ACUPUNCTURE MECHANISMS IN RABBITS
STUDIED WITH MICROINJECTION OF ANTIBODIES AGAINST \( \beta \)-ENDORPHIN, ENKEPHALIN AND SUBSTANCE P

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Summary—Injection of protein-A purified antibodies against Met-enkephalin and \( \beta \)-endorphin into the periaqueductal gray matter (PAG) was shown to decrease the analgesic effect of electroacupuncture (EA) in rabbits. Met-enkephalin antibodies were more potent than the \( \beta \)-endorphin antibodies in causing a statistically-significant effect on electroacupuncture analgesia. Antibodies to Met-enkephalin were also active at the spinal level, whereas antibodies against \( \beta \)-endorphin were without effect: this is in agreement with a rich enkephalinergic innervation and absence of \( \beta \)-endorphin-containing fibres in the spinal cord. Substance P, the other neuropeptide of this study, also seems to be important in mediating effects of electroacupuncture. Injection of antibodies into the periaqueductal gray caused decrease of the effect of electroacupuncture whereas intrathecal administration of Fab-fragment substance P antibodies caused a marked potentiation. The demonstration of site specificity of the neuropeptides in mediating analgesia induced by electroacupuncture supports the validity of this experimental approach.

Key words: antibodies, acupuncture, \( \beta \)-endorphin, enkephalin, substance P, rabbits.

Acupuncture is known to cause a number of changes in neurochemical variables in the CNS and its efficacy can be influenced by a wide range of neuroactive agents affecting several CNS transmitter systems (Han and Terenius, 1982). The variety of effects and the interaction between various transmitter systems makes it difficult to define which system is critically involved. Among the strongest candidates as mediators of the effects of acupuncture are the endogenous opioids, the endorphins. They are peptides, derived from three different precursors, each one with its characteristic distribution and each one able to generate several active species of peptide (Hughes, 1983; Smyth, 1983). The structural complexity and regional diversity is paralleled by a multiplicity of opioid receptors. The opiate antagonist naloxone differs in its affinity for various receptor subtypes and is no ideal drug, at least not for differentiation between the different endorphin systems. In this study, therefore, advantage has been taken of the specificity of the antibody–antigen interaction. Antisera with specificity for enkephalin and \( \beta \)-endorphin have been generated, and also for substance P, a neuropeptide which may be the transmitter at the first synapse for nociceptive transmission (Otsuka and Takahashi, 1977), and is also topographically associated with enkephalins at higher levels in the CNS (Hökfelt, Ljungdahl, Steinbusch, Verhofstad, Nilsson, Brodin, Fernow and Goldstein 1978). These antisera were injected intrathecally in order to understand spinal processes, as well as into the periaqueductal gray (PAG), which has been implicated as one of the most important areas of the CNS for endogenous modulation of pain (Fields and Basbaum, 1978).

METHODS

Male rabbits, 2–2.5 kg, were used. Stainless-steel cannulae (0.7 mm o.d.) were implanted bilaterally in the periaqueductal gray (P 9.5, L 1.0, H 10 according to Sawyer's atlas) under pentobarbital anaesthesia. One week later, the animals were prepared for injections through 0.3 mm (o.d.) tubing protruding 2 mm beyond the tip of the outer cannula. Bilateral injections were made slowly, each in 0.25 µl vol at 0.25 µl/min. Experiments were separated by one week and no animal was used for more than 5 experiments. At the end of the series of experiments, the position of the cannulae was verified by histological examination.

Using different rabbits, intrathecal injection was accomplished using an indwelling PE-10 polyethylene catheter, inserted via the foramen magnum and extending to segments L3–4. The free end was cemented to the skull (Yaksh and Rudy, 1976). Two weeks later, the animals were prepared for experiments.
Antibodies in 70 μl saline were infused at a rate of 25 μl/min. It was later found, by dye injection, that the extent of diffusion after injection was within 50 mm cranially and 10 mm caudally from the L3-4 area.

For acupuncture, the animals were placed in a sling which allowed their feet to protrude and be moveable. Electroacupuncture (EA) was induced via two stainless-steel needles inserted into the Zusanli (5 mm lateral to the anterior tubercle of the tibia) and the Sanyinjiao (between the medial meniscus and the Achilles tendon) points, respectively, and kept in position by a piece of tape. Radiant heat was directed against the skin around the nostrils or against the shaved tail; a head jerk or a tail-flick was taken as a nocifensive response. Response latencies, obtained during three sessions, were averaged and used as the basal pain threshold. This threshold was about 5–8 sec. Subsequent response latencies, after acupuncture treatment and injections were expressed as percentage changes from basal levels. An increase of 200% was used as the cut-off. If not stated otherwise the head-jerk response was used. For details see Han, Zhou and Xuan (1983).

Electroacupuncture stimulation (0.3 msec pulse duration at 2 to 15 Hz for 10 min) was given 10 min after the injection of antibodies. The change in pain threshold was then measured every 10 min for 50 min. All peptides were obtained from Peninsula (San Carlos, California, U.S.A.). The antisera were raised in rabbits using 10 mg of peptide conjugated to thyroglobulin, 50 mg by carbodiimide coupling. The conjugate was dissolved in 500 μl of physiological saline and emulsified in an equal volume of Freund’s complete adjuvant. One hundred micrograms of the immunogen was injected intradermally into 30–40 sites on the back. To obtain antisera of a reasonable titre 3 or more booster doses were given. Purification of IgG was accomplished by ammonium sulphate precipitation followed by affinity separation on a protein-A-Sepharose CL-4B (Pharmacia) column. The IgG fraction was eluted by a 0.2 M glycine- HCl buffer of pH 2.6. The eluate was dialyzed against physiological saline. The IgG from non-immunized animals, purified as above, served as control. The purified antibody to human β-endorphin was tested for its capacity to recognize β-endorphin extracted from the rabbit pituitary (Merin, Höllt, Przewlocki and Herz, 1980). The antiserum was found to recognize material extracted from a single rabbit pituitary diluted more than 500,000-fold. The affinity-purified IgG for substance P was also transformed into the Fab fragments by digestion for 4 hr at 37°C with insoluble papain (Sigma) in 0.01 M cysteine, 2 mM EDTA (antibody–enzyme ratio 100:1). After centrifugation at 10,000 rpm for 10 min the supernatant was dialyzed against 0.1 M phosphate buffer of pH 7.4. Purification of Fab fragments was done by a second protein-A chromatography where the Fab-fragments eluted in the unabsorbed peak. Tested for cross-reactivity with substance P fragments, the purified IgG and Fab-fragments cross-reacted 100% with substance P (3–11) and (4–11) fragments; 60% with the (5–11) fragment and 20% to substance P (6–11) fragment and less than 0.1% with the (1–7), (1–10) fragments. There was no cross-reaction with enkephalin, β-endorphin, somatostatin or eleodoisin.

For use in experiments, the antibodies were coded along with control solutions (saline or normal IgG). Experiments were run double-blind and results were analyzed for statistical significance using Student’s t-test (two-tailed) in order to compare the reaction times of rabbits treated with antibodies, with reaction times of rabbits treated with control solutions.

RESULTS

Electroacupuncture, applied for 10 min to each rabbit in a group of 32, caused elevation of the pain threshold. Injection of 4 μl of saline or 4 μl with 15 μg of control IgG into the periaqueductal gray did not cause statistically significant change in electroacupuncture-induced analgesia (not shown). However, bilateral microinjection of antibodies against enkephalin 15 μg (4 μl) decreased the effect of using a working dilution of 1/9000. The Kd in RIA was 4 × 10−10 M against β-endorphin.

β-Endorphin IgG was tested for its capacity to recognize β-endorphin extracted from the rabbit pituitary (Merin, Höllt, Przewlocki and Herz, 1980). The antisera was used in RIA in order to compare the reaction times of rabbits treated with antibodies, with reaction times of rabbits treated with control solutions. For use in experiments, the antibodies were coded along with control solutions (saline or normal IgG). Experiments were run double-blind and results were analyzed for statistical significance using Student’s t-test (two-tailed) in order to compare the reaction times of rabbits treated with antibodies, with reaction times of rabbits treated with control solutions.
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Fig. 2. The effect of intrathecal (T) injection of antibodies against enkephalin on electroacupuncture-induced analgesia (EA) as measured by the tail-flick and the head-jerk responses. **P < 0.01; *** < 0.001 (n = 7).

Fig. 3. The effect of injection of antibodies against SP into the periaqueductal gray (PAG) on analgesia induced by electroacupuncture (EA). *P < 0.05 (n = 12).

Bilateral microinjection of β-endorphin antibodies into the periaqueductal gray, in doses of 5 and 15 μg, blocked the effect of electroacupuncture in a dose-dependent manner (Fig. 3), with 15 μg causing a statistically significant decrease of electroacupuncture-induced analgesia as measured by head jerk response 10 min after the onset of electroacupuncture.

Injection of antibodies against substance P into the periaqueductal gray caused prompt and persistent inhibition of analgesia induced by electroacupuncture (Fig. 4). A similar effect was produced by the Fab-fragments generated from the substance P antibodies (Fig. 5). A dose comparison cannot be made on the basis of this experiment but the Fab-fragment was definitely not more potent than the antibodies. In contrast to the inhibitory effect of injection of the antibodies into the periaqueductal gray, intrathecal injection (30 μg in 70 μl) caused a marked potentiation of the effect of electroacupuncture measured by the tail flick latency (Fig. 6). The analgesic effect as measured by head twitch was not significantly different from that in the control groups.

Neither the intracerebral nor intrathecal injection of the different antibodies significantly altered effects of morphine given 1 hr later indicating that the antibodies used were ineffective in altering morphine-induced analgesia.

DISCUSSION

Involvement of chemical mediators in acupuncture-induced analgesia was proposed by a
Evidence for this suggestion was reported by Nar-
terius (1975), substance P does not bind to opiate
receptors in the brain nor does it inhibit the
proliferation of substance P. It has there-
fore been suggested that substance P produces anal-
gesia at the level of the periaqueductal gray and
spinal cord, whereas $\beta$-endorphin seems to be im-
portant only at the level of the periaqueductal gray
area. This demonstration of site specificity of the
antibodies to $\beta$-endorphin and to Met-enkephalin
supports the validity of the approach. Injection of
substance P antibodies or Fab-fragments into the
periaqueductal gray decreased the effect of elec-
troacupuncture but administration of substance P anti-
bodies or Fab-fragments at the spinal level increased
the analgesic effect. This suggests that the in-
volve ment of substance P is another important factor
with site-specificity. Han et al. (unpublished), have
found that injection of substance P into the peri-
aqueductal gray in rabbits increased the pain thresh-
old, whereas intrathecal injection caused a decrease.
The present results show that injection of antibodies
had the opposite effect. This makes substance P a
possible candidate as a mediator of analgesia pro-
duced by electroacupuncture at the level of the periaqueductal gray. At the spinal level the situation
might be different. Even if enkephalins are released
by electroacupuncture and suppress the release of
substance P (according to the model of Jessel and
Iversen, 1977) there appears to be sufficient release of
substance P, which can be inactivated by the anti-
body. This leads to potentiation of the analgesia
produced by electroacupuncture.

Although this study has demonstrated the poten-
tial use of antibodies as agents to discriminate be-
tween various endorphins and substance P in mediat-
ing responses to electroacupuncture, there are
experimental limitations. The antibodies used are of
polyclonal origin and naturally may have some disad-

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![Figure 6](image_url)

**Figure 6.** The effect of intrathecal injection of Fab-fragment antibodies against substance P on analgesia induced by electroacupuncture as measured by the tail-flick and the head-jerk responses. *P < 0.05 (n = 9).
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vantages, such as cross-reactivity to structurally
related peptides. For instance, although cross-
reaction between $\beta$-endorphin and enkephalin is neg-
ligible, the antibody preparations may react with
related peptides. On the other hand, the antibody
preparations had different effects when injected into
the periaqueductal gray or at the spinal level. This
differential effect and site specificity suggest that the
antibody preparations were indeed interacting with
the respective peptide systems.

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