**·Article·**

# Effect of acetylcholine on pain-related electric activities in hippocampal CA1 area of normal and morphinistic rats

Yu XIAO, Xiao-Fang YANG, Man-Ying XU

*Department of Physiology, Harbin Medical University, Harbin 150081, China*

**Abstract: Objective** To examine the effect of acetylcholine (ACh) on the electric activities of pain-excitation neurons (PEN) and pain-inhibitation neurons (PIN) in the hippocampal CA1 area of normal rats or morphinistic rats, and to explore the role of ACh in regulation of pain perception in CA1 area under normal condition and morphine addiction. **Methods** The trains of electric impulses applied to sciatic nerve were set as noxious stimulation. The discharges of PEN and PIN in the CA1 area were recorded extracellularly by glass microelectrode. We observed the influence of intracerebroventricular (i.c.v.) injection of ACh and atropine on the noxious stimulation-evoked activities of PEN and PIN in the CA1 area. **Results** Noxious stimulation enhanced the electric activity of PEN and depressed that of PIN in the CA1 area of both normal and addiction rats. In normal rats, ACh decrease the pain-evoked discharge frequency of PEN, while increased the frequency of PIN. These effects reached the peak value at 4 min after injection of ACh. In morphinistic rats, ACh also inhibited the PEN electric activity and potentialized the PIN electric activity, but the maximum effect appeared at 6 min after administration. The ACh-induced responses were significantly blocked by muscarinic receptor antagonist atropine. **Conclusion** Cholinergic neurons and muscarinic receptors in the hippocampal CA1 area are involved in the processing of nociceptive information and they may play an analgesia role in pain modulation. Morphine addiction attenuated the sensitivity of pain-related neurons to the noxious information.

**Keywords:** acetylcholine; hippocampal CA1 area; morphine; electric activity

## 1 Introduction

Drug addiction resulted from long-term exposure to opioid drugs usually poses serious social, medical and economic problems. It is commonly suggested that neural plasticity mechanisms underlying physiological phenomena such as learning and memory may be engaged in drug addiction. Hippocampus has been widely described to be involved in many important functions such as learning and memory, addiction and pain. Subiculum receives output of hippocampal CA1 neurons and projects glutamatergic synapses onto nucleus accumbens (NAc), the subicular-NAc pathway linking memory and reward system[1]. The change of hippocampal synaptic plasticity may be the key of drug addiction[2]. Recently, the study of pain neurobiology have revealed that neuronal plasticity mechanisms also apply to pain process[3,4]. The hippocampus is distributed with extensive pain-related neurons and the CA1 field interneurons process nociceptive-related information[5]. Evidence showed that nociceptive behaviors were significantly decreased by microinjection of ritanserin or MK-801 into the hippocampal CA1[6,7].

Acetylcholine (ACh) as a major excitatory neurotransmitter plays critical roles in perception and control of pain, and muscarinic acetylcholine receptors (mAChRs) are repeatedly reported as pharmacological targets for treatment of pain. An approximate 30% increase in basal ACh release produces antinociception and that a 30% decrease in basal release produces hyperalgesia[8]. In addition, the analgetic effect of morphine may also be mediated by components of cholinergic system[9]. It is believed that ACh released presynaptically from cholinergic neurons controls the induction and persistence

Corresponding author: Man-Ying XU
Tel: 86-451-86697507
Fax: 86-451-86697507
E-mail: manyingxu@sohu.com
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of morphine addiction[10]. However, the role of ACh in reinforcement and addiction of abusive drugs is not well understood because its agonists or antagonists generated widely effects on many brain regions.

Persistent nociceptive input (50 μL formalin, 5%) could increase the hippocampus ACh release[11]. However, presynaptic opioid receptors have been shown to inhibit the ACh release in the rat hippocampus and striatum[12]. In morphinistic rats, the regulative role of hippocampal cholinergic neurons to the noxious information is still not well known. So we performed this research to observe the effects of exogenous ACh and mAChRs antagonist atropine on the evoked discharges of pain-related neurons in hippocampal CA1 area, in order to elucidate the pain modulation mechanism in normal and morphinistic rats from electrophysiology standpoint.

## 2 Materials and methods

**2.1 Animals and group** All experimental procedures were carried out according to the experimental animal care and use guidelines. A total of 100 Wistar rats in either gender, weight from 200 g to 280 g, were obtained from the Center of Experimental Animals of the Second Affiliated Hospital of Harbin Medical University (Certificate No. 09-2-1). Under the condition of a 12-h light (7:00-19:00) and dark (19:00-7:00) cycle, animals get access to food and water *ad libitum*. Morphine hydrochloride was injected subcutaneously three times per day according to a gradually increasing dose scheme, in which the morphine dose from 5 mg/kg to 50 mg/kg during 5 d, to produce morphinistic rats[13]. The normal rats and morphinistic rats were randomly divided into three groups, with different physic liquors injected into the lateral ventricle: (1) control group ($n$ = 10): 0.9% sterile saline (10 μL) was injected; (2) ACh group ($n$ = 20): ACh (20 μg / 10 μL) was injected; (3) ACh+atropine group ($n$ = 20): atropine (5 μg / 10 μL) was injected 2 min after the ACh was injected. All physic liquors were i.c.v. administrated evenly by an automatic injector within 2 min.

**2.2 Surgical procedures** Routine operations were performed under general anesthesia (20% urethane by intraperitoneal injection, 1 g/kg). A stainless steel cannula with 0.8 mm in outer diameter was inserted into the lateral ventricle (AP: 0.1mm; ML: 1.5 mm; DV: 3.0 mm)[14] for drugs administration, and then was fixed with dental cement on the surface of skull. Rat head was fixed on the stereotaxic apparatus (SN-2, Narishige, Japan) and paralysed with tubocurarine (1 mg/kg) after rat regained consciousness. Meanwhile artificial ventilation was maintained at 60 times/min. Sterile 0.9% saline was injected (5.0 mL, i.p.) to prevent dehydration.

**2.3 Microelectrode record** The glass microelectrode (0.5-1.0 μm; DC resistance, 10-30 MΩ) filled with 3 mol/L KCl was fixed on a microelectrode manipulator (SM-11, Narishige, Japan). Discharges of pain-related neurons were induced by the microelectrode inserted into left or right hippocampal CA1 area (AP: 3.2-4.0 mm; ML: 2.5-3.0 mm; DV: 2.5-3.1 mm)[14]. The homotenous signals of electric discharge were displayed on the oscillometer (VC-10, Nihon Konden, Japan) through an amplifier and recorded by a bi-track tape recorder simultaneously. The stimulation acting on the right sciatic by trains of impulse (voltage strength, 28 V; wave width, 0.3 ms; interval 5 ms and every train impulse included 5 pulses) output from electronic stimulator (SEN-3301, Nihon Konden, Japan) was set as noxious stimulation. The electric discharge of pain-related neurons were continually observed and recorded for 30 min. At the end of the experiment, recording microelectrode filled with 2% Pontamine Sky Blue was given cathode direct current (25 μA) for 15 min to locate the tip of microelectrode (Fig. 1).

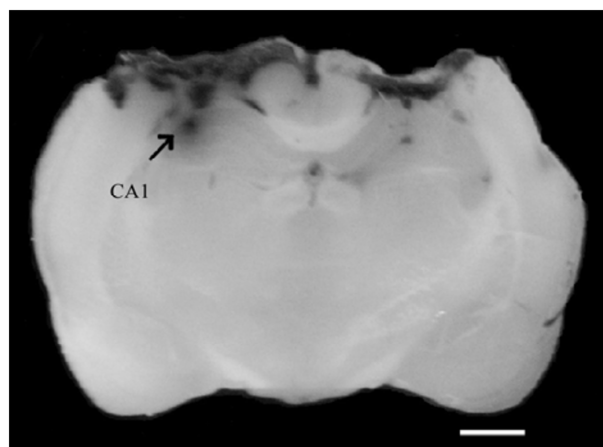


**Fig. 1 Location of microelectrode point in the hippocampal CA1 area. Scale bar, 1.6 mm.**

**2.4 Statistical analysis** Data were scanned into computer via Powerlab/8 s after the management and analyzed by Chart V5.3 software. Statistical analysis of data was handled with SPSS 13.0 software. All data are expressed as mean±SD. One-way ANOVA and $t$-test were introduced in analysis. The accepted value of statistical significance was $P < 0.05$.

## 3 Results

**3.1 Influence of noxious stimulation on the electric activi-**

**ties of pain-excitation neurons (PEN) and pain-inhibitation neurons (PIN) in hippocampal CA1 area** As Fig. 2 demonstrated, noxious stimulation increased the discharge frequency of PEN while decreased that of PIN. In normal rats, the discharge frequency net increased value (NIV, refers to the difference of PEN or PIN between the average frequency of evoked discharges changes after the electric stimulation and the average frequency of spontaneous discharges within 2 s before stimulation) of 12 PEN increased by (153.74±10.03)% after stimulation, while the frequency of 10 PIN decreased by (67.23±8.74)% ($P < 0.01$). In morphinistic rats, the average NIV of 11 PEN increased by (165.35±9.89)% after stimulation; on the contrary, the frequency of 10 PIN decreased by (67.85±7.69)% ($P < 0.01$).

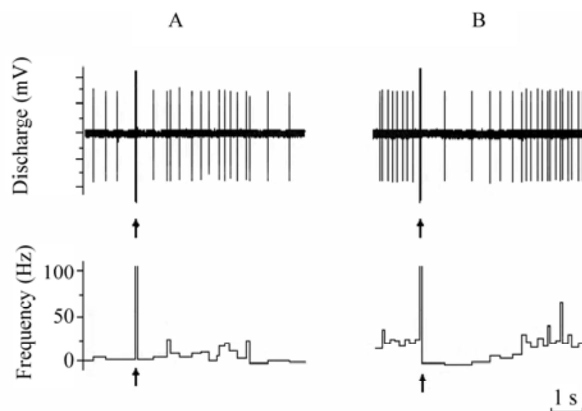


**Fig. 2 Responses of pain-related neurons in hippocampal CA1 area to noxious stimulation. A: Electric discharge of pain-excitation neurons; B: Electric discharge of pain-inhibition neurons. Arrows: Artificial stimulus.**

**3.2 Effects of ACh and atropine on the discharge frequency of PEN and PIN in the CA1 area of normal rats** The statistics analysis of 25 PEN and 22 PIN indicated that, the average frequency decreased at 2 min after injection of ACh, with a maximal effect at 4 min. The NIV of PEN decreased from (9.18±2.03) Hz to (2.75±1.03) Hz, reducing by (70.04±8.91)%. The NIV of PIN increased from (-6.12±1.78) Hz to (-1.45±0.82) Hz, increasing by (71.04±7.31)%. The average NIVs of PEN and PIN during 2-12 min after ACh injection had significances compared with control group ($P < 0.05$ or $P < 0.01$, Fig. 3). The discharge frequency of PEN and PIN started to recover at 14 min after administration of ACh.

The electric activities of PEN were weakened and that of PIN were enhanced after ACh administration, and these responses were interdicted by atropine. The average NIV of 23 PEN decreased from (8.90±2.43) Hz to (5.53±1.34) Hz, decreasing by (37.86±4.79)%. The average NIV of 21 PIN increased from (-6.43±1.22) Hz to (-3.53±0.83) Hz, increasing by (45.1±5.59)%, at 2 min after injected ACh. The effects of ACh on PEN and PIN electric activities started to attenuate soon after injection of atropine. The discharges of PEN and PIN recovered remarkably at 2 min after atropine administration, which meant that the NIV of PEN went back to (7.31±1.28) Hz and that of PIN returned to (-4.95±1.05) Hz. The average NIV of PEN or PIN during 0-8 min after injection of atropine had significance compared with ACh group ($P < 0.05$ or $P < 0.01$, Fig. 3).

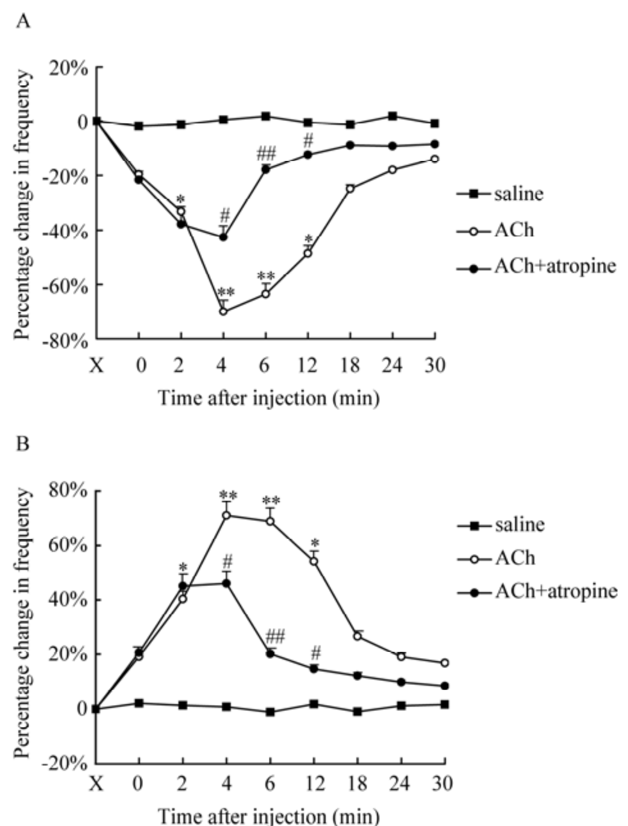


**Fig. 3 Effects of intracerebroventricular injection of ACh and atropine on the noxious stimulation-evoked discharge frequency of pain-related neurons in hippocampal CA1 area of normal rats. A: Atropine blocked the decreased effect of ACh on PEN discharge frequency. B: Atropine blocked the increasing effect of ACh on PIN discharge frequency. X, before injection. *$P < 0.05$, **$P < 0.01$ *vs* saline group; #$P < 0.05$, ##$P < 0.01$ *vs* ACh group.**

**3.3 Effects of ACh and atropine on the discharge frequency of PEN and PIN in the CA1 area of morphinistic rats** In morphinistic rats, 18 PEN and 17 PIN were completely recorded in ACh group. ACh obviously decreased the frequency of the evoked discharge of PEN and enhanced that

of PIN compared with control group. The change of frequency appeared at 2 min after injection of ACh and reached the peak value at 6 min. The average NIV of PEN decreased from (10.87±1.60) Hz to (3.28±0.59) Hz, decreasing by (71.84±3.65)%. The average NIV of PIN increased from (-7.38±0.95) Hz to (-2.23±0.53) Hz, increasing by (70.90±6.90)%. Differences between ACh group and control group during 2-14 min after ACh administration were significant ($P < 0.05$ or $P < 0.01$, Fig. 4). The discharge frequency started to recover at 16 min after ACh was injected.

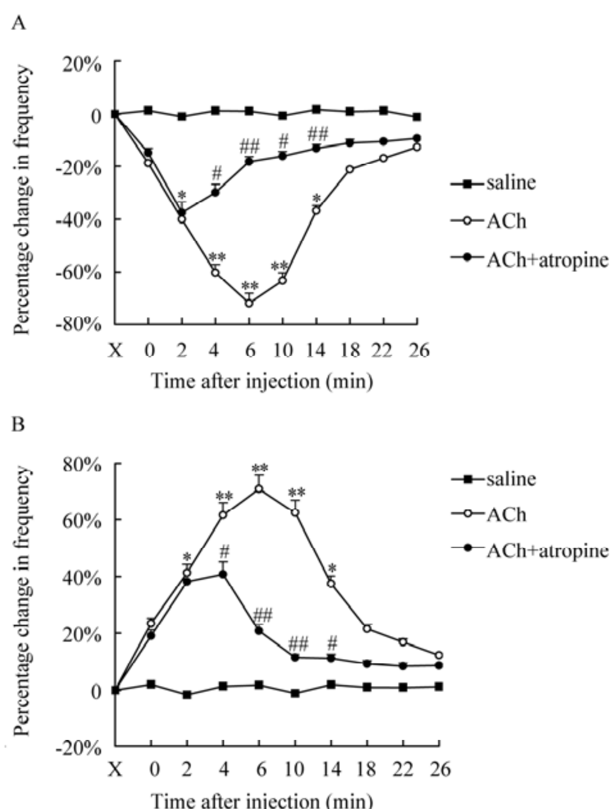


**Fig. 4 Effects of intracerebroventricular injection of ACh and atropine on the noxious stimulation-evoked discharge frequency of pain-related neurons in hippocampal CA1 area of morphinistic rats. A: Atropine blocked the decreased effect of ACh on PEN discharge frequency; B: Atropine blocked the increasing effect of ACh on PIN discharge frequency. X, before injection. *$P < 0.05$, **$P < 0.01$ *vs* saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* ACh group.**

The above mentioned changes of PEN and PIN discharges evoked by ACh were abolished after injection of atropine. The average NIV of 21 PEN decreased from (11.09±1.27) Hz to (6.84±1.38) Hz, decreasing by (37.5±8.34)% at 2 min after injected Ach; and the average NIV of 19 PIN increased from (-7.40±0.71) Hz to (-4.55±0.96) Hz, increasing by (38.20±7.52)%. The effects of ACh on PEN and PIN electric activities started to attenuate soon after injection of atropine. The discharges of PEN and PIN recovered remarkably at 2 min after administration of atropine, which meant that the NIV of PEN went back to (7.31±1.28) Hz and that of PIN returned to (-4.95±1.05) Hz. Differences between ACh+atropine group and ACh group during 0-10 min after atropine administration were significant ($P < 0.05$ or $P < 0.01$, Fig. 4 )

## 4 Discussion

In this study, the electric activities of PEN and PIN were recorded extracellularly in order to investigate the influence of ACh and atropine on hippocampal CA1 area pain-related neurons react to noxious stimulation. In normal rats, the discharge frequency of evoked PEN decreased, while the PIN frequency increased after i.c.v. administration of ACh, and these effects reached the peak value at 4 min. The above ACh-induced responses were blocked by muscarinic receptor antagonist atropine. These results support the hypothesis that the hippocampal formation may modify the processing of incoming nociceptive information and that ACh receptor sensitivity in the hippocampus may play a role in nociception.

The pain experience includes a sensory-discriminative and an emotional-affective component. The hippocampus may contributes to the negative affect and avoidance motivation experienced during pain[15]. Stimulation on the hippocampal formation can modulate nociceptive information, and painful stimuli can activate this structure[16,17]. As observed in our research, the discharges of PEN and PIN in the CA1 area were remarkably changed when peripheral traumatic stimulation signal was transferred to the hippocampus. Both systemically and intrathecally administered cholinergic agonists could produce antinociception[18]. Our results also suggested that i.c.v. injection of exogenous ACh has antinociceptive effect through the integration and coordination of PEN and PIN in the CA1 area. Meanwhile, noxious stimulus could induce ACh release in the apical dendrites of CA1 pyramidal cells[19]. We can deduce that the analgesia effect may result from the integration of exogenous and endogenous ACh.

Atropine as a non-selective antagonist of mAChRs abolished the effect of ACh, which indicated that the analgesia effect of ACh was mediated by the mAChRs in hippocampus CA1 region. Aside from a numerically sparse population of intrinsic cholinergic neurons, the hippocampus receives extensive cholinergic projection from the me-

dial septal nucleus and basal forebrain. ACh may direct or indirect activate the mAChRs to produce analgesia effect, therefore, further study is needed to determine the accurate pathway. A family of five muscarinic receptor subtypes are all expressed in hippocampus, and M1, M2, and M4 are the predominant subtypes in the CA1 area[20]. Activation of M1 muscarinic receptor subtype is fundamental to induce central cholinergic analgesia in mice[21]; the M2 is reported as the main receptor subtype participated in the morphine dependence and implicated in muscarinic pain pathways in centre[22]; and some findings suggest that the muscarinic M4 receptor subtype in spinal cord may be involved in cholinergic mechanisms of analgesia[23]. The research of selective M-receptor antagonist is necessary to expound whether the analgesic response of ACh is mediated by one subtype or results from the interaction between each other.

ACh also inhibited the PEN activity and enhanced the PIN activity, thus expressed analgesic effect in the morphonistic rats. But the maximal effect appeared at 6 after administration of ACh, delayed 2 min compared with normal rats. The result indicated that the sensitivity of pain-related neurons in the CA1 area to the noxious stimulation was attenuated. The affective state induced by drugs of abuse produces an indifference to pain, which may due to the adaptive changes of neurons to morphine, and the down-regulation of mAChRs evoked by morphine addiction was the most important[24,25]. Moreover, the change of neuronal structure may also induce the change of physiology function. Chronic morphine treatment may cause slight damage of ultrastructure of neurons in the CA1 area and NAc of rats[26]. In short, the pain perception and addiction is a complex problem. The underlying neurophysiology mechanisms are not fully understood, and more efforts are needed.

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# ACh 对正常大鼠和吗啡成瘾大鼠海马 CA1 区痛反应电活动的影响

肖宇，杨晓芳，徐满英
哈尔滨医科大学生理教研室，哈尔滨 150081

**摘要：目的** 研究 ACh 对正常大鼠和吗啡成瘾大鼠海马 CA1 区痛兴奋神经元(pain-excitation neurons，PEN)和痛抑制神经元(pain-inhibitation neurons，PIN)电活动的影响，进一步探讨 ACh 对正常和吗啡成瘾状态下 CA1 区痛觉调制的作用及机制。**方法** 电刺激坐骨神经作为伤害性电刺激，在细胞外用玻璃微电极记录CA1区PEN和PIN 的放电，观察 ACh 对正常大鼠和吗啡成瘾大鼠 CA1 区 PEN 和 PIN 电活动的影响。**结果** 伤害性刺激能够增强 PEN 的电活动，而减弱 PIN 的电活动。正常大鼠中，ACh 使 PEN 的痛诱发放电频率降低，PIN 的放电频率增加；ACh 的作用在注射后 4 min 达到峰值。吗啡成瘾大鼠中，ACh 同样也抑制了 PEN 的电活动，兴奋 PIN 的电活动，但是作用的高峰出现在注射后 6 min。胆碱能受体拮抗剂阿托品可阻断ACh的作用。**结论** 海马CA1区内的胆碱能神经元和毒蕈碱受体参与了伤害性信息的处理，并且起到了镇痛作用。吗啡成瘾可以降低CA1区痛反应神经元对伤害性刺激的敏感性。
**关键词：**ACh；海马 CA1 区；吗啡；电活动