

## Effect of acetylcholine receptors on the pain-related electrical activities in the hippocampal CA3 region of morphine-addicted rats

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### ABSTRACT

**Objective(s):** To determine the effect of acetylcholine (ACh), pilocarpine, and atropine on pain evoked responses of pain excited neurons (PEN) and pain inhibited neurons (PIN) in hippocampal CA3 region of morphine addicted rats.

**Materials and Methods:** Female Wistar rats, weighing between 230-260 g were used in this study. Morphine addicted rats were generated by subcutaneous injection of increasing concentrations of morphine hydrochloride for six days. Trains of electrical impulses applied to the sciatic nerve were used as noxious stimulation and the evoked electrical activities of PEN or PIN in hippocampal CA3 area were recorded using extracellular electrophysiological recording techniques in hippocampal slices. The effect of acetylcholine receptor stimulation by ACh, the muscarinic agonist pilocarpine, and the muscarinic antagonist atropine on the pain evoked responses of pain related electrical activities was analyzed in hippocampal CA3 area of morphine addicted rats.

**Results:** Intra-CA3 microinjection of ACh (2 µg/1 µl) or pilocarpine (2 µg/1 µl) decreased the discharge frequency and prolonged the firing latency of PEN, but increased the discharge frequency and shortened the firing inhibitory duration (ID) of PIN. The intra-CA3 administration of atropine (0.5 µg/1 µl) produced opposite effect. The peak activity of cholinergic modulators was 2 to 4 min later in morphine addicted rats compared to peak activity previously observed in normal rats.

**Conclusion:** ACh dependent modulation of noxious stimulation exists in hippocampal CA3 area of morphine addicted rats. Morphine treatment may shift the sensitivity of pain related neurons towards a delayed response to muscarinic neurotransmission in hippocampal CA3 region.

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## Introduction

Opioids are commonly prescribed for chronic cancer pain management (1). Opioids are also known to be extremely addictive. The addictive features of morphine lead to its inappropriate use which creates significant medical and socioeconomic problems worldwide. Opioids are co-stored with glutamate in several hippocampal pathways, including the lateral perforant path (LPP) input to granule cells in the dentate gyrus, the LPP input to CA3 pyramidal cells, and the mossy fiber projection to CA3 pyramidal cells (2). Several studies have shown that chronic exposure to morphine or heroin leads to the impairment of

hippocampal long-term potentiation (LTP) (3) and to the impairment of acquisition (4) or retention (5) of spatial memory.

Acetylcholine (ACh) is a major excitatory neurotransmitter in the central nervous system (CNS) of vertebrates and invertebrates (6). More recently, a study on the neurobiology of pain has revealed that the brain's cholinergic system, particularly muscarinic acetylcholine receptors (mAChRs), may be involved in the modulation of pain (7). This action of ACh is mediated by mAChRs (8). In addition, a vast amount of literature has appeared describing the antinociceptive action of both cholinesterase

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inhibitors and cholinomimetic drugs (9). Various mAChRs have also been shown to be potential mediators of pain related neuroplasticity. Intrathecal administration of cholinergic muscarinic agonists or acetylcholinesterase inhibitors produces analgesia in both animals and humans (10). Moreover, the analgesic effect of morphine may be mediated by components of the cholinergic system (11). Therefore, it can be postulated that morphine or heroin may affect nociceptive neurotransmission in the hippocampus. However, the precise role of ACh in the reinforcement and addiction of abusive drugs is not well understood.

The hippocampus is a region of the mammalian brain that has been extensively studied due to its role in many forms of learning, memory, and pain response. Electrophysiological, pharmacological, behavioral, and clinical data indicate that the hippocampal formation is an integral component of the limbic system and, as such, plays an important role in the affective and motivational aspects of pain perception (12). The hippocampus receives cholinergic projections from the medial septal nucleus and Broca's diagonal band, which terminate in the CA1, CA3, and dentate gyrus regions (13). Some scholars report that both morphine and AChR agonists can modulate hippocampal neuronal excitability (14). Moreover, previous studies have shown that the CA1 and dentate gyrus of the hippocampus are involved in nociception (6, 15, 16). However, the interplay of cholinergic and opioid effects for pain evoked responses of PEN and PIN, particularly in the hippocampal CA3, is not well studied. Therefore, in this study, we have evaluated the effects of ACh, the muscarinic agonist pilocarpine, and the muscarinic antagonist atropine on pain related electrical activities in the hippocampal CA3 region of morphine addicted rats.

## Materials and Methods

### Experimental animals

Female Wistar rats (Animal Centre of the Second Affiliated Hospital, Harbin Medical University, Certificate No. 09-2-1) weighing between 230-260 g were used in this study. Experiments complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23; revised, 1996). Morphine-dependent rats were produced by subcutaneous injection of morphine hydrochloride for 6 days at increasing concentrations (Table 1) as previously described (17). Morphine hydrochloride was injected subcutaneously three times per day according to a gradually increasing dose scheme, in which the morphine dose increased from 5 mg/kg to 50 mg/kg over five days to produce morphine addicted rats. Morphine-dependent rats were

randomly divided into four groups: 1) control group (n=14, 14 neurons of 8 rats were recorded): intra-CA3 administration of 1  $\mu$ l saline; 2) ACh group (n=15, 15 neurons of 15 rats were recorded): intra-CA3 administration of ACh (2  $\mu$ g/1  $\mu$ l); 3) atropine group (n=13, 13 neurons of 12 rats were recorded): intra-CA3 administration of atropine (0.5  $\mu$ g/1  $\mu$ l); and 4) pilocarpine group (n=16, 16 neurons of 8 rats were recorded): intra-CA3 administration of pilocarpine (2  $\mu$ g/1  $\mu$ l). All injections were completed within 4 min via a microliter syringe.

### Neurosurgery and electrophysiological studies

Rats were anesthetized with 20% urethane (IP, 1 g/kg). The right sciatic nerve was isolated and two skull windows were opened and covered with liquid paraffin. The head of the rat was fixed on a stereotaxic frame (SN-2, Narishige, Japan). After 4-10 min, the rat was paralyzed with tubocurarine chloride (IP, 1 mg/kg) and artificial ventilation was maintained. Single-unit recordings were performed with a glass microelectrode (0.5-1.0  $\mu$ m, DC resistance 10-30 M $\Omega$ ) filled with KCl (3 mol/l). According to the B coordinate system of Pellegrino's Atlas (18), the glass microelectrode was inserted by a micromanipulator (SM-21, Narishige, Japan) into the hippocampal CA3 (A: -3.2 - -3.8 mm; R or L: 5.0-5.5 mm, H: 6.4-7.3 mm) to record the discharges of the CA3 neurons. Another glass microelectrode filled with drugs (ACh, atropine, and pilocarpine) was inserted by a micromanipulator (SM-11, Narishige, Japan) into the hippocampal CA3 (A: -3.5 mm, R: 5.25 mm, or L: 5.25 mm, H: 6.85 mm) for administration of drugs. The electrical activity was amplified by a microelectrical amplifier (MEZ-820, Nihon Kohden, Japan) and recorded by an output isolator (SS-201J-type, Narishige, Japan) in combination with a tape recorder (ST-CH707X type, Panasonic, Japan). Electrical activity was simultaneously monitored with an oscilloscope (VC-9, Nihon Kohden, Japan). As the neural discharges were recorded, the electrical stimulation of the sciatic nerve was performed through a double stainless steel electrode (delay: 0, interval: 5 msec, duration: 0.3 msec, and train: 5; SEN-3301, Nihon Kohden, Japan) as noxious

**Table 1.** Dose (mg/kg) and time of morphine injection

	Dose (mg/kg) of morphine injection		
	8:00	12:00	16:00
1 <sup>st</sup> day	5	5	5
2 <sup>nd</sup> day	10	10	10
3 <sup>rd</sup> day	20	20	20
4 <sup>th</sup> day	40	40	40
5 <sup>th</sup> day	50	50	50
6 <sup>th</sup> day	60	60	60

stimulation. Articular movement and hair touching were set as the non-noxious stimulation to identify the pain reactive neurons. The discharge of each neuron was monitored continuously for 30 min, and typical discharges were recorded three times every two min.

### Histological identification of the hippocampal CA3 region

At the end of the experiment in order to identify the tip position, pontamine sky blue in the microelectrode was diffused out with a negative direct current (30 mA, 15 min) into the hippocampal CA3 (A: -3.2 - -3.8 mm; R or L: 5.0-5.5 mm, and H: 6.4-7.3 mm) (Figure 1).

### Definition of neurons

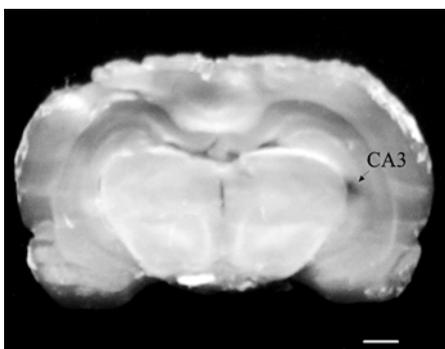
Recording PEN and PIN activity is widely considered as an appropriate electrophysiological read-out of pain (19, 20). PENs are defined as the neurons that responded by increasing the discharge frequency to noxious stimulus (21), while PINs are defined as the neurons that responded by decreasing the discharge frequency to noxious stimulus (22). PEN showed very few spontaneous discharges before noxious stimulation. In contrast, spontaneous discharges were common in PIN, which were readily inhibited by the application of noxious stimulation.

### Observation index

The net increased value (NIV, in Hz) refers to the difference in the PEN or PIN between the average frequency of evoked discharges after noxious stimulation and the average frequency of the discharges within 2 sec before noxious stimulation. The latency (s) refers to the latency time from the noxious stimulation to the appearance of the PEN discharges. Inhibitory duration (ID, in sec) refers to the latency time from the noxious stimulation to the appearance of the PIN discharges.

### Statistical analysis

Data were scanned to a computer with Powerlab/8sec (ADInstruments, Australia) and analyzed with Chart v5.3 software (ADInstruments, Australia). All data were expressed as mean±SEM and were analyzed using SPSS



**Figure 1.** Location of the microelectrode (arrow) in the hippocampal CA3 area of rat. Scale bar, 1.8 mm

16.0 software (SPSS, USA). Statistical differences were evaluated by repeated measures ANOVA, while the post hoc test was used to compare the differences between the two groups.  $P < 0.05$  was considered statistically significant.

## Results

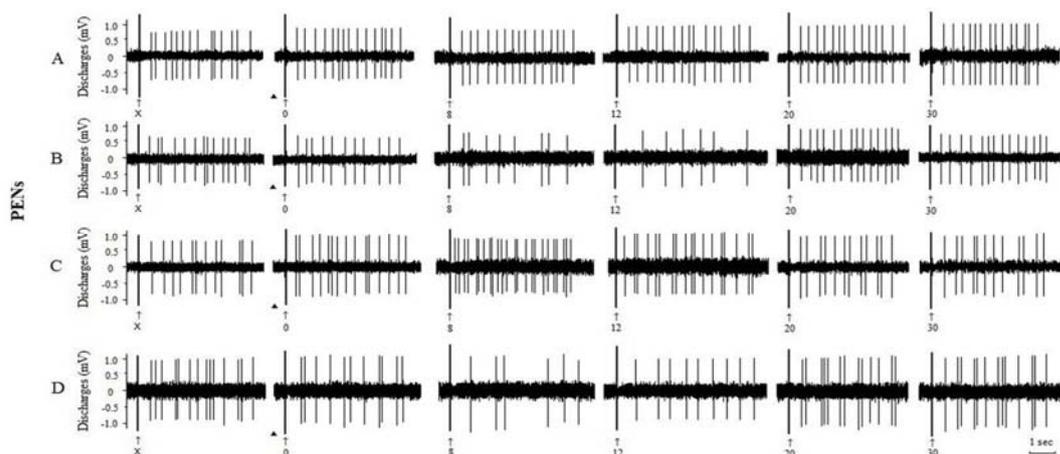
### Effects of saline on the electrical activities in hippocampal CA3 neurons of morphine addicted rats

In the control group, the noxious stimulation increased the discharge frequency of PENs while decreasing the frequency of PINs. The average NIV of PENs was  $3.15 \pm 0.15$  Hz, and the latency was  $0.26 \pm 0.03$  sec. The NIV of PENs and the latency showed no significant differences 0-30 min after the intra-CA3 administration of saline compared to values before injection (Figure 2A). The average NIV of PINs was  $-4.01 \pm 0.57$  Hz and the average ID of PINs was  $0.31 \pm 0.04$  sec. Zero to 30 min after the intra-CA3 administration of saline, the NIV of PINs and the ID showed no significant differences compared to values before injection (Figure 3A). Administration of saline as a control treatment produced no significant change in the electrical activity of PENs and PINs (Figure 2A and Figure 3A).

### Influence of ACh on the electrical activities of the CA3 neurons in morphine-addicted rats

In the ACh group, the average NIV of PENs was  $3.38 \pm 0.37$  Hz, and the latency was  $0.27 \pm 0.03$  sec. Immediately after the intra-CA3 administration of ACh, the NIV of PENs began to decline and the latency began to prolong (Figure 2B). These effects reached a peak at 12 min after administration of ACh, with the NIV decreasing to  $1.06 \pm 0.2$  Hz ( $F$ -statistic=990.268,  $P < 0.0001$ ) and the latency being prolonged to  $0.50 \pm 0.04$  sec ( $F = 359.312$ ,  $P < 0.0001$ ). Between the time points of 0-20 min after administration of ACh, the NIV ( $F = 105.045$ ,  $P < 0.0001$ ) and latency ( $F = 51.613$ ,  $P < 0.0001$ ) of PENs showed significant changes compared with those before administration. Between the time points of 2-18 min after administration, the NIV, and between the time points of 2-20 min after administration, latency showed significant changes ( $P < 0.05$  or  $P < 0.01$ ) compared with those of the saline control group (Figure 4). At 22 min after ACh administration, the NIV and latency of PENs gradually returned to the values observed before treatment.

The average NIV of PINs was  $-4.41 \pm 0.78$  Hz, and the average ID of PINs was  $0.31 \pm 0.06$  sec. Immediately after injection of ACh, the average NIV began to increase and ID began to shorten (Figure 3B). These changes reached a peak value at 10 min after injection; with the average NIV at  $-0.91 \pm 0.27$  Hz ( $F = 392.202$ ,  $P < 0.0001$ ) and the ID decreased to  $0.1 \pm 0.02$  sec ( $F = 257.19$ ,  $P < 0.0001$ ). The average NIV ( $F = 41.187$ ,  $P < 0.0001$ ) showed significant differences



**Figure 2.** Effects of intra-CA3 injection of different substances on the evoked discharges of PENs in the CA3 of morphine-addicted rats. Morphine-dependent rats were injected with (A) saline (1  $\mu$ l), (B) ACh (2  $\mu$ g/1  $\mu$ l), (C) atropine (0.5  $\mu$ g/1  $\mu$ l); and (D) pilocarpine (2  $\mu$ g/1  $\mu$ l).  $\uparrow$ , Stimulus artifact;  $\blacktriangle$ , injection substance; X, before injection; 0, 8, 12, 20, 30, time after injection (min)

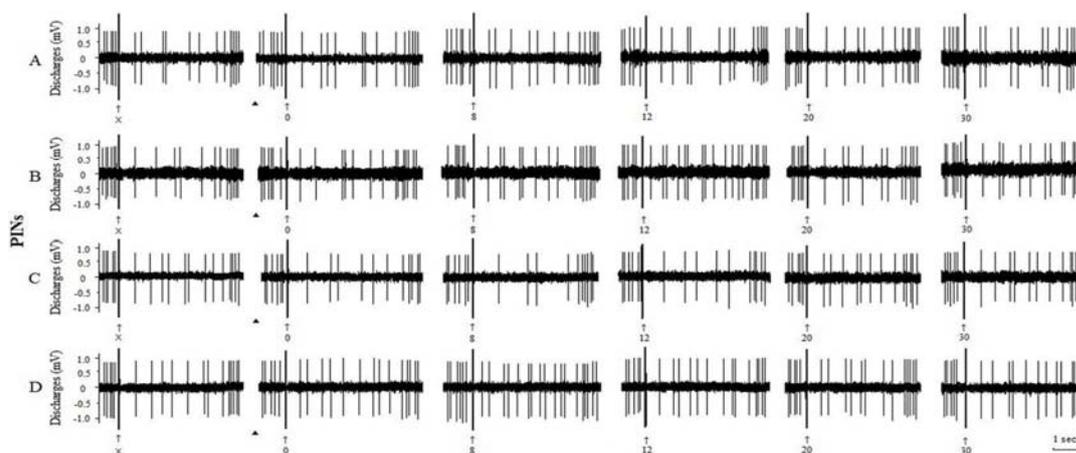
compared to values before injection between the time points of 0-18 min after injection. Between 0-20 min after injection, the ID ( $F=12.832$ ,  $P=0.006$ ) of PINs showed significant differences compared to values before injection. Between the time points of 2-16 min after administration, the NIV, and between the time points of 2-18 min after administration, ID showed significant changes ( $P<0.05$  or  $P<0.01$ ) compared with those of the control group (Figure 5).

#### **Effects of atropine on the electrical activities of the CA3 neurons in morphine addicted rats**

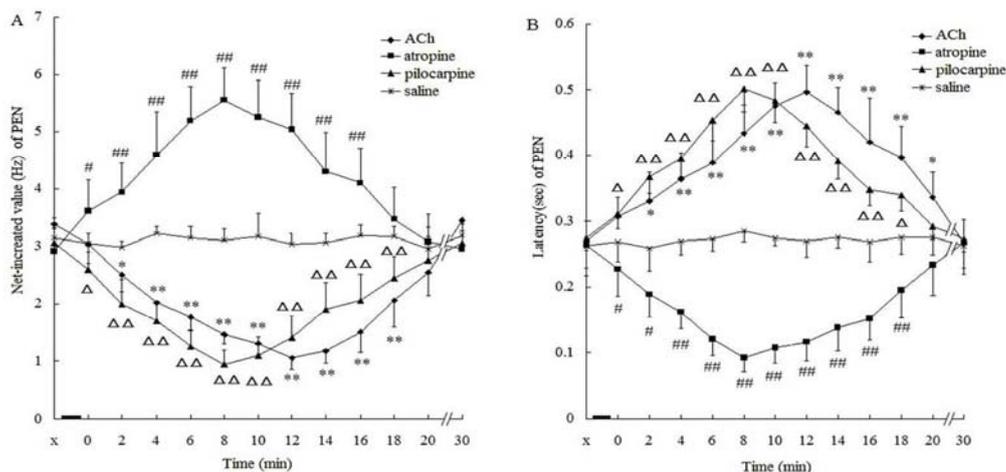
In the atropine group, at 8 min after the intra-CA3 administration of atropine, the average NIV of PENs significantly increased from  $2.92\pm 0.4$  Hz to  $5.56\pm 0.61$  Hz ( $F=515.756$ ,  $P<0.0001$ ) and the latency decreased from  $0.26\pm 0.05$  sec to  $0.09\pm 0.02$  sec ( $F=391.803$ ,  $P<0.0001$ ) (Figure 2C). During 0-18 min after the injection of atropine, the NIV ( $F=21.246$ ,  $P=0.001$ ) and the latency ( $F=24.994$ ,  $P<0.0001$ ) of PENs showed

obvious changes compared to those before administration. Between the time points of 0-16 min after administration, the NIV, and between the time points 0-18 min after administration, latency showed significant changes ( $P<0.05$  or  $P<0.01$ ) compared with those of the control group (Figure 4).

The average NIV of PINs significantly decreased 8 min after the intra-CA3 administration of atropine from  $-4.15\pm 0.47$  Hz to  $-6.81\pm 0.58$  Hz ( $F=119.403$ ,  $P<0.0001$ ) and the ID was prolonged from  $0.33\pm 0.04$  sec to  $0.54\pm 0.08$  sec ( $F=136.968$ ,  $P<0.0001$ ) compared to values before injection (Figure 3C). At the time points of 0-20 min after the injection, the NIV ( $F=7.242$ ,  $P=0.023$ ) and ID ( $F=17.395$ ,  $P=0.002$ ) of PINs showed significant changes compared to values before administration. Between the time points of 0-16 min after administration, the NIV, and between the time points of 0-18 min after administration, ID showed significant changes ( $P<0.05$  or  $P<0.01$ ) compared to the saline control group (Figure 5).



**Figure 3.** Effects of intra-CA3 injection of different substances on the evoked discharges of PINs in the CA3 of morphine-addicted rats. Morphine-dependent rats were injected with (A) saline (1  $\mu$ l), (B) ACh (2  $\mu$ g/1  $\mu$ l), (C) atropine (0.5  $\mu$ g/1  $\mu$ l); and (D) pilocarpine (2  $\mu$ g/1  $\mu$ l).  $\uparrow$ , Stimulus artifact;  $\blacktriangle$ , injection substance; X, before injection; 0, 8, 12, 20, 30, time after injection (min)

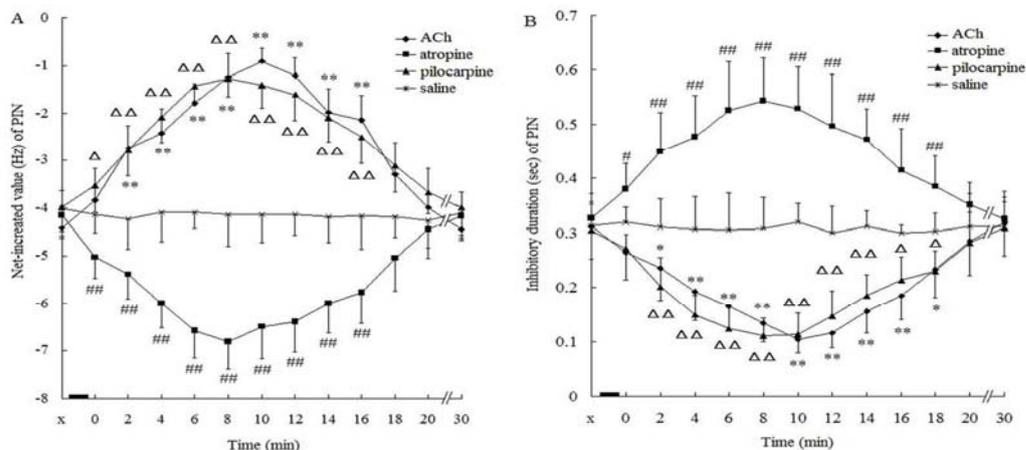


**Figure 4.** Influence of intra-CA3 injection of different substances on the NIV (A) and latency (B) of PEN in the CA3 of morphine-addicted rats. ■, injection of substance; X, before injection; 0, 2, . . . 30, time after injection (min); values are presented as means±SEM. \**P* < 0.05, \*\**P* < 0.01, #*P* < 0.05, ##*P* < 0.01, Δ*P* < 0.05, ΔΔ*P* < 0.01, when compared with the saline control group

**Influence of pilocarpine on the electrical activities of the CA3 neurons in morphine addicted rats**

In the pilocarpine group, the average NIV of PENs was 3.03±0.43 Hz and the latency was 0.27±0.04 sec before the injection of pilocarpine. After the intra-CA3 administration of pilocarpine, the NIV of PEN began to decrease and the latency began to prolong (Figure 2D). These effects peaked at 8 min after administration of pilocarpine. The average NIV decreased to 0.94±0.25 Hz (*F*= 550.604, *P* < 0.0001), and the latency was prolonged to 0.5±0.08 sec (*F*=193.24, *P* < 0.0001) compared to values before injection. The NIV (*F*=14.213, *P*=0.002) and the latency (*F*=4.999, *P*=0.041) showed significant differences during the time points of 0-20 min after the injection. Between the time points of 0-18 min after administration, the NIV and latency showed significant changes (*P* < 0.05 or *P* < 0.01) compared with those of control group (Figure 4).

After the intra-CA3 injection of pilocarpine, the NIV of PINs began to increase and ID began to shorten (Figure 3D). These effects also reached a peak value at 8 min after the injection, when the average NIV of PINs significantly increased from -3.97±0.52 to -1.27±0.39 Hz (*F*=507.714, *P* < 0.0001), and the average ID decreased from 0.30±0.05 to 0.11±0.03 sec (*F*=526.168, *P* < 0.0001) compared to values before injection. The time points of 0-20 min after the injection showed significant changes in the average NIV (*F*=21.813, *P*=0.001) and ID (*F*=5.772, *P*=0.04) of PINs compared with measurements obtained prior to injection. Between the time points of 0-16 min after administration, the NIV, and between the time points of 2-18 min after administration, ID showed significant changes (*P* < 0.05 or *P* < 0.01) compared with the control group (Figure 4). About 22 min after the administration, the NIV and ID of PINs returned to the values observed before treatment (Figure 5).



**Figure 5.** Influence of intra-CA3 injection of different substances on the NIV (A) and ID (B) of PIN in the CA3 of morphine-addicted rats. ■, injection of substance; X, before injection; 0, 2, . . . 30, time after injection (min); values are presented as means±SEM. \**P* < 0.05, \*\**P* < 0.01, #*P* < 0.05, ##*P* < 0.01, Δ*P* < 0.05, ΔΔ*P* < 0.01, when compared with the saline control group

## Discussion

In order to investigate the interplay of cholinergic and opioid effects for pain evoked responses in the CNS, we studied the effects of ACh on the electrical activities of PENs and PINs in hippocampal CA3 of morphine-addicted rats. Our study revealed that, in this animal model for morphine dependence, ACh and pilocarpine inhibited the electric activities of evoked discharges of PENs, but potentiated discharges of PINs. On the other hand, atropine potentiated the electric activities of evoked discharges of PENs and inhibited those of PINs. These results demonstrate that intra-CA3 administration of ACh or pilocarpine can produce antinociceptive effects, whereas intra-CA3 administration of atropine can facilitate nociceptive effects in morphine-dependent rats.

These results reveal that cholinergic neurons in the hippocampal CA3 region of morphine-dependent rats are involved in the response to noxious stimuli, and these results are similar to the previously studied effects in control rats (23). In the previous study in normal rats, cholinergic substances evoked a peak in electrical activity at 6 to 10 min after injection. In morphine-dependent rats, we observed a peak activity at 8 to 12 min after injection; 2 to 4 min later than in normal rats. Although further studies are needed comparing the effects in normal rats versus morphine-dependent rats, the rather large differences to the previous results (23) indicate that morphine treatment delays ACh-dependent electrical activities of PENs and PINs in hippocampal CA3. This may be a sign of delayed sensitivity of pain related neurons towards ACh in the hippocampal CA3 region after morphine treatment.

Early pharmacological experiments have shown that the microinjection of ACh or carbachol into specific brainstem nuclei can produce antinociceptive effects that can be reversed by muscarinic receptor antagonists (24). Previous studies, including three studies performed in our laboratory (6, 15, 23) provided evidence that the hippocampal formation is involved in pain modulation and that nociceptive stimuli modify the electrical activity of the hippocampus (25). Moreover, ACh can mediate some antinociceptive effects, which are produced by other types of receptors or drugs. For instance, the antinociceptive effect of crotoxin is attributed to activation of muscarinic receptors, by action of the toxin on central cholinergic neurons (26). Another study showed that atropine blocked the antinociceptive effect of the crotoxin, further confirming that central muscarinic receptors mediate the antinociceptive effect of the toxin (27). Our present results, demonstrating that atropine potentiated the electric activities of evoked discharges of PENs and inhibited those of PINs, are consistent with the notion that ACh participates in antinociception in the CNS. On the other hand, centrally

administered opioids increase the ACh concentration in cerebrospinal fluid and in spinal cord dorsal horn microdialysates. In this case, analgesia resulting from central opioid injection is partially reversed by spinal injection of mAChR antagonists (28). Therefore, it appears that ACh participates in analgesic effects after systemic or central administration of opioids.

The hippocampus receives cholinergic innervation from the medial septum-diagonal band complex. Every hippocampal region is connected by extensive fiber networks. The hippocampal CA3 area receives synaptic input from the granule cells of the dentate gyrus. The pyramidal cells in the CA3 area send their axon collaterals into the strata radiatum and strata oriens of the hippocampal CA1 area. Aside from these major fiber connections, the dendritic shafts of various types of interneurons can build connections with the spines of principal cells. Cholinergic suppression of synaptic transmission has been reported in the CA3 region (29). When synaptic transmission in the CA3 region was suppressed, the transmission of noxious stimulus was likely to be inhibited. Our previous study indicates that the hippocampal CA3 area is involved in the modulation of nociception (23); the effect in the hippocampal CA3 of morphinistic rats was similar to the effects in normal rats (23).

Compared to the results of our previous study performed in normal rats (23), the peaks of the pain evoked responses in morphine-dependent rats are shifted to later response times. These results are similar to the previously studied effects in the hippocampal CA1 area (16). The results indicate that the sensitivity of pain related neurons in the CA1 and CA3 areas to noxious stimulation is attenuated in morphine addicted rats. Thus, morphine addiction seems to attenuate the sensitivity of pain related neurons to the noxious information processed in several hippocampal areas. Altered synaptic transmission and plasticity in brain areas involved in reward and learning are thought to underlie the long-lasting effects of addictive drugs. Data by Kahn *et al* showed a strong reduction of cellular proliferation together with an increase of glutamate decarboxylase-67 mRNA transcription in the dentate gyrus-CA3 region of the adult rat hippocampus after repeated morphine treatment (30). It is likely that reducing cell proliferation and neurogenesis and altering neuronal phenotypes, have an impact on ACh-dependent antinociceptive neurotransmission in the hippocampal CA3. However, further investigation is required to determine the precise mechanisms underlying the changes in antinociceptive effects in morphine addicted rats.

## Conclusion

In conclusion, our results indicate that: 1) ACh or pilocarpine can produce antinociceptive effects, whereas intra-CA3 administration of atropine can facilitate nociceptive effects in morphine-dependent

rats; and 2) the hippocampal CA3 area is involved in the ACh-dependent modulation of noxious stimulation in morphine addicted rats. In comparison to ACh effects in normal rats (23), morphine addicted rats show delayed responses of pain related neurons to ACh in the hippocampal CA3, which is likely related to adaptive changes of synaptic transmission and plasticity. Further comparative studies on the underlying mechanisms will deepen our knowledge about pain modulation by ACh and morphine and the effects of morphine dependence on pain.

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## Conflicts of interest

The authors declare no conflicts of interest.

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