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Alpha-Calcitonin Gene-Related Peptide-Null Mice Shows Normal Responses to Various Noxious Stimuli

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Despite the wealth of data concerning the roles of α-CGRP in nociceptive behaviors, α-CGRP-null mice showed no obvious phenotypic differences in nociceptive behaviors from wild type. The present studies specifically demonstrate that α-CGRP null mice showed no CGRP immunoreactivity from the spinal cord, implying that CGRPs in the mice spinal cord are mainly a-isoforms. However, the nociceptive behaviors of the null mice are not significantly different from the wild type mice in thermal nociceptive behaviors on hotplate, chemical nociception tests to intraplantar capsaicin or formalin injection, and visceral pain behaviors to intraperitoneal acetic acid or magnesium sulfate injections. These data suggest that α-CGRP is dispensable for nociceptive behaviors or that compensatory mechanisms may exist to overcome the absence of this peptide.

Key Words: CGRP, Pain, Knockout mice, Spinal cord

INTRODUCTION

Based on its expression pattern and pharmacological studies, calcitonin gene-related peptide (CGRP) has been implicated to be involved in nociception. CGRP is expressed in dorsal root ganglion and dorsal horns of spinal cords (Gibson et al, 1988; McNeill et al, 1988) and noxious thermal or mechanical stimulations evoke release of CGRP in the superficial dorsal horn (Morton & Hutchison, 1989). Intrathecal injection of CGRP significantly reduces in the latency of hindpaw withdrawal in response to noxious thermal and mechanical stimulation (Oku et al, 1987), and an intrathecal injection of anti-CGRP antiserum and $CGRP_{8-37}$ inhibits thermal and mechanical nociception. These studies suggest that the endogenous CGRP has a facilitating function in nociceptive transmission in the spinal dorsal horn (Kuraishi et al, 1988; Yu et al, 1994).

Alpha-CGRP is a pleiotropic peptide neuromodulator that is widely expressed throughout the central and peripheral nervous systems (Rosenfeld et al, 1981). Alpha-CGRP is produced by tissue specific alternative RNA splicing in neurons from the calcitonin/αCGRP genomic locus (Amara et al, 1982). In addition to the calcitonin/αCGRP gene, a second CGRP gene has been identified and is referred to as β-CGRP (Amara et al, 1985). Alpha- and β-CGRP differ only by 1 amino acid in the rat and by 3 amino acids in both humans and mice. Because of overlapping expression patterns for α- and β-CGRP, the multiple CGRP receptor subtypes (Kapas & Clark, 1995; Aiyar et al, 1996), and the lack of specific CGRP antagonists (Dumont et al, 1997), the precise role of α-CGRP in nociception remains unclear. In the present study, we used α-CGRP-deficient mice to investigate the nociceptive function of α-CGRP.

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METHODS

Animals

Generation of α-CGRP knockout mice generation has been described previously (Lu et al, 1999) and backcrossed onto the C57BL/6 background for ten generations. Animals were housed in a temperature and humidity-controlled environment with 12-hour light-and-dark cycle, and had free access to food and water. Female mice were used (8~13 weeks of age). All experiments were performed between 9:00 a.m. and 3:00 p.m. Each mouse was acclimatized in the test apparatus for 30~60 min. All experiments were performed blind and no animals were used more than once.

Immunofluorescence

Mice were deeply anesthetized with pentobarbital sodium (100 mg/kg) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. The L4~5 segments of the spinal cord were removed and postfixed for 4 h in the same fixatives and then cryoprotected overnight in 30% sucrose. Frozen sections of the spinal cord were cut transversely at 30 μm on a freezing microtome. The sections were washed in PBS and incubated with 4% normal donkey

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; SP, substance P; DRG, dorsal root ganglion.

serum and 0.3% Triton X-100 in TBS for 2 hours. The sections were then incubated with primary antiserum (anti-CGRP, 1 : 2,000; anti-SP, 1 : 1,000) overnight at room temperature, washed in TBS containing 0.1% Triton X-100, and incubated at room temperature for 1 hour with anti-rabbit IgG conjugated with Cy-3 (1 : 1,000). Preparations were washed and mounted in Aqua Poly/Mount (Polysciences Inc.). All sections were examined under epifluorescence.

Responses to thermal noxious stimuli

We measured the latency to lick a hindpaw or jump from a hotplate as a sign of thermal nociceptive response. The cut-off time was 60 sec for 52.5°C hotplate and 30 sec for 55.5 and 58.5°C hotplates to prevent tissue damage. To evaluate C-fiber-mediated thermal nociceptive behavior, we performed incremental hotplate test. Mice were placed on a hotplate at 25°C and the temperature of hotplate was increased slowly at 6°C per min. The latency spent to lick or jump was measured as an index of pain response.

Responses to chemical noxious stimuli

For formalin test, mice were injected with 20 μl of dilute formalin (2 % in saline, Sigma) subcutaneously into the right hindpaw. Then, each animal was returned to the observation chamber and the duration of time licking the injected paw was recorded during phase 1 (0 to 10 min after injection) and phase 2 (10 to 45 min after injection) as an index of chemical nociception (Hunskaar & Hole, 1987). For the capsaicin test, 20 μl of capsaicin (4.5 mg in 10 ml saline/10% ethanol/0.5% Tween 80) was injected intraplantarly to the right hindpaw. The duration of time spent licking the injected paw was measured for 5 min after the injection.

Responses to visceral noxious stimuli

For visceral nociceptions, we used abdominal constriction models with minor modifications (Cao et al, 1998). Mice were placed in individual mouse cages with bedding for at least 30 min prior to testing. For nociceptive testing, a mouse was injected intraperitoneally in the right lower quadrant of abdomen with 0.3 ml of 0.6% acetic acid (5.0 ml/kg) for visceral pain with inflammation, or $MgSO_4$ (120 mg/kg) for visceral pain without inflammation. Then, the abdominal stretch was counted for 20 min after the injection. An abdominal stretch is characterized by contraction of the abdominal muscles, the arching of the back ventrally such that the abdomen touches the bedding surface, and extension of one or both hind limbs. Mice were used once and then immediately euthanized.

Statistical analysis

All results are expressed as mean±S.D. Statistical analysis of the results was performed using Student's t test or analysis of variance (ANOVA) where appropriate. Differences were considered to be significant if $p<0.05$.

RESULTS

CGRP-immunoreactivities in the spinal cords

In tissue sections taken from the lumbar spinal cord of wild-type mice, dense CGRP-immunoreactivities (ir) were observed in the superficial laminae (I and II) of the spinal cord dorsal horn bilaterally, while CGRP-ir was absent in the sections taken from α-CGRP knockout mice, indicating that α-CGRP is the predominant CGRP isoform in the dorsal horn of the spinal cord. (Fig.1). In both α-CGRP wild type and knockout mice, a dense network of SP-positive fibers was seen in the lumbar spinal cord dorsal horn (data not shown).

Responses to noxious stimuli

To determine the thermal nociceptive threshold, a cons-

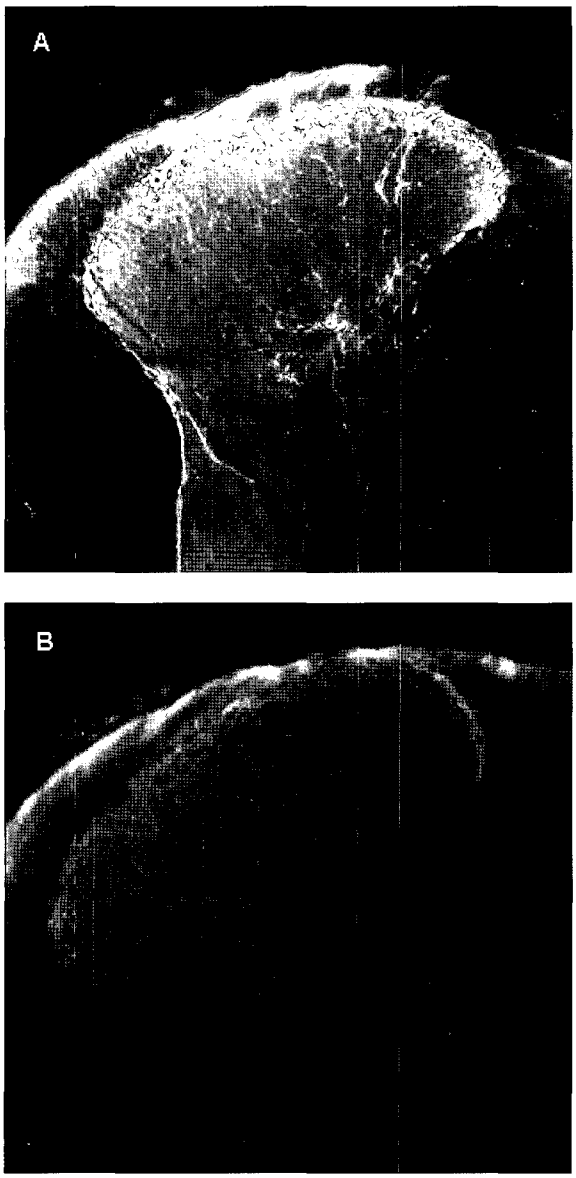

Fig. 1. Immunofluorescence of CGRP in the spinal cord of the wild type and mutant mice. (A) Superficial layers of the dorsal horn are stained with CGRP polyclonal antiserum in the wild-type mice. (B) Very faint CGRP-immunoreactivity is observed in the dorsal horn of the mutant mice.

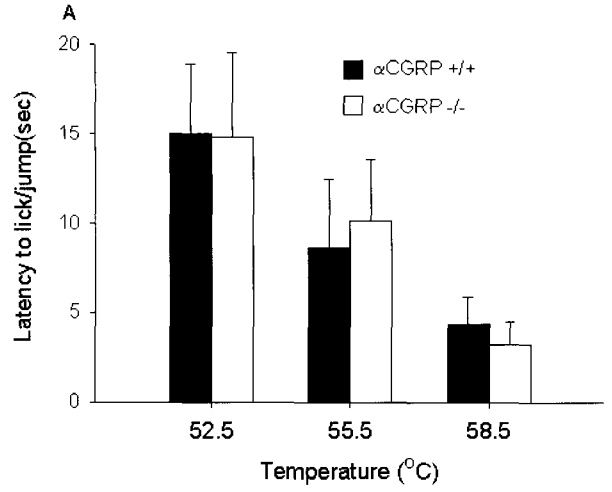

Fig. 2. Pain responses to different thermal stimulus intensities. (A) Licking/jump latency in the constant hot-plate assay (n=8). Wild type and mutant mice do not differ at 52.5, 55.5 and 58.5°C. (B) Lick/jump latency in the incremental hot-plate assay (n=8). Starting from room temperature (27°C), the unit was set to increase the plate temperature by 6°C/min. No significant difference was observed between wild type and the mutant mice during 5 test sessions.

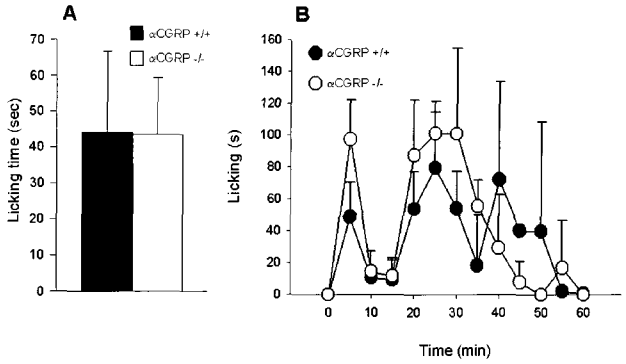

Fig. 3. Somatic pain responses to different chemical stimuli. (A) Chemical nociceptive responses (licking duration) induced by intraplantar injection of capsaicin. (B) The time course of chemical nociceptive responses induced by intraplantar injection of formalin. No significant difference was observed between wild type and the mutant mice.

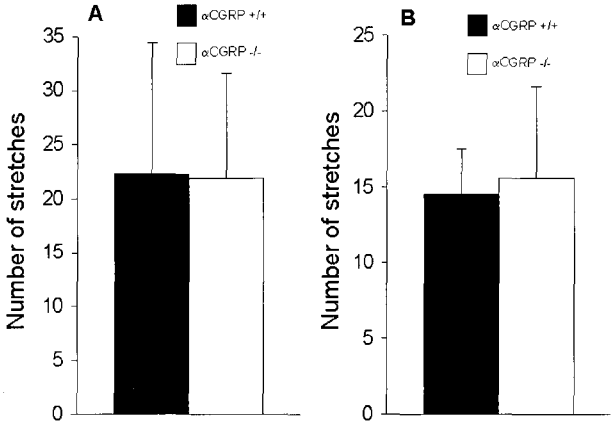

Fig. 4. Visceral pain responses to different chemical stimuli. (A) Visceral pain response (abdominal stretching) was assessed by intraperitoneal injection of dilute acetic acid, a stimulus associated with inflammation (wild type: n=19; mutant mice: n=14). (B) Visceral pain response was assessed by intraperitoneal injection of $MgSO_4$, a stimulus that produces pain without inflammation (n=13 for both wild type and mutant). There were no differences in the visceral pain responses.

tant hot plate test for Aδ-fiber mediated nociception and an incremental hot plate for slow conducting C-fiber mediated nociception were performed in wild type and mutant animals. The latency to jump or lick hindpaw showed no difference between wild type and mutant mice on 52.5, 55.5 and 58.5°C constant hot plate test (Fig. 2A). And no difference in latency to lick or jump on an incremental hot plate test was observed between wild type and mutant mice (Fig. 2B).

For chemical nociception studies, we injected capsaicin or formalin solution subcutaneously into hindpaw and measured hindpaw licking time. However, as seen in Fig. 3A, we found no difference between wild type and the null mutant in capsaicin induced nociception test during 10 min period. In the formalin-induced nociception test, we observed typical 2 phases of hind paw licking in both the wild-type and mutant mice, and there was no difference in licking durations between them, either (Fig. 3B).

To induce visceral pain, acetic acid or $MgSO_4$ were injected intraperitoneally and the number of abdominal stretches was counted as an index of pain response. Acetic acid induces visceral pain with inflammation and $MgSO_4$ evokes pain by directly stimulating nociceptive neurons. There was no difference in acetic acid and $MgSO_4$-induced abdominal stretch responses between the wild type and mutant mice (Fig. 4).

DISCUSSION

Because of anatomical distribution and activities on nociception, CGRP has been proposed to be a neurotransmitter

or neuromodulator for nociceptive transmission. Contrary to expected potential role of nociceptive transmission, results from our studies indicate that α-CGRP may not play a significant part in the nociceptive transmission: α-CGRP null mice had a similar profile to wild type mice in the thermal, chemical and visceral nociceptive behavioral tests.

In the present study, thermal nociception was examined by constant- and incremental-temperature hot plate test. CGRP and substance P are colocalized in the superficial dorsal horn and coreleased upon noxious thermal stimulations (Gibson et al, 1984; Skofitsch & Jacobowitz, 1985). Furthermore, intrathecal injection of CGRP can potentiate the effects of substance P (Wiesenfeld-Hallin et al, 1984; Biella et al, 1991), and PPT-A null mice show delayed latency to lick on 55.5°C hotplate test. Thus, it was expected that α-CGRP null mice show similar defect in the thermal nociceptive behavior as with PPT-A null animals. However, as seen in Fig. 2A, the thermal nociceptive behavior on 52.5, 55.5 and 58.5°C hotplates was comparable in both wild type and α-CGRP null mice.

The constant temperature hotplate test measures fast responding myelinated A δ-fibers. It has been reported that low rates of skin heating may evoke capsaicin-sensitive, C-fiber-mediated responses, whereas higher rates may recruit the involvement of capsaicin-insensitive, A δ-nociceptors (Yeomans et al, 1996). Since CGRP is mainly expressed in the unmyelinated C-fibers, we analyzed C-fibers response by slow incremental temperature hotplate test. As seen in Fig. 2B, the thermal nociceptive behavior on the incremental temperature hotplate was not significantly different between the wild type and α-CGRP null mice.

Nociceptive responses to chemical stimuli were assessed using capsaicin and formalin. Capsaicin injection to the hindpaw directly activates capsaicin-sensitive C-fibers. The duration of licking upon intraplantar injection of capsaicin was same between the two groups. We also found similar pain behavior in the first phase of the formalin test, which provides a different measure of the acute pain produced by direct chemical activation of C-fibers. There was no difference in pain behavior in the second phase, which has been proposed to result from a central sensitization process set up by activity during the first phase. This argues against a contribution of CGRP to central sensitization.

We tested mice by two models of acute visceral pain; one ($MgSO_4$) that induces an immediate pain response which is independent of inflammation, and the other (acetic acid) that is secondary to a delayed inflammatory response. In both models, we found no difference in pain behavior between the wild type and mutant mice.

These data suggest that endogenous α-CGRP is not required in mediating acute polymodal nociceptive information *in vivo* in contrast to SP. Or alternatively, the absence of α-CGRP in the null mice allowed compensatory mechanisms to develop. One of the candidate molecules for such compensatory mechanism would be β-CGRP. Despite the presence of α- and β-CGRP (3:1 ratio) in the DRG neurons, immunohistochemical studies on the spinal cord revealed no CGRP-ir in the α-CGRP null mice. However, it is quite possible that even a minimal amount of residual β-CGRP may be enough to carry out its role in the nociceptive transmission. Some molecules that have no structural similarity to CGRP may also substitute CGRP in a part of redundant pathway.

This lack of nociceptive phenotype in α-CGRP null mice is in contrast to the CGRP's proposed biological action suggested by its pharmacological studies. However, it should be recognized that α-CGRP null animals are not the same as the administration of pharmacological antagonists. With the genetic approach, there will be no functional receptors or ligands, as opposed to the administration of the antagonists, where only a proportion of receptors are likely to be blocked at effective doses. A further issue is the possibility that the knockouts alter aspects of higher central processing in a way that may modify pain reactions.

Changeux and colleagues found normal nociceptive response in the tail flick and hot plate tests in their α-CGRP null mice (Salmon et al, 1999). However, their knockout mice demonstrated decreased nociceptive behaviors in the capsaicin- and formalin-induced somatic chemical pain tests as well as acetic acid- and $MgSO_4$-induced visceral pain tests which were not observed in our α-CGRP null mice.

There is no simple explanation for the apparently inconsistent behavioral responses of different animals. The knockout approach is prone to a number of problems such as the genetic background of mice (Banbury Conference on Genetic Background in Mice, 1997), the developmental actions of deleted gene, and redundancy of function and compensatory changes, as well as the sensitivity, specificity, and reproducibility of the behavioral tests used to measure the phenotype of the animals. All of the above may contribute: For instance, there is a growing appreciation of enormous strain differences in the response of mice to noxious stimuli and analgesic drugs. Our α-CGRP knockout mice strain (C57/BL6) is different from Changeux's α-CGRP knockout strain (129/sv$\times$C57/BL6). Their mice have not been backcrossed to a point where genetic stability is present, therefore, the diverse hybrid genetic background of each animal raises a possibility that differences in modifier genes may alter the phenotype, independent of mutation. Thus, the animals and the tests used are not directly comparable. It would be helpful if all animals - knockouts and wild types- could be compared in a single laboratory.

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