行政院國家科學委員會補助專題研究計畫成果報告

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※ 調控脊髓基因對於針刺痛之影響(I) ※
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計畫主持人： 孫 維 仁

共同主持人：

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□赴國外出差或研習心得報告一份

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□國際合作研究計畫國外研究報告書一份

執行單位：

國立台灣大學醫學院麻醉科

中 華 民 國九十一年 一 月三十一日

行政院國家科學委員會專題研究計畫成果報告

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一、中文摘要

許多臨床經驗都可以觀察到：針刺鎮痛的效果與是否「得氣」有很大的關係，但是其「得氣」的真正意義及針刺本身是否引起疼痛至目前為止並不清楚。動物模型顯示，在四肢周邊的特定部位以電刺激會產生類似針灸止痛的效果，稱為「電針止痛」，主要是經由興奮及去極化周邊的感覺神經受器，誘發脊髓及脊髓以上中樞神經的「內源性下行疼痛抑制系統」而產生止痛作用。進一步的研究顯示不同的針刺參數可以對此鎮痛機轉有不同的調節作用；而過去大部分的研究集中於針刺頻率對止痛的影響，但只有很少的研究注意針刺強度與「得氣」間的關係。

我們在此年度的研究中，建立了我們的針刺止痛動物模型：大白鼠在輕度的氣體麻醉下，分別接受低強度及高強度的電針刺激，並觀察在分別在閃尾實驗及福馬林足掌注射的疼痛反應。我們發現：在閃尾實驗中(代表 pain threshold 的變化)，提高電針刺激強度，確實可以延長止痛反應的時間；而對足掌注射福馬林引起的化學性刺激，低或高電針強度都只能產生類似的輕度止痛效果。在對脊髓背角所做的免疫組織化學染色反應中，我們發現，電針刺激與福馬林刺激所引發脊髓背角的反應神經元的分布並不相同，代表著這兩種刺激是分別經由不同的次級感受神經元所參與。其差別在於，電針刺激可能是經由較粗的初級傳入神經所傳導，如 Aβ fibers (代表非傷害性感覺)，而福馬林刺激是由細的初級傳入神經所傳導，如 Aδ或 C-fibers (代表傷害性感覺)。這結果，暗示著電針刺激的鎮痛機轉並非經由 DNIC (diffuse noxious input control)的機制所參與。

關鍵詞：針灸、疼痛、得氣、c-*fos*、DNIC

Abstract

Clinical experience has observed that the analgesic effect of acupuncture depends largely on the presence of “Der-Chi”, a deep “aching” sensation evoked by point stimulation, but it remains unclear whether stimulation-evoked nociception is required for acupuncture–induced analgesia. Animal models showed peripheral electric stimulation at specific point, called electroacupuncture (EA), could produce acupuncture-like analgesia by depolarizing peripheral sensory receptors in the meanwhile activating endogenous pain inhibitory system at spinal and supraspinal levels. Further evidences revealed different EA parameters could participate in regulating analgesic mechanisms. Despite many studies had investigated the correlation between EA frequency and its analgesic effect, only a few experiments focused on the impact of EA intensity on "Der-Chi" phenomenon.

We developed an EA animal model with consistent EA analgesia in behavioral responses to tail-flick test and formalin injection under light inhalation anesthesia.

Intensity-dependent analgesia was recorded in rats receiving low and high EA stimulating intensity. Differential laminar distribution of Fos nucleoprotein in receptive spinal dorsal horn implied the different molecular processing between these two peripheral signals, i.e. transmission of EA and nociception was mediated by different afferent fibers, large-diameter and small diameter fibers respectively. We concluded that efficacy of EA analgesia was generated in proportion to its stimulation intensity within the threshold of recruiting primary noxious afferents. This finding contradicts the possible involvement of "diffuse noxious input control" (DNIC) in EA mechanism.

Keywords: Electroacupuncture, analgesia, pain, c-*fos*, DNIC

二、緣由與目的

Clinical experience has observed that the analgesic effect of acupuncture depends largely on the presence of Der-Chi, a deep, dull aching sensation evoked by point stimulation. Despite the well-known phenomenon of Der-Chi in clinical practice, it remains largely unclear whether stimulation-evoked nociceptive response is required for acupuncture–induced analgesia. Peripheral electric nerve stimulation at specific point, or electroacupuncture (EA), is believed to produce analgesia by depolarizing peripheral sensory receptors in the meanwhile activating endogenous pain inhibitory system at spinal and supraspinal levels in animal model[iii]. One consequence of the "Der-Chi" phenomenon, through altering EA stimulating parameters in animal model, is the finding that low frequency stimulation (less than 10 Hz) evokes the release of β-endorphin, enkephalin[iiiiv], and endomorphin-1 [v] and high frequency stimulation sti mulates non-opioidergic system[vivii]. However, the minimal intensity of EA stimulation required to elicit analgesia is still controversial and, activation of either innocuous large afferent fibers or small noxious afferents were advocated to serve as the primarily sensory nerves for transmitting peripheral EA signal to central relay neurons. Till now, only a few experiments[viiiix] had intentionally investigated the impact of EA intensity on analgesia.

It is generally believed that intense peripheral stimulation, which sufficiently excites the high-threshold primary sensory afferents, is required to produce longer and higher analgesic effect. Stimulation of hindlimb meridian points at high intensities that recruit Aδ and C afferents, but not lower intensities, leads to a persistent depression of the jaw-opening reflex elicited by electrical stimulation of the incisor pulp in rat[x]. In primates, electrical stimulation of tibial nerve at a strength that activated only Aα and Aβ fibers produced a minor inhibition of lumbar spinothalamic tract cells without post-stimulation effect, yet recruitment of Aδ and C fibers with higher intensities produced an inhibition lasting up to 30 min[xi]. Toda et al. reported EMG amplitude of jaw-opening reflex was maximally suppressed as increasing EA stimuli strength to activate Aβ fiber and Aδ fiber stimulation scarcely augmented suppression rate, but they did not mention their EMG recording time during or after EA stimulation. Nevertheless, long-lasting suppression effect was induced

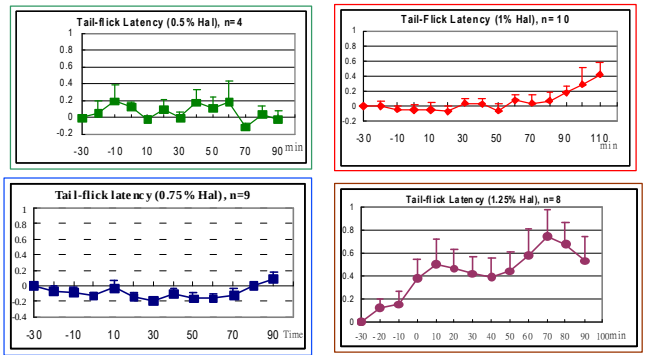
in another experiment using strong intensity sufficient to activate Aδ fiber[xii]. Recording of only Aα, but not Aδ, activities was documented in mice receiving high intensity, low frequency EA stimulation with post-treatment hypoalgesia for over 20 min. However, the addictive effect by stress analgesia was implicated because EA was applied to unanesthesized animals[xiii].

Expression of Fos nuclear protein, functioning as “third messenger” molecule coupling short-term extracellular events to long-term intracellular changes by regulating c-*fos* proto-oncogene[xivxvxvi] in the brain and spinal dorsal horn, has been widely used in animal nociception model as a reliable pain marker [xvii] . Various types of noxious stimulation, including thermal, mechanical, and chemical stimuli [xviii xix xx xxi] were characterized by rapid and transient c-*fos* expression 20-90 min following excitation [xxii xxiii] . Many studies have confirmed Fos activity in the spinal dorsal horn following noxious stimulation are distributed through lamina I, II, V, X in the same dermatone [xxiv] , and the number of Fos-labeled neurons increases approximately proportional to the stimulus intensity or to pain behaviors[xxvxxvi].. Innocuous stimuli, in contrast, scarcely induce c-*fos* expression at the above laminae except only few labeled neurons located at lamina III-IV[xxvii]. Using the characteristic of localizing and quantitative correlation, it is possible to test the neuronal activities at segmental spinal cord following electroacupuncture stimulation.

In this study, we hypothesized stronger EA intensity would result in higher analgesic effect, whereas, we do not have the idea of which types of nerve endings were evoked by EA stimulation. Thus, by identifying the prolongation of animal tail withdrawal latencies and detecting c-*fos* expression of spinal dorsal horn under EA stimulation, we could answer to the basic question of acupuncture: "How strong could a needle stimulus be effective?" Another aim of this study was to resolve the possible mechanism of EA analgesia by differentiating the activities of nociceptive neurons at dorsal horn. Animals in this study were anesthetized by halothane inhalation to decrease the influence of stress-induced analgesia. This method, as we know, is the first report of EA analgesia testing the tail flick latency of animals under inhalation anesthesia.

三、結果

1. Effect of Anesthetic Concentration on Tail-flick Latency

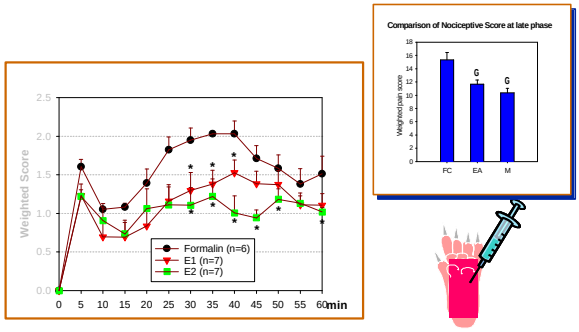


2. EA Analgesia in Tail-flick Test*2..1 Tail-flick test*

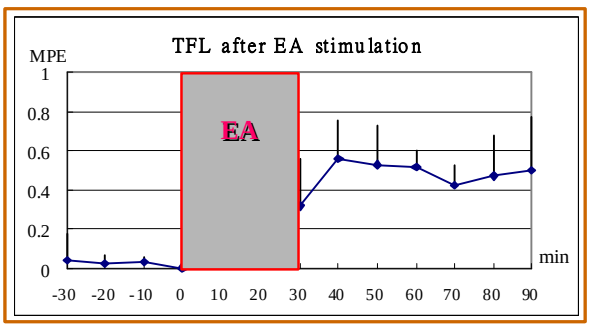
As illustrated in Fig 1 and 2, the control group kept considerably constant tail-flick latency throughout experiment without temporal influence of 2-hr inhalation anesthesia. Increase of tail-flick latency was shown after electrical stimulation of unilateral hindlimb at 4 Hz frequency for a duration of 30 min in the low and high

intensity groups comparing with that of control group. Nociception inhibited by low or high intensity EA was statistically significant since 50 and 20 min respectively after treatment (Fig. 1). In the 10T group, two peak MPEs appeared at 50 min (31.74 ± 8.4 %) and 70 min (28.58 ± 2.55 %), whereas, maximal MPEs of 20T group were at 60 min (72.50 ± 16.87 %) and 90 min (71.04 ± 15.25 %). The latter two values were located far beyond the EA treatment period. High inter-individual variations in MPE values could be observed in both EA groups.

Comparing two EA-treated groups, 20T



produced higher analgesia than 10T did for 30 min after cessation of electrical stimulation. It was important to note that the analgesia produced by 20T group did not decay throughout time, instead, steady increasing of analgesia was present till the end of study. The maximal inhibition of tail flick reaction of high intensity group was

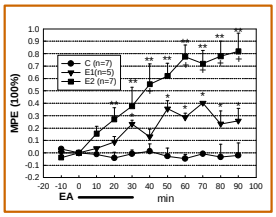


comparable to that of morphine-treated group (MPE= 66.29 ± 7.63 %, dose: 2.5 mg/kg), but peak effect of morphine appeared earlier. (Fig. 2)

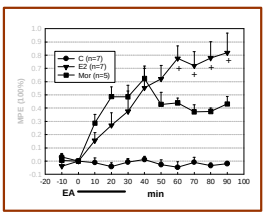
Tail-flick latency in low and high EA intensity

2..2 Formalin test

The typical biphasic response was



Analgesia produced by different EA intensities
■20×; ▼10×; ●control



Analgesic effect between EA and Morphine
■morphine; ▼20×;

illustrated in 3 groups receiving formalin injection (figure 3). Intense mechanical stimulation caused a barrage of primary afferents activation leading to an early phase for a period of 5 min, and followed by another 40-min of late phase representing ongoing inflammation of peripheral nociceptors and increased afferent activities[xxviiixxixxxx]. In EF and MF groups, similarly analgesia at late phase was evident when comparing with control group as illustrated in figure 3 and 4 ($p<0.05$), but no statistical difference could be detected between EF and MF groups. We can conclude that the analgesic potency of 20T EA was approximately equal to the strength of 2.5 mg/kg morphine in formalin-induced pain model. Besides, analysis of two summed scores at biphasic time courses, analgesia appeared only at late phase rather than early phase (figure 3, 4), indicating that EA fails to inhibit the brief, intense mechanical pain produced by mechanical injection. Since EA exerted a prolonged after-effect that outlasted its treatment period for over 60 min in tail-flick model, we

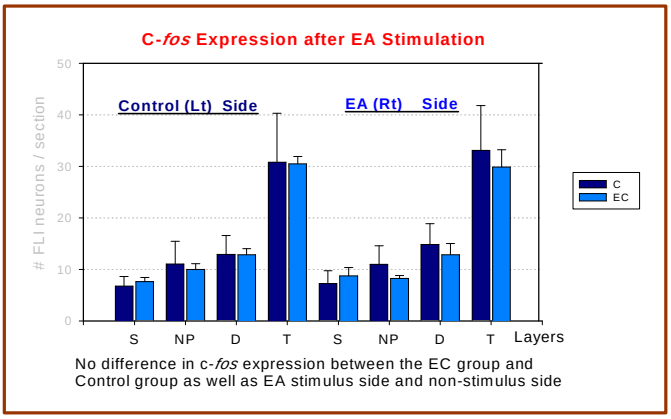
believe that the decreased pain scores at late phase of EF group was the analgesic effect produced by EA treatment.

Formalin Test in low and high EA intensity

3. Fos-like immunoreactivity

C-*fos* immunoreactivity was lightly detected in all laminae of spinal dorsal horn of rats in both control group and EC group (figure 5). Analyzing individual lamina of two sides of dorsal horn in figure 5, there is no significant difference in numbers of FLI cells can be demonstrated between side-to-side comparison in the same group or group-to-group comparison in the same side ($p<0.05$). Our results revealed that high voltage peripheral electrostimulation did not activate the significant expression of nociceptive neurons specifically localized at spinal lamina I, II, and V, though it was presumed that EA intensity to the extent of noxious level was required to obtain a analgesic effect. Further, typical induction of c-*fos* expression is predominantly shown at left dorsal horn of either FC group after 1 % formalin injection (figure 6), especially at superficial and deep laminae, but much less shown at right dorsal horn. Noxious response of formalin irritation, however, was significantly suppressed by either pretreatment with high intensity of EA or 2.5 mg/kg morphine for the consequence of decrease in total Fos-LI cells in all laminae of dorsal horn to the extent of 25 % and 20 % in group EF and MF respectively ($p < 0.05$, three columns of T at left panel of figure 6). More specifically, the decrease was mainly manifested at the location of deep layers but not superficial layer or nucleus proprius (figure 6). Similarly, no significant difference in FLI neurons is seen between EA and morphine treatment groups at right side dorsal horn.

C-fos Expression in Spinal Dorsal Horn in *EA* and *Control* group



No difference in c-*fos* expression between the EC group and Control group as well as EA stimulus side and non-stimulus side

四、討論

Our data demonstrated the low frequency peripheral electrical stimulation at meridian point prolongated the tail flick latency in an intensity-dependent manner, that is, the higher stimulation intensity the more inhibitory effect on tail withdrawal reflex is produced. We also demonstrated the analgesic effect on formalin-induced pain by high intensity EA with evidences of decreased pain scores and decreased number of Fos-LI neurons in spinal level. However, these strong peripheral electrostimulations did not cause detectable increase in c-*fos* expression of corresponding spinal neurons at sensory receptive field than baseline expression level. These results implied the efficacy of EA analgesia could be generated in proportion to its stimulation intensity within the strength not exceeding the threshold of recruiting pain pathway.

We did not record the sensory afferent fibers elicited by EA stimulation in our study, so it is impossible to differentiate which types of fibers had been activated by high intensity stimulation. It might be rationale to postulate the excitation of high-threshold non-myelinated nociceptors during electrical stimulation as we increased the EA intensity in order to enhance analgesia, however, our immunocytochemical measurements put forth a different result. Rats in groups without receiving formalin injection showed only low-grade expression of Fos-labeled neurons at spinal dorsal horn after intense EA stimulation. This increase was not significantly different when comparing with the background expression at the contralateral side of the same group or with ipsilateral side of the naïve group. The failure in activation of spinal nociceptive neurons can be explained by the reasons that, firstly, high-voltage intensity could possibly be insufficient to activate Aδ or C fibers, or secondly, the excitation of peripheral input is unable to depolarize spinal second neurons at dorsal horn. Though no direct evidence can be derived from our crude data, we highly suggest that the EA analgesia can be initiated by a peripherally strong but still sub-algesic stimulation, in view of our molecular (c-*fos* expression) and behavioral (unharmed appearance) findings during and after anesthesia. Further, it is a rational deduction that “pain sensation” on acupuncture needling is not a prerequisite at clinical practice because some advocated “diffuse noxious inhibitory control”, which always needs the drive of pain input, was involved in the mechanism of EA analgesia.

More details of complex EA mechanism need to be discussed. Formalin evoked nociception is mediated by an integrated consequence of both spinal and supraspinal structure, while tail-flick response is a different mechanism representing a withdrawal reflex at the level of spinal cord. Current studies have confirmed Fos activity in the spinal dorsal horn following nociceptive stimulation are distributed through lamina I, II, V in the same dermatone, and its expression can be suppressed by administrating analgesic treatments such as morphine [xxxi], noradrenalin [xxxii] and heterotopic noxious stimulation [xxxiii]. In inflamed tissue, peripheral nociception is mostly mediated via non-myelinated C fibers to neurons in lamina I-II at first[xxxiv], then activates deeper projection neurons or interneurons under conditions of barrages of

painful input or/and sensitization of interneurons. A broad population of initially "silence" neurons at nociception-responsive laminae could be potentiated or "wind-up" in view of neuroplastic characteristics, a phenomenon known as "central sensitization" which furtherly resulting in temporal prolongation and spatial expansion[xxxv] of original pain signals. Our findings demonstrated that EA stimulation could suppress Fos expression localized at the deep portion (Lamina V) but not in superficial layer (lamina I, II). The current result suggests that EA could indirectly yield analgesic effect by hyperpolarizing the deep layer neurons at EA-treated and formalin-injected sides through the activation of descending inhibitory modulation so as to inhibit the central desensitization at spinal cord level. We also hypothesize the predominant wide-dynamic-range neurons in lamina V, which can be converted from initially "silence" to exciting state by intense nociception, are possible the main targets being desensitized in this process.. However, more studies are needed to elucidate our putative inference.

Inhalation anesthesia was used in this expeiment to avoid the possible interference of stress-induced analgesia, a mechanism which was reported to be induced by intense peripheral stimulation, cold water swimming, or restraint stress in awake animal and also a mechanism which can insidiously potentiate acupuncture analgesia[xxxvixxxvii].. The present study, to our understanding, is the first report using inhalation anesthesia to evaluate EA effect on tail flick response. In control group, rats inhaling 1 % of halothane showed unchanged tail flick latencies for 120 min. It appears that behavioral response to noxious stimulation can be adequately preserved at this light anesthetic level. Also important is that the inhalational anesthetic possesses unique characteristics of rapid wash-in and washout, which can help to facilitate the easier control of anesthetic depth and faster awakening from anesthesia. We found almost all the rats in this study were fully awaked from anesthesia within 5 min after stop of anesthetic inhalation, it created an advantage for us to observe the behavior response of formalin test immediately following anesthesia free from the influence of anesthetics. In contrast, intravenous anesthetic/sedative agents can cause fluctuating drug concentration in blood by single or intermittent injection, as well as accumulate in fat tissues leading to delayed post-anesthetic emergence by prolonged continuous infusion. Accordingly, we recommended this feasible anesthetic model for EA study because of its better manipulation of animals than that of injection anesthesia in point of fine anesthetic level control and immediate recovery from anesthesia.

Our another distinctive finding is the delayed peak analgesic effect. It is noticed that the peak effect of the 20T and 10T groups appeared outside the electric stimulation period and a persistent elevated pain threshold occurred in 20T group. Most previous studies reported the maximal inhibition effect of tail flick test occurred at or near the endpoint of EA stimulation time, and the post-stimulation effect usually declined with time[xxxviiixxxixxlxli]. To explain the discrepancies, possible causes related to

study design are raised. First, inhalation anesthesia in rats still needed to be meticulous when considering its potential effect on noxious input and EA [xlii] [xliii] .. Though no evidence showing its influence on tail flick latency, inhaled anesthetic could probably modify the central nervous integration involved in acupuncture analgesia mechanism and postpones the peak inhibition response to noxious stimulation. Second, we introduced the electric stimulation at unilateral limb, which exerted less energy amount than bilateral electric stimulation did, so the maximal analgesic response during EA period is lowered. Third, we did not categorize the rats into high EA responder or low EA responder before study. Evidences have shown considerably individual differences in analgesic effect exist either by manually twisting or electrically stimulating the acupuncture needles[xlivxlv], so many reports enrolled only the EA responder into studies from the non- or low-responding rats in advance to improve their result efficacy[xlvixlvii].

In conclusion, our study proved low frequency peripheral EA stimulation prolonged the tail flick latency in an intensity-dependent manner, that is, higher intensity EA produced stronger and longer analgesic effect than lower intensity did. The analgesic effect of EA was weak to moderate, roughly equivalent to that of morphine 2.5 mg/kg. We also demonstrate the intense EA did not evoke the c-*fos* expression of nociceptive neurons at spinal cord, indicating EA stimulation is not a pain signal. This finding contradicts the possible involvement of "diffuse noxious input control" in mechanism of EA analgesia and thereby supports increasing stimulating intensity as much as those before causing pain on patients. Meanwhile, the feasibility of inhalation anesthesia in animals treated with EA analgesia was introduced for which is advantageous over injection anesthesia in better control of anesthetic level and immediate recovery from anesthesia.

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