathecal injections of 8% dextrose solution and ECT and TFL values were measured as above. These experiments were performed to establish that this vehicle had no effect on nociceptive thresholds and also to establish a mean and a scatter around the mean of nociceptive measurements at both skin sites. The latter could be used to test statistically if any particular threshold measurement taken after drug administration was significantly different from control.

Table 1: Vehicle controls, 8% dextrose Results from control experiments. ECT responses from tail and neck electrodes after 5 μl intrathecal injections of 8% dextrose.

	mean	*SEM*	*n*
ECT tail	1.07	0.01	26
ECT neck	1.03	0.01	26

Statistics and construction of dose response curves

Both agonist drugs proved difficult to confine in their actions to the caudal segments of spinal cord, i.e. rises in neck thresholds were observed frequently. This occurred in spite of dissolving the drug in a solution that was hyperbaric compared with CSF (8% dextrose) and performing injections with the rat inclined at 30° to the horizontal ("head up") to try to restrict the drug by gravity to caudal segments of spinal cord. However, the responses to both drugs were found to be long lasting, i.e. an antinociceptive effect occurred within five minutes of the intrathecal injection and it continued throughout the experimental period of 40 minutes thereafter. It was decided to employ a statistical method to extract those datum points for ECT (t) and TFL where there was no significant rise in the ECT (n) value taken at that time point and to use these data to construct dose response curves.

For each lignocaine positive experiment the %MPE for the TFL measurements and the values for ECT (t), ECT (n) and TFL were calculated for each 5 min time point. Thus each time point after all doses of agonist given in an experiment where the lignocaine test was positive yielded three values, an ECT (n) with the corresponding ECT (t) and TFL values. The values of ECT (n) obtained in the control experiments in which the vehicle was given intrathecally (8% dextrose, n = 26) were combined to calculate a mean and 95% confidence intervals. These values were used to identify ECT neck data that showed no significant change following intrathecal agonist (within the 95% confidence intervals around the mean) and thus the corresponding ECT tail and TFL changes that could be ascribed to a spinal cord action of the intrathecal drug. Logistic regression ($\gamma = a\ \log_e(x)+b$) using sums of squares was performed on these spinal data (where ECT neck value was within the 95% confidence intervals of the neck data in control experiments with 8% dextrose). The resultant line with 95%

confidence limits was plotted against dose of drug administered superimposed on a scatter plot of the TFL and ECT responses that were used to calculate the regression lines.

Authors' contributions

ML performed the experiments at the bench closely supervised by RN and CSG who conceived and planned the experiments.

Acknowledgements

The authors wish to thank Dr John Ludbrook for assistance with statistical analysis.

References

1. Chen G: **The anti tremorine effect of some drugs as determined by Hoffner's method of testing analgesia in mice.** *J Pharmacol Exp Ther* 1958, **124:**73-76
2. Herz A: **Actions of arecoline on the central nervous system.** *Naunyn-Schmiedebergs Arch Exp Pathol Pharmacol* 1962, **242:**414-429
3. Metys J, Wagner N, Metysova J, Herz A: **Studies in central antinociceptive action of cholinomimetic agents.** *Int J Neuropharmacol* 1969, **8:**413-425
4. Harris LS, Dewey WL, Howes JF, Kennedy JS, Pars H: **Narcotic antagonist analgesics:interactions with cholinergic systems.** *J Pharmacol Exp Ther* 1969, **169:**17-22
5. Ireson JD: **A comparison of the antinociceptive actions of cholinomimetic and morphine-like drugs.** *Br J Pharmacol* 1970, **40:**92-101
6. Pedigo NW, Dewey WL, Harris LS: **Determination and characterization of the antinociceptive activity of intraventricularly administered acetylcholine in mice.** *J Pharmacol Exp Ther* 1975, **193:**845-852
7. Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A: **Role of muscarinic receptor subtypes in central antinociception** *British Journal of Pharmacology* 1992, **105:**77-82
8. Dawson GR, Johnstone S, Boyley P, Iversen SD: **The effects of a novel muscarinic receptor agonist, L-689666 in the mouse tail-flick test of antinociception.** *Br J Pharmacol* 1991, **104:**459P
9. Iwamoto ET, Marion L: **Characterization of the antinociception produced by intrathecallyadministered muscarinic agonists in rats.** *J Pharmacol Exp Ther* 1993, **266:**329-338
10. Davies RH, Scholes HH, Virdi S, Broadley KJ: **Inhibition of field stimulation-induced contractions of rabbit vas deferens by muscarinic receptor agonists: selectivity of McN-A-343 for M1 receptors.** *J Pharm Pharmacol* 2001, **53(4):**487-496
11. Naguib M, Yaksh TL: **Characterization of muscarinic receptor subtypes that mediate antinociception in the rat spinal cord.** *Anesthesia & Analgesia* 1997, **85:**847-853
12. Goodchild CS, Serrao JM: **Intrathecal midazolam in the rat: evidence for spinally-mediated analgesia.** *Br J Anaesth* 1987, **59:**1563-1570
13. Abram SE, O'Connor TC: **Characteristics of the analgesic effects and drug interactions of interactions of intrathecal carbachol in rats.** *Anesthesiology* 1995, **83:**844-849
14. Abram SE, Winne RP: **Intrathecal acetyl cholinesterase inhibitors produce analgesia that is synergistic with morphine and clonidine in rats.** *Anesthesia and Analgesia* 1995, **81:**501-507
15. Detweiler DJ, Eisenach JC, Tong C, Jackson C: **A cholinergic interaction in alpha 2 adrenoceptor-mediated antinociceptionin sheep.** *J Pharmacol Exp Ther* 1993, **265:**536-542
16. Naguib M, Yaksh TL: **Antinociceptive effects of spinal cholinesterase inhibition and isobolographic analysis of the interaction with mu and alpha 2 receptor systems.** *Anesthesiology* 1994, **80:**1338-1348
17. Sheardown M, Shannon HE, Swedberg MDB, Suzdak PD, Bymaster FP, Olesen PH, Mitch CH, Ward JS, Sauerberg P: **M1 Receptor Agonist Activity Is Not a Requirement for Muscarinic Antinociception.** *J Pharmacol Exp Ther* 1997, **281:**868-875
18. Gomeza J, Shannon H, Kostenis E, Felder CC, Zhang L, Brodkin J, Grinberg A, Sheng H, Wess J: **Pronounced pharmacologic deficits in M2 muscarinic acetylcholine receptor knockout mice.** *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96(4):**1692-1697
19. Gillberg PG, Askmark H: **Changes in cholinergic and opioid receptors in the rat spinal cord, dorsal root and sciatic nerve after ventral and dorsal root lesion.** *Journal of Neural Transmission* 1991, **85:**31-39
20. Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A: **Role of muscarinic receptor subtypes in central antinociception.** *British Journal of Pharmacology* 1992, **105:**77-82
21. Ghelardini C, Malmberg-Aiello P, Giotti A, Malcangio M, Bartolini A: **Investigation into atropine-induced antinociception.** *British Journal of Pharmacology* 1990, **101:**49-54
22. Hoglund AU, Baghdoyan HA: **M2, M3 and M4, but not M1, Muscarinic Receptor Subtypes are Present in Rat Spinal Cord.** *J Pharmacol Exp Ther* 1997, **281:**470-477
23. Yaksh TL, Rudy TA: **Chronic catheterization of the spinal subarachnoid space.** *Phys Behav* 1976, **17:**1031-1036
24. Hu J, El-Fakahany EE: **Selectivity of McN-A-343 in simulating phosphoinositide hydrolysis mediated by M1 muscarinic receptors.** *Molecular Pharmacology* 1990, **38:**895-903
25. Gillberg PG, Gordh T Jr, Hartvig P, Jansson I, Pettersson J, Post C: **Characterization of the antinociception induced by intrathecally administered carbachol.** *Pharmacol Toxicol* 1989, **64:**340-343
26. Zimmerman M: **Ethical guidelines for investigation of experimental pain on conscious animals.** *Pain* 1983, **16:**109-110
27. Edwards M, Serrao JM, Gent JP, Goodchild CS: **On the mechanism by which midazolam causes spinally mediated analgesia.** *Anesthesiology* 1990, **73:**273-277
28. Nadeson R, Guo Z, Porter V, Gent JP, Goodchild CS: **$GABA_A$ Receptors and Spinally-Mediated Antinociception in Rats.** *J Pharmacol Exp Ther* 1996, **278:**620-626
29. Hoglund AU, Hamilton C, Lindblom L: **Effects of Microdialyzed Oxotremorine, Carbachol, Epibatadine, and Scopolamine on Intraspinal Release of Acetylcholine in the Rat.** *J Pharmacol Exp Ther* 2000, **295:**100-4
30. Fu-Yue Z, Jürgen W: **Identification and Molecular Characterization of M_3 Muscarinic Receptor Dimers.** *J Biol Chem* 1999, **274:**19487-497