Plastic Changes in Nociceptive Transmission of the Rat Spinal Cord With Advancing Age

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IntroduCtion

Psychophysical investigations have shown that perceptual pain threshold rises slightly or even remains unchanged with advancing age (Harkins and Scott 1996). On the other hand, chronic pain conditions are more prevalent in senescent individuals (Brena and Bonica 1970; Ferrell et al. 1991; Harkins et al. 1988). Recent animal studies on neuropathic pain have revealed that partial peripheral nerve injury induces more severe or prolonged hyperalgesia in aged rats as compared with young rats (Kim et al. 1995; Novak et al. 1999). The previous studies on age-related changes in morphology and conduction velocity of unmyelinated fibers, and neuropeptides in spinal ganglion cells (Bergman et al. 1999; Fundin et al. 1997; Ochoa and Mair 1969; Samorajski 1974; Sato et al. 1985) have indicated that aging does not affect peripheral nociceptive systems to a great degree. However, little is known about the response properties of neurons in nociceptive pathways in the CNS of the aged rats. Therefore we investigated age-related alterations in activity of spinal dorsal horn nociceptive neurons. To elucidate the mechanisms underlying this change and the outcome of this alteration, we further examined changes in descending control systems and behavioral responses to noxious thermal stimulation.

The results demonstrate an increased dorsal horn excitability and impaired descending modulation in the aged rats. These findings suggest that the aging process may affect central nociceptive pathways.

METHODS

The experiments were performed on male Fischer 344/DuCrj rats in two age groups: 7–13 mo old (adult, body weight: 408.8 ± 15.3 g, n = 26) and 29–34-mo old (aged, 366.6 ± 44.3 g, n = 25). The rats were raised under pathogen-free conditions and fed ad libitum. They were housed three per cage and maintained on a 12:12 light-dark schedule (lights on at 6:00 am) at 22°C. The mean survival time for the rats in the same colony is about 28 mo. Behavioral tests were done on 21 adult and 20 aged rats. Physiological experiments of dorsal horn neurons were performed using adult (n = 17) and aged (n = 15) rats that had been used for the behavioral tests. Five adult and five aged rats were perfused for immunohistochemical observation of fibers in the dorsal horn. The study was approved by the Animal Experimentation Committee at Osaka University Faculty of Dentistry and at the Tokyo Metropolitan Institute of Gerontology. The animals were treated according to the guidelines of the International Association for the Study of Pain (Zimmermann 1983).

Behavioral test

Nociceptive behavior was quantified by determining paw withdrawal latency to noxious heat stimulation of the hind paw. The animals were subjected to the behavioral test after 2 wk of handling. The radiant heat stimuli were applied to the plantar surface of the hind paw through a glass plate on which the animals stood (Hargreaves et al. 1988). The paw withdrawal latency to heat stimulus was measured four times at a minimum interval of 15 s in the afternoon (1:00–4:00 pm). This was repeated three to five times in different days. Furthermore, the occurrence of the paw licking was assessed following heating of the paw. The room temperature was maintained at 25°C during testing.
Recording activities of dorsal horn nociceptive neurons

ANIMAL PREPARATION. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and the trachea and left external jugular veins were cannulated to allow artificial respiration and intravenous administration of drugs, respectively. Anesthesia was maintained with halothane (2–3%) mixed with air during surgery. The rats were mounted on a stereotaxic frame, the L5-L6 spinal cord was exposed, and a mineral oil pool was made with the skin flaps surrounding the laminectomy. After surgery, anesthesia was maintained throughout the experiment by continuous inhalation of halothane (1–2%) mixed with oxygen. During recording sessions, the rats were immobilized with pancuronium bromide (1 mg · kg⁻¹ · h⁻¹ iv) and ventilated artificially. The expired CO₂ concentration was monitored and maintained between 3.0 and 4.0%. Rectal temperature was maintained at 37–38°C by a thermostatically controlled heating pad (FHC), and the electrocardiogram was monitored. Blood pressure was measured every 30 min indirectly from the tail and kept at 90–120 mm Hg during the experiments. If the heart rate or blood pressure increased after mechanical or thermal stimulation of the receptive fields, the percentage of halothane was increased (2–3%).

STIMULATION AND RECORDING. Enamel-coated tungsten microelectrodes (impedance = 10–12 MΩ, 1,000 Hz) were advanced into the spinal dorsal horn at the levels L₄ to L₅ in 1-μm steps. Spinal dorsal horn neurons were searched for by applying mechanical stimulation (pressure or brush) to the skin of the hip and leg region. When a single neuron was isolated, the responses to mechanical stimulation of the foot were carefully examined and the receptive field was mapped. Then graded mechanical stimuli (brushing with a camel brush, pressure produced by a large arterial clip and pinch produced by a small arterial clip) were applied to the most sensitive areas of the receptive fields. Each neuron was classified either as a wide-dynamic-response (WDR) neuron that responded to both noxious and nonnoxious stimuli and increased its firing frequency as stimulus intensity increased or as a nociceptive-specific (NS) neuron that responded exclusively to noxious mechanical stimulation of the receptive fields. After characterization with mechanical stimuli, the response to thermal stimuli were further examined by heating and cooling the most sensitive area of the mechanical receptive field. Before application of the thermal stimulus to the receptive field, the surface temperature was adapted to 38°C for 180 s. Skin heating and cooling ranged 42–55°C and 10–30°C, respectively, and lasted 30 s. The rate of temperature change was set at 10°C/s. The thermal stimuli were applied every 210 s to avoid sensitization of peripheral nociceptors (Beitel and Dubner 1976). The tip of the thermal probe was 5 mm in diameter. Neuronal activity was fed into a tape recorder (bandwidth DC to 20 kHz) for subsequent analysis. After evaluating the response properties of spinal dorsal horn neurons, lesions were made at the recording site by passing negative DC of 100 μA for 10 s for histological identification of the recording site.

To produce spinal block, additional laminectomy was performed at the thoracic (T₄) level. After examining response characteristics of spinal dorsal horn neurons to peripheral stimulation, 10% lidocaine was applied to the spinal cord at the T₄ level. The action of the local anesthetic on bulbospinal transmission was verified by monitoring the field potential elicited by electrical stimulation of the rostroventral medulla and recording from the dorsal surface of the L₅ spinal cord. The responsiveness of dorsal horn nociceptive neurons were re-examined during and after washing out of lidocaine.

HISTOLOGICAL CONFIRMATION OF RECORDING SITE. At the conclusion of the experiment, the rats were overdosed with pentobarbital sodium (100 mg/kg) and perfused transcardially with 50 ml 0.01 M PBS (pH 7.4) followed by 10% formalin in 0.1 M phosphate buffer. The spinal cord was removed and placed in cold fixative for a few days, then transferred to cold phosphate-buffered 30% sucrose for 48 h. Serial sections (50-μm thick) were cut along the path of the electrode penetration. The sections were counterstained with thionin for identification of the recording sites. Camera lucida tracings of the recording sites were drawn at ×400 magnification with a drawing tube.

DATA ANALYSIS. The waveform of single or multiple neuronal activity was analyzed off-line. The waveform of each neuron was identified using spike 2 microcomputer software (CED). Peristimulus time histograms (binwidth = 1 s) were generated in response to each stimulus. Background discharges were first recorded for 10 s before application of the mechanical or thermal stimulus and they were subtracted from the neuronal responses during analysis. Stimulus-response (S-R) functions of each nociceptive neuron were obtained in response to the mechanical (brush, pressure, pinch) or thermal (42–45°C) stimuli. The mechanical or thermal stimulation of the receptive fields was considered to have induced an effect when the peak firing frequency at 5 s after mechanical and 30 s (1 trial for each neuron with 180-s intervals) after thermal stimulation differed from the mean background discharge rate by ±2 SD. The receptive fields of all neurons were drawn to scale on standard diagrams of a rat leg. Areas of the receptive fields were calculated using image analysis software (National Institutes of Health image 1.61).

Immunohistochemistry for serotoninergic and noradrenergic fibers in the dorsal horn

Rats were overdosed with pentobarbital sodium and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The lumbar spinal cord was dissected and post fixed in the same fixative at 4°C overnight, followed by immersion in 20% sucrose in 0.1 M phosphate buffer at 4°C for cryoprotection. After a few days the tissues were frozen in powdered dry ice and cut transversely with a cryostat at 30 μm. Sections collected from the aged and adult animals were processed for serotonin (5-HT) or dopamine β-hydroxylase (DBH) immunohistochemistry. Sections were incubated in 1.5% normal goat serum (NGS, Sigma, St Louis, MO) for 24 h at 4°C and then in rabbit anti-5-HT polyclonal antisemur (1:50000; DiaSorin) or anti-DBH polyclonal antisemur (1:1000; Eugene Tech International) for 48 h at 4°C with agitation. The sections were further incubated in biotinylated goat anti-rabbit immunoglobulin G (1:200; Vector Labs, Burlingame, CA) for 1 h at 37°C and peroxidase-conjugated avidin-biotin complex (1:200; ABC, Vector Labs) for 2 h at 4°C. To develop the ABC reaction product, the sections were incubated in 0.035% 3,3′-diaminobenzidene-tetra HCl (DAB; Sigma), 0.2% nickel ammonium sulfate, and 0.05% peroxide in 0.05 M Tris-buffer (TB, pH 7.4). Between incubations, sections were rinsed three times by 0.01 M PBS for 15 min. Every other section was counterstained with 1.0% thionin or cresyl violet.

For measuring areas occupied by 5-HT and DBH-ir fibers, areas (100 × 200 μm) in the superficial (I-II) and intermediate (III-IV) laminae of the middle mediolateral portion of the L₅ dorsal horn were processed using a microcomputer system (National Institutes of Health image 1.61).

Statistical analysis

Statistical analysis was performed by using ANOVA followed by post hoc Fisher’s protected least-significant-difference (PLSD) or Scheffe F tests. Student t-test was also used as appropriate. Differences were considered significant at P < 0.05. Results are presented as means ± SE.

RESULTS

Nocifensive behavior

The aged (29-mo old) rats exhibited significantly shorter paw withdrawal latency [6.70 ± 1.08 (SD) s, n = 20] to noxious thermal stimulation of the hind paw as compared with
adult (7-mo old) rats (7.34 ± 0.86 s, n = 21; t-test, \( P < 0.05 \)) (Fig. 1A), suggesting a reduction of nociceptive threshold in aged animals. On the other hand, the occurrence of paw licking was less in the aged animals (51.7 ± 21.6% total trials, \( n = 20 \)) as compared with that in the adult rats (88.4 ± 9.2% total trials, \( n = 21 \); t-test, \( P < 0.001 \); Fig. 1B).

**Excitability of nociceptive neurons in the aged rats**

A total of 104 nociceptive neurons in the spinal dorsal horn (25 WDR and 13 NS neurons in 10 adult rats; 47 WDR and 19 NS neurons in 11 aged rats) were analyzed. Figure 2 illustrates the distribution of the histologically identified recording sites of WDR and NS neurons in the dorsal horn. Most NS neurons were located in the superficial laminae, and WDR neurons were in the superficial and deep laminae of the lumbar 4–5 spinal cord. No difference in distributions of NS and WDR neurons was observed between aged and adult rats. Figure 3, A–F, illustrates a typical WDR neuron recorded from a 32-mo old rat. The neuron was located in the superficial dorsal horn (Fig. 3B), and the high-threshold area of the receptive field was relatively large, extending from the thigh to hind paw (Fig. 3A). This neuron exhibited graded responses to mechanical stimulation of the low (Fig. 3C) and high (Fig. 3D) threshold areas of the receptive field and to graded heat stimulation (Fig. 3E), indicating coding of stimulus intensity. The neuron also responded to cooling of the receptive field and graded its responses following decreases in stimulus temperature (Fig. 3F).

We found no difference in responses of dorsal horn nociceptive neurons to graded mechanical stimulation between the adult and aged rats (data not shown). The relationship between responses and intensity of heat stimuli is shown in Fig. 4. A repeated-measure ANOVA for the responses indicated a significant between (age) effect \([F(1,120) = 5.160, P < 0.05]\) and a significant within (temperature) effect \([F(6,120) = 20.888, P < 0.0001]\). A significant age-by-temperature interaction was also found \([F(6,120) = 2.463, P < 0.05]\). The responses to cooling were not significantly different between the aged and adult rats (data not shown). However, 28% (13/47) of WDR neurons recorded from the aged rats, whereas only 12% (3/25) of neurons in adult rats, responded to both heating and cooling \((\chi^2 \text{ test, } P < 0.05)\). The responses of dorsal horn nociceptive neurons to 50 and 54°C heating of the skin in aged animals were significantly greater as compared with those of adult animals (Fisher’s PLSD, \( P < 0.05 \)). The mean receptive field size of WDR neurons in the aged animals was significantly larger for the high-threshold area but smaller for the low-threshold area when compared with the adult animals (Fig. 5).

The receptive fields of NS neurons were significantly larger in the aged animals than in the adult animals as well (Fig. 5). Background activity of WDR and NS neurons in aged animals \((3.67 ± 0.76 \text{ and } 5.28 ± 1.22 \text{ imp/s, respectively})\) was significantly higher than that of adult animals \((0.93 ± 0.31 \text{ and } 0.43 ± 0.21, \text{ respectively}; \text{Fig. } 6A)\). We also noticed that afterdischarges occurred more frequently in the aged animals following the cessation of the noxious stimulus (Fig. 6B).

**Effect of spinal block on the activity of nociceptive neurons**

To assess the contribution of descending pathways to increased activity of dorsal horn neurons in aged rats (4 rats), a
A reversible spinal block was produced by application of a local anesthetic, lidocaine (10%, 0.1 ml), onto the dorsal surface of the thoracic cord (T1) that was rostral to the recording site. In the adult rats (7 rats), neuronal responses to heat stimulus were significantly increased during the spinal block (Fig. 7, A and B). A repeated-measure ANOVA showed a significant within (stimulus temperature) effect \( F(3,60) = 27.744, P < 0.0001 \) and between (block) effect \( F(1,60) = 7.904, P = 0.02 \). A significant spinal block-by-temperature interaction was also found \( F(3,60) = 3.934, P = 0.02 \). These results indicated that the net effect of the descending system was inhibitory in the adult rats. On the contrary, the spinal block did not have an effect on neuronal responses in the aged rats (Fig. 8 A) except there was a reduced response to 50°C heating during the block (Fig. 8 B). No significant between (block) effect was found for neurons in the aged rats \( F(1,96) = 0.893, P > 0.3 \); Fig. 7B), although the spinal block-by-temperature interaction was significant \( F(3,96) = 5.490, P < 0.002 \). Thus there appeared to be a reduced net descending inhibition or an increased descending facilitation, in aged rats.

Background activity was significantly increased in both aged and adult animals during spinal block (Fig. 9A). Afterdischarges following noxious stimulation were significantly increased in the adult rats (Fig. 9B).

**Changes in dorsal horn histochemistry in aged rats**

Serotonin and norepinephrine (NA) are two major neurotransmitters mediating descending modulation of nociception at the spinal level (Willis and Coggeshall 1991). We further compared the immunohistochemical distribution of 5-HT and DBH, an essential enzyme in NA synthesis, in the spinal cord of aged and adult rats. A large number of 5-HT and DBH-immunoreactive (ir) fibers were observed in both superficial (laminae I-II) and deep laminae (laminae III-IV) of the dorsal horn of the adult animals (Fig. 10, A and E), whereas they were much sparser in aged animals (Fig. 10, B and F). The total area occupied by 5-HT-ir fibers was significantly smaller in the aged animals than those in the adult rats (Fig. 10D). The areas occupied by DBH-ir fibers were significantly smaller in laminae I-II of the aged rats as compared with those of the adult rats (Fig. 10H). Furthermore, aberrant (i.e., swollen and/or meandering) 5-HT-ir and DBH-ir fibers were observed in the spinal dorsal horn of the aged animals (arrowheads in Fig. 10, C and G), suggesting degenerative changes of these fibers.

**DISCUSSION**

In the aged rat, there are fewer nerve fibers in the subepidermal plexus and fewer radiating calcitonin gene-related peptide (CGRP)/substance P (SP)/galanin-ir nerve endings in the epidermis (Fundin et al. 1997). There are minimal changes in the number and cross-sectional area of unmyelinated fibers in both human and experimental animals with aging (Ochoa and Mair 1969; Samorajski 1974). The axonal conduction velocity of unmyelinated fibers in the peripheral nerve remains unaltered in the aged rat (Sato et al. 1985). The population of primary afferent terminals containing...
SP and CGRP has been shown to be slightly decreased in the dorsal horn of the aged animals compared with young adults (Bergman et al. 1996). Because substance P is contained in the small-diameter fibers that transmit nociceptive information to the dorsal horn, a decrement of SP- and CGRP-ir terminals in the dorsal horn would be indicative of a deficiency in transmission of nociceptive information from primary afferent fibers to spinal secondary neurons in aged animals. Thus peripheral nociceptive mechanisms are likely to be unaltered or tend to decline slightly with advancing age rather than increasing in activity. Nevertheless the present experiments demonstrated that the responses to noxious thermal stimulation and background activity of dorsal horn nociceptive neurons in the aged rats were significantly higher as compared with adult animals.

Activity of spinal nociceptive neurons is modulated by descending pathways (Handwerker et al. 1975; Mayer and Price 1976). Such descending modulation includes both inhibitory and excitatory components (Mayer and Price 1976). In the present experiments, spinal cord block resulted in a significant increase in neuronal responses to noxious stimulation as well as background activity in adult rats. However, neuronal activity in the aged rats was not affected. These findings indicate that tonic descending inhibition declined in the aged rats. This is also consistent with previous findings using HPLC. The 5-HT content in the dorsal horn is less in the aged rats compared with the young rats (Goicoechea et al. 1997; Ko et al. 1997). Serotonergic and noradrenergic fibers originating in the brain stem are components of descending modulatory systems terminating in the dorsal horn (Gomes et al. 1999; Jones 1991; Jones and Gebhart 1988). Other studies support our findings of a decline in endogenous pain inhibitory systems in aged rats after tissue and nerve injury (Hamm et al. 1986; Novak et al. 1999). The balance between descending inhibitory and facilitatory systems is dynamically altered after nerve injury (Urban et al. 1999; Wei et al. 1999). The decline in descending inhibition in the aged rat could be considered as a functional compensation for a decreased activity and/or a loss of neurons in the periphery and the spinal cord. However, the present 5-HT and DBH immunohistochemical data show the decreased density and abnormal profiles of these fibers in the aged rat dorsal horn, indicating that a decline in descending inhibition is not just functional adaptation but rather due to degenerative changes in these neurons. The loss of descending inhibitory effects in aged animals will upset the dynamic balance after injury leading to a relative absence of descending inhibition and greater enhancement of spinal dorsal horn excitability as demonstrated in the present study. An imbalance of these modulatory systems may also be one mechanism underlying variability in acute and chronic pain conditions, especially those involving deep tissues such as muscle and viscera. For patients suffering from temporomandibular disorders, fibromyalgia, low back pain, or irritable bowel syndrome, the diffuse nature and amplification of persistent pain may in part be the result of such an imbalance.
It has been well documented that nociceptive neurons in the dorsal horn mediate the flexor reflex as well as nociception (Ellrich and Treede 1998; Wall et al. 1988; Woolf and Wall 1986). The increased activity in these neurons is very likely to contribute to a shorter withdrawal latency to noxious heat stimulation in the aged rats (Fig. 1A) (see also Crisp et al. 1994; Novak et al. 1999). Bergman and Ulfhake (1998) also examined the withdrawal behavior using a similar method and have reported longer response latency in the aged rats compared with the younger rats (see also Jourdan et al. 2000). The reason for the discrepancy between our present findings and their results is unclear. The present results coincide with the finding that pain tolerance is lower in the elderly (Schludermann and Zubek 1962). However, there are discrepancies between the motor response and pain sensation after noxious stimulation. In fact, we observed that the occurrence of licking was significantly lower in the aged rats as compared with the adult rats (Fig. 1B). It has been reported that both licking behavior and the paw withdrawal latency change with a similar time course following peripheral inflammation in the young animals (Hargreaves et al. 1988). These two responses share common neural pathways, although the higher CNS was more involved in producing the licking behavior than the paw withdrawal response (Chapman et al. 1985). This suggests that the differential effects of aging on paw withdrawal reflex and licking behavior may result from the differential effects of aging on the higher CNS and the spinal cord. The underlying mechanisms for these differential effects of aging on pain sensation require further study.

Our results indicate the complexity of the effects of aging on nociceptive pathways. In fact, despite the increased response to noxious thermal stimulation, no alteration of mechanical responses was found in the aged rats. Nevertheless, the afterdischarge following strong mechanical stimulus was increased. Furthermore after spinal block, the background activity of dorsal horn neurons was enhanced while the stimulus-response function to noxious stimuli unaltered. We know little about differences in processing thermal versus mechanical nociceptive information neither the difference between small myelinated versus unmyelinated fibers in the aged rat. We recently demonstrated that the wind-up of the flexor reflex was induced with longer stimulus intervals in aged rats than in adult rats (Kanda et al. 2001). However, the magnitude of the reflex response to noxious stimuli was unaltered (unpublished observation). These findings suggest that the direct and after effects of C-fiber activities on the spinal nociceptive pathways are regulated differently.

Another interesting finding in the present experiments is that the high- and low threshold portions of the receptive fields of

![Figure 7](http://jn.physiology.org/)

**FIG. 7.** Effects of spinal cord block on responses of dorsal horn nociceptive neurons in adult animals. **A:** examples of responses of a WDR neuron following graded heat stimulation of the hind paw before (intact), during (block), and after washing out of the lidocaine (recover) in adult rats (9-mo old). The efficiency of the local anesthetic block was verified by monitoring the field potential elicited by electrical stimulation of the rostroventral medulla and recording from the dorsal surface of the L5 spinal cord (insets; ↑, the onset of brain stimulation). **B:** stimulus-response functions of WDR neurons following graded heat stimulation of the receptive fields in the adult rats (7–9-mo old). *P < 0.05 (Fisher’s PLSD).

![Figure 8](http://jn.physiology.org/)

**FIG. 8.** Effects of spinal cord block on responses of dorsal horn nociceptive neurons in aged animals. **A:** examples of responses of WDR neurons following graded heat stimulation of the hind paw before (intact), during (block), and after washing out of the lidocaine (recover) in an aged rat (31-mo old). **B:** stimulus-response functions of WDR neurons following graded heat stimulation of the receptive fields in the aged rats (30–34 mo old). *P < 0.05 (Fisher’s protected least-significant-difference).
WDR neurons in the aged rats were differentially affected. The size of the peripheral high-threshold area of WDR neurons was significantly larger, whereas the size of the central, low-threshold area was smaller, in the aged rats than in the adult rats. After peripheral inflammation, dorsal horn nociceptive neurons show an expansion of the receptive fields that is explained by dorsal horn hyperexcitability (Hylden et al. 1989; Iwata et al. 1999). It is tempting to conclude that the expansion of high-threshold receptive fields of nociceptive neurons in aged rats also reflects plastic changes in central pain pathways. Our results suggest that a reduced descending inhibition may contribute to hyperexcitability, including the expansion of the receptive fields, of dorsal horn neurons in aged rats. Previous studies have shown that large-diameter myelinated fibers were more strongly affected by aging as compared with small-diameter nerve fibers (Ochoa and Mair 1969; Samorajski 1974). Because a WDR neuron receives input from both large and small primary afferents for its low-threshold receptive field and only input from small fibers for the high-threshold portion of the receptive field, it is likely that the differential changes in small and larger fibers contribute to alteration in the receptive field. These data also suggest that the total amount of innocuous information from the peripheral structures was decreased in aged animals.
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