Antagonist of nicotinic acetylcholine receptors (nAChR) enhances formalin-induced nociception in rats: tonic role of nAChRs in the control of pain following injury

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Abstract

Following tissue injury, spinal neurons increase in spontaneous activity and in responsiveness to peripheral stimulation. These changes in spinal neurons may underlie abnormal pain behavior. Nicotinic acetylcholine receptor (nAChR) agonists are analgesic when evaluated in animal models of pain, but it is not known if the nAChRs differentially modulate acute and tonic pain. To test this, mecamylamine, a non-subtype selective nAChR antagonist, was systemically injected into rats prior or after hind paw injection of formalin. Formalin injection results in biphasic pain-related behaviors, characterized by a first phase (i.e. acute pain) immediately following formalin injection, then by a second phase (i.e. tonic pain) 15–60 min after formalin injection. Either pre- or post-formalin treatment with mecamylamine decreased phase 1 behaviors and significantly increased phase 2 pain behaviors in a dose-dependent manner. These results suggest that nAChRs may exert opposing effects on acute versus tonic pain and, as such, may have implications for the potential development of nAChR ligands for the treatment of pain.

1. Introduction

Recent studies have shown that the nicotinic acetylcholine receptor (nAChR) ligands nicotine and epibatidine have analgesic effects in animal models of pain, which is reduced with the ion channel blocker mecamylamine [1,3,4,14]. Binding sites for nAChR ligands and mRNA for nAChRs have been found in regions that modulate pain perception including dorsal root ganglia, spinal cord dorsal horn and medullary nuclei [11,17]. Elevating cerebrospinal fluid (CSF) levels of acetylcholine with the acetylcholine esterase inhibitor neostigmine also leads to analgesia, which is partially attenuated by mecamylamine [3,7]. These observations suggest that the analgesia following activation of the cholinergic system is mediated, at least in part, through activation of nAChRs.

Tissue injury is known to result in persistent or abnormal pain perception. For instance, formalin injection into the rat hind paw results in biphasic pain-related behaviors. The first phase (i.e. acute pain) is observed immediately following formalin injection and lasts only a few minutes, whereas the second phase (i.e. tonic pain) appears about 15 min after formalin injection and lasts at least 60 min post-formalin. Both phases are characterized by an increase in pain-related behaviors (such as licking, flinching and biting of the injured paw) but seem to involve two distinct mechanisms. The initial pain behaviors are believed to be driven by primary afferent nociceptor activity, as opposed to the pain behaviors in phase 2 which are thought to arise from nociceptive spinal neuron hyperactivity, a consequence of the injury-induced excitatory barrage from primary afferents [5]. It has been hypothesized that a decrease of inhibitory input to spinal neurons...
also underlies spinal neuron hyperactivity. Activation of GABA$_\text{A}$, serotonergic and $\alpha$-adrenergic receptors leads to analgesia. Conversely, blockade of each of these receptors with antagonists prior to hind paw formalin injection results in increased pain behaviors [9,13]. The elevation of pain behaviors following blockade of endogenous inhibitory systems suggest that these systems tonically modulate pain following tissue injury.

Acute noxious stimulation increases CSF levels of acetylcholine, but it is not known if the cholinergic system is activated during prolonged pain states [6]. It is hypothesized that the cholinergic system, particularly via activation of nAChRs, may play an inhibitory role in tonic pain. Thus, prevention of acetylcholine activity by blockade of nAChRs with mecamylamine, either pre- or post-formalin, should increase formalin-induced pain behaviors. The role of nAChRs in acute pain is also not well explored and this study will compare the effect of blockade of nAChRs on acute versus tonic pain.

2. Materials and methods

All experiments followed guidelines of the National Institutes of Health and were approved by the institutional animal care committee. Male Sprague–Dawley rats (275–325 g; Sasco–Harlan) were group-housed, on a 12 h light–dark cycle and had free access to food and water.

2.1. Mecamylamine pre-treatment

Individual rats were acclimated to Plexiglas observation chambers for 30–60 min prior to behavioral evaluation. Rats were then treated with either mecamylamine hydrochloride (1.5, 3, 6 mg/kg; Research Biochemicals, Inc.) or saline vehicle, injected subcutaneously (s.c.) in a volume of 1 ml/kg.

Five minutes following injection of either mecamylamine or vehicle, 50 µl of formalin (0.25, 0.5, 1, 2.5%) was injected s.c. into the left plantar hind paw. Low concentrations of formalin (0.25–2.5%) were tested in order to better observe increases in pain behaviors.

The number of pain behaviors (flinches and licking of the hind paw) were counted for 1 min, every 5 min for the duration of the 60 min observation period. The phases were defined as: phase 1: 0–5 min, interphase: 5–15 min, and phase 2: 15–60 min. Upon completion of the observation period, rats were promptly euthanized.

To determine if mecamylamine affected the formalin-evoked phase 1 and phase 2 pain behaviors, formalin concentration response curves were constructed and $A_{50}$ values were compared. $A_{50}$ is defined as the formalin concentration needed to produce 50% maximal total pain behaviors [16]. The $A_{50}$ values were calculated based on the total number of pain behaviors from phase 1 and phase 2, converted to a percent of maximal effect (%MPE) according to the following:

$$\%\text{MPE} = \frac{(\text{Total pain behaviors})}{E_{\text{max}}} \times 100,$$

where $E_{\text{max}}$ was the observed maximum total pain behaviors. The $E_{\text{max}}$ in phase 1 and 2 were 48 and 277, respectively.

2.2. Mecamylamine post-treatment

Robust enhancement by mecamylamine of formalin-induced pain behaviors was observed in rats treated with 0.5% formalin. In a separate group of rats, mecamylamine (1.5, 3, 6 mg/kg) was injected s.c. 6 min following hind paw injection of 0.5% formalin. Pain behaviors were recorded as described above. Upon termination of the experiment, rats were promptly euthanized.

2.3. Statistics

Calculations and comparisons of the mecamylamine pre-treatment and vehicle pre-treatment $A_{50}$ values were performed using computer software based on Tallarida and Murray [16] and statistical significance was evaluated by $t$-test. Comparisons of the total pain behaviors in each phase were made between mecamylamine- and saline-treated rats using one-way analysis of variance, with post-hoc analysis using Dunnett’s test when appropriate. $P$ values less than 0.05 were considered significant.

3. Results

3.1. Mecamylamine pre-treatment

Pain behaviors as a function of formalin concentration in mecamylamine and saline-treated rats are illustrated in Fig. 1. Following formalin injection, the saline-treated group exhibited significant biphasic pain behaviors over time, suggesting that the concentrations of formalin that were used for this study were adequate to induce painful tissue injury. Regardless of the pre-treatment (i.e. saline or mecamylamine), formalin increased total pain behaviors in a dose-dependent manner (Fig. 1).

Overall, mecamylamine treatment altered both phase 1 and phase 2 pain behaviors induced by formalin administration (Fig. 1). Since no discernible effect of mecamylamine was observed on interphase pain behaviors, the results will focus on the effects on phase 1 and 2.

Mecamylamine dose-dependently decreased pain behaviors induced by formalin in phase 1, with the most effective dose being the lowest tested (1.5 mg/kg). At this dose, the formalin concentration that elicited 50% of maximal observable pain behaviors in phase 1 ($A_{50}$) was
mecamylamine, the \( A_{50} \) was shifted from 1.4 to 0.6%, which is a 1.6-fold leftward shift compared to saline-treated rats (\( P<0.05 \); Table 1, Fig. 1B).

The effect of mecamylamine was dependent on the concentration of formalin tested. Thus the effect of mecamylamine on phase 1 was more pronounced at a concentration of 1% formalin whereas the effect of mecamylamine on phase 2 was more pronounced at a concentration of 0.5% of formalin (Fig. 2).

3.2. Mecamylamine post-treatment

To evaluate the effect of blocking the cholinergic ion channel following tissue injury, the highest dose of mecamylamine was injected during the interphase (i.e. 6 min after injection of 0.5% formalin). Rats treated with mecamylamine after formalin displayed a slight, but significant, increase of total pain behaviors in phase 2 (\( P<0.05 \) versus vehicle; Fig. 3).

4. Discussion

The present study demonstrates that blockade of nAChRs by mecamylamine, before formalin injection in the rat hind paw, leads to an enhancement of phase 2 pain behaviors, whereas phase 1 pain behaviors tended to be reduced. The increase in pain behaviors was also observed when mecamylamine was administered after phase 1. This suggests that in the formalin test, nAChRs may have opposing roles in modulating the initial and long-term response to tissue injury.

The current study utilized different concentrations of formalin to evaluate the effect of antagonizing nAChRs on acute and tonic pain. Effects on pain behaviors, particularly in phase 2, may not have been apparent if a common stimulus, a high concentration of formalin (e.g. 5%), was utilized. A weaker stimulus, 0.5% formalin, which was enough to evoke long lasting pain behaviors, did reveal an enhancement by mecamylamine. Increases in pain behavior with mecamylamine were not evident at the higher concentrations (1–2.5%) of formalin, likely due to a ceiling effect. On the other hand, a decrease in pain

Table 1

<table>
<thead>
<tr>
<th>Mecamylamine (mg/kg)</th>
<th>Phase 1 Potency ratio(^a)</th>
<th>Phase 2 Potency ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.1 (1.6–2.9)</td>
<td>1.4 (1.0–1.9)</td>
</tr>
<tr>
<td>1.5</td>
<td>14.0 (6.4–30.8)*</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>3.4 (2.1–5.5)</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>1.8 (1.1–3.1)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

\(^a\) The \( A_{50} \) is the formalin concentration that will elicit 50% maximal pain behaviors.

\(^*\) Potency ratio: The ratio of the \( A_{50} \)'s of vehicle to mecamylamine. A potency ratio of greater than 1 suggests a leftward shift of the formalin concentration curve and a potency ratio of less than 1 suggests a rightward shift. Thus, mecamylamine tended to increase the phase 1 \( A_{50} \) and decrease phase 2 \( A_{50} \).

\(^{Significant difference between mecamylamine treatment and vehicle treatment, in the corresponding phase (\( P<0.05 \)).
behavior was only evident at concentrations of formalin greater than 0.5%. Thus, the effect of mecamylamine on pain behavior is directly dependent on the level of pain induced by formalin. Similarly, the effect of mecamylamine is dose-dependent. Mecamylamine appears to increase pain behaviors at doses that are lower than the doses that decrease pain behavior. Although not proven, the differential dose-dependent effect of mecamylamine may be related to different levels of ACh released during different pain states (acute versus chronic).

In the current study, systemic antagonism of nAChRs on formalin-induced pain leads to conflicting results. On one hand, blockade of nAChRs tended to decrease acute pain behaviors and on the other hand, blockade of nAChRs enhanced tonic pain behaviors. These data suggest that activation of nAChRs may lead to a transient increase in pain. Indeed, nicotine has been shown to activate primary afferent nociceptors and spinal cord nociceptive neurons, which evokes brief pain-related behaviors in rats [8,10,15]. The current data also suggests that the tonic pain state is modulated by the cholinergic system and that blockade of...
nAChRs will lead to increased pain behaviors. The dual effects observed in the current study may be due to blockade of nAChRs at different sites. Also, the decrease/increase in pain behaviors following mecamylamine treatment may be due to altered properties of nAChRs in the acute versus the tonic pain state. It is possible that following injury, hyperactivity of spinal or brain neurons may induce alterations in the cholinergic system, such as changing acetylcholine release or receptor function. To test this hypothesis, mecamylamine was administered after formalin injection. As with injection prior to formalin, mecamylamine injection after formalin resulted in an increase in phase 2 pain behaviors. This result suggested that activity of the cholinergic system is perceptibly modified, as assessed by pain behavior.

Alternatively, as mecamylamine is a non-competitive ion channel blocker, it may act at receptors other than the nAChR, thus complicating any interpretation of mecamylamine’s effects on pain behavior. As such, mecamylamine may inhibit the activity of NMDA receptors [12]. However, blockade of the NMDA receptor channel by NMDA receptor antagonists such as MK-801 has been shown to decrease, rather than to increase phase 2 pain behaviors [2]. This rules out NMDA receptors but it cannot be excluded that increased pain behaviors caused by mecamylamine may be due, at least in part, to blockade of other inhibitory ion channel receptors, such as the GABA_A receptor [18]. This will have to be further investigated.

In conclusion, these data support the hypothesis that nAChRs play a tonic role in the control of tonic pain in rats. Inhibition of nAChRs with the antagonist mecamylamine, either before or after formalin injection, results in an increase in pain behaviors and suggests that the endogenous cholinergic system, mediated through nAChRs, has, at least in part, an inhibitory role in tonic pain as measured in the formalin test in rats. These data also indicate that nAChRs or certain nAChR subtypes may have opposing effects on the modulation of acute and tonic pain. Consequently, it is extrapolated that the treatment of acute pain may be more amenable to nAChR antagonists, whereas the treatment of chronic pain may be more amenable to nAChR agonists or partial agonists. Although the identity of the nAChR subtypes that regulate pain are currently unknown, modulating the function of these receptors with subtype selective ligands holds promise as a novel therapeutic approach for certain forms of pain.

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References