NMDA Receptor Involvement in Neuroplastic Changes Induced By Neonatal Capsaicin Treatment in Trigeminal Nociceptive Neurons

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Chiang, Chen Yu, James W. Hu, and Barry J. Sessle. NMDA receptor involvement in neuroplastic changes induced by neonatal capsaicin treatment in trigeminal nociceptive neurons. J. Neurophysiol. 78: 2799–2803, 1997. This study examines whether 1) the neonatal loss of C-fiber afferents results in neuroplastic changes in the mechanoreceptive field (RF) properties and spontaneous activity of nociceptive neurons in trigeminal subnucleus caudalis (medullary dorsal horn) of adult rats, and that 2) N-methyl-D-aspartic acid (NMDA) receptor mechanisms are involved in these neuroplastic changes. Compared with vehicle-treated (i.e., control, CON) rats, capsaicin-treated (CAP) rats showed a marked increase in neuronal spontaneous activity and RF size per se, but these neuroplastic changes could be significantly reduced by MK-801 (1 mg/kg, iv), a noncompetitive NMDA receptor antagonist; RF size and spontaneous activity remained unchanged in CON rats after MK-801 administration and in CAP rats after vehicle (saline, iv). Administration of 7-chlorokynurenic acid intrathecally (5 µg/10 µl), an antagonist of strychnine-insensitive glycine binding sites on the NMDA receptor, also significantly reduced neuronal RF size and spontaneous activity in CAP rats, but not in CON rats. These data provide evidence that C-fiber afferents play a role in shaping the properties of nociceptive neurons and that the neuroplastic changes involve NMDA receptor mechanisms.

INTRODUCTION

Capsaicin administered to neonatal rats (CAP rats) results in destruction of up to 90% of C-fiber afferents with only a minimal effect on myelinated afferents (see Buck and Burks 1986; Fitzgerald 1983; Holzer 1991). In adulthood, CAP rats also show a considerable reduction in substance P and somatostatin associated with C-fiber afferents, a marked reduction in nociceptive responses to noxious thermal and chemical stimuli, and impairment of neurogenic plasma extravasation evoked by cutaneous application of mustard oil, a C-fiber excitant and inflammatory irritant. The finding that expansion of the mechanoreceptive field (RF) of both nociceptive and nonnociceptive neurons occurs in CAP rats has led to suggestions that, in addition to their role in nociception, C-fiber afferents might also be involved in shaping the RF properties of somatosensory neurons in the central nervous system (Kwan et al. 1996; Wall et al. 1982). The neurochemical mechanisms associated with these neuroplastic changes have received little attention. N-methyl-D-aspartic acid (NMDA) receptor mechanisms, however, appear to be involved in a variety of neuroplastic changes, including nociceptive neuronal hyperexcitability and RF expansion (see Dubner and Basbaum 1994; Urban et al. 1994; Woolf 1991). Therefore the aims of the present study were to test whether 1) the neonatal loss of C-fiber afferents results in neuroplastic changes in the RF properties and activity of nociceptive neurons in trigeminal (V) subnucleus caudalis (medullary dorsal horn) of adult rats and 2) that NMDA receptor mechanisms are involved in these neuroplastic changes. Preliminary data have been presented in abstract form (Chiang et al. 1995; Sessle et al. 1995a).

METHODS

This study was carried out in 52 male Sprague-Dawley rats. The rats were injected subcutaneously after 36–48 h of birth either with capsaicin (50 mg/kg, Sigma, dissolved in saline containing 10% Tween 80 and 10% ethyl alcohol, n = 30) or with vehicle only (i.e., control, CON rats, n = 22) (Gamse et al. 1980; Hammond and Ruda 1991; Kwan et al. 1996); both groups were studied electrophysiologically 2–4 mo later. The effectiveness of neonatal capsaicin treatment in markedly reducing the number of C-fiber afferents was assessed by spectrophotometric analysis of the plasma extravasation of injected 1% Evan’s blue dye (20 mg/kg, iv). The Evan’s blue was infused into the CAP or CON adult rat after termination of neuronal recording (see next paragraph). Extravasation was induced by cutaneous application of 20% mustard oil (allyl-isothiocyanate, BDH, Toronto, Canada; diluted in mineral oil; 0.02 ml) to the shaved skin of the left hindlimb. Then, 20 min later this hindlimb skin, as well as the analogous part of the contralateral hindlimb, was excised and the amount of extravasated Evan’s blue was determined spectrophotometrically (Gamse et al. 1980; Kwan et al. 1996). We also compared RF and response changes of some caudalis nociceptive neurons in CAP versus CON rats after 20% mustard oil was applied on their cutaneous RF.

The methods used for animal preparation and anesthesia, stimulation, and neuronal recording and classification were similar to those detailed previously (Chiang et al. 1994; Hu 1990). Briefly, rats were anesthetized with urethan (1 g/kg ip) ± chloralose (50 mg/kg, ip), immobilized with gallamine triethiodide, and artificially ventilated. Percentage expired CO2, heart rate, and rectal temperature were maintained at 3.5±4.5%, 330±420 beats/min, and 37.0±37.5°C, respectively. Single neuronal activity was recorded extracellularly from histologically confirmed sites in V subnucleus caudalis. A wide range of graded mechanical stimuli and noxious radiant heat (51–53°C) was used to classify units according to previously outlined criteria (Hu 1990) into wide dynamic range (WDR), nociceptive-specific (NS) (see results), or low-threshold mechanoreceptive (LTM) neurons or primary afferents (not studied further). Mechanical stimuli included a hair brush (<0.005 N), a blunt probe for application of low (<0.2 N) and high (2.0–5.0 N) forces, and calibrated forceps for pinching the RF skin (2.0–3.0 N). To test for possible NMDA mechanisms, two antagonists (or their vehicle, as control) were used. Either (−)-MK-801 (Research Biochemicals, Natick, MA), a noncompetitive blocker of NMDA receptor–ion channel, was administered systemically (1 mg/kg in saline, iv) or else 7-chlorokynurenic acid (7-CK; Research Biochemicals, Natick, MA), an antagonist of the strychnine-insensitive glycine-binding site on the NMDA receptor, was applied to the medullary surface overlying subnucleus caudalis.
RF size was 5.8 neonomous RF properties similar to those described previously in tion with the age of the animal (Yu et al. 1993). This was done 5 min before drug administration, and thereafter at 5-min intervals for the first 10 min and at 10-min intervals for the subsequent 50-min observation period. Because the tactile-sensitive area of the RF of WDR neurons in CAP rats often had an unclear boundary (see RESULTS), only the pinch-sensitive area of the RF was studied in detail. The RF areas were measured by a computer-aided device (SigmaScan, Jandel, CA). Any changes in RF area after completion of either antagonist or vehicle administration were expressed as a percentage relative to the control value (100%). Spontaneous activity of caudalis nociceptive neurons was regularly recorded for a 2-min period before each RF size determination. For statistical comparison of values (mean ± SE), one of the following was used: Student’s t-test, Mann-Whitney U test, Fisher’s exact probability test, two-way analysis of variance (ANOVA) with repeated measures, Friedman repeated measures ANOVA, or linear regression test. P < 0.05 was considered statistically significant.

RESULTS

The amount of Evan’s blue extravasation induced by application of mustard oil to the ipsilateral (left) hindlimb skin of CAP rats (0.95 ± 0.14 μg, SE, n = 20) was significantly less (P < 0.0001, t-test) than that of CON rats (7.07 ± 0.55 μg, n = 21). The Evan’s blue amount in contralateral skin samples that received no application of mustard oil was not significantly different between CAP (0.55 ± 0.06 μg) and CON (0.63 ± 0.08 μg) rats. Linear regression based on the age of individual CAP rats and their postmortem Evan’s blue value revealed no significant correlation (r = 0.331, P > 0.10). Similarly, there was no significant correlation with age in the neuroplastic changes induced in nociceptive neurons, so the CAP rats were pooled into a single group for analysis of the electrophysiological data.

A total of 108 caudalis neurons were functionally identified as nociceptive neurons in both CON and CAP rats: 67 as WDR neurons (CON, n = 27; CAP, n = 40), 41 as NS neurons (CON, n = 21; CAP, n = 20). Seventy-eight of these neurons were histologically retrieved in caudalis laminae IV–VI and 14 neurons in laminae I–II. Nociceptive neurons (7 WDR, 3 NS) tested in CON rats with mustard oil applied to their facial RF showed an immediate increase in their firing rate (peak response at 30 s: 1.169 ± 699% of the background level) that lasted for 3–5 min. Agreeing with Yu et al. (1993), we noted that the neuron’s RF size was also significantly increased throughout the 30-min observation period (peak expansion at 10 min; 192 ± 19% of control). In contrast, 11 of 13 nociceptive neurons (9 WDR, 4 NS) similarly tested in CAP rats showed no mustard oil–induced increase in spontaneous activity, and 9 of 11 neurons showed no RF expansion. These differences between CON and CAP rats were statistically significant (P < 0.001, t-test; P < 0.05, Mann-Whitney U test).

The 48 nociceptive neurons studied in CON rats had cutaneous RF properties similar to those described previously in the rat caudalis (Chiang et al. 1994; Hu 1990; Yu et al. 1993). Compared with CON rats, however, CAP rats showed a marked enlargement of RF size (Fig. 1, A and B). RF size was 5.8 ± 0.6 cm² (WDR, n = 40) and 2.6 ± 0.5 cm² (NS, n = 20) in CAP rats and 2.3 ± 0.4 cm² (WDR, n = 27) and 1.6 ± 0.3 cm² (NS, n = 21) in CON rats. This increase was statistically significant (P < 0.0001, Mann-Whitney U test) for WDR neurons. Many WDR neurons indeed had a pinch RF that encompassed two or three V divisions without discontinuity (24 of 40 in CAP rats vs. 8 of 27 in CON rats; P < 0.05, Fisher’s test) and that often encroached upon the contralateral face (8 of 40 in CAP rats vs. 1 of 27 in CON rats; P < 0.05, Fisher’s test). This enlargement of the pinch RF of WDR neurons had no significant correlation with the age of the animal (r = 0.05; P > 0.70). The tactile sensitive area of the RF of WDR neurons was also enlarged in CAP rats and sometimes comprised two or three discontinuous areas. However, the lack of a clearly delineated RF boundary did not permit accurate quantification of tactile RF area.

The incidence of spontaneous activity was comparable in CAP (37 of 40 and 14 of 20 for WDR and NS neurons, respectively) and CON (21 of 27 and 10 of 21 for WDR and NS neurons, respectively) rats. However, the mean spontaneous firing rate of WDR neurons in CAP rats (12 ± 4.8 spikes per 30 s) was significantly higher than that in CON rats (2.4 ± 1.1 spikes per 30 s; P < 0.05, Mann-Whitney U test). The mean rate for NS neurons in CAP rats (3.4 ± 1.9 spikes per 30 s) was also higher than that in CON rats (2.5 ± 1.1 spikes per 30 s), but this difference was not significant (Fig. 1C). The higher spontaneous firing rate of WDR neurons in CAP rats had no significant correlation with the age of the animal (r = 0.13; P > 0.30). There was no indication that the increases in RF size and spontaneous activity were related to the location (laminae I–II or V–VI) of the neurons tested.

A profound reduction in RF size occurred in all eight WDR and one NS neurons tested with MK-801 (1 mg/kg iv) in CAP (MK-801/CAP) rats. This reduction peaked at
10 min and remained around this level throughout the 60-min observation period. No notable reduction of neuronal RF size occurred in six WDR and three NS neurons tested with MK-801 in CON (MK-801/CON) rats or in five WDR and three NS neurons tested with the vehicle saline (SAL) in CAP (SAL/CAP) rats (Fig. 2A and Table 1). A two-way ANOVA with repeated measures demonstrated that there was a significant difference ($P < 0.002$) among the three groups; a post hoc analysis revealed a significant difference between MK-801/CAP group and the other two groups. A significant interaction was found between groups and values at different times ($P < 0.02$) and a post hoc analysis revealed significant differences among preinjection values and those at 10, 20, and 60 min postinjection time-points in the MK-801/CAP group ($P < 0.05$, Bonferroni method) but no significant differences were found in the MK-801/CON or SAL/CAP groups (see Fig. 2A).

MK-801 also markedly reduced the spontaneous firing rate in all nine neurons tested in CAP rats (Table 1). A Friedman repeated measures ANOVA revealed that there were significant differences in spontaneous firing rate between the MK-801/CAP group and the other two groups ($P < 0.05$). The spontaneous firing rate of nine nociceptive neurons in the MK-801/CAP group was significantly higher ($P < 0.05$, Dunn method) before MK-801 administration than the firing rate 10 min after drug administration. Neither MK-801 administered to CON rats nor saline administered to CAP rats produced any significant changes in spontaneous activity (Table 1).

As shown in Fig. 2B, 7-CK (5 µg/10 µl it) also produced a profound decrease in RF size of four WDR and one NS neurons tested in CAP (7-CK/CAP) rats. This reduction peaked at 5–10 min after drug application to caudalis and returned to initial levels by 40 min. The same dose of 7-CK resulted in no changes to neuronal RF size of two WDR and three NS neurons tested in CON (7-CK/CON) rats. A two-way ANOVA with repeated measures revealed significant differences between 7-CK/CAP and 7-CK/CON groups ($P < 0.005$) as well as an interaction between groups and values at different times ($P < 0.0001$). In addition, the differences between these two groups in the mean values at postapplication times of 5, 10, and 20 min were also significant ($P < 0.05$, Bonferroni method). Neuronal spontaneous activity was also significantly decreased by 7-CK in CAP rats ($P < 0.05$, Dunn method; see Table 1).

**DISCUSSION**

The dosage of capsaicin that we used (50 mg/kg) is generally accepted to be effective for depleting C-fiber afferents at the age (36–48 h postnatal) that it was administered (see Buck and Burks 1986; Fitzgerald 1983; Holzer 1991). We verified the effectiveness of neonatal capsaicin treatment by documenting the reductions in mustard oil–induced plasma extravasation and neuronal RF and activity changes in CAP rats. We have also shown in a recent electronmicroscopic analysis (Hu et al. 1996) that these rats also show >80% depletion of C-fiber afferents. Our findings that caudalis nociceptive neurons in CAP rats show neuroplastic alterations that include high spontaneous activity and RF expansion are consistent with previous observations of alterations in LTM neurons (Kwan et al. 1996; Sessle et al. 1995b; Wall et al. 1982) and in spinal nociceptive neurons (Wall et al. 1982). Our findings also provide further support to the view that C-fiber afferents may have a role in shaping the RF properties of somatosensory neurons in the CNS (Wall et al. 1982).

Anatomic findings that the central terminals of A-fiber afferents invade regions in the spinal dorsal horn of CAP rats where C-fiber afferents normally terminate (Nagy and Hunt 1983; Shortland et al. 1990) may explain our findings in CAP rats that the RF enlargement of WDR neurons that received both large- and small-fiber convergent inputs is more pronounced than that of NS neurons that received only...
small-fiber inputs. Hammond and Ruda (1991) have recently reported that neonatal capsaicin treatment induces reductions in substance P and especially in calcitonin gene-related peptide-like immunoreactivity and fluoride-resistant acid phosphatase activity in the rat spinal dorsal horn, and these changes are associated with decreases in mechanical, thermal, and chemical nociceptive sensitivity. The changes in calcitonin gene-related peptide-like immunoreactivity and fluoride-resistant acid phosphatase (but not substance P) activity originating in primary afferents gradually recover to vehicle-treated control levels by 8–12 wk of age in association with the recovery of mechanical and thermal sensitivity. However, Hammond and Ruda’s (1991) finding that the CAP rats remain insensitive to ophthalamic noxious chemical stimulation is consistent with our current data of the ineffectiveness of mustard oil application to facial skin in inducing caudalis neuronal activity or RF changes in CAP rats. Furthermore, the Hammond and Ruda (1991) data of age-related changes in early postnatal life, plus our findings of a lack of correlation with age for the caudalis neuroplastic changes in CAP rats tested at age 8–16 wk, suggest that permanent changes in RF properties induced by the depletion of capsaicin-sensitive C-fibers may have occurred in the early postnatal life (<8 wk) of our CAP rats.

Neuronal RF expansion and high spontaneous activity in the spinal and medullary dorsal horns have usually been attributed to the hyperexcitability of central neurons generated by excessive small-fiber inputs, especially from C-fiber afferents or by various pathological conditions (see Dubner and Basbaum 1994; Woolf 1991; Yu et al. 1993). Our findings, however, reveal that the loss of C-fiber afferents resulting from neonatal capsaicin treatment can also produce RF expansion and high spontaneous activity. Thus both enhancement and depletion of C-fiber afferent inputs appear to be conditions resulting in central neuronal hyperexcitability. It is also noteworthy that the increased C-fiber drive that may result from noxious stimuli, injury, and inflammation produces an NMDA-mediated nociceptive neuronal hyperexcitability and associated hyperalgesia (Dubner and Basbaum 1994; Mao et al. 1995; Ren et al. 1992, 1994; Woolf 1991; Yu et al. 1996). In contrast, C-fiber depletion induces an NMDA receptor–mediated enhancement of RF size and spontaneous activity (see RESULTS) that is not associated with hyperalgesia (Buck and Burks 1986; Fitzgerald 1983; Hammond and Ruda 1991; Holzer 1991). Our data suggest that NMDA receptor activation alone may not be sufficient to result in hyperalgesia and that additional factors (e.g., ongoing nociceptive afferent inputs) are likely to be required for the development of hyperalgesia.

Our findings that MK-801 administration did not affect nociceptive neuronal RF size in CON rats are consistent with previous studies (Ren et al. 1992). The finding that CAP rats showed a long-lasting and significantly depressive effect of MK-801 on both RF size and spontaneous activity of nociceptive neurons appears consistent with the slow dissociation feature of the action of this noncompetitive, ion-channel blocker of the NMDA receptor (McDonald and Nowak 1991). Moreover, these data are supported by our additional findings that 7-CK can also significantly decrease RF size in CAP rats but not in CON rats, because 7-CK is a potent antagonist that blocks the glycine binding sites on the NMDA receptor (Dingledine et al. 1990; Johnson and Asher 1987). The greater depressive effect and shorter time course of 7-CK on RF size might be explained by a more potent pharmacological action of 7-CK (Kemp et al. 1988) than MK-801 or the different modes of drug delivery (it for 7-CK vs. iv for MK-801).

The high spontaneous activity and expanded RFs of neurons in CAP rats could be attributed to presynaptic alterations (e.g., enhancement of afferent inputs to the neurons and increased neurotransmitter release) or ion changes in the local environment of the neurons. They may also be explained by postsynaptic changes (e.g., membrane depolarization that removes the Mg$^{2+}$ blockade of the NMDA receptor–ion channel) or alterations in sensitivity of the NMDA receptor per se and subsequent intracellular second messenger cascades. With respect to the possible presynaptic changes, the basal release of excitatory amino acids including glutamate in the dorsal horn in CAP rats has, however, been found comparable to that in normal rats (Kanggra and Randic 1990; Skilling and Larson 1993). Data relevant to changes in extracellular concentrations of cations (e.g., potassium, calcium, and magnesium) in the spinal cord of CAP rats are not available. For the possible postsynaptic mechanisms, activation of the NMDA receptor produces a marked increase in intracellular Ca$^{2+}$ concentration that would facilitate activation of protein kinase C and, as a consequence, the phosphorylation of the NMDA receptor in turn could

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**Table 1. Summary of RF size and spontaneous activity of caudalis nociceptive neurons before and 10 min after MK-801, saline, or 7-CK application**

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>RF Size, cm$^2$ Before</th>
<th>RF Size, cm$^2$ After</th>
<th>Spontaneous Activity, spikes/30 s Before</th>
<th>Spontaneous Activity, spikes/30 s After</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-801 experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK-801/CON</td>
<td>9</td>
<td>4.27 ± 1.23</td>
<td>4.59 ± 1.39</td>
<td>1.63 ± 1.18</td>
<td>1.48 ± 0.96</td>
</tr>
<tr>
<td>MK-801/CAP</td>
<td>9</td>
<td>5.44 ± 1.03</td>
<td>3.72 ± 0.58*</td>
<td>5.5 ± 3.16</td>
<td>0.41 ± 0.24</td>
</tr>
<tr>
<td>Saline/CAP</td>
<td>8</td>
<td>4.62 ± 1.21</td>
<td>4.72 ± 1.31</td>
<td>4.66 ± 2.25</td>
<td>4.4 ± 2.07</td>
</tr>
<tr>
<td>7-CK experiment</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>7-CK/CON</td>
<td>5</td>
<td>3.58 ± 0.82</td>
<td>3.25 ± 0.98</td>
<td>1.56 ± 0.83</td>
<td>0.38 ± 0.24</td>
</tr>
<tr>
<td>7-CK/CAP</td>
<td>5</td>
<td>5.53 ± 0.89</td>
<td>1.48 ± 0.39*</td>
<td>3.81 ± 1.33</td>
<td>0.25 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, number of nociceptive neurons included in the analysis. RF, mechanoreceptive field. * Significant differences between values before and after either MK-801 iv or 7-CK local application as determined by two-way repeated measures analysis of variance (ANOVA) with Bonferroni methods ($P < 0.05$). † Significant differences between values before and after either MK-801 iv or 7-CK local application as determined by Friedman repeated measures ANOVA on ranks with Dunn methods ($P < 0.05$).
result in removal of the Mg\(^{2+}\) blockade in the NMDA receptor–ion channel (Chen and Huang 1992). Under these conditions, even small amounts of endogenous glutamate/aspartate released from primary afferents, supraspinal descending pathways, and/or dorsal horn local interneurons may activate the NMDA receptor (Mao et al. 1995; Urban et al. 1994). Findings that the NMDA receptor in the dorsal horn is selectively activated by small-diameter capsaicin-sensitive fibers (Urban and Dray 1992) also raise the possibility that C-fiber depletion is associated with a denervation-type supersensitivity of the NMDA receptor. Indeed, neonatal treatment with capsaicin can significantly delay the developmental decline of the NMDA-induced increase in intracellular free Ca\(^{2+}\) concentrations in dorsal horn neurons (Hori and Kanda 1994), thus suggesting that more NMDA receptors survive and/or are sensitized in CAP rats.

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