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# Rats with decreased brain cholecystokinin levels show increased responsiveness to peripheral electrical stimulation-induced analgesia

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**Abstract**

Using the P77PMC strain of rat, which is genetically prone to audiogenic seizures, and also has decreased levels of cholecystokinin (CCK), we examined the analgesic response to peripheral electrical stimulation, which is, in part, opiate-mediated. A number of studies have suggested that CCK may function as an antagonist to endogenous opiate effects. Therefore, we hypothesized that the P77PMC animals would show an enhanced analgesic response based on their decreased CCK levels producing a diminished endogenous opiate antagonism. We found that the analgesic effect on tail flick latency produced by 100 Hz peripheral electrical stimulation was more potent and longer lasting in P77PMC rats than in control rats. Moreover, the potency of the stimulation-produced analgesia correlated with the vulnerability to audiogenic seizures in these rats. We were able to block the peripheral electrical stimulation-induced analgesia (PSIA) using a cholecystokinin octapeptide (CCK-8) administered parenterally. Radioimmunoassay showed that the content of CCK-8 in cerebral cortex, hippocampus and periaqueductal gray was much lower in P77PMC rat than in controls. These results suggest that low CCK-8 content in the central nervous system of the P77PMC rats may be related to the high analgesic response to peripheral electrical stimulation, and further support the notion that CCK may be an endogenous opiate antagonist.

*Keywords:* Cholecystokinin octapeptide CCK-8; Acupuncture; Seizure; Opioid; Neuropeptide

## 1. Introduction

Considerable experimental evidence [1,7,17] suggests that cholecystokinin (CCK) may be a selective antagonist of opioid-induced analgesia. For example, in rats, CCK administration attenuates antinociception evoked by systemic morphine [7,18], intraventricular-endorphin [14,23], electroacupuncture (PSIA) [10], restraint stress [4], classical conditioning of front-paw foot-shock, but not hind-paw foot-shock which is non-opioid mediated [7]. In addition, CCK antagonists or active immunization against CCK potentiate exogenous opiate-induced antinociception [15,26]. This opioid facilitatory effect is preferentially mediated by $CCK_B$ receptors [5,27,28].

Anatomical findings are also consistent with such an opioid-antagonistic function, since the CNS distribution of CCK [2,22] and its binding sites [13,21,24] parallels that of opioids and their receptors within pain processing areas of both the brain (e.g., periaqueductal gray (PAG), nucleus raphe magnus) [2,21,22,24] and spinal cord (i.e., substantia gelatinosa) [13,24].

While substantial evidence supports an interaction between CCK and opiate systems, little is known of the mechanisms of this effect. Moreover, few studies have been performed to evaluate whether *endogenous* CCK can alter the antinociceptive responses induced by *endogenous* opioid system activation. Thus, we propose to study this interaction using antinociceptive stimulation produced by PSIA in P77PMC rats. The P77PMC rat were developed at Beijing Medical University and were originally derived from Wistar rats. They demonstrate congenital audiogenic seizures (AS) [34], and previous data from our laboratory indicate a reduced CCK-8 content in the brains of P77PMC rats [33]. Acupuncture is an ancient Chinese method of inducing analgesia by peripheral stimulation, which is thought to be mediated by the release of endogenous opioid peptides [8]. In the present study, we used tail flick latency to measure the analgesic effect induced by peripheral electrical stimulation. We then correlated this with

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susceptibility to audiogenic seizures and measured the CCK-8-immunoreactivity (-IR) in the brains of P77PMC rats and controls. We observed an increase in responsiveness of the P77PMC rats to peripheral electric stimulation-induced analgesia compared to controls which correlated in the P77PMC rats with seizure susceptibility. Furthermore, we found decreased CCK-8-immunoreactivity in the P77PMC rats in the cerebral cortex, hippocampus and periaqueductal gray (PAG). These results lend further support to the idea that CCK may be an antagonist to endogenous opiate-induced analgesia, and demonstrate a functional enhancement of analgesic responsiveness in animals with deficient CCK levels in brain.

## 2. Materials and methods

### 2.1. Animals

Adult Wistar rats and P77PMC rats of both sexes, weighing 250–300 g were obtained from the animal center in Beijing Medical University. They were kept six to a cage with food pellets and water available ad libitum.

### 2.2. Nociceptive test

The animals were restrained in a plastic holder with their tails protruding for the assessment of tail flick latency in response to a heat stimulus as described in detail elsewhere [20]. The tail flick latency for most of the animals was within the range of 4–6 s with room temperature maintained at $20 \pm 1$°C. The mean value of the first three assessments taken 5 min apart was considered baseline pain threshold. Subsequent values were expressed as percent change over the baseline level with +150% as the cut-off to avoid damage to the tail skin.

### 2.3. Peripheral electric stimulation

Two stainless steel pins of 0.2 mm width were inserted into each hind leg, one at the origin of anterior tibial muscle and the other in the front of the achilles tendon. Electrical impulses of 0.3 ms width, at a frequency 100 Hz, were applied through the pins. The intensity was increased stepwise from 1 to 2 to 3 mA, each lasting for 10 min. Tail flick latency was tested every 10 min during and after the stimulation for a total of 90 min in the initial study and for varying times in subsequent experiments.

### 2.4. Assessment of audiogenic seizures in P77PMC rats

The rats were maintained in a quiet environment prior to the testing. The experiment was performed at 09.00–11.00 h for three consecutive days. Each rat was placed in a plastic observation device, exposed to an electric ring at 120 dB for 60 s and seizure activity monitored. Seizures were scored according to the criteria described in a previous study [34]. Briefly: (stage 1) no significant change of behavior; (stage 2) running but no convulsive movement; (stage 3) two phases of violent running and jumping with startling, masticatory movements, and mild facial clonus; (stage 4) two phases of wild running and jumping followed by generalized clonus of forelimbs; (stage 5) two phases of wild running and jumping followed by complete tonic extension of hindlimbs and then generalized clonus of all limbs. To avoid the interaction between the analgesia testing and the audiogenic seizure testing, the analgesia testing was performed 3 days after the audiogenic seizure testing.

### 2.5. Pharmacological manipulation of CCK levels in P77PMC and Wistar rats

Several manipulations were performed to test the functional effects of CCK-8 and its antagonist L-365,260 on electrical stimulation-induced analgesia in P77PMC. CCK-8 was administered in doses of 0.5–2.0 $\mu$g/kg i.p., the tail flick latency of the rats were detected 30 min later, and the $CCK_B$ antagonist was administered i.c.v. immediately before a peripheral injection of CCK-8 at a dose of 4 ng/4 $\mu$l, which was shown to have no effect on its own on the electrical stimulation induced analgesia. For the latter studies, surgery was conducted under chlorohydrate anesthesia. Stainless-steel guide cannulae of 0.8 mm outer diameter were implanted into the brain 1 mm posterior to bregma and 2 mm lateral to the midline on one side and to a depth of 3.7 mm from the surface of the skull, and then were fixed on the skull with dental acrylic. One week later, a stainless steel injection tube of 0.4 mm outer diameter was inserted into the guide cannula and extended 1 mm beyond its lower end to reach the lateral ventricle. L-365,260 dissolved in 4 $\mu$l saline was infused over 5 min.

### 2.6. Radioimmunoassay of CCK-8-immunoreactivity (-IR)

CCK-8-IR was measured in the two strains of rats. The brains of rats were removed quickly after chlorohydrate anesthesia and decapitation, and immediately put into boiling water for 10 min. The cerebral cortex, hippocampus and PAG were then dissected according to the rat brain atlas of Paxinos and Watson [19] on a metal plate placed on ice. Brain tissue was homogenized with distilled water, and centrifuged ($10\,000 \times g$, 4°C, 30 min), and the supernatant was immediately frozen and lyophilized. Samples were redissolved with phosphate buffer (0.05 M, pH 7.6) and assayed with antisera raised against CCK-8. The radioactive ligand [$^{125}$I]BH-CCK-8 was obtained from Amersham, UK. The antisera against CCK-8 (kindly provided by Dr. J.S. Hong, NIEHS, USA) show very mild cross-reactivity with the following peptides: unsulfated CCK-8, 0.15%; CCK-7, 0.08%; CCK-4, 0.05% and gastrin, 0.1%. CCK-8 was a gift from Squibb and Sons.

Antibody antigen reaction was carried out to equilibrium at 4°C for 72 h and free labeling was separated using a goat anti-rabbit IgG combined with PEG. The absolute sensitivity of the assay was 0.8 fmol/tube with 9.9% inter-assay and 4.6% intra-assay variation.

### 2.7. Statistical analysis

Data were analyzed using a multifactorial analysis of variance (ANOVA). When the ANOVA revealed a significant difference between groups, the Duncan test was used for further analysis. Student's *t*-test was used for group comparisons where appropriate. The correlation between the vulnerability of epileptic seizure and the effect of 100 Hz analgesia was analyzed using correlation coefficient. A probability level of 0.05 was regarded as significant.

## 3. Results

### 3.1. Analgesia induced by 100 Hz peripheral electric stimulation in Wistar rats and P77PMC rats

In the Wistar control group ($n = 20$), electrical stimulation for 30 min elicited an increase in tail flick latency by $66.8 \pm 14.3\%$ ($\mu \pm$ S.E.M.), which subsided in 30 min. In the P77PMC rats ($n = 19$) tail flick latency increased to $118.5 \pm 11.1\%$ of baseline, which persisted for 60 min (Fig. 1). The difference between the two groups was significant ($P < 0.01$, ANOVA).

We attempted to block the analgesic effect in the P77PMC rats using the opioid antagonist naltrexone at 1 ($n = 10$) and 25 ($n = 10$) mg/kg vs. saline ($n = 20$) injected subcutaneously. A significant effects was observed only with the higher dose of naltrexone ($P < 0.01$, ANOVA; Fig. 2) and this represented a partial, but not complete, reversal of the stimulation-induced analgesia.

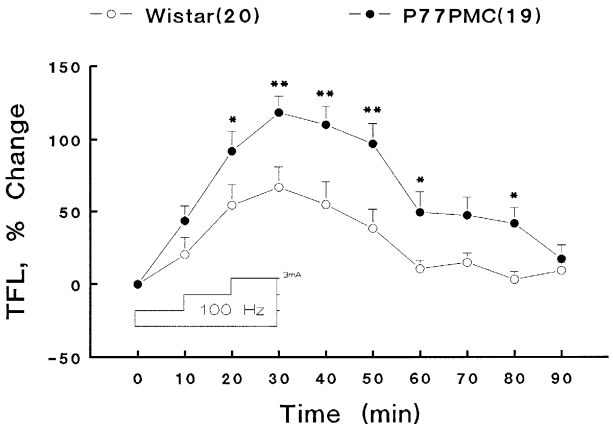


Fig. 1. The analgesic effect induced by peripheral electrical stimulation at 100 Hz is illustrated in P77PMC and Wistar rats. On the ordinate is plotted the percentage change in tail flick latency (TFL) over baseline (mean value of three separate tests prior to the stimulation). The group mean and standard errors are plotted over time (abscissa). The time period and the intensity of the stimulation is shown at the bottom of the figure. Significant differences between groups (ANOVA followed by Duncan test for comparison between two groups in the same time point) are illustrated with asterisks: * $P < 0.05$; ** $P < 0.01$.

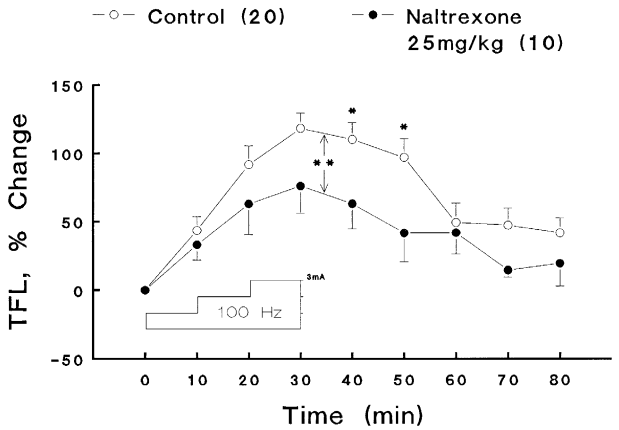


Fig. 2. The effect of naltrexone (25 mg/kg) on the analgesic effect elicited by 100 Hz electric stimulation in P77PMC rats. Naltrexone was administered, s.c., 2 h prior to 100 Hz stimulation. The group mean ($\mu \pm$S.E.M.) analgesic response is plotted over time. Naltrexone significantly, but only partially reversed the electrically stimulated analgesia in P77PMC rats. * $P < 0.05$ and ** $P < 0.01$ (ANOVA, between-groups comparison).

### 3.2. Audiogenic seizures

In a separate group of 47 P77PMC rats, electrical stimulation (100 Hz) was administered to evaluate analgesia in rats that were also assessed for seizures. The increase in tail flick latency in each rat (taking the average of six assessments obtained during the stimulation period

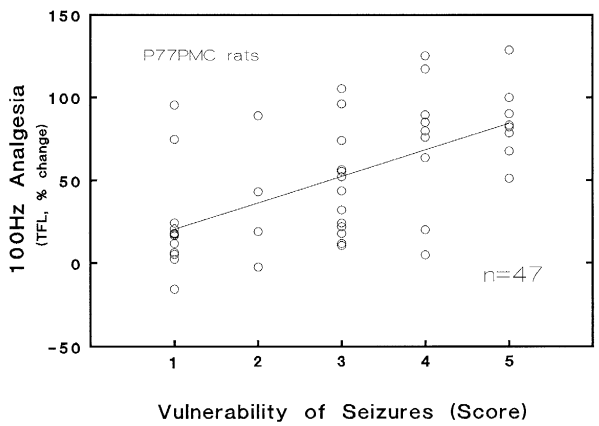


Fig. 3. The correlation between 100 Hz stimulation-induced analgesia and seizure scores in P77PMC rats. Abscissa: scores showing the vulnerability and severity of seizures; ordinate: percentage change in tail flick latency (TFL, average of six assessments taken during and after the electrical stimulation) as a result of 100 Hz electrical stimulation. A significant positive correlation was observed such that greater seizure response was associated with a greater analgesic response. $r^2 = 0.57$, $n = 47$, $P < 0.01$.

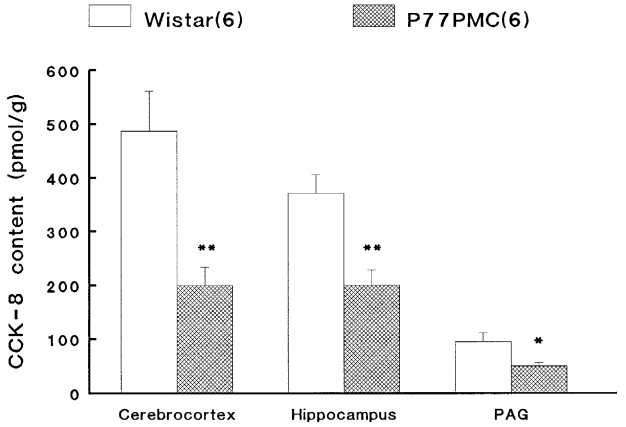


Fig. 4. CCK-8-immunoreactivity in cerebral cortex, hippocampus and PAG of P77PMC rat brain. The group mean ($\mu \pm$S.E.M.) CCK-8 content measured by radioimmunoassay is plotted in pmol/g tissue for P77PMC and Wistar rats. In each brain region, decreased CCK-8-immunoreactivity was observed in the P77PMC rats compared to Wistar controls. $^{*}$ $P < 0.05$; $^{**}$ $P < 0.01$; $n = 6$ in each group.

and within 30 min after the termination of stimulation, compared to baseline) was plotted against the seizure score, as shown in Fig. 3. A positive correlation was found between the two parameters ($r^2 = 0.57$, $P < 0.01$), indicating that the higher the vulnerability to audiogenic seizures, the greater the percent change in tail flick latency.

### 3.3. CCK-8-immunoreactivity in cerebral cortex, hippocampus and PAG of P77PMC rat brain

Six P77PMC or Wistar rats which were all naive were sacrificed. Their brains were removed and cerebral cortex, hippocampus and PAG were dissected out for measuring

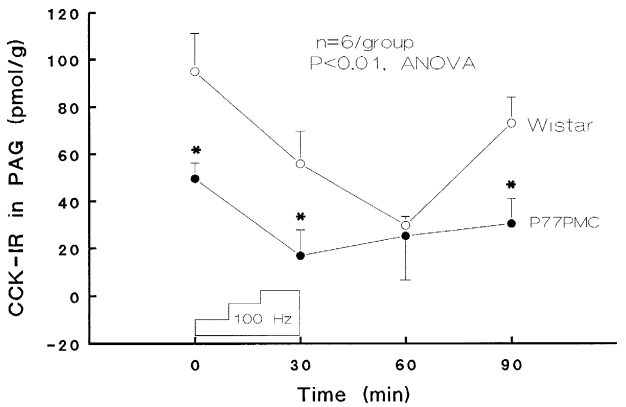


Fig. 5. Changes of CCK-8-immunoreactivity in the PAG of rats after 100 Hz peripheral electrical stimulation is plotted for different groups of rats ($n = 6$/group) sacrificed at various time points following stimulation. Although Wistar rats have higher levels of CCK initially and at most other time points, both groups show a similar pattern of a decrease in CCK-8-immunoreactivity following stimulation that returns toward baseline by 60 min. $^{*}$ $P < 0.05$ compared to the corresponding point of Wistar control rats. $^{**}$ $P < 0.01$ between the two groups (ANOVA). One-way ANOVA for Wistar rats: $P < 0.05$ at 30 min and $P < 0.01$ at 60 min; for P77PMC rats: $P < 0.05$ at 30 min.

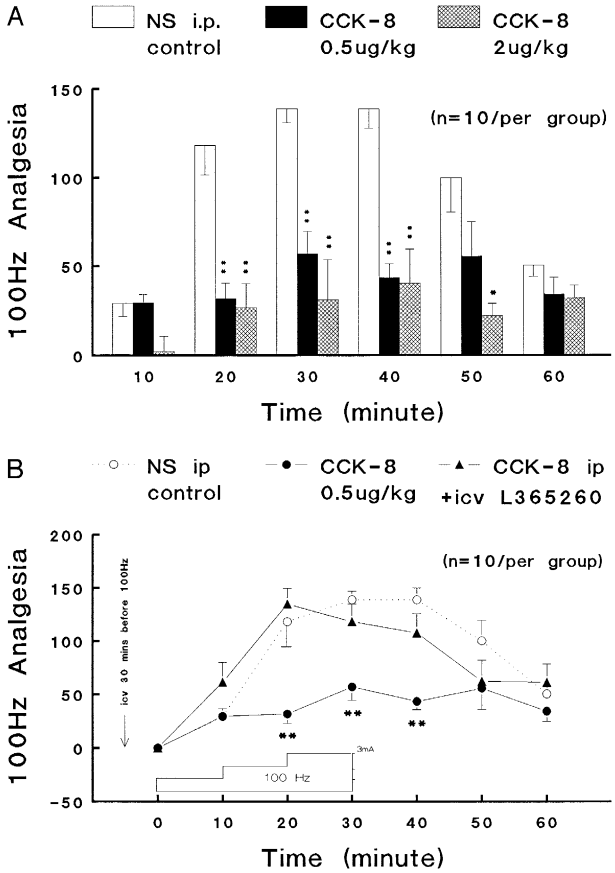


Fig. 6. A: antagonism of 100 Hz stimulation-induced analgesia by CCK-8 in P77PMC rats. CCK-8 at two doses (0.5 and 2 $\mu$g/ kg) was injected i.p. 30 min prior to stimulation. CCK-8 markedly reduced the analgesia in P77PMC rats. $n = 10$ per group. $^{*}$ $P < 0.05$, $^{**}$ $P < 0.01$ compared to the normal saline-injected (NS) control group at the same timepoint. B: the effect CCK-8 in P77PMC rats was reversed by i.c.v. injection of the CCK antagonist L365,260 (4 ng) administered immediately before CCK-8. $n = 10$ per group. The antagonist returned the P77PMC animals to their initial potentiated level of analgesia. $^{**}$ $P < 0.01$ compared to CCK-8-injected P77PMC rats.

CCK-8-IR by radioimmunoassay. The results are shown in Fig. 4. The CCK-8-IR in cerebral cortex, hippocampus and PAG of P77PMC rats were about 40.9%, 53.7% and 52.2% of Wistar control, respectively.

### 3.4. Changes of CCK-8-immunoreactivity in PAG after 100 Hz stimulation

To determine whether CCK-8-IR would be altered by PSIA, we stimulated four groups of P77PMC and Wistar rats ($n = 6$ group) and sacrificed these animals at four time points relative to the stimulation: before, immediately after, 30 or 60 min after the termination of 30 min of stimulation at 100 Hz. Their brains were removed and the PAG was dissected out as described above for measuring CCK-8-IR. The results are shown in Fig. 5.

In Wistar rats, electrical stimulation caused a 41% decrease in the content of CCK-8-IR immediately after the

stimulation, suggesting a release of CCK-8. This trend continued for 30 min after the stimulation was terminated and began to return to baseline levels by 60 min. While the content of CCK-8-IR in the PAG of P77PMC rats was only 52.2% that of the Wistar rats, the changes that occurred in CCK-8-IR were similar to those seen in Wistar rats, i.e., an immediate drop after the stimulation, followed by returning toward baseline.

### *3.5. Antagonistic effect of exogenously administered CCK-8 on peripheral stimulation-induced analgesia (PSIA)*

To further evaluate the functional importance of CCK-8 in the PSIA, we administered exogenous CCK-8 to P77PMC rats. Three groups of rats ($n = 10$/group) were given i.p. injections of (a) normal saline, (b) 0.5 $\mu$g/kg, or (c) 2.0 $\mu$g/kg of CCK-8, followed by electrical stimulation at 100 Hz 30 min later. Fig. 6A illustrates the analgesia induced during the period of stimulation and the subsequent 30 min under each condition. Electrical stimulation produced a $130 \pm 15\%$ maximal increase in TFL in the normal saline control group, whereas in the groups treated with CCK-8 (b and c), the stimulation-produced analgesia was markedly attenuated (ANOVA, $P < 0.01$).

To demonstrate that the effect of peripherally administered CCK-8 was related to brain $CCK_B$ receptors, we administered the $CCK_B$ antagonist L-365,260 (4 ng) intracerebroventricularly immediately before a peripheral injection of CCK-8 (0.5 $\mu$g, i.p.), followed by 100 Hz stimulation 30 min later, and then measured tail flick latency. We compared this with controls (P77PMC rats) that received saline or just CCK-8 (0.5 mg/kg, i.p.). Previous studies have shown that the $CCK_B$ antagonist L-365,260, at doses between 3 and 50 ng i.c.v., has no significant effect on tail flick latency on its own [3]. As shown in Fig. 6B, the effects of i.p. CCK-8 were completely reversed by the $CCK_B$ antagonist.

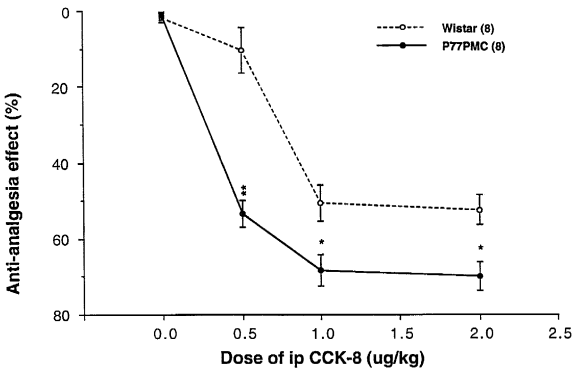


Fig. 7. The comparison of the antagonism of CCK-8 i.p. injection between P77PMC rats and Wistar rats. On the ordinate is the percent suppression of TFL by i.p. injection of CCK-8, which are mean value of six time points; on the abscissa are the doses of i.p. CCK-8. * $P < 0.05$, * * $P < 0.01$.

### *3.6. Comparison of the antagonism of i.p. CCK-8 between P77PMC rats and Wistar rats*

Four groups of P77PMC and Wistar rats ($n = 8$/group) were each given i.p. injection of (a) normal saline, (b) 0.5 $\mu$g/kg, (c) 1.0 $\mu$g/kg, or (d) 2.0 $\mu$g/kg of CCK-8, followed 30 min later by 100 Hz electrical stimulation. The anti-analgesia effect was averaged among the six time points. The results are illustrated in Fig. 7. In both groups of rats, a suppression of electrical stimulation-induced analgesia was observed. However, for the Wistar rats the overall suppression of analgesia by CCK-8 was less: 2.00%, 10.46%, 50.56%, 52.34% for saline, 0.5, 1.0, and 2.0 $\mu$g/kg, respectively; while for P77PMC rats, CCK-8 caused 1.35%, 53.45%, 68.33%, 69.79% suppression, respectively. The difference between the two groups was significant ($P < 0.01$, ANOVA). The difference between each dose among these two groups were also significant, as indicated in Fig. 7.

## 4. Discussion

The main result of these studies is that P77PMC rats exhibit a much stronger analgesic response to peripheral electrical stimulation than Wistar rats. Moreover, the relationship of this observation to deficient CCK-8 function in P77PMC rats was supported through measures of endogenous CCK and through exogenous manipulations of this system in both strains of rats. As in Wistar rats, the analgesia induced by 100 Hz stimulation can be partially blocked by the opiate antagonist naltrexone at a high dose (25 mg/kg, Fig. 2) but not at a low dose (1 mg/kg), which fits well with the documented finding that 100 Hz high frequency stimulation-induced analgesia was mediated mainly by dynorphin via $\kappa$ opioid receptors [9,12,25].

The previous evidence in favor of the hypothesis that increased analgesia in the P77PMC rats is related to a deficiency in the central content of cholecystokinin octapeptide (CCK-8) which has been shown to be a potent anti-opioid peptide [7] included: (a) our preliminary data suggesting that low cerebral cortex and hippocampal content of CCK-8 may account for the high susceptibility of audiogenic seizures in P77PMC rats [33]; (b) the CCK receptor antagonist L-365,260 potentiated the 100 Hz PSIA in Wistar rats but not in P77PMC rats [3], suggesting that in P77PMC rats minimal amounts of CCK-8 were released in response to 100 Hz stimulation (but see below for discussion of our results in this context); (c) an increased concentration of CCK-8 was detected in spinal perfusates following peripheral electric stimulation of 100 Hz in rats [35]; and (d) data indicating that the effectiveness of PSIA depends upon a delicate balance between the functional availability of endogenous opioids on one hand and the anti-opioid peptides on the other hand [8].

In the present study we measured the CCK-8-IR in

three different areas of rat brain including cerebral cortex, hippocampus and PAG, which were consistently lower than the Wistar rats. In control rats, 100 Hz stimulation produced a dramatic decrease in CCK-8 content in PAG during and 30 min after the stimulation, implying that the CCK-8 existing in nerve terminals was released. This release of CCK-8 appears to limit the PSIA since administration of the CCK receptor antagonist L-365,260 enhanced the PSIA in Wistar rats [3]. Since the CCK-8 content in brain of P77PMC rats is only half of that in Wistar rats, one would expect a much smaller release of CCK-8 in these rats. As a consequence there is a disinhibition (potentiation) of the stimulation-produced endogenous opioid analgesia. Since we know that the spinal cord is one of the most important sites for CCK's anti-opioid effects [16,27], we still need to determine whether CCK content is also low in this region in the audiogenic seizure-prone rats.

What was surprising to us was that even in the P77PMC rats, there was a significant decrease in the CCK content following the 100 Hz stimulation, suggesting that there should be some CCK-8 being released, and that the CCK receptor antagonist L-365,260 should be have been able to further potentiate the stimulation-produced analgesia in the P77PMC rats. However, the experimental results do not confirm this; instead L-365,260 was found to be totally ineffective in potentiating stimulation-produced analgesia in P77PMC rats. These results suggest that mechanism in addition to the reduced biosynthesis of CCK may exist in these animals, e.g., an accelerated degradation of CCK-8 or a functional change in CCK receptor effects. To evaluate the latter possibility, we administered CCK-8 intraperitoneally at a dose range of 0.5–2.0 $\mu$g/kg [1,7]. A marked suppression of the stimulation-produced analgesia was observed (Fig. 6A), which was antagonized by the central administration of the CCK antagonist L-365,260 (Fig. 6B). The amount of CCK-8 needed to suppress PSIA in P77PMC rats was smaller than that of the control group (Fig. 7). Thus, it appears that the CCK receptor may be upregulated or more sensitive to exogenous CCK.

CCK is not only an anti-opioid peptide, but also an anticonvulsant substance [30,31,33]. Therefore, another issue of interest was whether a defect in CCK production serves as a common denominator for the high susceptibility to epileptic seizures as well as for a highly effective stimulation-produced analgesia. The positive correlation found in the current study between these two measure supports this contention. We have also recently shown that transfection of CCK gene into the brain of P77PMC rats can inhibit the vulnerability of these animals to epileptic seizures [32], further demonstrating the functional importance of CCK to seizure behavior in these animals. And most recently, we have demonstrated that the effectiveness of peripheral electrical stimulation-induced analgesia could be decreased by the overexpression of CCK in the CNS (Zhang et al., unpublished observations).

CCK is the first neurochemical substance analyzed in these audiogenic seizure-prone rats. We have known that the analgesia effects induced by peripheral electric stimulation could be mediated by nonopiate neurotransmitters, such as serotonin [29], norepinephrine [11], GABA [6], etc. In this paradigm, the anti-opiate CCK has a much greater antagonistic effect on 100 Hz PSIA than even 25 mg/kg naltrexone did. This may imply that CCK as a potential antagonist of non-opiate analgesia (at least in this genetically altered rats).

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