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# Muscarinic receptor subtypes modulate the release of [$^3$H]-noradrenaline in rat spinal cord slices

Eiichiro Umeda*, Yoshihiko Aramaki, Tomohisa Mori, Tomiei Kazama

*Department of Anesthesiology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan*

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**Abstract**

Spinal muscarinic receptors are involved in the mediation of antinociceptive effects. The modulation of noradrenaline (NA) release on muscarinic receptor subtypes in the rat spinal cord was investigated in *in vitro* perfusion experiments. After rat spinal cord slices were preincubated in [$^3$H]NA, the slices were perfused with a superfusion apparatus. The slices were field stimulated during the 4th ($S_1$) and 11th ($S_2$) superfusion collection periods. Perfusion of drugs was initiated at the 8th collection period and was maintained until the 14th collection period. Fractional release was calculated as the percentage of the radioactivity present in the slices at the beginning of the stimulation period. Drugs were administered between $S_1$ and $S_2$. The following drugs were used: [$^3$H]NA, neostigmine, pirenzepine ($M_1$ antagonist), AFDX116 ($M_2$ antagonist), atropine. Neostigmine significantly increased the release of [$^3$H]NA in a concentration-dependent manner. Pirenzepine (1 μM) and atropine (0.3 μM) significantly reduced the release of [$^3$H]NA, but AFDX116 (1 μM) did not significantly reduce release in the presence of neostigmine (1 μM). The results of this study indicate that neostigmine can enhance noradrenergic neurotransmission, and that acetylcholine can stimulate spinal cord NA release via $M_1$ muscarinic receptor subtypes.


*Keywords:* Muscarinic receptor; Muscarinic receptor subtypes; Modulation of noradrenaline release; Spinal cord; Antinociception

## 1. Implication statemant

The modulation of noradrenaline release on muscarinic receptor subtypes in the rat spinal cord was investigated in *in vitro* perfusion experiments. This study indicated that neostigmine can enhance noradrenergic neurotransmission, and that acetylcholine can stimulate spinal cord NA release via $M_1$ muscarinic receptor subtypes.

## 2. Introduction

The spinal muscarinic receptors in antinociception and the functional roles of muscarinic receptor subtypes are well established. It has been reported that the stimulation evoked NA release from rat hippocampal slices is modulated via muscarinic cholinergic receptors subtypes [9]. But no investigations of the modulation of noradrenaline (NA) release on muscarinic receptors subtypes have been reported for the spinal cord. There is increasing evidence that intraspinal administration of muscarinic receptor agonists produces analgesia [7,5,14].

Muscarinic cholinergic receptors play important roles in the regulation of noradrenergic neurotransmission in the central nervous system [11]. It has been showed that there are four main subtypes of muscarinic cholinergic receptors ($M_1$, $M_2$, $M_3$, $M_4$) in the spinal cord according to basic radioligand binding and analysis studies [17]. It has been reported that the stimulation evoked NA release from rat hippocampal slices is modulated via muscarinic cholinergic receptors subtypes. The neostigmine-evoked NA release was not affected by the $M_2$-selective muscarinic antagonist gallamine but was completely blocked by the $M_1$-selective muscarinic antagonist pirenzepine [9]. There are some autoradiographic and immunocytochemistry studies of muscarinic receptors subtypes in spinal cord [18,12,15]. However, the roles of muscarinic receptors subtypes in the regulation of noradrenergic neurotransmission are not fully understood in spinal cord.

Therefore, this study was designed to evaluate the hypothesis that muscarinic cholinergic receptor subtypes modulate NA release in rat spinal cord slices.

* Corresponding author. Tel.: +81 4 2995 1692; fax: +81 4 2992 1215.
*E-mail address:* eume@ndmc.ac.jp (E. Umeda).

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We hypothesized that muscarinic receptors subtypes modulated NA release for the spinal cord.

## 3. Methods

### 3.1. Preparation of spinal cord slices

The protocol was approved by the local Animal Care and Use Committee. Male Sprague–Dawley rats, weighing 325–470 g, were killed by decapitation. The vertebral column of each rat was removed and put into ice-cold Krebs solution. After the tissue was chilled, the adhering meninges were removed, and the cord was dissected at full length and cut into 1.0 mm thick transverse slices with a steel blade.

### 3.2. Experimental procedure

The slices were preincubated for 30 min at 37 °C in 1 mL oxygenated medium containing 0.1 mM [$^3$H]NA. After incubation, the slices were placed into a superfusion apparatus [16]. Single slices were randomly placed in each of six chambers. The slices were superfused at 37 °C with oxygenated medium containing 0.2 mM ascorbic acid and 0.02 mM pargyline at 0.2 mL/min for 90 min to prevent breakdown of NA. After an initial perfusion of 90 min, 5-min fractions were collected with a fraction collector. NA release was induced by electrical stimulation (3 Hz, 2 ms, 40 V for 3 min). The slices were field stimulated for 3 min during the 4th ($S_1$) and 11th ($S_2$) 5-min superfusion collection periods. Perfusion of drugs was initiated at the 8th collection period (15 min before $S_2$) and was maintained until the 14th collection period. The slices were solubilized at the end of the experiment. The incubation and superfusion medium contained (mM) NaCl 118, KCl 4.7, $CaCl_2$ 1.4, $NaHCO_3$ 25, $KH_2PO_4$ 1.25, $MgSO_4$ 1.25, glucose 11.5, ascorbic acid 0.2, and pargyline 0.02 and the medium was saturated with 5% $CO_2$ in $O_2$.

### 3.3. Measurement and calculation of radioactivity

The radioactivity of the fractions and of the tissues was measured by liquid scintillation spectrometry after the addition of appropriate scintillation fluids. Evoked release was calculated by subtracting basal release values from the total release during the stimulation period. Fractional release (FR) was calculated as the percentage of the radioactivity present in the slices at the beginning of the stimulation period. The effect of drugs on evoked release was expressed as the ratio of the FR values for the first ($S_1$) and second ($S_2$) stimulations ($FRS_2/FRS_1$). Drugs were administered between $S_1$ and $S_2$ and maintained in the perfusion fluid until the end of experiment. Each drug was tested in a separate slice session.

### 3.4. Drugs

The following drugs were used: levo-[7-$^3$H]norepinephrine (NEN$^{TM}$ Life Science Products, Inc., Boston, MA), neostigmine bromide (Sigma Chemical Co., St. Louis, MO), pirenzepine ($M_1$ antagonist) (Sigma Chemical, St. Louis, MO), AFDX116 ($M_2$ antagonist) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), atropine (muscarinic antagonist) (Sigma Chemical, St. Louis, MO). The drugs were dissolved in distilled water.

### 3.5. Statistical analysis

Results are expressed as mean ± S.D. The data were analyzed by one-way ANOVA followed by Dunnett's test. Statistical significance was set at $p < 0.05$.

## 4. Results

A 42 spinal cord slices were included in this study. The $FRS_2/FRS_1$ ratio of control was $0.99 \pm 0.10$ ($n = 6$). The $FRS_2/FRS_1$ ratio of the cholinesterase inhibitor neostigmine were $1.30 \pm 0.07$ (0.01 μM, $n = 6$), $1.53 \pm 0.37$ (0.1 μM, $n = 6$), $1.63 \pm 0.03$ (1 μM, $n = 6$) and $2.20 \pm 0.50$ (10 μM, $n = 6$), respectively. Neostigmine significantly increased the release of [$^3$H]NA in a concentration-dependent manner (Table 1). The $FRS_2/FRS_1$ ratio of the $M_1$-selective muscarinic receptor antagonist pirenzepine (1 μM), non-selective muscarinic receptor atropine (0.3 μM) and the $M_2$-selective muscarinic receptor antagonist AFDX116 were $0.67 \pm 0.05$ ($n = 6$), $0.61 \pm 0.08$ ($n = 6$), $1.35 \pm 0.13$ ($n = 6$), respectively. Pirenzepine and atropine significantly reduced the release of [$^3$H]NA in the presence of neostigmine (1 μM), but AFDX116 did not significantly reduce [$^3$H]NA release in the presence of neostigmine (1 μM). (Table 2).

Table 1
Effect of neostigmine on stimulation-evoked release of [$^3$H]NA

| Drug | μM | FRS2/FRS1 | n |
|---|---|---|---|
| Control | 0 | 0.99 ± 0.10 | 6 |
| Neostigmine | 0.01 | 1.30 ± 0.07 | 6 |
| Neostigmine | 0.1 | 1.53 ± 0.37* | 6 |
| Neostigmine | 1 | 1.63 ± 0.03* | 6 |
| Neostigmine | 10 | 2.20 ± 0.50* | 6 |

Value are mean ± S.D.; $n$ = number of spinal cord slices recorded.
* $p$-Value < 0.05 vs. control (neostigmine 0 μM).

Table 2
Effect of muscarinic agonist or antagonist on the neostigmine-evoked release of [$^3$H]NA

| Drug | FRS2/FRS1 | n |
|---|---|---|
| Neostigmine (1 μM) | 1.63 ± 0.03 | 6 |
| Neostigmine (1 μM) + atropine (0.3 μM) | 0.61 ± 0.08* | 6 |
| Neostigmine (1 μM) + pirenzepine (1 μM) | 0.67 ± 0.05* | 6 |
| Neostigmine (1 μM) + AFDX116 (1 μM) | 1.35 ± 0.13 | 6 |

Value are mean ± S.D.; $n$ = number of spinal cord slices recorded.
* $p$-Value < 0.05 vs. neostigmine 1 μM.

## 5. Discussion

There is convincing evidence that supports the conclusion that stimulation of the noradrenergic neurons produces antinociception that can be blocked by intrathecal injection of neostigmine [19]. Clinically, intrathecal and epidural administrations of neostigmine has been used for spinal analgesia to treat intractable pain [10,8]. Clinical trials of other cholinesterase inhibitors are in progress. Neostigmine has been tested as an anesthetic adjuvant to reduce the amount of anesthetic used [1,2]. Negative feedback modulation of NA release by muscarinic receptor is a common feature of many tissues of central and peripheral origin [13]. Modulation of NA release by muscarinic receptors is common in the spinal cord [3,4]. In our study, neostigmine enhanced the release of NA from spinal cord slices. The $M_1$-selective muscarinic antagonist pirenzepine inhibited the release of NA from spinal cord in the presence of neostigmine, but the $M_2$-selective muscarinic antagonist AFDX116 did not inhibit. Some authors have shown that $M_1$ muscarinic receptor subtypes are present in rat spinal cord [7,18]. The results obtained from this study indicate that $M_2$ muscarinic receptor subtypes are not involved in the presynaptic modulation of