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Original Article

Effects of Iontophoretically Applied Substance P, Calcitonin Gene-Related Peptide on Excitability of Dorsal Horn Neurones in Rats

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Spontaneous pain, allodynia and hyperalgesia are well known phenomena following peripheral nerve or tissue injury, and it is speculated that secondary hyperalgesia and allodynia, are generally thought to depend on a hyperexcitability (sensitization) of neurons in the dorsal horn. It is supposed that the sensitization may be due to various actions of neurotransmitters (SP, CGRP, excitatory amino acids) released from the primary afferent fibers. In this study, we examined effects of the iontophoretically applied SP and CGRP on the response to EAA receptor agonists (NMDA and non-NMDA) in the WDR dorsal horn neurones and see if the effects of SP or CGRP mimic the characteristic response pattern known in various pain models. The main results are summarized as follows : 1) SP specifically potentiated NMDA response. 2) CGRP non-specifically potentiated both NMDA and AMPA responses. Potentiation of NMDA response, however, was significantly greater than that of AMPA response. 3) 50% of SP applied cells and 15.8% of CGRP applied cells showed reciprocal changes(potentiation of NMDA response and suppression of AMPA response). These results are generally consistent with the sensitization characteristics in diverse pain models and suggests that the modulatory effects of SP and CGRP on NMDA and non-NMDA (AMPA) response are, at least in part, contribute to the development of sensitization in various pain models.

Key Words: Pain, microiontophoresis, substance P, calcitonin gene-related peptide excitatory amino acid

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INTRODUCTION

Hyperalgesia is an altered state of cutaneous sensation characterized by an increase in the pain evoked by a noxious stimulus. Hyperalgesia following injury is composed of two components: primary hyperalgesia, which occurs at the site of injury, and secondary hyperalgesia, which develops in surrounding undamaged tissue.[1] Allodynia is pain evoked by normally innocuous stimuli. Primary hyperalgesia can be explained by the sensitization of primary afferent fibers.[1,2] However, secondary hyperalgesia and allodynia are generally thought to depend on a sensitization of neurons in the dorsal horn.[3-5] Sensitization of spinal cord neurons causes the cells to become hyperexcitable and to respond more vigorously than normally to both innocuous and noxious peripheral stimuli.

A series of experiments supporting the idea that sensitization of the dorsal horn cells may, at least in part, account for secondary hyperalgesia have been reported.[2,6-9] Intradermal injections of capsaicin, which is known to produce primary and secondary hyperalgesia in human subjects,[2] were made in monkeys while recording from the spinothalamic tract (STT) neurons.[8,9] Injections of capsaicin, but not vehicle, into the receptive field caused an increase in the discharges of wide dynamic range (WDR) STT cells.[8,9] This discharge paralleled the known time course of the pain evoked by such injections in humans.[2]

Sensitization of dorsal horn neurons has also

been demonstrated following the induction of inflammation by intraplantar injection of Freund's complete adjuvant (FCA) in rats,[10] by intraplantar injection of formalin in rats[11,12] or by injection of kaolin and carrageenan into the knee joints of cats or monkeys.[13-15]

Additionally, central hyperexcitability has been demonstrated in models of experimental neuropathy[16-18] during procedures that enhance flexor withdrawal reflexes[3-5,19] and during 'wind-up'.[20-22] In these studies, the receptive fields of the dorsal horn neurons expand and the cells often respond more vigorously to innocuous stimuli.

The central question in each of these diverse models is: what is (are) the mechanism (s) by which the various painful stimuli can produce a long-lasting sensitization of the dorsal horn neurons?

Since discharges in primary afferent fibers are required for the induction of sensitization and excitatory amino acid (EAA) and neuropeptides are released into the dorsal horn following noxious mechanical or chemical stimulation in the skin,[23-30] we believe that the neurotransmitters released by primary afferent fibers into the dorsal horn following noxious stimulation play an important role.

A role for excitatory amino acids in neurotransmissions and for SP or CGRP in their modulation particularly of nociceptive information, from primary afferent fibers to neurons within the dorsal horn is supported by the work of several laboratories.[31-36]

In this study, we examined the effects of iontophoretically applied SP and CGRP on the response to EAA receptor agonists (NMDA and non-NMDA) in WDR dorsal horn neurones and see if the effects of SP or CGRP mimics the characteristic sensitization pattern which was known in diverse pain models.

MATERIALS AND METHODS

Young adult male rats (Sprague-Dawley, 150-200g) were used in the study.

Animals were anesthetized with sodium pentobarbital (40 mg/kg i.p. with supplementary doses of 5 mg/kg every 3 h). A laminectomy was performed at vertebral levels of T13-L3 to expose the lumbar enlargement and the spinal cord was covered with a pool of warmed mineral oil. The animals were paralyzed with pancuronium bromide (1 mg/kg, i.v.) and ventilated artificially. The end-tidal CO_2 level was maintained between 3.5 and 4.5%, and the rectal temperature was maintained close to 37℃ by a servo-controlled heating blanket.

A seven-barreled micropipette was inserted into the spinal cord, and its central barrel filled with a carbon filament was used to record extracellularly the single unit activities of dorsal horn neurons, whereas the surrounding outer barrels were used for iontophoretic ejection of substances.

Neurons were characterized based on their responses to three consecutive mechanical stimuli applied to the somatic receptive field. Mechanical stimuli consisted of brushing (brushing the skin across the center of the receptive field with a camel-hair brush at a frequency of about 3 Hz), pressing and pinching(grabbing the skin fold with a calibrated forceps at forces of 1 and 3.5 N, respectively). Only wide dynamic range neurones that responded well to these stimuli and more vigorously with increasing stimulation intensity were studied since these cells are known to be associated with pain sensation.

Extracellular single unit activity was monitored on storage and digital oscilloscopes and led to a data acquisition system (CED 1401) via a window discriminator for the construction of peristimulus time histograms of the firing rate. Spike configuration and size were continuously monitored on the digital oscilloscope to confirm that the same cell was registered by the window discriminator throughout the experiment.

The following substances were applied by microiontophoresis in the experiments: N-methyl-D-aspartate (NMDA; 50 mM, 60-90 nA) as a NMDA receptor agonist, (S)-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [(S)-AMPA; 50 mM, 30-50 nA] as a non-NMDA agonist, substance P (SP, 1 mM, 50-80 nA) and calcitonin gene-related peptide (CGRP, 1 mM, 50-80 nA). All substances were dissolved in 200 mM NaCl. One outer barrel of a micropipette was filled with 200 mM NaCl for current balance. Retaining currents (5-15 nA) were used between applications to prevent material

leakage. Net current at the electrode tip was continuously monitored and neutralized with the aid of a Medical Systems iontophoresis pump (Model BH-2).

EAA receptor agonists were applied by 10-s-long current pulses. Once the baseline responses were obtained, the effects of combined application of SP or CGRP were examined.

SP was administered alone for 2 min while background activity was recorded. While SP delivery continued, pulses of EAA receptor agonists were delivered. Application of SP was terminated immediately following the final agonist response.

The stored digital record of unit activity was retrieved and analyzed off-line. Accumulated frequency histograms of all records were generated and background activity was subtracted from all drug evoked responses. All results are presented as mean ± standard error (SE), unless otherwise stated. Student's t-test was used for data analysis. P-value of less than 0.05 was regarded as statistically significant.

RESULTS

General observations

The responses of dorsal horn neurons to NMDA, AMPA, SP and CGRP were analyzed. The activity of a total of 80 WDR cells (42 cell for testing the effects of SP and 38 cells for testing the effects of CGRP) were recorded in 50 experiments.

Average spontaneous activity increased from 2.6 ± 0.6 to 3.3 ± 0.6 spikes/sec by co-application of SP and from 3.4 ± 0.9 to 3.8 ± 0.8 spikes/s by CGRP but these changes were statistically insignificant (Fig. 1).

Total average NMDA responses increased to 161.3 ± 16.5% after co-application of SP but AMPA responses was not significantly changed by SP, indicating that SP specifically enhances NMDA responses (Fig. 1).

Total average NMDA and AMPA responses were increased to 161.8 ± 14.6% and 127.8 ± 11.8% respectively by the co-application of CGRP, indicating that CGRP enhances both responses non-specifically (Fig. 1).

Changes of NMDA and AMPA responses by SP or CGRP application were variable. According to the response pattern, we classified the cells into three types.

1) Type 1 cells (Fig. 2) showed enhancement of both NMDA and AMPA responses after the co-application of SP or CGRP (global facilitation). 2) Type 2 (Fig. 3) showed enhancement of NMDA evoked responses but suppression of AMPA responses (reciprocal NMDA facilitation). 3) Type

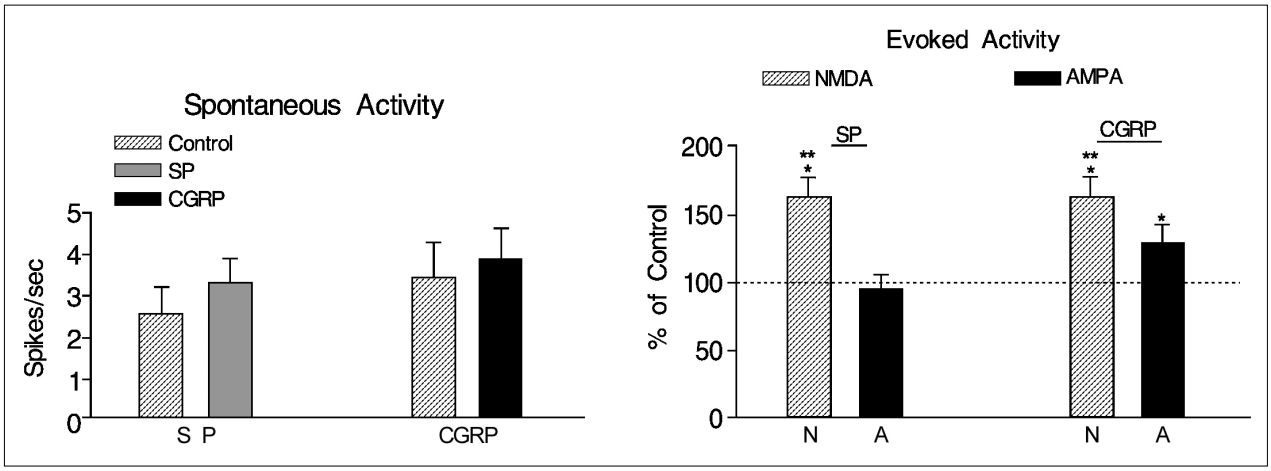

Fig. 1. Effects of SP and CGRP on the spontaneous activity and NMDA, AMPA evoked activities in WDR dorsal horn neurones. Changes of spontaneous activity following SP or CGRP application were statistically insignificant. NMDA evoked responses increased to 161.3 ± 16.5%, whereas AMPA response showed a slight changes following SP application. NMDA, AMPA evoked responses increased to 161.8 ± 14.6 and 127.8 ± 11.8% respectively. *p<0.05; comparison of the evoked responses between pre- and post-application of the neuropeptides (SP, CGRP). **p<0.05; comparison between NMDA and AMPA evoked responses after application of neuropeptides (SP, CGRP).

3 (Fig. 4) showed suppression of both NMDA and AMPA responses by co-application of SP or CGRP (global inhibition).

Effects of SP on NMDA and AMPA evoked responses.

When analyzed the proportion of the dorsal horn neurones which showed characteristic response patterns by co-application of SP, it was found that 26.1%, 50.0%, and 21.5% of the cells recorded were belonged to types I, II, and III (Fig. 5). respectively, indicating the largest proportion were type II (Fig. 6).

According to the response type I, II and III, we further analyzed the response characteristics for the changed NMDA and AMPA responses by co-applying SP or CGRP (Fig. 7). In type I cells, NMDA and AMPA responses after SP application significantly increased to 274.5 ± 43.5% and 180.5 ± 16.8% respectively ($p<0.05$), and the degree of change of the NMDA response was significantly larger than that of the AMPA response($p<0.05$). In type II cells, NMDA and AMPA responses after SP application significantly increased to 143.0 ± 9.3% and decreased to 71.9 ± 4.8% respectively($p<0.05$). In type III cells, NMDA and AMPA responses after SP application significantly decreased to 74.4 ± 8.5% and 48.2 ± 12.2% respectively ($p<0.05$). Moreover, the degree of change of the AMPA response was significantly larger than that of the NMDA response ($p<0.05$).

Effects of CGRP on NMDA and AMPA evoked responses.

When we analyzed the proportion of the dorsal horn neurones which showed characteristic response patterns after the co-application of CGRP, 60.6%, 15.8%, and 21.0% of the recorded cells were found to be of types I, II, and III. respectively, indicating that the largest proportion was type I (Fig. 5, and 6).

According to the response type I, II and III, we further analyzed the response characteristics for the changed NMDA, AMPA responses caused by the co-application of CGRP (Fig. 7). In type I cells, NMDA and AMPA responses after CGRP application significantly increased to 194.9 ± 19.3% and 165.8 ± 14.0% respectively ($p < 0.05$). Difference

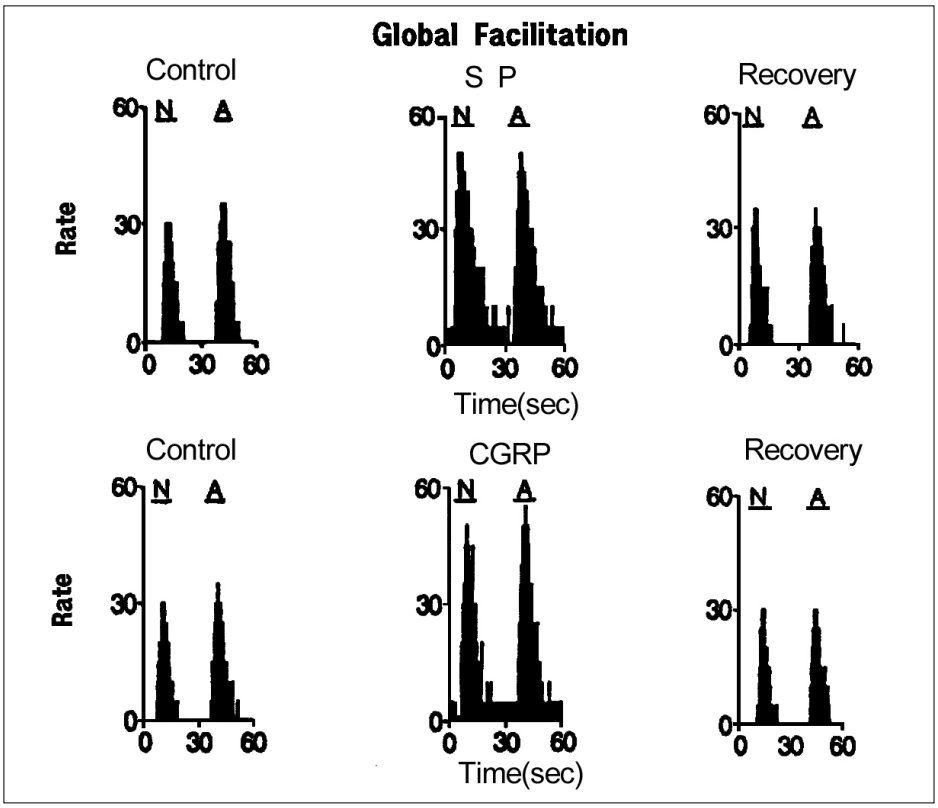

Fig. 2. A typical rate histogram showing enhancement of both NMDA and AMPA responses (Global Facilitation) by application of SP, CGRP. N, NMDA; A, AMPA. The bar represents the duration of iontophoretic application.

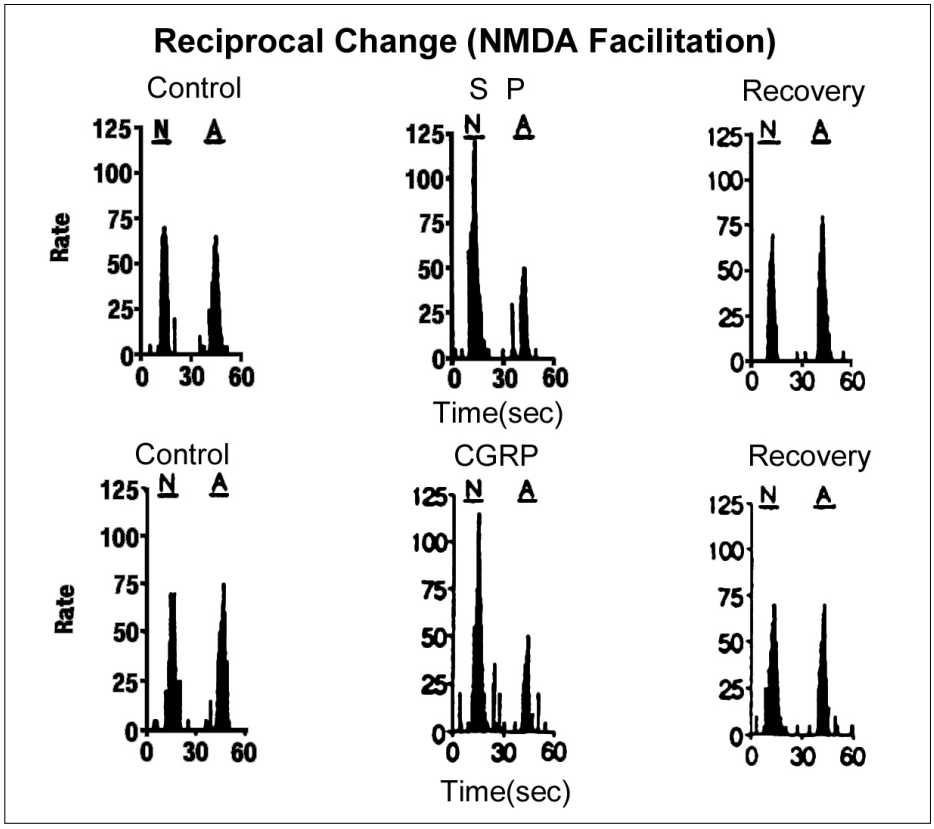

Fig. 3. A typical rate histogram showing enhancement of NMDA and suppression of AMPA responses (reciprocal NMDA facilitation) by application of SP, CGRP. N, NMDA; A, AMPA. The bar represents the duration of iontophoretic application.

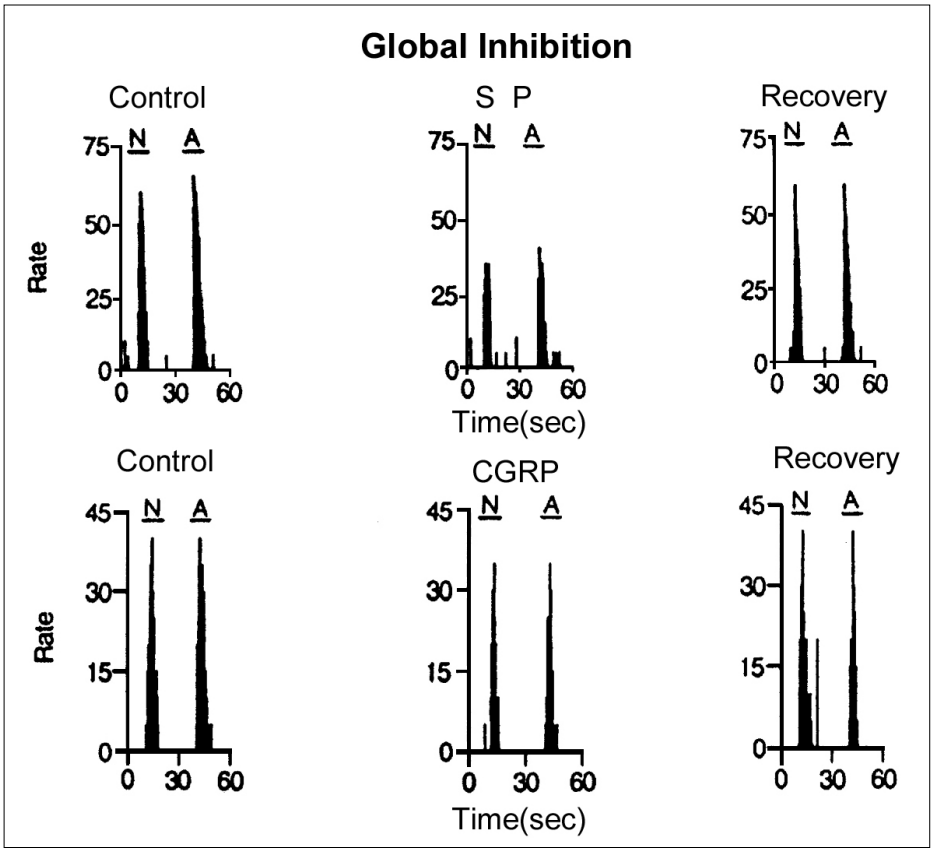

Fig. 4. A typical rate histogram showing suppression of both NMDA and AMPA responses (Global inhibition) by application of SP, CGRP. N, NMDA; A, AMPA. Bar represents duration of iontophoretic application.

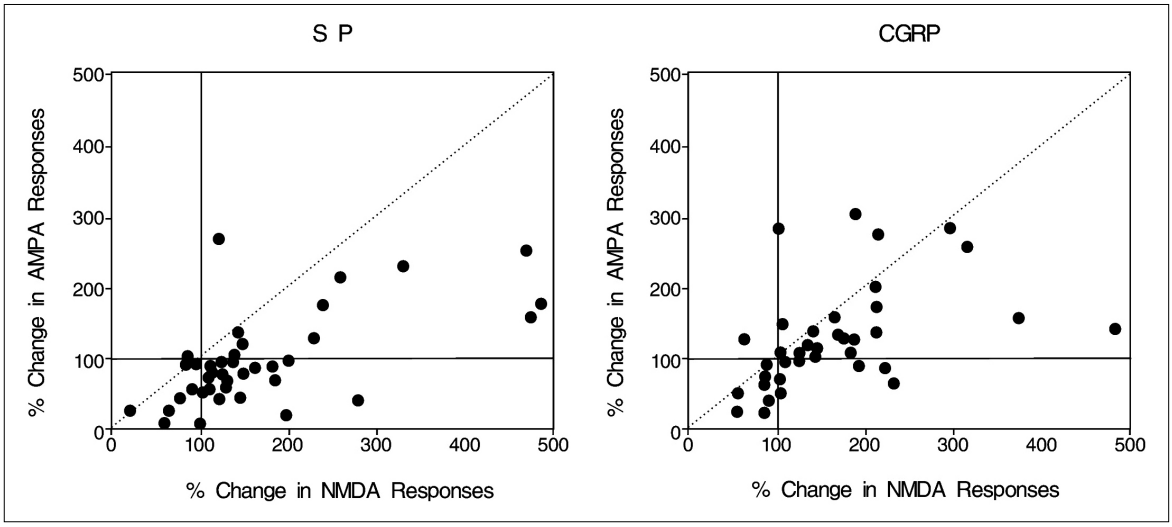

Fig. 5. Scatter plot showing % change in NMDA and AMPA responses by application of SP and CGRP. Most of the cells recorded belonged to the partitions for the enhancements of both NMDA, AMPA responses, reciprocal change (NMDA facilitation) and global inhibition.

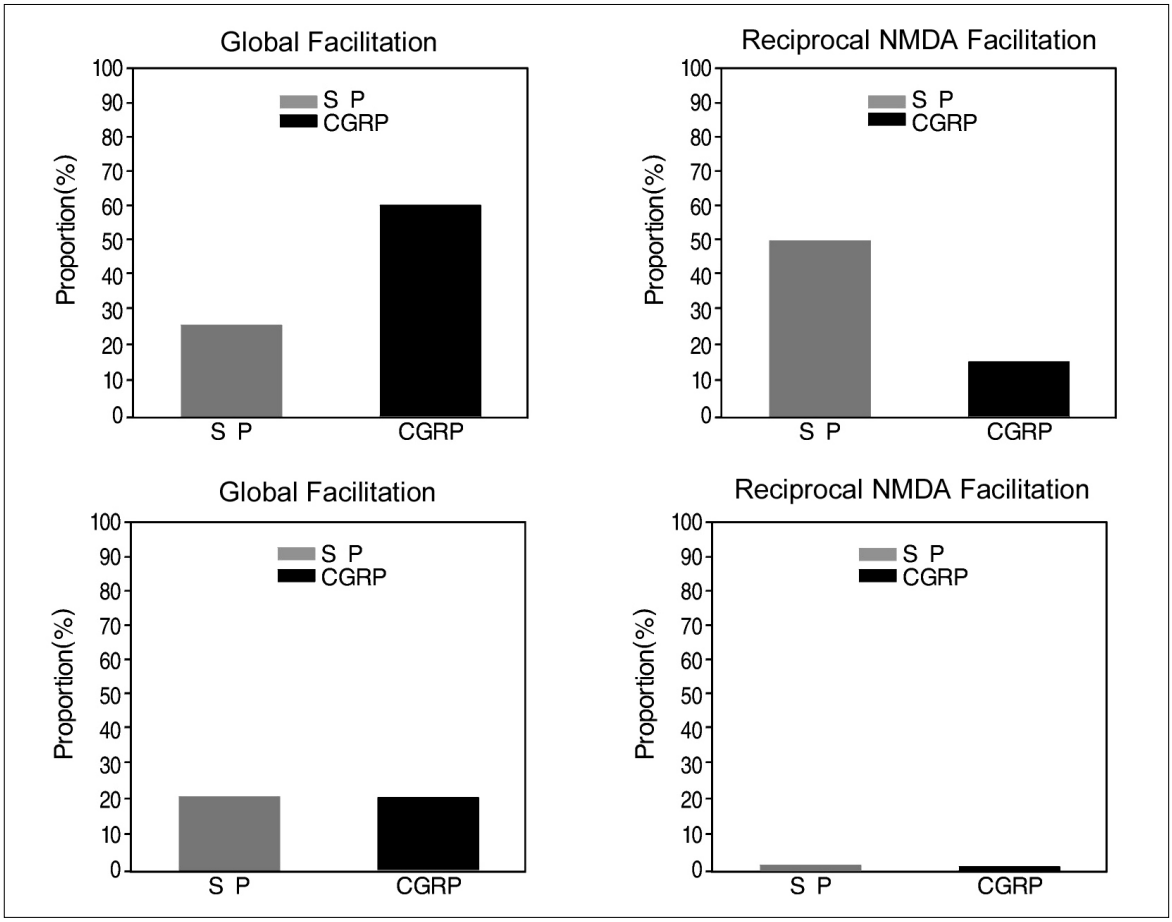

Fig. 6. Analysis of the proportion of dorsal horn neurones that showed characteristic response patterns by application of SP, CGRP. Recorded cells showed enhanced responses (26.1% and 60.6%) for both EAA receptor agonists (NMDA, AMPA) by SP and CGRP respectively (global facilitation). 50.0% and 15.8% of recorded cells showed reciprocal NMDA facilitation by SP and CGRP respectively. 20% of cells showed global inhibition by SP or CGRP.

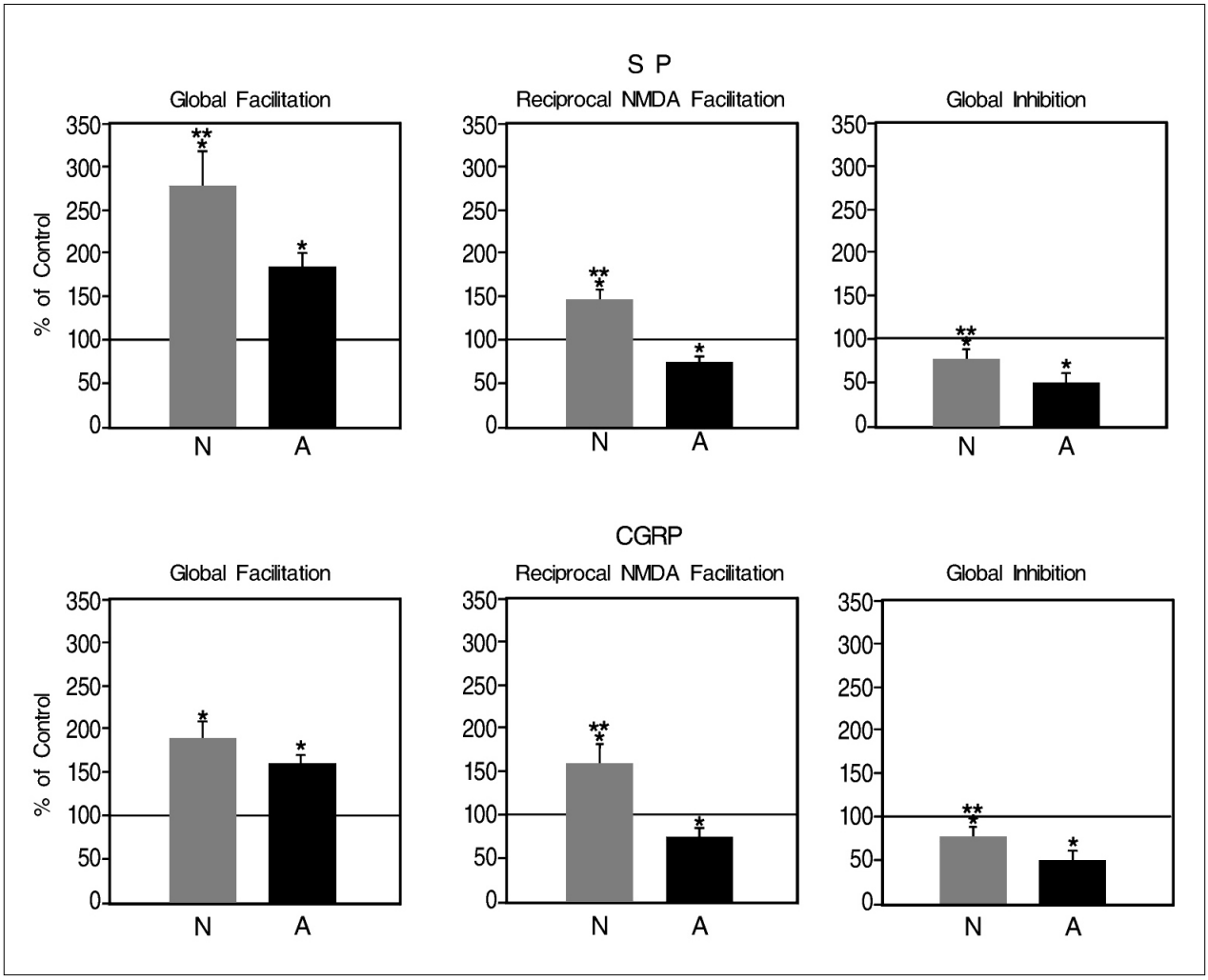

Fig. 7. Analysis of response patterns according to response type I, II, and III. In cells which belong to type I(global facilitation), NMDA and AMPA responses by SP or CGRP application significantly increased ($p < 0.05$). In case of SP application, degrees of changes for the NMDA responses were significantly larger than those of the AMPA responses ($p < 0.05$). In type II cells (reciprocal NMDA facilitation), NMDA significantly increased and AMPA responses significantly decreased after SP or CGRP application ($p < 0.05$). In type III cells (global inhibition), NMDA and AMPA responses were significantly decreased by SP or CGRP ($p < 0.05$). N, NMDA; A, AMPA. *$p < 0.05$; comparison between pre- and post-application of neuropeptides (SP, CGRP). **$p < 0.05$; comparison between NMDA and AMPA responses following application of neuropeptides (SP, CGRP).

in the changes between the NMDA and AMPA response were not statistically significant. In type II cells, NMDA and AMPA responses caused by CGRP application significantly increased to 162.2 ± 23.7% and decreased to 79.9 ± 7.4% respectively ($p < 0.05$). In type III cells, NMDA and AMPA responses caused by CGRP application significantly decreased to 79.0 ± 5.9% and 54.7 ± 8.5% respectively ($p < 0.05$), and these changes in AMPA response were significantly larger than the NMDA responses ($p < 0.05$).

DISCUSSION

The experiments reported here serve as a direct test of the possibility that SP and CGRP contribute to the sensitization of dorsal horn WDR neurons.

It has been reported that there is an increase in the responses of STT neurons in monkeys to iontophoretically applied EAAs following the intradermal injection of capsaicin or intra-articular injection of kaolin and carrageenan.[9,15] However, there was an interesting difference between the

hanges in the EAA responses in these two models. During the sensitization of STT cells following intradermal capsaicin application, an increase in response to both NMDA and non-NMDA agonists was observed. In contrast, during sensitization of STT cells in acute arthritis, there was an increase in responses to non-NMDA agonists, but no change in responses to NMDA.

A distinction should be made between the reciprocal inhibition (reciprocal NMDA facilitation) of the responses to one type of EAA agonist concomitant with an increase in the response to another type of EAA agonist versus the global inhibition of all EAA agonist responses by SP or CGRP. Reciprocal changes in EAA agonist responses are not novel and have in fact been observed in at least three *in vitro* preparations: dissociated chick spinal cord, cultures of retinal horizontal cells and slice preparations of the hippocampus in rats and mice[37-39] and also in some *in vivo* studies.[40,41]

The reciprocal changes in EAA agonist responses following SP or CGRP application may represent a switching of the dominant input to a particular cell from one type of EAA receptor to another. It has been suggested that AMPA-kainate non-NMDA receptors mediate the responses of the dorsal horn neurons to monosynaptic inputs while NMDA receptors mediate the responses of the dorsal horn neurons to polysynaptic inputs.[42] Assuming that this proposal is correct, sensitization of the NMDA responses of a cell by SP or CGRP would correspond to an increase in the responses to polysynaptic inputs. On the other hand, sensitization of the AMPA responses by SP or CGRP might reflect enhanced monosynaptic transmission

Reciprocal changes in EAA responses were found only occasionally following sensitization of STT cells by intradermal capsaicin.[9] Generally, responses to both NMDA and non-NMDA agonists increased in parallel.

Although we found that not all tested cells showed increased responses to both NMDA and non-NMDA agonists after the co-application of SP or CGRP in this study, it may be that sensitization in various chronic pain models is mediated in part by other sensory neuropeptides. One candidate is neurokinin A, which is released by stimuli similar to those that evoke SP release[27] and which causes an increase in EAA-evoked currents following bath applications to dissociated dorsal horn neurons.[43]

The cases of global inhibition of EAA agonist responses by SP or CGRP pose a more difficult problem for the hypothesis that neuropeptides mediate the sensitization of dorsal horn neurons. The global inhibitions that we observed, are not novel. Similar effects have been reported in studies conducted *in vitro* on acutely dissociated dorsal horn neurons[43] and on neurons recorded in the spinal cord.[44] Indeed, Rusin et al,[43] found that almost 30% of their NMDA responses were inhibited by SP. Thus, it is possible that the EAA agonist responses of some WDR cells are directly inhibited by SP, representing a condition analogous to long-term depression. Indeed, recent studies have shown that, depending on the extracellular concentration of calcium, the same conditioning stimuli may result in the generation of either long-term potentiation or long-term depression.[45] The induction of a long-term depression of some WDR cells may be related to the induction of zones of hypo-responsiveness to some cutaneous stimuli after injury, as observed by Raja et a1.[1]

Another explanation of global inhibition is the action of SP or CGRP on the inhibitory interneurons situated near and synapsing onto WDR neurons under observation. We do not have direct evidence that the effects of SP or CGRP that we observed on the EAA responses are due strictly to postsynaptic effects on WDR neurons.

Thus, it is plausible that some inhibitory in addition to excitatory interneurons are influenced by neuropeptides in our experiments and that these interneurons are sensitized by the application of SP or CGRP.

In conclusion, the present study has shown that the responses of WDR cells to EAA acting at either NMDA or non-NMDA receptors may be altered by the co-application of neuropeptides (SP, CGRP). We propose that this enhanced response (sensitization) of the cells, at least in part, may contribute to the development of secondary hyperalgesia or allodynia in some persistent pain models. The significance of the inhibition of EAA agonist responses produced by neuropeptides

remains unclear. And additional experiments will be required to determine the detailed mechanism of the observed changes in excitability, and to determine the criteria that predict whether the responses of a given cell to EAA agonist will increase or decrease following the co-application of SP or CGRP.

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