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Selective Antinociceptive Effect of Excitatory Amino Acid Antagonists in Intact and Acute Spinal Rats

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Selective Antinociceptive Effect of Excitatory Amino Acid Antagonists in Intact and Acute Spinal Rats[1]

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Running Head: EAA Antagonists in Spinal Rats

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While it is now fairly well established that the excitatory amino acids (EAAs), primarily glutamate and aspartate are involved in spinal pain processing, the specific role of the various EAA receptor subtypes in nociception is not well-defined. On the basis of their response to selective agonists, these receptors have been categorized as either NMDA (activated by N-methyl-D-aspartate), or non-NMDA subtypes, which include the AMPA/kainate (activated by alpha-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid and kainate) and metabotropic receptors. The present view is that AMPA/kainate receptors mediate acute, "fast", transmission in the spinal cord, elicited by low threshold, probably monosynaptic afferents. Conversely, NMDA receptor activation is preferentially involved in polysynaptic pathways, which mediate responses to high threshold (presumably nociceptive) inputs (9,10,11,18).

The physiological distinction between the AMPA and NMDA receptors is due to their respective functional characteristics. While AMPA receptors exhibit conventional, ligand-gated conductances, the NMDA receptor has some unique properties. Under normal physiological conditions, the NMDA channel is blocked by Mg++, and the receptor complex is not activated. Only after this blockade is removed by excessive, intense or repetitive depolarizing stimulation are receptor channels opened and conductances generated (9,10,11,18).

This characteristic of the NMDA receptor complex is believed to be responsible for the neurophysiological phenomenon of "wind-up", observed in recordings of dorsal horn neurons which occur in response to repeated electrical stimulation of nociceptive, C-fibre afferents. In this situation, the initial neural response to the first few stimuli suddenly increases dramatically, to a level that

is much greater than the original reaction. As a result, the responses of certain dorsal horn neurons increase substantially, in spite of the fact that the input into the spinal cord remains constant (9).

These sensitized responses of spinal dorsal horn neurons that have undergone "wind-up" are believed to be responsible for hyperalgesic reactions (increased responses to noxious stimulation) that characterize a variety of pain states, which are clinically referred to as neuropathic pain conditions. As a result, the possibility that antagonists of EAA receptors may provide a new class of analgesic agents has generated much interest (9,10,11).

Neurophysiological studies of such drugs generally support this suggestion. Electrical stimulation of myelinated primary afferent (i.e., below C-fibre activation) evoked monosynaptic responses in dorsal horn cells that were selectively blocked by the competitive AMPA/kainate antagonist, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; 19,20) but not an NMDA antagonist (26, see also 41). Conversely, direct, spinal (intrathecal;IT), application of the selective competitive NMDA antagonist, APV (AP5 (±)-2-amino-5-phosphonopentanoic acid) preferentially blocked the facilitated response (wind-up) of dorsal horn neurons, without affecting evoked responses. This was observed after a variety of noxious treatments, including electrical stimulation of the receptive field (13,32), injection of formalin into the hindpaw (17), tail ischemia (38), or prior administration of NMDA (1,37). Facilitation produced by activation of AMPA/kainate receptors was not blocked by APV (1).

Several recent studies have examined both CNQX and AP5 (or the similar drug AP7) in a variety of neurophysiological preparations

(3,14,29,44). They have demonstrated a consistent antagonism of spinally administered CNQX against all types of innocuous and noxious stimulation (and blockade of mono- and polysynaptic responses). The effects of AP5 or AP7, however, varied. In some cases this NMDA antagonist blocked responses to noxious input as well as wind-up (14,29), while in others there was minimal (3) or no effect (44) even on polysynaptic responses.

This pattern of results leads to the prediction that CNQX should reduce behavioral reactions to a variety of stimuli, while AP5 should have a selective action against intense or chronic noxious stimulation. Present evidence supports the latter but not the former hypothesis. Behavioral studies have shown that IT injection of NMDA or AMPA agonists themselves will produce aversive reactions in mice and rats (biting, scratching and squealing) which are selectively blocked by the respective antagonists (2,25,32,33). Intrathecal injections of low doses of AMPA or NMDA potentiate the hyperalgesic response to noxious chemical (7,8), thermal (2,21,24, see also 6), mechanical (23,42) and ischemic (36) stimulation, which is also blocked by the respective antagonists. Non-"potentiated" hyperalgesic reactions are also reduced by these agents (27,34). In these studies CNQX and AP5 alone had minimal, if any analgesic effect, unless given at doses that produced motor impairment (for AP5, 5,31,32,33). Long-term, chronic pain syndromes, produced by ligation or section of peripheral nerve are also reduced by either pre- or posttreatment with AP5 or CNQX, in the range of 1 to 30 μg (22,35,43). In recent reports, not only did AP7 and CNQX block the spinal release of EAAs in an animal model of arthritis (39) but CNQX showed a dramatic effect against both the hyperalgesia and the inflammation (40).

The fact that CNQX alleviates the potentiated pain reaction in these hyperalgesic conditions, without affecting the acute pain response of the uninjured limb may be inconsistent with the expectation from neurophysiological paradigms, which indicated that CNQX blocked neural reactions to innocuous as well as a variety of noxious stimuli. At present, we are aware of only one study that found an analgesic effect of CNQX on the acute thermal tail flick and hot plate tests, as well as the tonic, nociceptive formalin test (28). Furthermore, the presumed selective effect of AP5 against hyperalgesic input in neurophysiological paradigms is inconsistent with some behavioral studies which show that AP5 has analgesic effects even in the acute tail flick test (5,27,31).

With one exception, these drugs have only been assessed in peripheral models of chronic injury. One early report however (31) suggested that AP5 was also effective against hyperalgesia following central damage, produced by spinal transection. AP5 increased TF latencies of intact rats and this effect was markedly potentiated after spinalization, although those data were not shown. Yet, this result is similar to the effect of the non-competitive NMDA antagonist, ketamine, which does not alter the TF reflex in intact rats, but produces an antinociceptive increase after spinal transection (16,30).

The present study, therefore, specifically examined the effect of both CNQX and AP5 administered intrathecally to Intact and acute Spinal rats. The results show that CNQX is analgesic in Intact rats and that this effect is significantly potentiated after spinal transection. In contrast, AP5 did not significantly affect the TF response of Intact rats, but substantially inhibited this reflex in the Spinal condition.

METHODS AND PROCEDURE

Subjects

A total of 61 male albino Holtzman rats (Harlan Sprague-Dawley Laboratories, Madison, WI), weighing 300-500 g were used as subjects. All rats were individually housed in suspended wire mesh cages in a colony room maintained on a 12 L: 12 D cycle, with access to food and water throughout the experiments.

Surgical Procedure

Intrathecal catherization

Animals were anesthetized with a mixture of isoflourane (AErrane, Anaquest, Madison, WI) and oxygen and placed in a stereotaxic frame. An incision was made behind the ears and the neck muscles were scraped to expose the back of the skull. An incision of the atlanto-occipital membrane allowed insertion of an 8 cm long catheter of PE-10 polyethylene tubing filled with sterile saline into the spinal subarachnoid space. Prior to insertion, a loose knot was tied in the catheter and coated with dental cement so that it could be held in place against the skull with adhesive (Superglue; Bel-Art Products, Pequannock, New Jersey). The incision was closed and the exposed tip of the catheter was heat sealed. Any rat showing a post-operative neurological deficit (i.e., a crippled limb) was eliminated from the study.

Spinal Transection

In addition to the catheter implantation, several groups of rats (N=28) also sustained a spinal transection. The skin incision was extended and after retraction of the paraspinal muscles, a

laminectomy was performed between thoracic vertebrae 6 and 9. A 1 to 2 mm portion of the spinal cord was crushed and severed, leaving the catheter intact. The space left in the spinal cord was replaced with gelfoam to reduce bleeding, after which the incision was closed in layers and the cages placed under a heat lamp to maintain body temperature. On the morning after surgery, the hindquarters of each rat were washed with soap and warm water and their urine expressed manually by the application of pressure to their bladders. All experiments with Spinal animals were completed 24 h after surgery, whereas experiments with Intact animals were completed five days after surgery.

Behavioral Tests

Tail Flick

The tail flick (TF) was used for nociceptive assessment (IITC Life Sciences, Woodland Hills, CA). Noxious stimulation was provided by a beam of high-intensity light focused on the tail. The response time was measured automatically and was defined as the interval between the onset of the thermal stimulus and the abrupt flick of the tail. Each determination consisted of 3-5 trials; the mean score was taken as the response latency. Animals not responding within a 14 s limit were removed from the apparatus to prevent tissue damage, and assigned a score of 14 s.

Drug Administration and Procedure

For intrathecal (IT) injections, the tip of the catheter was cut, a 30 gauge needle was inserted into the catheter and 10 μl of the drug solution was infused followed by a 10 μl wash of the saline vehicle. Injections were performed manually with a 50 μl

Hamilton syringe (Hamilton Co., Reno, NV) over a 2-3 min period, after which the catheters were again heat sealed.

The AMPA antagonist, CNQX (Research Biochemicals International, Natick, MA) was dissolved in a solution of 50-60% DMSO (dimethylsulfoxide)/50-40% saline. Individual groups of Intact and Spinal rats were injected with either this vehicle or a dose of 10 μg (43 nM) or 20 μg (86 nM) CNQX, dissolved in the vehicle. AP5 was administered to Intact and Spinal rats in a saline vehicle at a dose of either 5 (25.4 nM), 10 (51 nM), or 30 μg (152 nM; given to Intact rats only). On the day of the experiment all rats were pre-tested on the tail flick apparatus for baseline assessment. Animals were then tested at 15, 30, and 60 min after injection. Any rat with a score of 14 s at the 60 min test was retested for recovery of the TF reflex to determine whether loss of the reflex was permanent. In the event that recovery did not occur autopsies were performed the next day to determine whether the catheter was inadvertently placed within the spinal cord. Each animal was used only once, and contributed a single pre and post-drug data set.

Statistical Analyses

The effects of the drugs were assessed by Student's t-test and one-way or two-way Analyses of Variance (ANOVAs) performed with the aid of a computer program (Sigma-Stat, Jandel, San Rafael, CA), followed by post-hoc, Neuman-Kuels and Dunnett's tests. Analyses were performed on the Area Under the Curve (AUC). This value was obtained with the aid of a computer program (PHARM/PCS). For each animal the AUC was determined by entering each xy data pair, in which x = the difference in TF latency between the pretest score

and the latency obtained at each time point and y = 15, 30, and 60 min. The computer program calculated the total area (i.e., the integral) based on an approximation using the trapezoidal rule. Statistical tests were performed on the AUC values that comprised each experimental group. Results were considered significant at $p < 0.05$.

RESULTS

The left side of Figure 1 summarizes the results obtained with CNQX. The mean AUC scores are plotted as a function of dose for each of the two experimental conditions, Intact (open circles) and Spinal (filled circles). A one-way ANOVA performed on the AUC scores of the Intact rats showed that there was an overall effect of dose ($n = 18$; $F(2,17) = 14.1$, $p < 0.001$). Post-hoc tests indicated that the effect of the 20 μg dose of CNQX was significantly greater than that of either the vehicle or the 10 μg dose ($p < 0.05$). A one-way ANOVA performed on the AUC scores of the Spinal rats showed that there was an overall effect of dose ($n = 18$; $F(2,17) = 13.5$, $p < 0.001$). Post-hoc tests indicated that the effect of the 20 μg dose of CNQX was significantly greater than that of the vehicle or the 10 μg dose, and that the effect of the 10 μg dose was significantly greater than the vehicle ($p < 0.05$).

A two-way ANOVA performed on the AUC scores of Intact and Spinal rats that were injected with the two doses of CNQX, indicated that there was a significant effect of spinal transection ($n = 23$; $F(1,22) = 8.2$, $p = 0.01$) and of dose ($F(1,22) = 10.9$, $p = 0.004$), with no interaction. Post-hoc comparisons, did not support a significant difference between Intact and Spinal

rats at either of the two doses separately. However, there was no overlap of AUC values between these two conditions at a dose of 20 μg. A nonparametric Mann-Whitney-U test performed on those data showed in fact that the groups differed significantly ($U(5,5) = 0$; $p < 0.008$).

The right side of Figure 1 summarizes the results obtained with the competitive NMDA antagonist AP5. The mean AUC scores are plotted as a function of dose for each of the experimental conditions. A one-way ANOVA performed on the AUC scores of the Intact rats showed that there was no difference among the three doses of 5, 10, and 30 μg ($n = 15$; $F(2,14) = 2.1$, NS). A t-test on the data of the two Spinal groups showed that there was no difference between the two doses of 5 and 10 μg ($t(8) = 0.7$, NS).

A two-way ANOVA on the scores of the Intact and Spinal rats that received the two common doses (5 and 10 μg) showed a significant effect of spinal transection ($n = 20$; $F(1,19) = 29.4$, $p < 0.001$) but not of dose ($F(1,19) = 2.2$, NS), with no interaction. Post-hoc comparisons showed that the scores of Spinal rats were significantly greater than those of Intact rats at each of the two doses ($p < 0.05$ in each case).

In this study there was no saline control included with the drug treatments. Therefore, in order to further evaluate the effect of AP5 in Intact rats, a repeated measures one-way ANOVA was performed on the TF latencies obtained across all time points (pre-test, 15, 30, and 60 min). The results showed no significant effect of either 5 or 10 μg at any time point in this condition.

However, an additional effect was noted after intrathecal injection of AP5 in Spinal rats. The response of 3 of the 5 rats that received 5 μg was still 14 s at 60 min post injection. One of

these animals did not recover the reflex by the next day. The response of 5 of the 6 rats that received 10 μg was also 14 s at the final 60 min test and 4 did not recover the reflex by the next day. In each case, the autopsies showed that the catheter was correctly placed outside of the spinal cord. In contrast, every rat injected with CNQX that reached the 14 s cutoff recovered the TF reflex during the day of the test.

DISCUSSION

The development of selective agents for the nonNMDA and NMDA types of EAA receptors made it possible to examine their respective roles in spinal pain processing. Neurophysiological evidence indicated that nonNMDA, particularly AMPA receptors, mediated fast excitatory transmission involving both innocuous and acute nociceptive input, while NMDA receptors were implicated specifically in nociceptive reactions, particularly those induced by intense, prolonged stimulation, sufficient to produce the hyperalgesic state underlying neuropathic pain. This dichotomy suggested that antagonism of AMPA receptors might produce a nonspecific blockade of sensory input, which would have limited therapeutic benefit (10). Antagonism of NMDA receptors, however, appeared to provide a novel mechanism for the development of analgesic drugs. The application of these agents in behavioral paradigms has not entirely supported this distinction. In particular, the AMPA antagonist CNQX has been shown to reduce behavioral indices of hyperalgesia without affecting the normal response to noxious stimulation. We are aware of only one study which has reported an analgesic effect of CNQX alone on non-hyperalgesic, acute, (TF and hot plate) behavioral pain assays

(28).

Results of AP5 in these same procedures provide support for the efficacy of NMDA antagonism in chronic, nociceptive conditions. Although AP5 has also been shown to have analgesic effects in some acute reflex assays, these are often transient (occuring within 5 min; 27,32) or require doses which are near threshold for motor impairment (250 nM; 5).

The present study assessed the effect of intrathecal administration of CNQX and AP5 on the acute, thermally elicited TF withdrawal reflex. In previous investigations a hyperalgesic reaction of this response (and of the comparable hindlimb response) was induced by a variety of peripheral treatments, such as injection of noxious agents into the hindlimb or on the spinal cord. However, in the present study a hyperalgesic facilitation of the TF was produced by central injury, i.e. spinal transection. Spinalization produces several characteristics of a hyperalgesic condition, namely, behavioral facilitation (decrease in latency) to constant nociceptive input (16); an increase in excitation of dorsal horn neurons (15) and a selective antinociceptive effect of the noncompetitive NMDA antagonist, ketamine (16, 30). Spinalization also potentiates the reflex scratching response elicited by IT L-glutamic acid (4).

The involvement of EAAs in these phenomena is further supported by the observation that excitation of dorsal horn convergent neurons, produced by IT NMDA, is reduced by the application of diffuse noxious inhibitory controls (i.e., noxious pinch of areas outside the neuron's receptive field; 37).

The results of this study are generally consistent with those obtained in paradigms which involved peripheral injury. Intrathecal

administration of CNQX significantly increased TF latencies in Intact rats at the higher, 20 μg dose, and the antinociceptive effect was potentiated after spinal transection. As predicted, intrathecal administration of AP5 did not significantly alter the latencies of Intact rats but produced a substantial antinociceptive effect after spinalization. This is consistent with previous reports showing a selective effect of AP5 after peripheral injury, and with the observation that the noncompetitive antagonist, ketamine, also has preferential effects in the spinal animal. This implies that ketamine-induced antinociception in this preparation is not due to its anesthetic property.

However, in contrast to both CNQX and ketamine, intrathecal injection of AP5 produced an apparent toxic reaction, in that the TF reflex did not recover in some of the spinal rats. This apparently permanent neurological deficit was most likely produced by the drug because 1) autopsies indicated that the catheter was correctly sited outside the spinal cord 2) more rats were affected by the higher dose than the lower dose 3) only AP5 produced this reaction and 4) only Spinal rats were affected. The fact that this toxic reaction was not previously noted in procedures involving peripheral injury, suggests that it is a consequence of the transection. We do not presently have an explanation for this phenomenon. However, if it is a specific effect of AP5 it might be blocked by pretreatment with the agonist, NMDA. The possibility of such a serious side effect suggests caution in regard to possible clinical applications of these drugs.

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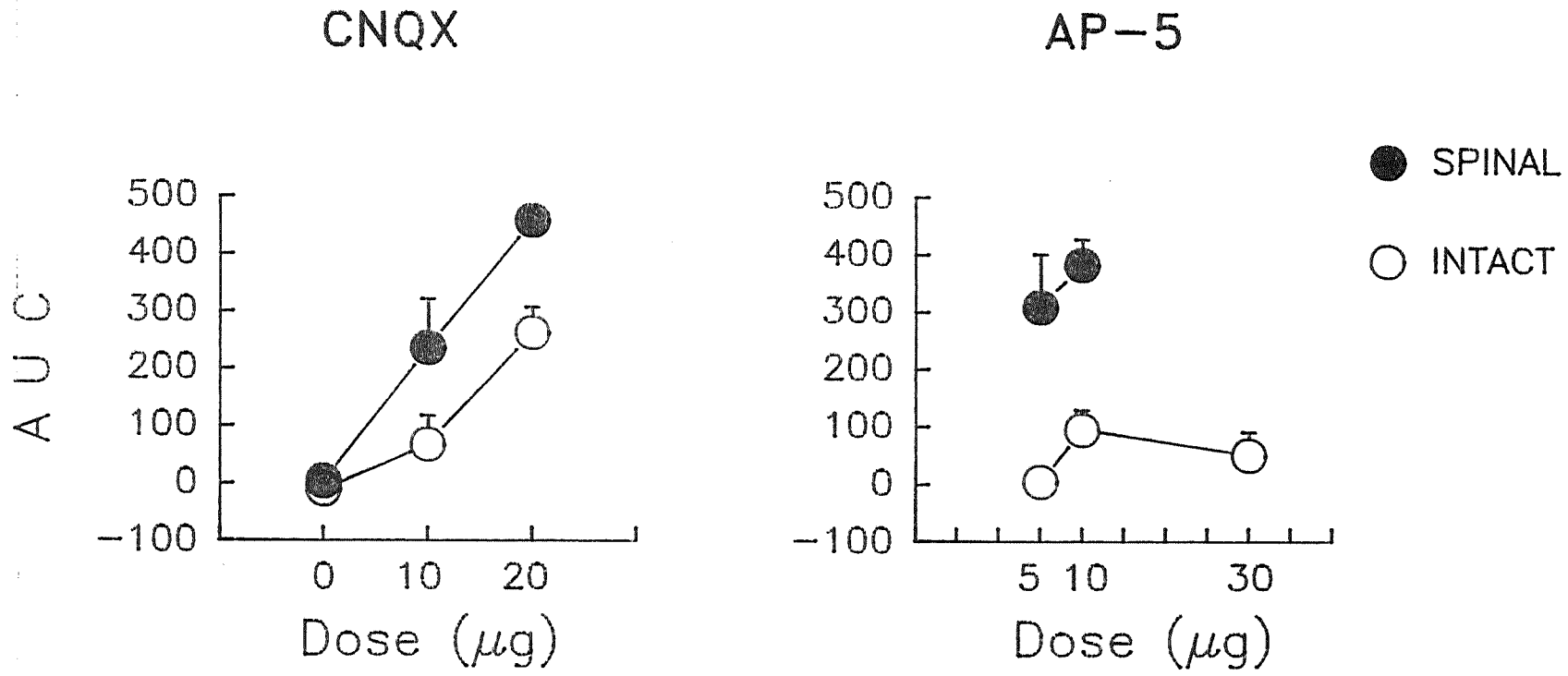
FIGURE 1

Dose response effect of intrathecally administered CNQX and AP5 on the thermally elicited tail flick reflex of Intact (open circles) and Spinal (filled circles) rats. Each symbol represents the mean ± SEM AUC (Area Under the Time Effect Curve) for each of the indicated doses, obtained from separate groups of rats (N=5-7), that were assessed prior to and 15, 30, and 60 min after the respective injections.

DOSE RESPONSE



General Discussion of The Selective Anti-Nociceptive Effect of the NMDA Antagonist, AP5 In Acute Spinal Rats

Honors Thesis

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Introduction: Chronic Pathological Pain

Although many painful conditions can be successfully treated with opiate analgesics, there are a significant number of pain syndromes which are not relieved by these drugs. In particular, the opiates do not help in many cases of long-term chronic pain. Such chronic pain syndromes are usually the result of direct damage to the peripheral nervous system (e.g., after limb amputation), damage to the cardiovascular system (e.g., ischemia), or long-term illness (e.g., arthritis). These chronic pain conditions often produce a syndrome which is characterized by, 1.) a lower threshold for pain (increased nociception), 2.) hyperalgesia, an increased sensitivity to noxious (painful) stimuli beyond the area of the initial injury, and 3.) allodynia, which is pain that is produced by previously innocuous (non painful) stimuli. Research in the field of pain has recently produced a number of promising results that may offer a physiological explanation for these symptoms and the possibility of effective treatment.

Role of Excitatory Amino Acids in Chronic Pain

Recently, a newly discovered transmitter system, the Excitatory Amino Acids (EAAs) has been linked to the production of pain sensation. The EAA transmitter system includes the substances glutamate, aspartate, and perhaps other amino acids. Research has produced three pieces of evidence to support this role of the EAA's in pain processes.

First, in laboratory experiments, when pain is produced

in animals there is a measurable increase in the levels of the EAA's in the spinal cord. Second, when EAA's are directly applied to the pain transmission neurons in the spinal cord, the neurons are excited, responding as if they were being activated by pain signals from the body. Both of these responses, as well as neural responses to painful stimulation are blocked by EAA antagonists. Last, if you inject an EAA on the spinal cord of an awake animal pain activity is increased. That is, at high doses rats and mice will scratch and bite the injection site, and vocalize in a way that indicates the drug was unpleasant.

Excitatory Amino Acid Antagonists

The EAA system has two primary receptor types which are designated as 1.) NMDA, which is activated by N-methyl-D-aspartate and 2.) non-NMDA type which includes the AMPA receptor, which is activated by alpha-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid. These receptor names are derived from the fact that synthetic analogues to glutamate (which is an EAA), such as NMDA and AMPA will produce the same effect as glutamate at these receptors. AMPA receptors are viewed to mediate "fast", acute pain such as a pin prick or a heat stimulus. NMDA receptors are viewed to mediate chronic pain states.

The AMPA receptor is a regular ligand-gated receptor and is excited by the EAA's. However, the NMDA receptor is different. NMDA's channel is normally blocked by Mg++, and only after intensive, repetitive depolarization is it activated.

In other words there has to be a constant release of EAA's to depolarize NMDA.

This characteristic block of the NMDA channel by Mg++ is believed to be responsible for the phenomenon of wind-up. Wind-up is a state where neurons are firing faster although the input stays the same (pain input is constant but, the neurons fire faster). This phenomenon of wind-up is believed to cause chronic pain syndrome symptoms, such as hyperalgesia.

We believe that the EAA antagonists may work in this transmitter system to produce a new class of analgesics. The two EAA antagonists that are used in the current study are 1.) CNQX (6-cyano-7-nitroquinoxaline-2,3 dione) which is a competitive AMPA antagonist and 2.) AP5 (±-2-amino-5-phosphonopentanoic acid), which is an competitive NMDA antagonist.

A number of studies have used AP5 and CNQX as well as a number of other EAA antagonists to study pain states. These studies have looked at peripherally produced chronic pain by doing things such as causing arthritis in a joint. What is interesting about the current study is that the chronic pain model used is produced with central damage (transection of the spinal cord). The spinalization of the rat causes the same chronic pain symptoms as peripheral chronic pain states.

Procedure

Male, albino Holtzman rats were implanted with an intrathecal catheter under isoflurane anesthesia. The intrathecal catheter ensures that the drug is injected directly

onto the spinal cord. Approximately one-half of these rats also sustained a spinal transection. Intact rats were tested five days after surgery and spinal rats were tested twenty-four hours after surgery. The behavioral measure of pain which was used is the tail-flick reflex. A beam of high intensity light is focused on the tail. The time elapsed until the animal removes or withdraws its tail is automatically recorded in seconds. The mean of three tests will represent a baseline value. After this baseline measure is obtained all rats will be injected intrathecally with their respective drug. All animals will be tested again at 15, 30, and 60 minutes.

For drug administration the tip of the catheter was cut and a 30 gauge needle was used to insert 10 microliters of drug and 10 microliters of a saline wash. The two doses of CNQX which were used were 10 and 20 Mg. All rats received both doses, The three doses of AP5 were 5, 10, and 30 Mg (30 Mg was given to Intact rats only).

Results

The effects of the drugs were assessed with Student's t-test, one-way and two-way ANOVA's and post-hoc Neuman-Kuels and Dunnets Tests. Analyses were performed on the Area Under the Curve (AUC). The AUC is determined by entering each x,y data pair where, x= the difference between pre-test tail-flick latency and the tail-flick at each of the time points, and y= each of the time points (15, 30, and 60 min.)

The results obtained showed in general that CNQX, which is the AMPA competitive antagonist, significantly increased

tail-flick latency in both Intact and Spinal rats. This means that CNQX helped both "fast" or acute and chronic pain. However, AP5, which is the NMDA competitive antagonist, was selective in only increasing the tail-flick latency in Spinal rats. These findings are supportive of the current neurological findings of antagonists of the NMDA and AMPA receptors.

DOSE RESPONSE

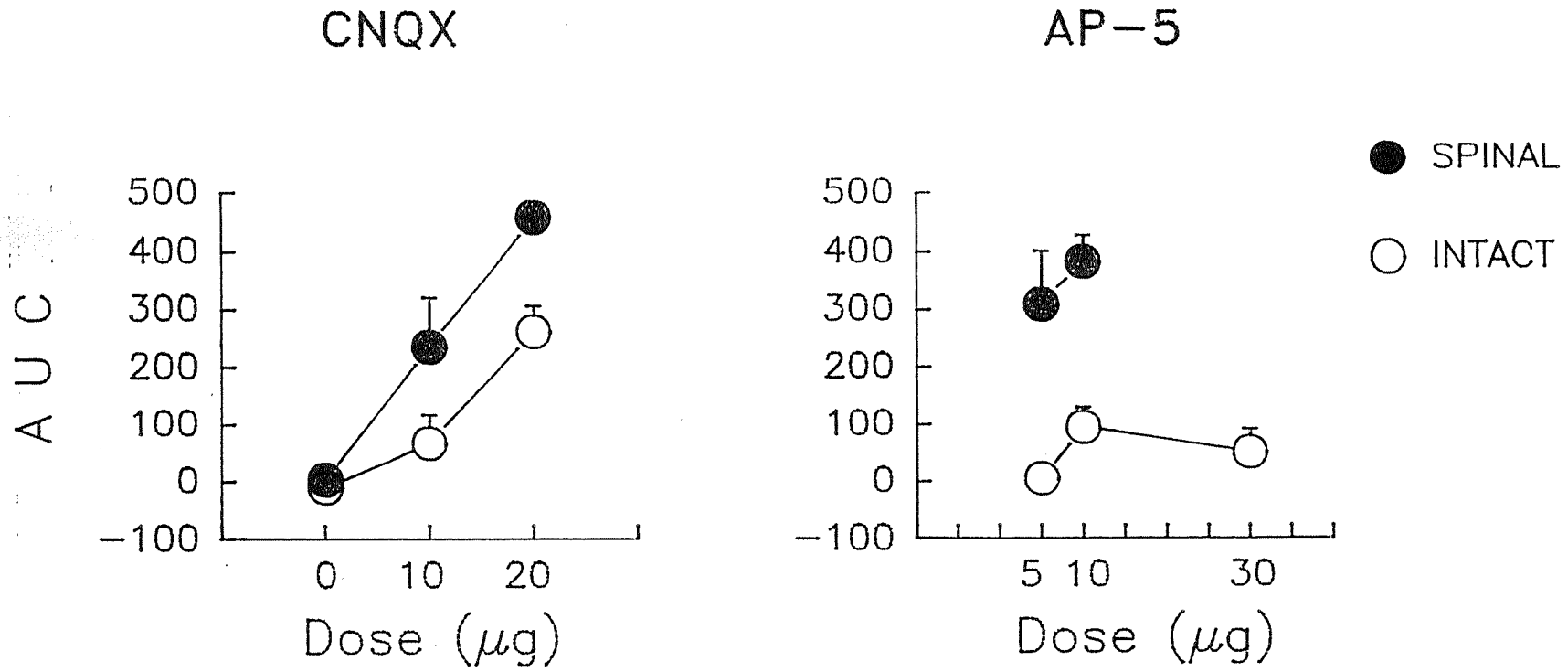

FIGURE 1

Dose response effect of intrathecally administered CNQX and AP5 on the thermally elicited tail flick reflex of Intact (open circles) and Spinal (filled circles) rats. Each symbol represents the mean ± SEM AUC (Area Under the Time Effect Curve) for each of the indicated doses, obtained from separate groups of rats (N=5-7), that were assessed prior to and 15, 30, and 60 min after the respective injections.