The effect of chronic inflammation on dorsal horn nociceptive neurons in aged rats

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Abstract

To elucidate the effect of chronic inflammation on spinal nociceptive neurons in the elderly, we compared nocifensive behavior, peripheral inflammatory responses and spinal dorsal horn neuronal activities between the aged (29-34 months) and adult (7-12 months) male rats after injection of CFA into the hind paw. Aged rats exhibited a significantly lower mechanical paw withdrawal threshold before inflammation. However, after CFA injection mechanical allodynia developed in both adult and aged rats after CFA injection. The changes of foot temperature and thickness following CFA injection were greater and lasted longer in aged than adult rats. One hundred twenty-four wide dynamic range (WDR) neurons (aged: 59, adult: 65) and 26 nociceptive specific (NS) neurons (aged: 13, adult: 13) were recorded from the lumber spinal dorsal horn. NS neurons from the inflamed adult rats showed significantly higher responses to noxious mechanical stimulation than aged rats, while WDR neurons from inflamed adult and aged rats were similar. Background activity of WDR neurons from the adult rats increased after CFA, whereas WDR neurons of aged rats and NS neurons from either group were not. The afterdischarge followed by noxious mechanical stimulation was significantly greater for WDR neurons in both adult and aged rats, whereas no significant differences were observed in NS neurons. Two days after CFA, Fos expression increased similarly in aged and adult rats. Thus, the aged rats showed enhanced peripheral inflammatory responses to CFA injection with only a slight change in dorsal horn neuronal activity. Together with our previous finding that nociceptive neurons in aged rats exhibit hyperexcitability, these results suggest that the dorsal horn
nociceptive system becomes sensitized with advancing age and its excitability can not be further increased by inflammation.
Introduction

Previous studies have indicated that many physiological properties are different between aged and adult people (Hayflick 1998; Janssens et al. 1999; Ribera-Casado 1999). Specifically, the pain perception mechanism is strongly affected by age (Harkins and Scott 1996; Schludermann and Zubek 1962). It has been reported that the chronic pain conditions are more prevalent in senescent individuals (Brena and Bonica 1970; Ferrell 1991; Harkins et al. 1988) and it is very difficult to relieve chronic pain form senescent patients because of their physical conditions (Wijeratne et al. 2001; Corran et al. 1977). Some animal studies on neuropathic and chronic inflammatory pain revealed that partial peripheral nerve injury and chronic inflammation induce more severe or prolonged hyperalgesia in aged rats as compared to young rats (Kim et al. 1995; Novak et al. 1999). The expression profile of a number of neuropeptides is changed with advancing age in the peripheral and central pain pathways (Ko et al. 1997; Goicoechea et al. 1997).

We have studied physiological properties of nociceptive spinal dorsal horn neurons in aged rats, and reported that nociceptive neurons in the aged rats showed stronger excitability to noxious heat stimulation than that observed in the adult rats.
(Iwata et al. 2002). The background discharge frequency and the afterdischarges following strong mechanical stimulation of the receptive fields in aged rats were significantly higher compared to those in adult rats. This hyperexcitability of nociceptive neurons may be induced by a deficit in the descending inhibitory modulation system in aged rats, since the density of noradrenergic and serotonergic immunoreactive fibers in the dorsal horn was reduced in aged rats (Iwata et al. 2002). These findings suggest that the central nervous system is highly involved in pain modulation with advancing age.

It is known that peripheral inflammation induces various changes in nociceptive neurons in young adult rats. The responsiveness of medullary dorsal horn nociceptive neurons to mechanical and heat stimuli, background activity, afterdischarge and receptive fields are significantly enhanced after inflammation (Iwata et al. 1999). It seems that the changes we have seen in the aged rats without inflammation were similar to those reported for adult rats with peripheral inflammation. Inflammation, especially chronic inflammation prevails among the elderly. The chronic inflammation is known to produce a variety of changes in the peripheral, as well as central nervous system. The physiological responses to chronic inflammation may be different between aged and adult rats. However, the peripheral and central mechanisms of changes in pain pathways with advancing age were unknown.

Thus, the present study was undertaken to examine changes in the central nervous system induced by peripheral inflammation with advancing age. We compared nocifensive behavior, peripheral inflammation and alterations in activity of spinal dorsal
horn nociceptive neurons between the aged and adult rats after hind paw inflammation.
Materials and Methods

The experiments were performed on male Fischer 344/DuCrj rats in the two age groups: 29-34-months old (aged, neuronal recording experiments: 244 ± 11 g, n=13) and 7-12-months old (adult, neuronal recording experiments: 232 ± 9 g, n=15). The rats were raised under pathogen free conditions and fed ad libitum. They were housed 3 per cage and maintained on a 12:12 light-dark schedule (lights on at 6:00 am) at 22 °C. The study was approved by the Animal Experimentation Committee at Nihon University School 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).

Complete Freund’s Adjuvant (CFA) injection

Aged and adult rats were anesthetized with pentobarbital Na (50 mg/kg, i.p.). The inflammatory agent, CFA was suspended in an oil/saline (1:1) emulsion and a volume of 0.05 ml was injected into the left hind paw subcutaneously.

Behavioral test

In daily sessions, rats (aged: 245 ± 6 g, n=6, adult: 228 ± 4 g, n=5) were trained to stay in the plastic cage during mechanical stimulation of the hind paw with von Frey filaments (Stoelting, Wood Dale, LI, USA). The maximum intensity used in
this study was 28.2 grams for behavioral test. One day before CFA injection, von Frey hair mechanical stimulation was applied to the hind paw and the escape threshold was measured in aged and adult rats. After CFA injection, the mechanical escape threshold was measured every day. Quantitative mechanical stimuli were applied to the hind paw in ascending and descending orders to evaluate the escape threshold. Each von Frey filament was applied 5 times. When rats showed one escape response to a filament, the bending force of that filament was defined as the escape threshold intensity. The median threshold intensity was calculated from the values following two ascending and one descending trials.

**Measurement of the paw temperature and thickness**

The paw temperature and thickness were measured in naïve aged and adult rats for 3 days before CFA injection to obtain consistent baseline data. The paw temperature and thickness were measured daily after CFA injection into the left hind paw (age: 215 ± 9 g, n=5, adult: 283 ± 5 g, n=5).

1) **Measurement of the paw temperature**

The experiments were conducted under the thermo-neutral condition (room temperature 26 ± 0.5 °C). Rats were anesthetized with pentobarbital Na (50mg/kg, ip). Thirty min after the pentobarbital injection, rats were gently held and placed on the
platform. The skin temperature was measured by a computer-assisted infrared thermograph (Thermo tracer TH3100ME, NEC-SANEI, Japan). The area of thermographic recording covered the whole hind paw region.

2) Measurement of the paw thickness

Paw thickness was measured just after the measurement of paw temperature. The dorso-ventral thickness of the middle portion of the hind paw was measured using a micrometer caliper as illustrated in Fig. 3C.

Recording from dorsal horn nociceptive neurons

1) Animal preparation

Since the paw temperature and paw thickness peaked at 1-2 days after CFA injection into the hind paw, the recording and Fos protein immunohistochemical experiments were done at two days after CFA injection. Two days after CFA injection, rats (aged: 250±9 g, n=7, adult: 233±8 g, n=8) were introduced for extracellular recording experiments. Naïve rats without CFA injection (aged: 237±9g, n=6, adult: 231±10 g, n=7) were used for recording experiments as controls. Animals were anesthetized with pentobarbital Na (50 mg/kg, i.p.) 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 L3-6 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, rats were immobilized with pancuronium bromide (1 mg/kg/h, i.v.) and ventilated artificially. The expired CO₂ concentration was monitored and maintained between 3.0-4.0%. Rectal temperature was maintained at 37-38°C by a thermostatically controlled heating pad (ATB-1100, Nihon Kohden, JAPAN) and the electrocardiogram was monitored. If the heart rate increased after mechanical or thermal stimulation of the receptive fields, the percentage of halothane was increased (2-3%).

2) Stimulation and recording

Enamel-coated tungsten microelectrodes (impedance = 10-12 MΩ, 1000Hz) were advanced into the spinal dorsal horn at the L4 to L5 levels in 2 µm steps. Spinal dorsal horn neurons were searched for by applying mechanical stimulation (pressure or brush) to the skin or the hip and leg regions. 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 using von Frey filaments and pinch with small arterial clip were applied to the most sensitive areas of the receptive fields for 5 s. Each neuron was classified either as 1) a wide-dynamic-range (WDR) neuron that responded to both non-noxious and noxious mechanical stimuli and increased its firing frequency as stimulus intensity increased, or as 2) a nociceptive-specific (NS) neuron that responded exclusively to noxious mechanical stimulation of the receptive fields.
After characterization with mechanical stimuli, responses to thermal stimuli were further examined by heating the most sensitive area of the mechanical receptive fields. Before application of the thermal stimulus to the receptive field, the surface temperature was adapted to 38 °C for 180 seconds. The skin heating ranged 42-50°C and lasted 10 s. The rate of temperature change was set at 10 °C/s. The thermal stimuli were applied every 190 s to avoid sensitization of peripheral nociceptors (Beitel and Dubner 1976). The tip of the thermal probe was 10 mm in diameter. Neuronal activity was fed into a computer disk for subsequent analysis. After evaluating the response properties of spinal dorsal horn neurons, lesions were made at the recording site by passing negative direct current of 10 µA for 10 s for histological identification of the recording site. Two-three recording sites were chosen for making lesions, where nociceptive neurons were encountered.

3) **Histological confirmation of the recording site**

At the conclusion of the experiment, rats were overdosed with sodium pentobarbital (100 mg/kg) and perfused transcardially with 50 ml 0.01M PBS (pH 7.4) followed by 10% formalin in 0.1 M phosphate buffer. The spinal cord was removed, placed in cold fixative for a few days, and 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 400X magnification with a drawing tube.
4) Analysis of neuronal activities

The waveform of single neuronal activities was analyzed off-line. The waveform of each neuron was identified using spike 2 microcomputer software (CED, Cambridge, UK). Peristimulus time histograms (bin-width = 1s) 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 quantitative mechanical (1.2, 5.4, 15.1, 28.2 and 75.8g) and pinch or thermal (44-50 °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 10 s (one trial for each neuron with 180s intervals) after thermal stimulation differed from the mean background discharge rate by ± 2 S.D. 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 (NIH image 1.61).

Fos protein immunohistochemistry

At 2 days after the CFA injection, rats (aged: 223 ± 3 g, n=5, adult: 283 ± 7 g, n=5) were anesthetized with sodium pentobarbital (80 mg/Kg, i.p.) and perfused through the aorta with 500 ml 0.02 M phosphate-buffered saline (PBS, pH 7.4) followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4-5
spinal cords were removed and post-fixed in the same fixative for 3 days at 4 °C. The tissues were then transferred to 30% sucrose (w/v) in phosphate buffered saline for several days for cryoprotection. Thirty micrometer thick sections were cut with a freezing microtome and every fourth section was collected in PBS. Free-floating tissue sections were rinsed in PBS, 10% normal goat serum in PBS for 1 hour, and then incubated in rabbit anti-Fos (1:10000) for 24 hours at room temperature. Subsequently, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector Labs, Burlingame, CA, USA) for one hour at 37 °C. After washing, the sections were incubated in peroxidase-conjugated avidin-biotin complex (1:100; ABC, Vector Labs) for 4 hours at 37°C. After washing in 0.05M Tris Buffer (TB), the sections were incubated in 0.035% 3,3’-diaminobenzidine-tetra HCl (DAB, Sigma), 0.2% nickel ammonium sulfate, and 0.05% peroxide in 0.05M TB (pH 7.4). After washing in PBS, the sections were serially mounted on gelatin-coated slides, dehydrated in alcohols and cover slipped. The number of Fos protein-like immunoreactive (LI) cells in the L4-5 spinal dorsal horn was analyzed.

**Statistical analysis**

Statistical analysis was performed by using analysis of variance (ANOVA) followed by Dunnet test. Student t-test or Mann-Whitney’s U-test was also used as appropriate. Differences were considered significant at $p < 0.05$. Results are presented as means ± SEM.
Results

Nocifensive behavior

Before CFA injection, the paw withdrawal threshold was significantly lower in the aged rats than adult rats as illustrated in Fig. 1A (aged, ipsi: 4.89 ± 0.24g, contra: 4.89 ± 0.29g) and B (adult, ipsi: 8.93 ± 0.33g, contra: 9.27 ± 0.56g) (p<0.01). After CFA injection into one hind paw, mechanical withdrawal threshold became significantly lower in both aged and adult rats (aged, ipsi: 3.27±0.30 g, contra: 6.11±0.25g, adult, ipsi: 5.60 ± 0.21g, contra: 14.61±0.33g at 1 day after CFA injection). The reduction of the threshold persisted during the observation period. We also observed a slight increase in paw withdrawal threshold on the contralateral side to CFA injection in both aged and adult rats as illustrated in Fig. 1. The paw withdrawal threshold of the inflamed paw was not significantly different between the aged and adult rats after CFA injection, except at 1 day after CFA (Fig. 1). Since the paw withdrawal threshold of the adult rats was significantly higher than that of the aged rats, these results suggest that the nocifensive behavior of the adult rats were more strongly affected by inflammation.
Changes in paw temperature and thickness following CFA injection

Following an injection of CFA into the hind paw, the surface temperature of the injected paw was significantly increased in all rats (Fig. 2). The increase in surface temperature started at the injection site and spread gradually to include the whole paw. Although the peak increase in paw temperature was similar in the aged and adult rats (n=5 per group), the time course of the temperature change was different. The significant increase in the paw surface temperature lasted for 5 days in the aged group (Fig. 2A), whereas it lasted for only 3 days in the adult rat group (Fig. 2B). There was a slight change in the paw temperature on the contralateral side in the aged rats.

The paw thickness, a measure of inflammation-induced edema, peaked at 1 day after CFA injection and lasted for more than 6 days in both aged and adult rats as illustrated in Fig. 3A (aged, 1 day: 7.23 ± 0.15 mm, 6 days: 6.29 ± 0.18 mm, n=5) and B (adult, 1 day: 6.85 ± 0.06 mm, 6 days: 5.69 ± 0.13 mm, n=5). However, the increase in paw thickness was significantly larger in the aged rats at 2 and 4-6-day time points as compared to the adult rats (Fig. 3). We could not observe any significant effect of CFA injection on the contralateral hind paw as illustrated in Fig. 3.

Spatial distribution of nociceptive neurons in the spinal dorsal horn

A total of 150 nociceptive neurons in the spinal dorsal horn (65 WDR and 13 NS
neurons in 15 adult rats; 59 WDR and 13 NS neurons in 13 aged rats) were analyzed (Table 1). Most nociceptive neurons were distributed in the superficial and deep laminae of the L4-5 spinal dorsal horn as illustrated in Fig. 4. We did not observe differences in distribution of nociceptive neurons in the aged and adult rats. Consistent with literature, WDR neurons were encountered in the superficial and deep laminae and NS neurons were mainly distributed in the superficial laminae of the spinal dorsal horn (Fig. 4).

**Mechanical and thermal responses**

Fig. 5 illustrates a typical example of a WDR neuron recorded from superficial lamina of the L5 spinal dorsal horn in a 32 month-old rat after CFA injection. This neuron increased firing frequency with graded mechanical stimulation of the hind paw (Fig. 5A). High frequency afterdischarges were elicited following pinch of the center of the receptive fields (Fig. 5A, right). This neuron also responded to heating of the receptive field (Fig. 5B). The firing frequency was increased following an increase in stimulus temperature in a graded manner. The recording site of this neuron is illustrated in Fig. 5C. The lesion site was indicated by the arrow. The receptive field of this neuron covered the ventral and dorsal surface of the hind paw (Fig. 5D).

The mechanical responses of WDR and NS neurons are summarized in Fig. 6. All nociceptive neurons increased firing frequency following the graded increase in mechanical stimulus intensity. It is interesting that NS neurons in the adult rats exhibited greater responses after CFA treatment to strong mechanical stimulation (28.2g, 75.8g and pinch stimuli in Fig. 6C). However, the responses of NS neurons in the aged rats did...
not show an increased response to mechanical stimulation after inflammation (Fig. 6A). The response of WDR neurons to mechanical stimulation was similar in the aged and adult groups and there was no significant change after inflammation (Fig. 6B and D).

The relationship between responses and intensity of heat stimulation is shown in Fig. 7. The inflammation did not have any effect on heat responses of WDR neurons in the aged rats, whereas there was a significant effect on responses in the adult rats following graded heat stimulation of the receptive fields.

**Background activity and afterdischarge**

In the naive aged rats, the background activity of WDR neurons (2.99 ± 0.74, n=35) (open colom in Fig. 8A) was significantly higher than that of the adult rats (1.17 ± 0.31, n=33) (open colom in Fig. 8C), (p<0.05). The background activity of WDR neurons was significantly higher in the adult rats (3.24 ± 0.84, n=32) (p<0.01) as compared with naïve adult rats, but not in the aged rats (3.75 ± 0.94, n=24) as compared with aged naïve rats, (p=0.55, Fig. 8A and C). The background activity of NS neurons in the aged rats was slightly higher than that in the adult naïve rats as compared with aged naïve rats (aged: 0.43 ± 0.38, n=4, adult: 0.24 ± 0.18, n=9, p=0.83, Fig. 8B and D). Although there was a trend for an increase in background activity of NS neurons in adult and aged rats, the increase was not significant for both age groups (aged: 2.00 ± 1.77, n=7, adult: 1.20 ± 1.20, n=3, p=0.81, Fig. 8B and D).

The afterdischarge of WDR neurons was significantly higher in the inflamed aged rats as compared with naïve aged rats (naïve: n=35, inflammed: n=24, p<0.01), but
not significantly higher in inflamed adult rats as compared with naïve adult rats (naïve: n=33, inflamed: n=32, \( p=1.00 \)) (Fig. 9A and C) and there was no difference between the two age groups. Compared to adult naïve rats, the afterdischarges of NS neurons were generally higher in the aged naïve rats, but inflammation did not induce significant increases in afterdischarges in either group (Fig. 9B and D).

**Receptive field property**

In the present study, the receptive fields of WDR neurons were designated as low and high threshold areas according to their responses to mechanical stimuli as illustrated in Fig. 5D. The low threshold areas were activated by both noxious and innocuous mechanical stimuli, whereas the high threshold areas were only activated by noxious stimulation. In general, the low threshold areas were surrounded by the high threshold areas. Figure 10 illustrates the effect of CFA injection on receptive fields size of WDR neurons (A) and NS neurons (B). We observed that the high threshold portion of the receptive fields of WDR neurons was significantly larger in the aged naïve rats than adult naïve rats \( (p<0.01, \text{Fig. 10A}) \). However, the receptive fields of WDR neurons were significantly larger in adult inflamed rats than naïve adult, but not in aged rats (Fig. 10A). There was no difference in the low threshold areas of the receptive fields between the aged and adult rats in both naïve and inflamed rats. For NS neurons, the receptive field was significantly larger only in the adult inflamed rats than adult naïve \( (p<0.05, \text{Fig. 10B}) \). However, the receptive field of inflamed aged rats was at the similar size as that in the aged naïve rats (Fig. 10B).
Expression of the Fos protein-LI cells

Two days after CFA injection into the hind paw, Fos protein-LI cells were observed in the superficial spinal dorsal horn of the aged and adult rats ipsilateral to the injection site as illustrated in Fig.11. Most Fos protein-LI cells were distributed in the superficial laminae of the dorsal horn and sparse in the deep laminae. The Fos protein-LI cells were restricted in the medial portion of the superficial dorsal horn, while those in deep laminae were widely distributed (Fig. 11A and B). The distribution pattern of Fos protein-LI cells to the aged and adult rats was similar. As illustrated in Fig. 11C, the number of Fos protein-LI cells on the ipsilateral side to CFA injection was slightly larger in the aged rats than that of adult in the superficial and deep dorsal horn (laminae I-II, aged: 8.02 ± 0.57, adult: 6.37 ± 0.85; laminae III-V, aged: 2.37 ± 0.68, adult: 2.30 ± 0.79, n=5 in each group, p>0.05), but the difference did not reach the significant level.

Discussion

The present study showed that mechanical paw withdrawal threshold was significantly lower in aged rats than adult rats before CFA. The structure difference of the paw skin between aged and adult rats has been reported previously (Delp et al. 1998; Jung et al. 1997; Oku et al. 1992; Tzaphlidou et al. 2004). The accumulation of fat under the paw skin was less in the aged than adult rats. The skin is also much thinner in the aged than adult rats. These suggest that the structure difference in paw skin between aged and adult rats may contribute to the decrement of mechanical paw withdrawal.
threshold in the aged rat.

After CFA injection, the difference in mechanical thresholds between the inflamed and noninflamed paws was apparently larger in adult rats than that in aged rats. The magnitude of nocifensive reflex activity has been known to depend on sensitization of the peripheral nociceptors and central sensitization of noxious pathways (Hylden et al. 1989; Schaible and Schmidt 1988). While present data suggest that the peripheral inflammation produced stronger effect on nocifensive behavior in adult rats compared to aged rats, one should be cautious about this conclusion since the contralateral paw of the aged rats showed reduced baseline threshold. A ceiling effect may have prevented a further reduction in threshold in the aged rats.

A significant increase in the paw temperature and thickness was observed in both aged and adult rats following CFA injection. However, judged from the degree of hyperthermia and swelling, the inflammatory reaction to CFA in the aged rats was significantly stronger and longer lasting when compared to the adult rats. These differences in response to CFA in the aged and adult rats may be explained by alterations in autonomic function in the aged rats. It is well known that the peripheral autonomic functions, such as temperature control and circulation, do not work well in the aged human (D'Esposito et al. 2003; Kenny et al. 1996). It is highly probable that the differences in inflammatory responses in aged and adult rats are a result of differences in peripheral circulation system. The peripheral circulation is significantly affected by age and the blood flow is much slower in the aged rats as compared to the adult (Khalil Z et al. 2000). The slower blood flow rate may produce a delay of healing
of the peripheral inflammation in the aged rats, resulting in a longer lasting inflammation. The longer lasting peripheral inflammation as observed in the aged rats may produce stronger sensitization of peripheral nociceptors. The sensitized nociceptors have been known to produce a barrage of action potentials in primary afferent fibers, which are conveyed to spinal dorsal horn neurons (Hylden et al. 1989; Schaible and Schmidt 1988; Schaible et al. 1991).

Peripheral inflammation produces an extensive increase in the excitability of primary afferent small-diameter nerve fibers, leading to a significant increase in the activity of nociceptive neurons in the CNS of young adult rats (Hylden et al. 1989; Iwata et al. 1999; Schaible and Schmidt 1988; Schaible et al. 1991). After peripheral inflammation, dorsal horn nociceptive neurons also showed an expansion of the receptive fields that was explained by the hyperexcitability of dorsal horn neurons (Hylden et al. 1989; Iwata et al. 1999). We observed similar results in the adult rats in the present and previous reports (Iwata et al. 1999, 2002). The nociceptive neurons in the adult rats increased their background activity, mechanical and heat responses following peripheral inflammation. The increment of the excitability of nociceptive neurons in the adult rats as observed after CFA injection may be a result of sensitization of primary afferent neurons as well as altered dorsal horn processing. The activity of C-fiber primary afferents seems not to be much affected by age (Sato et al. 1985). The conduction velocity of C-fiber afferents was not significantly changed with advancing age. This suggests that the hyperexcitability of peripheral nociceptors could be relayed to dorsal horn nociceptive neurons in the aged rats similar to that observed in the adult
rats. Accordingly, dorsal horn nociceptive neurons in aged rats may show similar excitability following peripheral inflammation, such as that observed in the adult rats. In fact, Fos protein expression in aged and adult dorsal horn neurons following CFA injection was not significantly different (Fig. 11).

Thus, it seems that nociceptive information fully reaches the spinal cord after inflammation in both aged and adult rats. However, we did not find significant effects of inflammation on responsibility of dorsal horn nociceptive neurons to noxious mechanical and heat stimuli, and their background discharge and the receptive field size in the aged rats while inflammation induced significant increases in activity in adult rats. One explanation for these observations seems to be the altered sensitivity of dorsal horn neurons to inflammation-induced afferent inputs in the aged rats. It has been reported that the synaptic density of spinal neurons is significantly reduced in aged rats as compared with young rats (Chen et al.1997; Santer et al. 2002). It is possible that the reduction of synaptic density in dorsal horn neurons may produce changes in sensitivity of dorsal horn neurons to primary afferent inputs. We noticed that noxious stimulus-evoked responses tended to be higher, and background activity and receptive fields were significantly larger in the non-inflamed aged rats compared to those in the non-inflamed adult rats. Thus, the effect of inflammation seems to have been occluded in the aged rats. In other words, since nociceptive neurons of the aged rats already exhibited hyperexcitability, inflammation could not produce a further increase in excitability. It is likely that age-related and inflammation-induced changes are underlined by common mechanisms.
The involvement of the descending modulator system could be considered as an alternative mechanism to explain the effect of inflammation on dorsal horn nociceptive neuronal activity in the aged rats. The descending system has been known as a powerful pathway to modulate pain transmission in adult rats (Carpenter et al. 1965; Ren et al. 1996a,b). It has been reported that an extensive increase in excitability of rostral ventromedial medulla neurons are induced following hind paw inflammation (Miki et al. 2002). This enhancement of function of the descending modulatory system may induce an effect on nociceptive transmission in the spinal dorsal horn. We previously reported that descending inhibition was reduced in the aged rats (Iwata et al. 2002). The descending serotonergic and adrenergic systems were significantly impaired in the aged rats and the spinal block did not affect nociceptive responses in aged rats. If there is a dysfunction of the descending system in the aged rats, the spinal dorsal horn neuronal activity should be significantly increased following peripheral inflammation. However, we observed only a small change in heat and mechanical responses in the aged rats, while dorsal horn nociceptive neurons in the adult rats showed a significant increase in activity after inflammation. These data suggest that the aged rats lack ability to further increase dorsal horn neuronal activity following peripheral inflammation. The afterdischarges are strongly modulated by the descending pathways. The descending system inhibits the long lasting afterdischarges produced by strong peripheral stimulation (Robinson et al. 2002). The modulation of afterdischarges should be a good indicator of the function of the descending modulation system. It is likely that a deficit of the descending system in the aged rats resulted in a significant increment of the
afterdischarges of dorsal horn neurons following peripheral inflammation.

Relatively small changes in pain threshold for aged human and fairly large alterations in response properties of nociceptive neurons in the aged rat spinal cord suggest that degenerative alterations and reorganization are taking place in the pain processing system with advancing age. Therefore, the descending and ascending systems as well as the peripheral nervous system in the aged rats deserves further study to elucidate pain system in the aged. This may lead to new ways for treatment of pain in the elderly.

Although the effect of aging on dorsal horn nociceptive neurons in the inflamed rats was discussed in the present study, it should be cautious that the sex differences and sensitivity to anesthetics used in aged rats may contribute to the difference in the responsiveness of dorsal horn nociceptive neurons of the inflamed aged rats.
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Figure legends

Fig. 1
Change in mechanical paw withdrawal escape threshold in aged (A) and adult rats (B) following CFA injection into one hind paw. pre: the day before CFA injection, a: ipsi vs. contra, b: pre-CFA injection vs. post-CFA injection, c: aged (ipsi) vs. adult (ipsi), d: aged (contra) vs. adult (contra), **: $p<0.01$, *: $p<0.05$ (Mann-Whitney’s U test, Dunnet test for pre-CFA injection vs. post-CFA injection).

Fig. 2
The time-course of changes in foot temperature following CFA injection into the hind paw. A and C: data from the aged rats, B and D: data from the adult rats. Note that the temperature change of the paw is larger in the aged than that in the adult rats. ipsi: ipsilateral side to CFA injection, contralateral side to CFA injection, a: ipsi vs. contra, b: pre vs. post, c: aged (ipsi) vs. adult (ipsi), d: aged (contra) vs. adult (contra), **: $p<0.01$, *: $p<0.05$ (Mann-Whitney’s U test, Dunnet test for pre vs. post).

Fig. 3
The time-course of changes in foot thickness following CFA injection into the hind paw.
A and C: data from the aged rats, B and D: data from the adult rats. Paw thickness was measured in the middle portion of the hind paw (as illustrated in C). Note that the paw thickness is slightly larger in the aged than that of the adult rats. The arrows in C (pre) indicate the region where the paw thickness was measured. a: ipsi vs. contra, b: pre vs. post-CFA injection, c: aged (ipsi) vs. adult (ipsi), **: $p<0.01$, *: $p<0.05$ (Mann-Whitney’s U test, Dunnet test for pre vs. pre vs. post-CFA injection).

Fig.4
Illustration of the recording sites of WDR and NS neurons in the aged (A: naïve, B: inflammation) and adult rats (C: naïve, D: inflammation).

Fig.5
Response properties of a typical nociceptive neuron in the dorsal horn of a 32-month-old rat. A: PST histograms of neuronal discharges to graded mechanical stimulation of the low threshold area of a WDR neuron. After pinching of the receptive field, high frequency afterdischarges were observed. B: PST histograms of neuronal discharges to graded heat stimulation of the low threshold area of the receptive field of a WDR neuron. C: The photomicrograph of the recording site in the dorsal horn. The lesion site was indicated by the arrow. D: Low (solid area) and high threshold (shaded area) areas of the receptive fields indicated by the arrows.

Fig.6
The stimulus-responses function of responses to graded mechanical stimuli in the aged (A: NS, B: WDR) and adult (C: NS, D: WDR) rats. PI: pinch. Note that the mechanical response was significantly larger in NS neurons after the inflammation. BG activity: background activity in this and following figures. *, #: p<0.05 (t-test).

Fig.7
The stimulus-response functions of the WDR neurons following graded heat stimuli in the aged and adult rats. Note that the heat response was significantly larger in adult rats after inflammation. **: p<0.01, *: p<0.05 (t-test).
#: p<0.05 (Mann-Whitney’s U-test), aged vs. adult rats with inflammation.

Fig.8
The mean background activity of WDR (A: aged, C: adult) and NS neurons (B: aged, D: adult). Open column indicates the naïve rat’s data and the solid column indicates the data from the inflamed rats. Note that only in adult WDR neurons showed significant increase in background activity after the CFA injection. **: p<0.01 (t-test).
#: p<0.05 (Mann-Whitney’s U-test), WDR neurons in aged vs. adult rats.

Fig.9
The mean afterdischarges of WDR (A: aged, C: adult) and NS neurons (B: aged, D: adult). Significant increase in afterdischarge was observed in WDR neurons of aged rats with CFA treatment. Open column: data from naïve rats, solid column: data from
inflamed rats. **: $p<0.01$ (t-test).

Fig. 10

The mean sizes of the receptive fields of WDR (A) and NS (B) neurons in the aged and adult rats (open column: naïve rat, solid column: inflamed rats). The receptive fields of WDR neurons include both low and high threshold areas. *: $p<0.05$, **: $p<0.01$ (t-test).

Fig. 11

Fos protein expression in dorsal horn neurons of the aged and adult rats at two days after CFA injection into the hind paw. A and B: Photomicrographs of L4 dorsal horn ipsilateral to the CFA injection in the aged (A) and adult (B) rats. A large number of Fos protein-LI cells were observed in the superficial laminae of the dorsal horn in aged and adult rats. C: The mean number of Fos-LI cells. The number of Fos-LI cells was not significantly different in the aged and adult rats ($p>0.05$, t-test).
# TABLE 1

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*Incidence of dorsal horn nociceptive neurons in aged and adult rats*
Fig. 1

A aged

B adult

---

38
Fig. 2

A

B

C

D

1 day

2 days

4 days

1 day

2 days

4 days
Fig. 3

A

B

C  aged  pre

D  adult  pre

1 day  1 day

2 days  2 days
Fig. 4

A  B  C  D

L4  L5

①: WDR neuron
●: NS neuron
Fig. 5

A

spikes/s
100 |
75 |
50 |
25 |
0 |
1.2 |
5.4 |
15.1 |
28.8 |
75.8 (g) |
pinch |

5s

afterdischarge

B

spikes/s
100 |
75 |
50 |
25 |
0 |
44 |
46 |
48 |
50 (°C) |

10s

C

D

high threshold area

low threshold area

dorsal surface

ventral surface
Fig. 6

(A) NS (aged)  
- : inflammation  
- : naive  
# : aged vs adult, p<0.05  
* : naive vs inflamed, p<0.05  

(B) WDR (aged)  

(C) NS (adult)  

(D) WDR (adult)  

peak freq. - BG activity (spikes/s)  

stimulus intensity (g)
Fig. 7
Fig. 10

A

- Bar graph showing mean area (cm²) for inflammation and naïve groups in low and high threshold areas for aged and adult subjects.
- Data indicates significant differences between inflammation and naïve groups with "**" indicating p<0.01 and "*" indicating p<0.05.

B

- Bar graph showing mean area (cm²) for aged and adult subjects in high threshold area.
- Data indicates significant differences with "*" indicating p<0.05.
Fig. 11

A  aged

B  adult

C

mean number of Fos-LI cells (section)

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500 μm