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Antinociceptive effects of the marine snail peptides conantokin-G and conotoxin MVIIA alone and in combination in rat models of pain

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Abstract

There are a number of neurologically active ion channel blocking peptides derived from cone snail venom, such as conantokin-G and ω -conotoxin MVIIA. Conantokin-G inhibits NMDA receptors containing the NR2B subunit whereas ω -conotoxin MVIIA blocks N-type Ca^{2+} channels. Separately, these peptides induce antinociceptive effects in pre-clinical pain models following intrathecal injection. In the current study, the efficacies of these peptides were determined separately and in combination by intrathecal injection into rats with a spinal nerve ligation, in rats with a spinal cord compression injury and in the formalin test. Separately, both conantokin-G and ω -conotoxin MVIIA dose-dependently attenuated nociceptive responses in all of these models. However, at high antinociceptive doses for both formalin and nerve injury models, ω -conotoxin MVIIA evoked untoward side-effects. Using isobolographic analysis, the combination of sub-antinociceptive doses of peptides demonstrated additive antinociception in rats with a nerve ligation and in the formalin test, without apparent adverse side-effects. In a model of neuropathic spinal cord injury pain, which is clinically difficult to treat, the combination of conantokin-G and ω -conotoxin MVIIA resulted in robust synergistic antinociception. These data suggest that a combination of these peptides may be analgesic across diverse clinical pains with limited untoward side effects, and particularly potent for reducing spinal cord injury pain.

1. Introduction

A number of voltage-gated and ligand-gated ion channels have been implicated in both normal pain processing and pathological pain (Millan, 1999). In the normal, uninjured state, at the level of the spinal dorsal horn, cation influx through voltage-gated ion channels accompanies depolarization at the primary afferent nociceptor central terminal, followed by neurotransmitter release. The released excitatory neurotransmitters, in turn, depolarize post-synaptic dorsal horn interneurons and neurons that project to the brain, sending the nociceptive “signal” supraspinally. Following tissue injury, the functioning of dorsal horn ligand-gated as well as voltage-gated ion channels is altered such that neurons exhibit persistent spontaneous firing or activation to normally non-noxious stimuli. The abnormal activity of primary afferents and dorsal horn neurons are in part responsible for the pain and cutaneous hypersensitivity that accompanies post-injury recovery and persists long after healing. Thus, one possible method to treat pathological pain is to block cation channels at the level of the dorsal horn.

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There are several peptide venoms that have been isolated from *Conus* marine snails that have significant biological effects. These peptides have been extensively characterized in *in vitro* assays and have been found to have selectivity to particular ligand- and voltage-gated ion channels (Olivera and Teichert, 2007). For example, intrathecal (i.t.) injection of ω -conotoxin MVIIA (MVIIA; ziconotide) potently blocks neuronal N-type voltage-gated Ca^{2+} channels ($\text{Ca}_v 2.2$) and is antinociceptive in a number of preclinical pain models (Chaplan et al., 1994b; Malmberg and Yaksh, 1994). Ziconotide (Prialt®) has been recently approved by the FDA for use in severe pain. However, the therapeutic index of intrathecal MVIIA is narrow and the severity of adverse side-effects appears dose-dependent (Horvath et al., 2002; Scott et al., 2002; Wermeling et al., 2003).

The N-methyl-D-aspartate (NMDA) receptor is a ligand-gated ion channel complex that is sensitive to block by conantokins which are also found in *Conus* venom (Layer et al., 2004). Activation of spinal dorsal horn NMDA receptors following tissue injury appears to be a key mechanism in tissue injury-associated pain and hypersensitivity (Millan, 1999). There are several binding sites that modulate NMDA receptor function that are potential targets for analgesic drugs, such as the glutamate binding site and the PCP binding site within the channel itself. Antagonists that bind within the channel and block ion influx are analgesic in neuropathic pain (Kristensen et al., 1992; Kvarnstrom et al., 2004; Max et al., 1995). However, psychomimetic side-effects do appear even after intrathecal injection. Antagonists that bind to a specific subunit of the NMDA receptor, such as the NR2B subunit, appear to decrease pathological pain but not normal pain perception and do not have the adverse side-effects associated with other subunit non-selective NMDA receptor antagonists (Boyce et al., 1999; Taniguchi et al., 1997). *In vitro*, conantokin-G (conG), isolated from *C. geographus*, potently inhibits NMDA receptor function, particularly to receptors containing the NR2B subunit (Layer et al., 2004). In mice, intrathecal injection of conG significantly suppresses the tonic phase (late phase) in the formalin test and neuropathic and inflammation-evoked hind paw hypersensitivity (Malmberg et al., 2003). The antinociceptive doses were well below that which caused motor impairment as measured in the rotarod test.

The current study evaluated the effects of conG and MVIIA in rat models of chronic pain. In particular, pain following spinal cord injury (SCI) is a notoriously difficult therapeutic indication. Since there are numerous mechanisms maintaining the chronic pain state, a combination pharmacological approach may lead to greater pain relief than a drug given alone and result in fewer side effects if lower doses can be used. Thus, the current study also evaluated the efficacy of sub-antinociceptive doses of the peptides combined.

2. Methods

Male Sprague-Dawley rats (Harlan, IN) were used for these experiments. Upon arrival at the vivarium, rats were 100–125 g, were housed two per cage and allowed free access to food and water. Procedures were reviewed and approved by the University of Miami Animal Care and Use Committee and followed recommendations of the “Guide for the Care and Use of Laboratory Animals”. At the end of the studies, rats were euthanized with CO_2 .

2.1 Surgical procedures

2.11 Spinal nerve ligation—Ligation of the left L₅ and L₆ spinal nerves was performed as described elsewhere (Kim and Chung, 1992). Rats were anesthetized in isoflurane in O_2 , and the back of the rat was shaved. Using aseptic technique, the transverse process of the L₆ vertebrae was identified and removed. The L₅ and L₆ spinal nerves were tightly ligated with 4-0 silk. The musculature was sutured shut and the skin incision closed with wound clips. One week following spinal nerve ligation surgery, rats underwent intrathecal catheter surgery.

2.12 Spinal cord injury—Rats were anesthetized with isoflurane in O₂ (Hama and Sagen, 2007). Their backs were shaved and, using aseptic surgical technique, a laminectomy was performed to expose spinal segment T₆-T₇. A micro-vascular clip (Harvard Apparatus, MA) was placed vertically on the exposed thoracic spinal cord and then left in place for 60 sec. Care was taken not to cut the dura or disturb near-by spinal nerve roots. Following spinal compression, the clip was removed, the muscles sutured shut and the skin closed with wound clips. Three weeks following compression surgery, rats underwent intrathecal surgery.

2.13 Intrathecal catheters—Rats were anesthetized with isoflurane in O₂, the back of the rat's head was shaved and the head was secured in a stereotaxic unit. Rats were maintained on isoflurane in O₂ via a nose cone. Using aseptic surgical technique, the atlantooccipital membrane was exposed and cut (Yaksh and Rudy, 1976). An intrathecal catheter (8 cm; ReCathCo, PA) was threaded down the intrathecal space and secured to the neck muscles with sutures. The skin incision was closed with cyanoacrylate and the externalized catheter was melted shut. Rats were allowed to recover at least three days following intrathecal surgery prior to use in experiments. At the end of testing, prior to euthanasia, 10 µl of 1.5% lidocaine was intrathecally injected to assess the location of the catheter tip. A bilateral flaccid paralysis of the hind limbs indicated that the catheter tip was in the correct spinal position.

2.2 Testing procedures

2.21 Tactile sensitivity—Hind paw responsiveness to innocuous tactile stimulation were measured with von Frey filaments. Rats were placed on an elevated wire mesh surface and enclosed in Plexiglas. Using the up-down method, filaments were pressed on the plantar hind paw until they slightly bent (Chaplan et al., 1994a). The response series determined the withdrawal threshold (g). In uninjured rats, the highest filament, 15 g, did not evoke a response. Thresholds were measured prior to i.t. injection and 30, 60, 90, 120 post-injection.

2.22 Formalin test—Uninjured rats were given i.t. catheters and allowed at least three days of recovery. Either the peptides or the vehicle was intrathecally injected in a volume of 5 µl, followed by a 5 µl flush with saline. Ten min. after injection, 50 µl of 5% formalin was subcutaneously injected into the plantar left hind paw and rats were immediately placed in a clear Plexiglas chamber (Wheeler-Aceto and Cowan, 1991). The number of hind paw flinches and licking occurring in one min were counted in five min intervals up to 60 min post-formalin injection. Phase 1 was defined as the first min following formalin injection (0–1 min) and phase 2 was defined as 15–61 min post-formalin injection.

2.23 Rotarod test—The effect of the peptides alone and in combination on coordinated motor function was assessed with an accelerating rotarod apparatus (Columbus Instruments, OH; (Hama et al., 2003)). A group of uninjured rats (n = 12) with i.t. catheters were trained once a day for two days on the apparatus prior to testing. The rotarod accelerated 5–25 rpm over 60 sec. Rats were tested prior to and 30, 60, 90, 120 min following i.t. injection. At each time point, the latency to fall (sec) off the rotarod was recorded. After a two day wash-out period, rats were reused. Following the second test session, placement of the i.t. catheter was confirmed and the rats were euthanized.

2.3 Peptides

Peptides were obtained from Bachem, Inc. (CA) and dissolved in saline (vehicle). The peptides, both separately and in combination, were injected in a volume of 5 µl, followed by a 5 µl vehicle flush.

2.4 Statistical analysis

To plot the dose-response curves, the behavioral effects of the peptides were converted to percent effect.

The withdrawal thresholds were converted to percent maximum possible effect:

$$\text{MPE \%} = (\text{Drug threshold} - \text{Baseline threshold}) / (15 \text{ g} - \text{Baseline threshold}) * 100$$

The formalin-evoked pain-related behaviors of peptide-treated were converted to a percent of vehicle:

$$(\text{Number of Vehicle behaviors} - \text{Number of Drug behaviors}) / \text{Number of vehicle behaviors} * 100$$

The A_{50} (50% antinociceptive dose) of the peptides alone were calculated from the dose-response curves using a computer program (Tallarida and Murray, 1981). In testing the combination, multiple doses were used, based on the fixed ratio of the A_{50} values of the peptides. Although the A_{50} of the peptides alone differed according to the test, the same dose-ratio was used for each test in order to minimize the amount of MVIIA used and to be able to make comparisons across the tests. It is possible that other ratios for each test may lead to super-additive effects (Tallarida and Raffa, 1996).

To assess whether the effect of a peptide combination was greater than that of either peptide alone an isobologram was constructed on a Cartesian plane. The A_{50} of each peptide was plotted on the horizontal and vertical axis. The line connecting the two A_{50} was the line of additivity, the theoretical effect locus of the combination "dose pairs". A dose-response curve of the combination was constructed and the A_{50} was calculated. The combination was merely additive if the experimentally determined A_{50} fell on the line of additivity (or within its S.E.M.). An A_{50} that was below the line of additivity indicated that lesser amounts were needed to obtain the effect whereas an A_{50} that was above the line of additivity indicated that greater amounts were needed (Tallarida, 2001). Thus, if the A_{50} of the combination was less than the theoretical additive A_{50} , the combination was superadditive (synergistic) but if the A_{50} was greater, then the combination was sub-additive. The theoretical and experimentally determined A_{50} were compared using Student's *t*-test to determine statistical significance ($P < 0.05$). Details of the statistical analysis of drug synergism have been elaborated elsewhere (Tallarida et al., 1989).

3. Results

3.1 General behavioral observations

Following i.t. injection of 300 and 1000 pmol MVIIA, movement-evoked tremors and tail writhing were observed for the duration of the observation period (Malmberg and Yaksh, 1994). Visible adverse side-effects were not apparent at lower doses of MVIIA. Although performance on the rotarod test was diminished (see 3.5 *Rotarod test*), no visible adverse side-effects were observed with conG at any of the doses tested.

3.2 Formalin test

Increasing doses of both MVIIA and conG resulted in significantly decreased formalin-evoked pain related behaviors in both phases (Table 1 Fig. 1 A,B). Although there were no significant differences in potencies of the peptides between phases, ConG was more potent than MVIIA in both phase 1 and phase 2 ($P < 0.05$). A high dose of MVIIA (1000 pmol) was needed to

produce substantial antinociception in the formalin test (both phases), but this dose resulted in tremors and tail writhing.

The theoretical additive A_{50} values for phase 1 and phase 2 were 116 pmol and 138 pmol, respectively. The A_{50} values of the combination (phase 1: 70 pmol, phase 2: 99 pmol) were not significantly different from the theoretical additive A_{50} . Therefore, the combination was additive (Fig. 1C, Fig. 4). The highest doses of the combination used (62.5 pmol conG and 25 pmol MVIIA), which produced robust antinociception in both phases, did not result in either tremors or tail writhing.

3.3 Spinal nerve ligation

In a peripheral neuropathic pain model, increasing doses of both MVIIA and conG resulted in significantly increased withdrawal thresholds (Table 1, Fig. 2 A, B). Since the peak antinociceptive effects were observed at 60 min post-injection, the A_{50} s were calculated at that time point. The potencies of conG and MVIIA, 183 pmol and 277 pmol, respectively, were not significantly different, but effective doses of MVIIA also results in untoward side effects. ConG appeared to be markedly less effective in reducing peripheral neuropathic pain in this model compared with effects using the formalin test (Table 1, over 5-fold difference in potency compared with phase 1 and 2). Using sub-antinociceptive doses of both peptides in combination, the A_{50} of the combination (125 pmol) was not statistically different from the theoretical additive A_{50} (203 pmol), indicating that the combination was additive (Fig 2C, Fig. 5).

3.4 Spinal cord injury

Increasing doses of both MVIIA and conG resulted in significantly increased withdrawal thresholds (Table 1, Fig. 3 A,B). The peak antinociceptive effect of conG was reached at 60–90 min post-injection, and sustained for at least 120 min. A significant antinociceptive effect was observed for MVIIA as early as 30 min post-injection, which was maintained for the two hr observation period. In contrast with both the formalin test and nerve ligation models, MVIIA was markedly more potent in reducing nociception in the spinal cord injury model (by 3- to 5-fold). Antinociceptive potency of conG alone was similar in both the peripheral and central neuropathic pain models (Table 1). In contrast to the additive antinociceptive effects of the peptide combination in the other models, the A_{50} of the combination (19 pmol) in the spinal cord injury pain model was significantly less than the theoretical additive A_{50} (118 pmol; $P < 0.05$). Therefore, the combination was synergistic (Fig. 3C, Fig. 6).

3.5 Rotarod test

Rats that did not fall off the rotarod prior to the 60 sec. cut-off were assigned a latency of 60 sec. At baseline, prior to i.t. injection, the rats were able to stay on the accelerating rotarod for the full 60 sec (Fig. 7). Intrathecal injection of vehicle did not significantly disrupt coordinated locomotion. By contrast, beginning at 30 min post-injection, antinociceptive doses of either MVIIA (300 pmol) or conG (300 pmol) significantly decreased the latency to fall from the rotarod ($P < 0.05$ vs. baseline). At 60, 90, and 120 min post-injection, the latencies of MVIIA and conG-treated rats were significantly reduced compared to that of vehicle ($P < 0.05$). With the highest tested dose of the peptide combination, slight decreases in the latency to fall were observed but these were not significantly different from either baseline or vehicle latencies ($P > 0.05$).

The highest tested dose of MVIIA (1000 pmol; Fig. 1) in this study was not used in the rotarod test due to the long duration (6–12 hrs) of movement-evoked tremor at this dose.

4. Discussion

Intrathecal injection of peptides with distinct molecular targets, MVIIA, a N-type voltage-gated Ca^{2+} channel blocker, and conG, a NMDA receptor antagonist, led to robust antinociceptive effects in rat models of persistent pain. In the formalin test and in rats with peripheral neuropathic pain, combination of the two peptides resulted in additive antinociceptive effects, but in rats with neuropathic SCI pain, a significant synergistic interaction was obtained. The potencies of the peptides differed across models which suggest varying degrees of involvement of the N-type Ca^{2+} channel and the NR2B-containing NMDA receptor across the models. The differential involvement of these molecular targets could also explain the additive antinociceptive effect of the peptides in some pain states compared to the super-additive (synergistic) effect in SCI pain. The current data also suggest that combining these peptides may be a novel approach to clinical pain control.

Persistent pain following tissue injury, including SCI, is a significant clinical problem and few pharmacological treatments are effective (Finnerup et al., 2001; Namaka et al., 2004). There are drugs that are effective for peripheral neuropathic pain, although the same drugs may not be as effective for neuropathic SCI pain, suggesting that drugs may have varying efficacies depending on the pain state (Finnerup and Jensen, 2004). Thus, despite similarities in symptoms, the various chronic pain states have distinct underlying mechanisms. Greater clarification of the pain mechanisms may lead to drugs with improved efficacy and safety and possibly activity across a spectrum of pain states.

Lesioning of the spinal cord leads to extensive neural and gliotic responses including the formation and release of excitatory amino acids, neuropeptide transmitters, inflammatory substances and cations (Yeziarski, 2005). The initial release of excitatory substances triggers further exocytosis, spreading to adjacent intact tissue, mediated by voltage-gated and ligand-gated ion channels (Millan, 1999). The elevated influx of cations not only leads to exocytosis but also stimulation of Ca^{2+} dependent protein kinases, which have a number of modulatory effects on gene expression and receptor function (Millan, 1999). These abnormal and persistent changes in spinal cord homeostasis, in turn, underlie cutaneous hypersensitivity and spontaneous pain (Yeziarski, 2005). Thus, blocking injury-associated ion entry into neurons may significantly reduce neuropathic SCI pain.

N-methyl-D-aspartate receptor antagonists markedly attenuate spinal cord tissue pathology and accompanying cutaneous hypersensitivity caused by spinal vascular ischemia (Hao et al., 1991). Systemic NMDA receptor antagonists have been shown to be antinociceptive in other models of chronic pain and in some types of clinical neuropathic but not acute pain (Eide et al., 1994; Nelson et al., 1997). However, the maximum tested doses were accompanied by adverse side-effects, such as dizziness and fatigue, discouraging widespread clinical use. N-methyl-D-aspartate receptors are localized in the dorsal horn of the spinal cord, so i.t. antagonist delivery attenuated pain with greatly diminished side-effects compared to systemic drug administration (Bennett et al., 2000; Sato et al., 1993; Tolle et al., 1993). Alternatively, antagonists selective for the NR2B subunit of the NMDA receptor may be useful for the pain treatment. Selective antagonists attenuate pain-related symptoms in rat neuropathic pain models, with few observable side-effects following systemic administration and are also neuroprotective (Boyce et al., 1999; Chazot, 2004; Chizh et al., 2001). The effects of small molecule selective antagonists have not been evaluated in neuropathic SCI pain.

A previous study utilizing various mouse pain models demonstrated robust antinociception following i.t. conG injection (Malmberg et al., 2003). The current study also demonstrated antinociceptive effects in rats, confirming the notion that antagonism of spinal NR2B-containing NMDA receptors with conG leads to antinociception across chronic pain models

with diverse etiologies. In the dorsal horn, the NR2B subunits are found on primary afferent central terminals, which suggests that the antinociceptive effect of conG is due to reducing neurotransmitter release (Furuyama et al., 1993; Marvizon et al., 2002; Monaghan and Cotman, 1985; Tolle et al., 1993). The exact role, however, of spinal NR2B-containing NMDA receptors in nociception is controversial. Despite unambiguous localization of the subunit protein central terminals, i.t. injection of NR2B-selective small molecule antagonists do not attenuate nociception (Coderre, 1993; Nakazato et al., 2005). Also, in the case of SCI, NMDA receptor function is decreased, as measured by ^3H MK-801 binding (Krenz and Weaver, 1998). These observations imply that conG may have mechanisms of action beyond NMDA receptor antagonism (Barton et al., 2004).

Similar to antagonism of pre-synaptic central terminal NMDA receptors, block of pre-synaptic N-type Ca^{2+} channels inhibit transmitter release and decreases spinal cord tissue pathology due to ischemia (Burns et al., 1999; Smith et al., 2002). Also, N-type Ca^{2+} channel blockers applied to post-synaptic dorsal horn neurons in rat models of chronic pain reduce spontaneous activity and abnormal responses to peripheral stimuli (Heinke et al., 2004; Matthews and Dickenson, 2001; Takemura et al., 1989; Vanegas and Schaible, 2000; Yusuf et al., 2001). Blocking other Ca^{2+} channel subtypes, such as P/Q- and L-type, in the spinal cord has little effect on chronic pain behavior (Chaplan et al., 1994b). Although i.t. ziconotide has been FDA approved for use in severe chronic pain, serious side-effects have been reported, including psychosis, dizziness and hypotension, which appear to be drug-mediated (Olivera and Teichert, 2007; Penn and Paice, 2000). N-type Ca^{2+} channel block may not be efficacious against some types of neuropathic pain (Yamamoto and Sakashita, 1998). To circumvent these issues, a low dose of MVIIA may be combined with other analgesics, the resulting mixture having significantly greater efficacy with markedly reduced side-effects compared to an equivalent dose of MVIIA (Wallace et al., 2008; Wang et al., 2000).

In the formalin test, the pain-related behaviors of the first phase is mediated through an acute activation of primary afferent nociceptors and the second phase is mediated by a sustained dorsal horn neuron activity (Yaksh et al., 1999). Block of dorsal horn N-type Ca^{2+} channels and NMDA receptors prior to formalin injection results in a slight antinociceptive effect in the first phase but a significant attenuation in the second phase, indicating that these channels and receptors are involved in the initiation of injury-induced persistent nociception (Coderre et al., 1993; Malmberg and Yaksh, 1994). The current data suggests that a combination of a NR2B-selective antagonist with a N-type Ca^{2+} channel blocker may prevent post-injury persistent nociception in an additive manner. Thus, such pretreatments would be useful prior to surgical procedures to diminish post-operative pain (Atanassoff et al., 2000; Choe et al., 1997; Ong et al., 2005).

Although numerous clinical analgesic drugs have been tested in rat models of neuropathic SCI pain, MVIIA has not been extensively evaluated (Hama and Sagen, 2007; Xu et al., 1992). In the current study MVIIA dose dependently ameliorated below-level hypersensitivity. Likewise, a recent case report demonstrated efficacy on below-level, but not at-level, neuropathic SCI pain (Saulino, 2007). Thus, block of spinal dorsal horn N-type Ca^{2+} channels may be effective for some types of SCI pain. Large-scale clinical trials are needed to confirm the efficacy of N-type Ca^{2+} channel blockers in neuropathic SCI pain.

Interestingly, MVIIA was significantly more potent than conG on SCI below-level hypersensitivity. The possible underlying source of increased potency of MVIIA includes increased channel affinity to MVIIA, but the exact functional disposition of N-type Ca^{2+} channels following SCI is not known. Characterizing changes in other ion channel function and distribution following SCI may also lead to the development of novel analgesic drugs.

Conversely, it appears that conG is more potent in the formalin test than in long-term neuropathic pain models. In fact, it appears that i.t. NMDA receptor antagonists are more potent in the formalin test than in spinal nerve injury pain (Chaplan et al., 1997). The dissimilar potencies suggest significant mechanistic differences between the models. Perhaps the inactivity of the NMDA receptors, prior to formalin injury, allows for antinociception to be obtained at low doses. As mentioned earlier, drugs that are applied prior to injury, as they are in the formalin test, may have utility as pre-operative analgesics (Atanassoff et al., 2000; Choe et al., 1997; Ong et al., 2005).

Similar to a previous study in mice, the highest tested doses of conG and MVIIA (300 pmol) in the current study resulted in impaired coordinated locomotion (Malmberg et al., 2003), thus, it is possible that motor impairment masqueraded as antinociception. However, motor impairment cannot entirely explain the effects of the peptides since the antinociceptive effects observed in the current study were dose-dependent and not limited to the highest dose. Motor impairment without antinociception can be observed with other drugs. For example, diazepam (3 mg/kg), which disrupts rotarod performance, is not antinociceptive (data not shown; (Carter, 1991; Franklin and Abbott, 1993)).

It is possible that in addition to enhanced antinociception of the combination, the side-effects are comparably worsened. Co-administration of conG and MVIIA resulted in an additive antinociception and in the case of neuropathic SCI pain, synergism. However, in the rotarod test, the combination did not significantly disrupt performance, in contrast to the markedly degraded performance with antinociceptive doses of the peptides alone. The lack of either additivity or synergism with the combination suggests distinct roles of NR2B-containing NMDA receptors and N-type Ca^{2+} channels in locomotion and sensation. It is unknown if other adverse side effects of ziconotide, which are difficult to discern in rats (e.g. dizziness), are affected by the addition of conG -- further studies are needed.

The current data suggest that acute i.t. injection of cone snail peptides, especially in combination, leads to robust antinociception. It has yet to be determined if efficacy is maintained with extended i.t. infusion of conG (Olivera and Teichert, 2007). Although these are peptides, which limits the route of administration, there are a number of potential ways that they could be continuously delivered for pain relief, including novel gene therapy (Glorioso et al., 2003) or cellular transplantation approaches (Eaton, 2006). Such methods may be of utility especially for chronic neuropathic SCI pain since few pharmacotherapies have long term efficacy.

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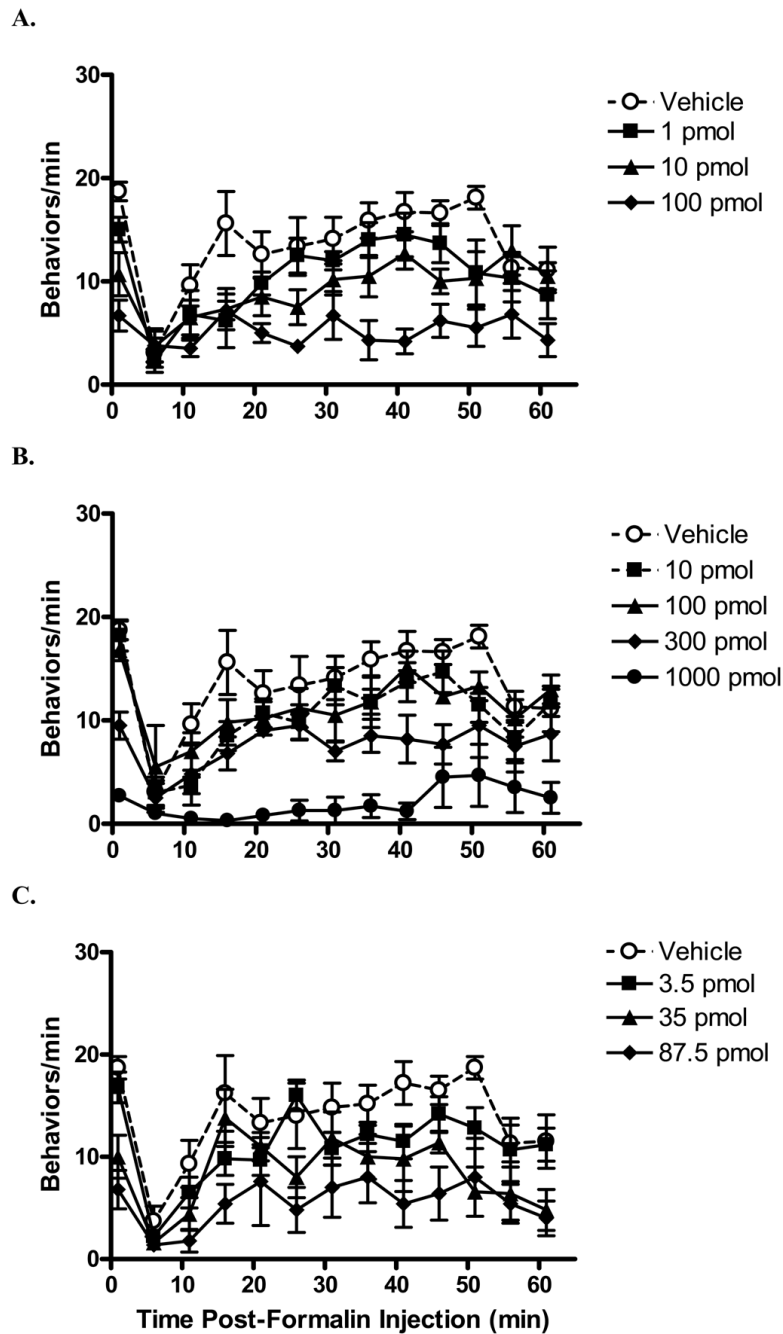


Fig. 1. Time course of the intrathecally injected peptides in the formalin test. Peptide or vehicle was injected 10 min prior to hind paw formalin injection. Following formalin injection, the numbers of flinches and hind paw licks within one minute were counted at five minute intervals. In rats pretreated with vehicle, an initial increase in formalin-evoked behaviors was observed, followed by prolonged period of pain-related behaviors. Pre-treatment with either conantokin-G (conG; A) or conotoxin MVIIA (MVIIA; B) markedly attenuated in a dose-dependent manner the formalin-evoked behaviors. C: Intrathecal injection of the combination of conG and MVIIA dose-dependently decreased formalin-evoked behaviors. The total dose (pmol) of the combination is shown. $N = 6-7$ /group. Data are expressed as mean \pm S.E.M.

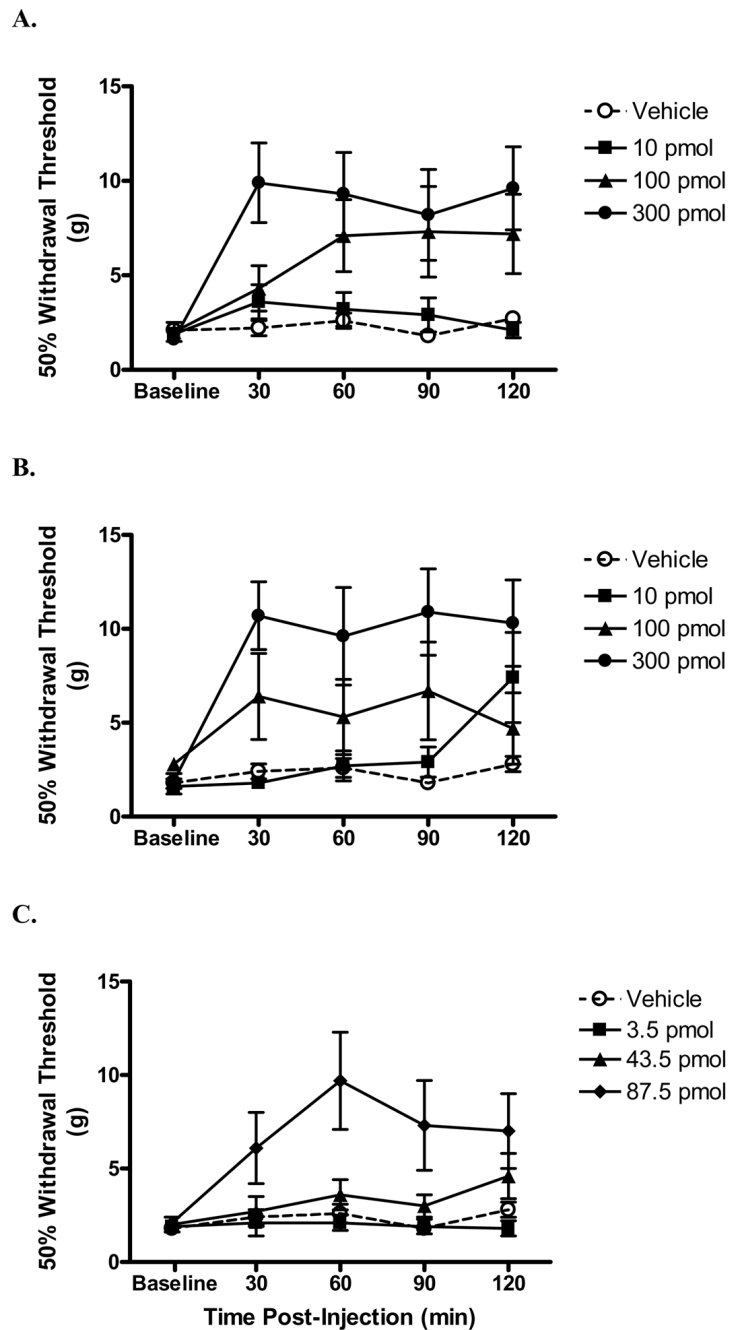


Fig. 2. Effect of intrathecally injected peptides over time on tactile hypersensitivity in rats with a spinal nerve ligation. Following measurement of hind paw withdrawal thresholds (g), rats were i.t. injected with either peptide or vehicle and tested once every 30 min. Intrathecal injection of either conG (A) or MVIIA (B) dose-dependently increased withdrawal thresholds. By contrast, vehicle did not alter withdrawal thresholds. C: Intrathecal injection of the highest dose of the combination of conG and MVIIA increased withdrawal thresholds. The total dose (pmol) of the combination is shown. $N = 7/\text{group}$. Data are expressed as mean \pm S.E.M.

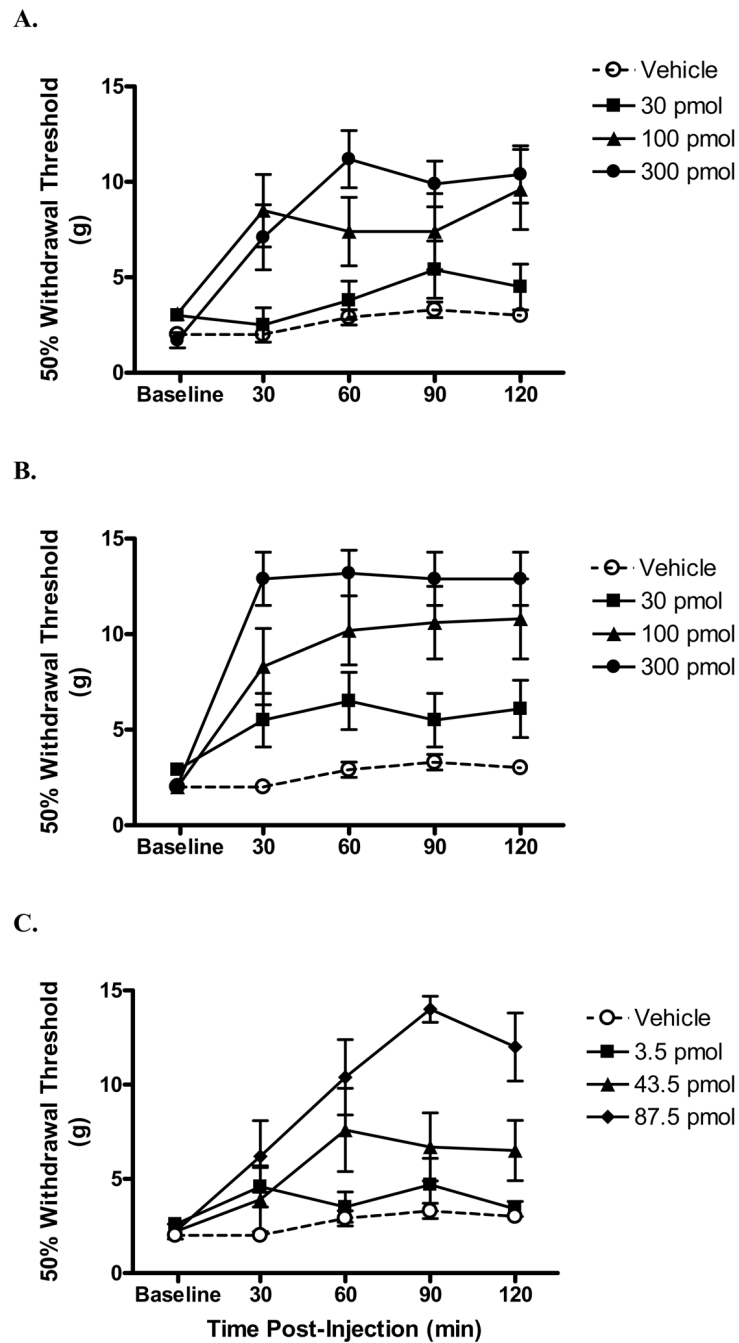
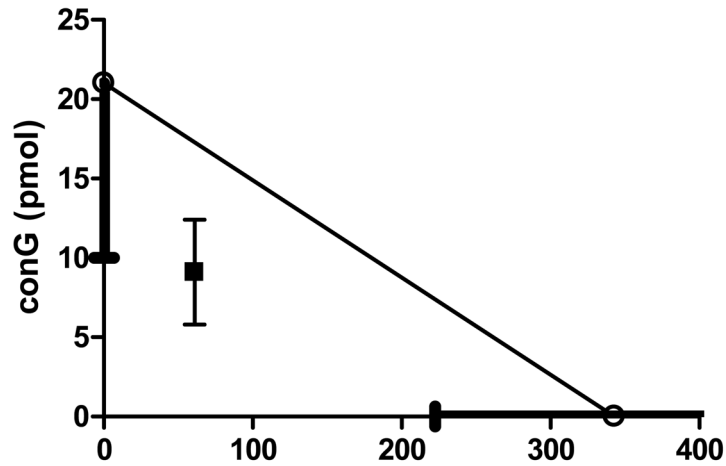


Fig. 3. Effect of intrathecally injected peptides over time on tactile hypersensitivity in rats with a spinal compression injury. Following measurement of withdrawal thresholds (g) of the hind paws, rats were i.t. injected with either peptide or vehicle and tested once every 30 min. Intrathecal injection of either conG (A) and MVIIA (B) dose-dependently increased withdrawal thresholds. By contrast, vehicle injection did not alter withdrawal thresholds. C: Intrathecal injection of the combination of conG and MVIIA dose-dependently increased withdrawal thresholds. The total dose (pmol) of the combination is shown. $N = 6/\text{group}$. Data are expressed as mean \pm S.E.M.

A.

Phase 1



B.

Phase 2

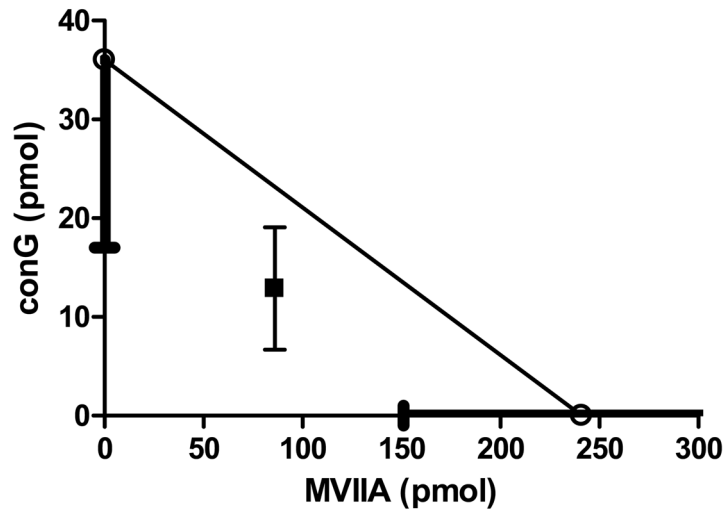


Fig. 4.

Isobolograms of the combination of conantokin-G and conotoxin MVIIA in the formalin test. The A_{50} of MVIIA (pmol) is demarcated on the horizontal axis and the A_{50} of conG (pmol) is marked on the vertical axis. The S.E.M. is the thick line overlying the axis. The line connecting the A_{50} values is the theoretical additive line. A: Isobologram of phase 1. The experimentally determined A_{50} of the combination falls within the S.E.M. of the line of additivity. Thus, the combination in phase 1 is merely additive. B: Isobologram of phase 2. The combination in phase 2, similar to phase 1, is merely additive.

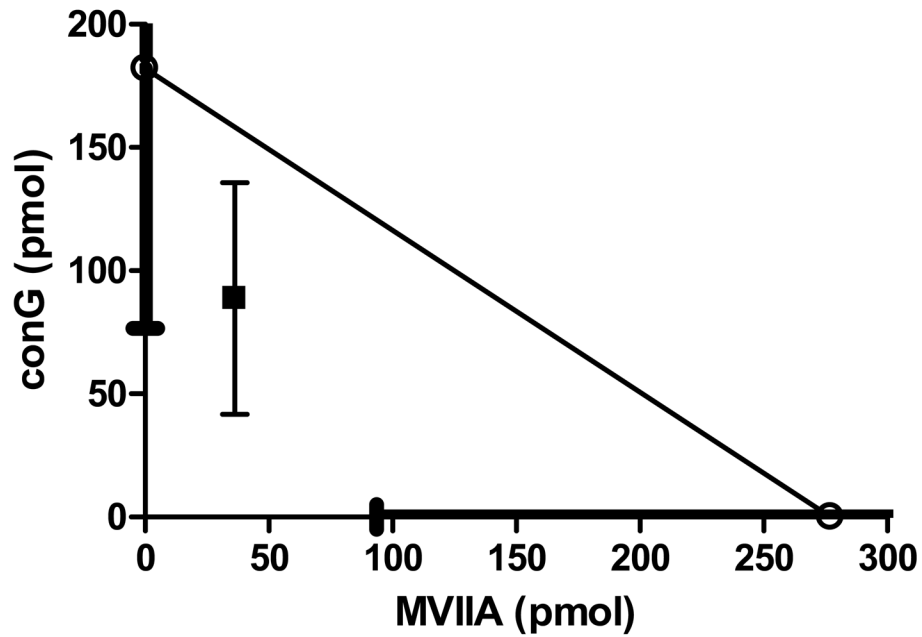


Fig. 5.

Isobologram of the combination of conantokin-G and conotoxin MVIIA in rats with a spinal nerve ligation. The A_{50} of MVIIA is demarcated on the horizontal axis and the A_{50} of conG is marked on the vertical axis. The S.E.M. is the thick line overlying the axis. The line connecting the A_{50} values is the theoretical additive line. The experimentally determined A_{50} of the combination falls within the S.E.M. of the line of additivity. Thus, the combination is merely additive.

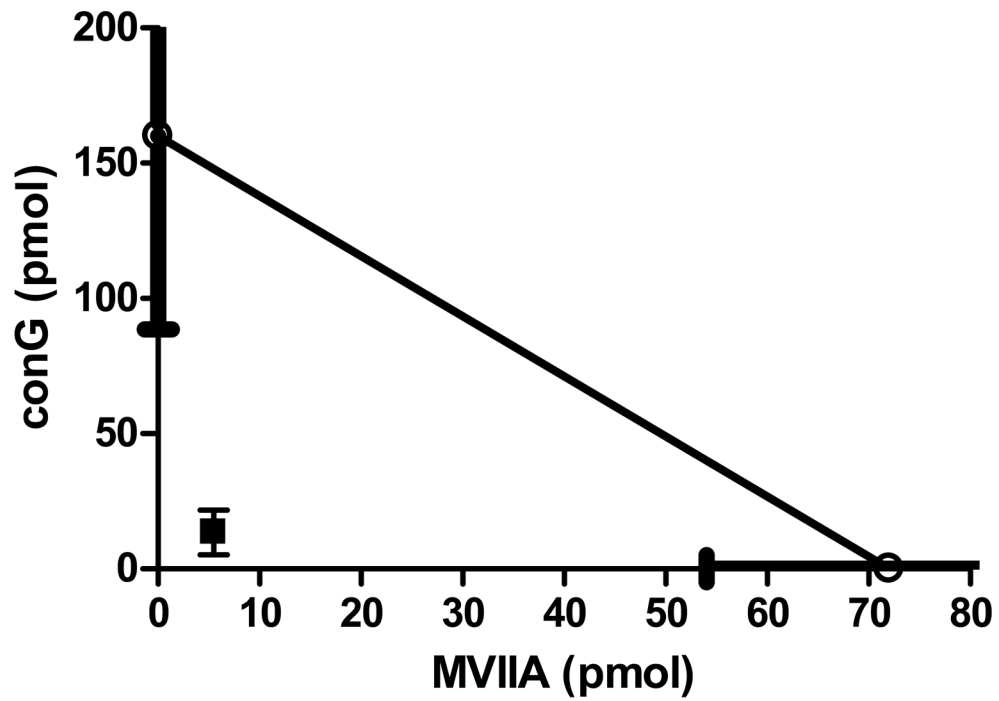


Fig. 6. Isobologram of the combination of conantokin-G and conotoxin MVIIA in rats with a spinal compression injury. The A_{50} of MVIIA is demarcated on the horizontal axis and the A_{50} of conG is marked on the vertical axis. The S.E.M. is the thick line overlying the axis. The line connecting the A_{50} values is the theoretical additive line. The experimentally determined A_{50} of the combination falls below the S.E.M. of the line of additivity. Thus, the combination is synergistic.

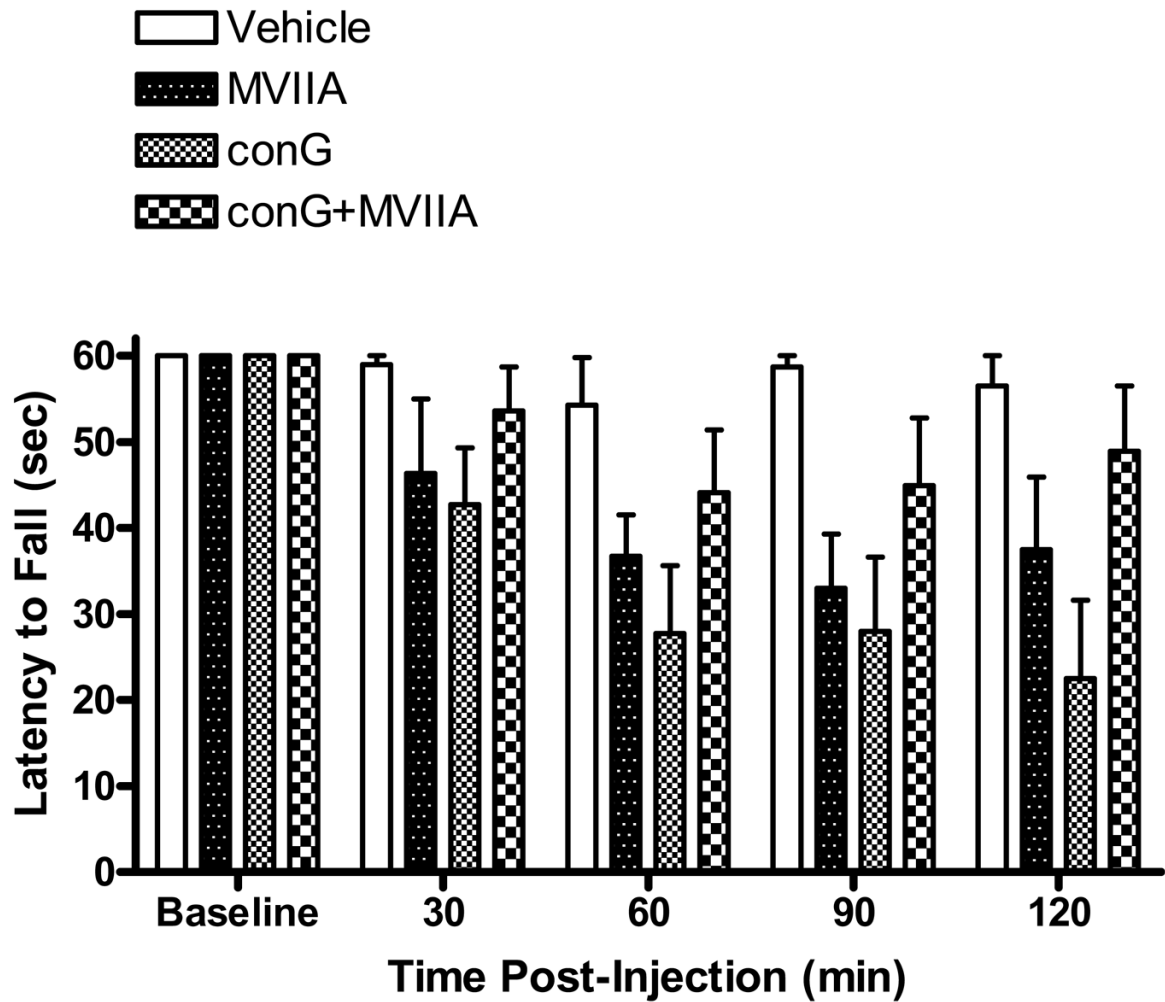


Fig. 7. Effect of intrathecally injected peptides over time on coordinated locomotion in the rotarod test. Following baseline measurement of the latency to fall (sec) from the rotarod, rats were i.t. injected with either peptide or vehicle and tested once every 30 min. Injection of either conG (300 pmol) or MVIIA (300 pmol) significantly decreased the latency to fall. By contrast, vehicle did not affect locomotion. Also, injection of the combination conG+MVIIA (87.5 pmol) did not significantly impair locomotion. $N = 6/\text{group}$. Data are expressed as mean \pm S.E.M.

Table 1

50% antinociceptive doses of the peptides in rat models of pain.

	ConG	MVIIA	Theoretical additive	Combined
Formalin test				
Phase 1 (0–1 min)	21 ⁺ (22)	343 (241)	116 (90)	70 (51)
Phase 2 (15–61 min)	36 ⁺ (38)	241 (180)	138 (91)	99 (95)
Spinal nerve ligation 60 min	183 (211)	277 (367)	203 (192)	125 (132)
Spinal cord injury 90 min	160 (143)	72 (36)	118 (64)	19 [*] (23)

50% antinociceptive doses (A₅₀) are expressed in pmol. Values in parenthesis are 95% confidence limits. The A₅₀ were determined from the dose response curves. The “theoretical additive” A₅₀ were calculated based on the dose response curves of the peptides alone. The “combined” A₅₀ were determined from the experimentally determined dose response curves of conG+MVIIA.

* Synergistic interaction ($P < 0.05$ vs. theoretical additive).

⁺ $P < 0.05$ vs. A₅₀ for MVIIA.