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Decrease in the descending inhibitory 5-HT system in rats with spinal nerve ligation

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ABSTRACT

The descending serotonergic (5-HT) system is shown to be plastically altered under pathological conditions such as inflammation or peripheral nerve lesion. Although much evidence indicates that the potentiation of descending facilitatory 5-HT pathways may contribute to the development of chronic pain, the inhibition of descending inhibitory 5-HT system may be functionally more important to the development of central sensitization and neuropathic pain. In the present study, we observed that the inhibitory effects of 5-HT and its receptor agonists including 1A, 1B, 3, 4, and probably 2C receptor agonists, on the C-fiber responses of dorsal horn wide dynamic range (WDR) neurons in the spinal cord decreased significantly following spinal nerve ligation (SNL). Furthermore, we found that the antagonistic effects of 5-HT 1B, 2C, 3, and 4 receptor antagonists on the 5-HT-induced inhibition of C-fiber responses of WDR neurons were also attenuated after SNL. In consistent with these observations, we also found an obvious decrease in the content of 5-HT and 5-HIAA, and a marked increase in the turnover rate of 5-HT (5-HIAA/5-HT) in the ipsilateral dorsal half of the lumbar spinal cord after SNL. These data indicate that a loss or decrease in the descending inhibitory 5-HT system upon the spinal processing of nociceptive information appears to occur following spinal nerve injury, and this kind of decrease in the descending inhibitory 5-HT system is proposed to be involved in the development of central sensitization and ultimately to the nerve injury-induced neuropathic pain.

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Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); WDR, dorsal horn wide dynamic range; SNL, spinal nerve ligation; LTD, long-term depression; LTP, long-term potentiation; GABA, γ-amino-butyric acid; HPLC, high performance liquid chromatography; PWT, paw withdrawal threshold; 8-OH-DPAT, (2R)-(+)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide; CGS 12066, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo-[1,2-a]quinoxaline; α-m-5-HT, α-methyl-5-hydroxytryptamine maleate; MK 212, 6-chloro-2-(1-piperazinyl) pyrazine hydrochloride; mCPBG, 1-(3-chlorophenyl)biguanide hydrochloride; BZTZ, 2-[1-(4-piperonyl)piperazinyl]benzothiazole; WAY 100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinyl-cyclohexanecarboxamide maleate salt; GR55562, 3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyrid-dinyl)phenyl]benzamide dihydrochloride; RS102221, 8-[5-(2,4-dimethoxy-5-(4-trifluoromethylphenylsulfonamido)phenyl-5-oxopentyl]-1,3,8-triazaspiro[4.5]decane-2,4-dione hydrochloride; MDL 72222, 3-tropanyl-3,5-dichlorobenzoate; GR113808, [1-[2-[(methylsulfonyl)-amino]-ethyl]-4-piperidinyl]methyl1-methyl-1H-indole-3-carboxylate; DMSO, dimethyl sulfoxide; NS, normal saline; ANOVA, analysis of variance; AUC, area under the time–course curve

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1. Introduction

The mechanisms underlying the development of neuropathic pain are still not fully understood. Compelling evidence now shows that a combination of increased activity in the excitatory and a concomitant decreased activity in the inhibitory systems within the spinal cord contribute to the central sensitization and, ultimately, to the development of pathological pain (Finnerup et al., 2007; Jarvis and Boyce-Rustay, 2009; Vanderah, 2007). It has been established that the descending serotonergic (5-hydroxytryptamine, 5-HT) pathways exerts an inhibitory or facilitatory influence upon the spinal processing of nociceptive information, and the effects of 5-HT depend on the cell type and type of receptor it acts on (Bardin et al., 2000a; Campbell and Meyer, 2006; Dogrul et al., 2009; Jeong et al., 2004; Sommer, 2006). Although much evidence indicates that the descending facilitatory 5-HT pathways are likely to be involved in the development of neuropathic pain (Apkarian et al., 2009; Bee and Dickenson, 2007; Saade and Jabbur, 2008; Sanoja et al., 2008), roles of the descending inhibitory 5-HT pathways in governing the sensitivity of dorsal horn neurons as well as the pain transmission may be functionally more important (Braz and Basbaum, 2008; Heinricher et al., 2009; Jeong et al., 2004; Sommer, 2006; You et al., 2005). Dysfunction of these descending inhibitory pathways produces hypersensitivity to pain and even lower the pain threshold so that normally non-noxious stimuli become painful (Nakae et al., 2008b; Stahl and Briley, 2004).

Currently, there are seven families of 5-HT receptors (5-$HT_{1–7}$), comprising at least 14 distinct receptor subtypes (Barnes and Sharp, 1999; Hoyer et al., 1994; Hoyer and Martin, 1996; Hynie, 1995). Several receptors, including the 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 receptors, which are proposed to be involved in the modulation of spinal nociceptive transmission, have been identified in the spinal cord dorsal horn (Bardin et al., 2000a; Faerber et al., 2007; Huang et al., 2008; Jeong et al., 2004; Liu et al., 2007). Accordingly, intrathecal (i.t.) administration of 5-HT or selective 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, or 5-HT_3 receptor agonist produces significant spinal antinociceptive effects in intact rats (Crisp et al., 1991), whereas the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, or 5-HT_3, 5-HT_4 receptor antagonist reverses or attenuates this antinociception induced by 5-HT (Bardin et al., 2000a; Jeong et al., 2004). These results suggest that activation of these receptors expressed on the dorsal horn neurons can produce an inhibitory effect on spinal nociceptive transmission (Yoshimura and Furue, 2006), although facilitatory effects have also been reported less frequently (Sommer, 2006; Zeitz et al., 2002). In line with these behavioral observations, we and others have previously found that 5-HT inhibits the C-fiber responses of the spinal wide dynamic range (WDR) neurons in normal rats, and multiple 5-HT receptor subtypes, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_3, and 5-HT_4 receptors, are likely to be involved in mediating the inhibitory effects of 5-HT (Liu et al., 2007; Lu and Perl, 2007; Rivot et al., 1987; You et al., 2005). The C-fiber responses of WDR neurons are usually regarded as related to nociception (Lu and Perl, 2007; Qu et al., 2009; Rygh et al., 2000). However, the roles of the descending inhibitory 5-HT pathways in the development of neuropathic pain and, particularly, the underlying mechanisms of the potential actions, still remain largely unclear.

Considerable evidence has accumulated that the descending serotonergic system seems to be plastically altered under pathological conditions (Goettl et al., 2002; Hains et al., 2002; Heinricher et al., 2009; Nakae et al., 2008a; Sounvoravong et al., 2004). For example, decreases in basal release of serotonin have been reported in several models of neuropathic pain (Goettl et al., 2002; Hains et al., 2002; Sounvoravong et al., 2004). Selective lesion of descending 5-HT system on 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, and 5-HT_3 receptors in the spinal cord have also been shown in rats with peripheral nerve injury (Laporte et al., 1995; Nakae et al., 2008a). In support of these findings, the behavioral studies have confirmed that i.t. administration of 5-HT attenuate mechanical hyperalgesia in rats with neuropathic pain at a much higher dose than that required in normal rats (Bardin et al., 2000b); intrathecal transplantation of serotonergic precursor cells, which secrete 5-HT, not only alleviate mechanical allodynia and thermal hyperalgesia but also reduce the bilateral hyperexcitability of dorsal horn neurons following spinal hemisection injury in rats (Hains et al., 2001b, 2003). Moreover, we have previously demonstrated that electroacupuncture (EA) at 100 Hz induces dorsal horn long-term potentiation (LTP) instead of long-term depression (LTD) in SNL rats (Xing et al., 2007). We speculate the reason is probably because of a loss or decrease in the endogenous inhibitory system following spinal nerve injury, and this kind of decrease is likely to be involved in the development of pathological pain (Heinricher et al., 2009; Xing et al., 2007; Zeilhofer, 2008; Zeilhofer and Zeilhofer, 2008). In the present study, we investigated whether the inhibitory effects of 5-HT and its receptor subtypes on the nociceptive responses of dorsal horn WDR neurons in the rat spinal cord decreased following SNL. The aim of this study was to determine whether decrease in the descending inhibitory 5-HT pathways occurred in rats associated with neuropathic pain.

2. Results

2.1. *Decrease in the inhibitory effects of 5-HT and its receptors agonists on the C-fiber responses of WDR neurons after SNL*

To determine whether loss or decrease in the descending inhibitory 5-HT system occurred in rats associated with neuropathic pain, we first investigated if the inhibitory effects of 5-HT or its receptor subtype agonists on the nociceptive responses of dorsal horn WDR neurons were attenuated following spinal nerve injury. 5-HT was spinally applied at the doses of 0.5, 1.5, and 5.0 μg in normal rats and, 1.0, 5.0, 10, and 100 μg in SNL rats, respectively. As shown in Figs. 1 and 2, the significant inhibitory effects of 5-HT on the C-fiber responses of WDR neurons appeared at 1.5 μg in normal rats but at a higher dose of 5.0 μg in SNL rats ($P<0.001$, two-way ANOVA, $n=5–8$; Figs. 2A–D). The maximal inhibitory rate of 5-HT (at 5.0 μg) on the C-fiber responses was much lower in SNL rats (54.4±6.3%, $n=8$) than that in normal rats (77.3±5.6%, $n=6$; $P<0.001$, two-tailed unpaired Student's *t*-test; Fig. 2E), and the ID_{50} of 5-HT was markedly increased from 1.9 μg in normal rats ($r^2=0.7960$, Sy.=13.62, $n=7$) to 10.1 μg in SNL rats ($r^2=0.6768$, Sy.x=16.25, $n=8$; Fig. 2F).

Furthermore, as shown in Fig. 3, all applied 5-HT receptor agonists including 5-HT_{1A} receptor agonist 8-OH-DPAT at 5 μg,

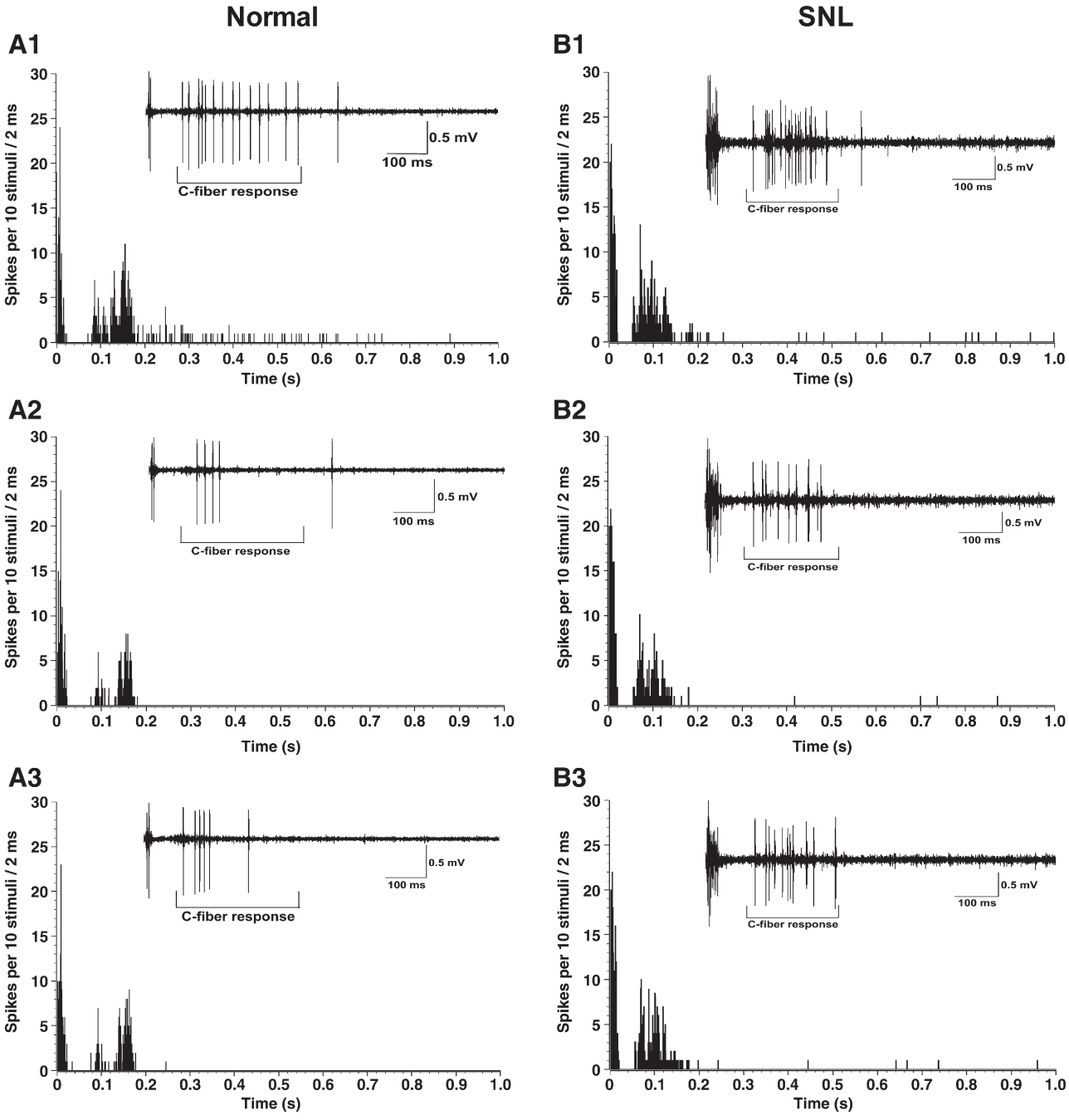


Fig. 1 – Examples represent the inhibitory effects of 5-HT (5.0 μg) on the C-fiber responses of dorsal horn WDR neurons in normal (A1–A3) and SNL (B1–B3) rats. Panels illustrate the poststimulus histogram of the electrically evoked neuronal responses in a WDR neuron at the time point of 15 min before 5-HT application (A1 and B1), 20 min (A2 and B2), and 60 min (A3 and B3) after 5-HT application, respectively. Inset shows the original recordings of the first electrically evoked neuronal responses corresponding to each time point, respectively.

5-HT_{1B} receptor agonist CGS 12066 at 50 μg, 5-HT_{2A} receptor agonist α-m-5-HT at 3 μg, 5-HT_{2C} receptor agonist MK 212 at 10 μg, 5-HT_3 receptor agonist mCPBG at 10 μg, and 5-HT_4 receptor agonist BZTZ at 30 μg significantly inhibited the C-fiber responses of WDR neurons in normal rats ($P<0.001$, two-way ANOVA, $n=5$–8, Figs. 3A–F), whereas only 5-HT_{2A} receptor agonist α-*m*-5-HT exerted inhibitory effects on the C-fiber responses of WDR neurons in SNL rats ($P<0.01$, two-way ANOVA, $F_{(3,4,12/145)}=(42.86, 3.97, 1.95)$, $n=7$, Fig. 3C). As summarized in area under the time–course curve (AUC) values of C-fiber responses (–15 to 60 min of the analysis time), the inhibitory effects of 5-HT_{1A} receptor agonist 8-OH-DPAT ($P<0.05$, $n=6$), 5-HT_{1B} receptor agonist CGS 12066 ($P<0.05$, $n=6$), 5-HT_3 receptor agonist mCPBG ($P<0.05$, $n=7$), and 5-HT_4 receptor agonist BZTZ ($P<0.01$, $n=8$) decreased significantly in SNL rats as compared with those in normal rats (two-tailed unpaired Student's *t*-test; Fig. 3G). Similar decrease was also observed in the maximal inhibitory effects of these 5-HT receptor agonists in SNL rats, in

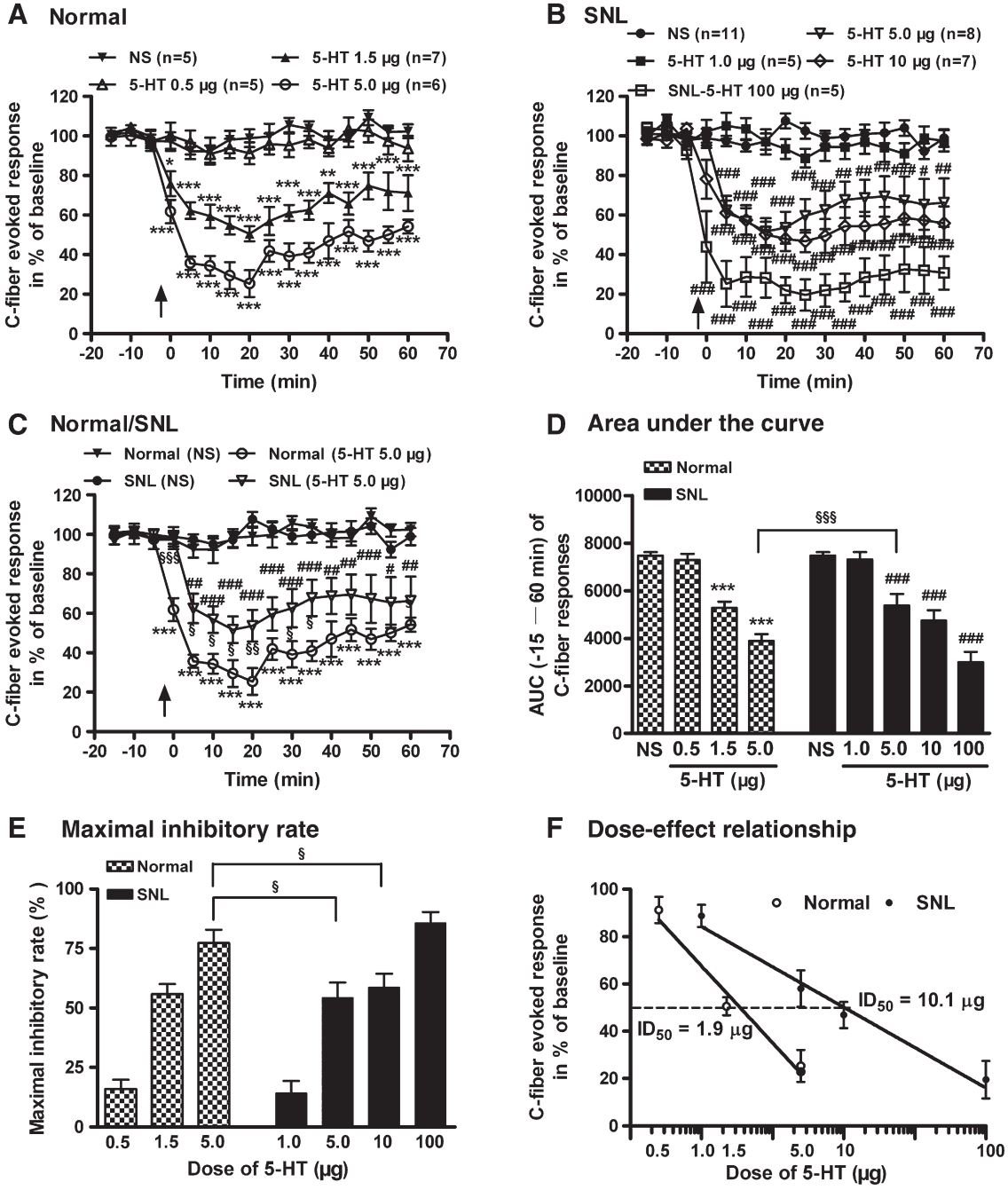


Fig. 2 – Decrease in the inhibitory effects of 5-HT on the C-fiber responses of WDR neurons in SNL rats. (A) Effects of spinal application of 5-HT (0.5, 1.5, and 5.0 μg) on the C-fiber responses of WDR neurons in normal rats. (B) Effects of 5-HT (1.0, 5.0, 10, and 100 μg) on the C-fiber responses of WDR neurons in SNL rats. Note that 5-HT inhibited the C-fiber responses in a dose-dependent manner either in normal rats or in SNL rats. However, the effective dose of 5-HT on the C-fiber responses was much higher in SNL rats (5.0 μg) than that in normal rats (1.5 μg). (C) Comparison of the inhibitory effects of 5-HT (5.0 μg) on the C-fiber responses of WDR neurons between normal and SNL rats. (D) AUC of 5-HT on the C-fiber responses in normal and SNL rats. (E) The maximal inhibitory rate of 5-HT on the C-fiber responses in normal and SNL rats. Note that the inhibitory effects of 5-HT on the C-fiber responses decreased significantly in SNL rats as compared with those in normal rats. (F) Dose–effect relationship of 5-HT on the C-fiber responses of WDR neurons. Note that the ID_{50} of 5-HT on the C-fiber responses was markedly increased in SNL rats. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ as compared with normal saline (NS) control in normal rats; $^{\#\#\#}P<0.001$ as compared with NS control in SNL rats, one- or two-way ANOVA followed by Bonferroni *post hoc* test, $n=5–8$; $^{\S}P<0.05$, $^{\S\S}P<0.01$, and $^{\S\S\S}P<0.001$, compared between normal and SNL group, two-tailed unpaired Student's *t*-test, $n=6$ in normal group; $n=8$ in SNL group. Data are expressed as mean±SEM. Arrow, application of NS or 5-HT.

which the maximal inhibitory rate of 5-HT_{1A} receptor agonist 8-OH-DPAT was decreased from 57.5±13.0% in normal rats to 21.2±4.3% in SNL rats ($P<0.01$, two-tailed unpaired Student's t-test, $n=6$). Likewise, the maximal inhibitory rates of 5-HT_{1B} receptor agonist CGS 12066, 5-HT_3 receptor agonist mCPBG, and 5-HT_4 receptor agonist BZTZ were also decreased from 57.4±9.2%, 53.0±8.3%, and 74.0±9.9% in normal rats to 25.0±6.3% ($P<0.01$, $n=6$), 28.6±6.2% ($P<0.01$, $n=7$), and 28.3±2.7% ($P<0.01$, $n=8$) in SNL rats, respectively (two-tailed unpaired Student's t-test). No significant decrease was observed in the maximal inhibitory rate of either 5-HT_{2A} receptor agonist α-m-5-HT (from 62.9±7.9% in normal rats to 51.3±4.9%, $P>0.05$, $n=7$), or 5-HT_{2C} receptor agonist MK 212 (from 36.6±10.7% in normal rats to 32.5±6.2%, $P>0.05$, $n=8$) in SNL rats (two-tailed unpaired Student's t-test; Fig. 3H). These results suggested that the inhibitory effects of 5-HT and its multiple receptor agonists on the C-fiber responses of WDR neurons decreased significantly following SNL, in which the 5-HT receptor subtypes including 1A, 1B, 3, 4, and probably 2C were likely to be involved in mediating the decrease in the inhibitory 5-HT system.

2.2. *Effects of 5-HT receptor antagonists on the 5-HT-induced inhibition of C-fiber responses of WDR neurons after SNL*

To further investigate whether the antagonistic effects of 5-HT receptor antagonists on the 5-HT-induced inhibition of C-fiber responses of WDR neurons were also decreased following SNL, various antagonists of 5-HT receptor subtypes were spinally administrated 5 min before 5-HT (1.5 μg in normal rats and 10 μg in SNL rats, respectively) application. As shown in Fig. 4, in normal rats, all applied antagonists of 5-HT receptor subtypes including 5-HT_{1B} receptor antagonist GR 55562 at 30 μg ($P<0.05$, one-way ANOVA, $F_{(5,48)}=22.30$, $n=5$; Figs. 4B1 and B3), 5-HT_{2A} receptor antagonist ketanserin at 15 μg ($P<0.001$, one-way ANOVA, $F_{(5,48)}=20.54$, $n=7$; Figs. 4C1 and C3), 5-HT_{2C} receptor antagonist RS 102221 at 30 μg ($P<0.001$, one-way ANOVA, $F_{(5,48)}=13.70$, $n=5$; Figs. 4D1 and D3), 5-HT_3 receptor antagonist MDL 72222 at 15 μg ($P<0.01$, one-way ANOVA, $F_{(5,48)}=9.49$, $n=5$; Figs. 4E1 and E3), and 5-HT_4 receptor antagonist GR 113808 at 15 μg ($P<0.001$, one-way ANOVA, $F_{(5,48)}=11.47$, $n=6$; Figs. 4F1 and F3), but not 5-HT_{1A} receptor antagonist WAY 100635 at 10 μg ($P>0.05$, one-way ANOVA, $F_{(5,48)}=17.93$, $n=5$; Figs. 4A1 and A3), significantly antagonized the 5-HT-induced inhibition on the C-fiber responses of WDR neurons. Whereas in SNL rats, at the same doses as used in normal rats, only 5-HT_{2A} receptor antagonist ketanserin could significantly antagonize the 5-HT-induced inhibition ($P<0.01$, one-way ANOVA, $F_{(5,48)}=20.54$, $n=6$; Figs. 4C2 and C3). At two-fold higher doses as used in normal rats, all of the receptor antagonists except 5-HT_{2C} receptor antagonist RS 102221 and 5-HT_3 receptor antagonist MDL 72222 still showed significant antagonistic effects on 5-HT-induced inhibition in SNL rats (Figs. 4A3–F3). As basal controls, we also examined whether the 5-HT receptor antagonist alone had any effect on the C-fiber responses of WDR neurons in SNL rats. Similar to our previous observations in normal rats (Liu et al., 2007), spinal application of all above 5-HT receptor antagonists alone had no significant inhibitory effects on the C-fiber responses of WDR neurons in SNL rats ($P>0.05$, one-way ANOVA, $n=5$–8; Fig. 5). These data indicated that the antagonistic effects of 5-HT 1B, 2C, 3, and 4 receptor antagonists on the 5-HT-induced inhibition of C-fiber responses were likely attenuated after SNL.

2.3. *Decrease in the 5-HT and 5-HIAA content in the spinal cord after SNL*

As shown in Fig. 6, the content of 5-HT in the dorsal half of the lumbar spinal cord decreased significantly after SNL. In the ipsilateral dorsal half of the lumbar spinal cord, the content of 5-HT decreased significantly from 154.3±17.8 pg/mg tissue before operation to 63.1±7.3 pg/mg tissue at 7 days after operation ($P<0.001$, one-way ANOVA, $F_{(5,36)}=6.72$, $n=7$). Whereas in the contralateral dorsal half of the lumbar spinal cord, no significant change was observed in the content of 5-HT at day 7 after SNL (158.8±19.0 versus 124.1±14.1, $P>0.05$, compared between before and after operation, two-tailed unpaired Student's t-test). The 5-HT content was significantly decreased in the ipsilateral dorsal half of the lumbar spinal cord as compared with that in the contralateral spinal cord tissue ($P<0.05$, one-way ANOVA, $F_{(5,36)}=6.72$, $n=7$; Fig. 6A). Similar decrease was also observed in the content of 5-hydroxyindoleacetic acid (5-HIAA), a main metabolite of 5-HT in the body, which could also be used to determine the body's levels of 5-HT. In the ipsilateral dorsal half of the lumbar spinal cord, the content of 5-HIAA decreased significantly from 223.7±13.7 pg/mg tissue before operation to 166.5±12.0 pg/mg tissue at 7 days after operation ($P<0.01$, one-way ANOVA, $F_{(5,36)}=3.41$, $n=7$). However, in the contralateral dorsal half of the lumbar spinal cord, no significant change was observed in the content of 5-HIAA at day 7 after SNL (217.0±13.1 versus 214.1±12.8, $P>0.05$, compared between before and after operation, two-tailed unpaired Student's t-test). The 5-HIAA content was also markedly decreased in the ipsilateral dorsal half of the lumbar spinal cord as compared with that in the contralateral spinal cord tissue ($P<0.05$, one-way ANOVA, $F_{(5,36)}=6.72$, $n=7$; Fig. 6B). As assessed by 5-HIAA/5-HT, the turnover rate of 5-HT in the ipsilateral dorsal half of the lumbar spinal cord increased significantly at day 7 after SNL (from 1.6±0.2 before operation to 2.9±0.4 after operation, $P<0.01$, one-way ANOVA, $F_{(5,36)}=6.49$, $n=7$), whereas no significant change was observed in the turnover rate of 5-HT in the contralateral dorsal half of the lumbar spinal cord (1.5±0.2 versus 1.8±0.2, $P>0.05$, compared between before and 7 days after operation, two-tailed unpaired Student's t-test). The turnover rate of 5-HT in the ipsilateral spinal cord tissue was markedly increased as compared with that in the contralateral spinal cord tissue ($P<0.05$, one-way ANOVA, $F_{(5,36)}=6.49$, $n=7$; Fig. 6C).

3. Discussion

In the present study, we observed that the inhibitory effects of 5-HT and its multiple receptor agonists including 1A, 1B, 3, 4, and probably 2C receptor agonists, on the C-fiber responses of WDR neurons decreased significantly in rats with neuropathic pain. Furthermore, we found that the antagonistic effects of 5-HT 1B, 2C, 3, and 4 receptor antagonists on the 5-HT-induced inhibition of C-fiber responses of WDR neurons were also attenuated after SNL. In line with these observations,

we also found a significant decrease in the content of 5-HT and 5-HIAA, and a marked increase in the turnover rate of 5-HT (5-HIAA/5-HT) in the ipsilateral dorsal half of the lumbar spinal cord after SNL. These data suggest that a loss or decrease in the descending inhibitory 5-HT system occurs following spinal nerve injury, and this kind of decrease is proposed to be involved in the development of central sensitization and, ultimately, to the nerve injury-induced neuropathic pain.

3.1. *Decrease in the inhibitory effects of 5-HT and its receptors agonists on the C-fiber responses of WDR neurons following spinal nerve injury*

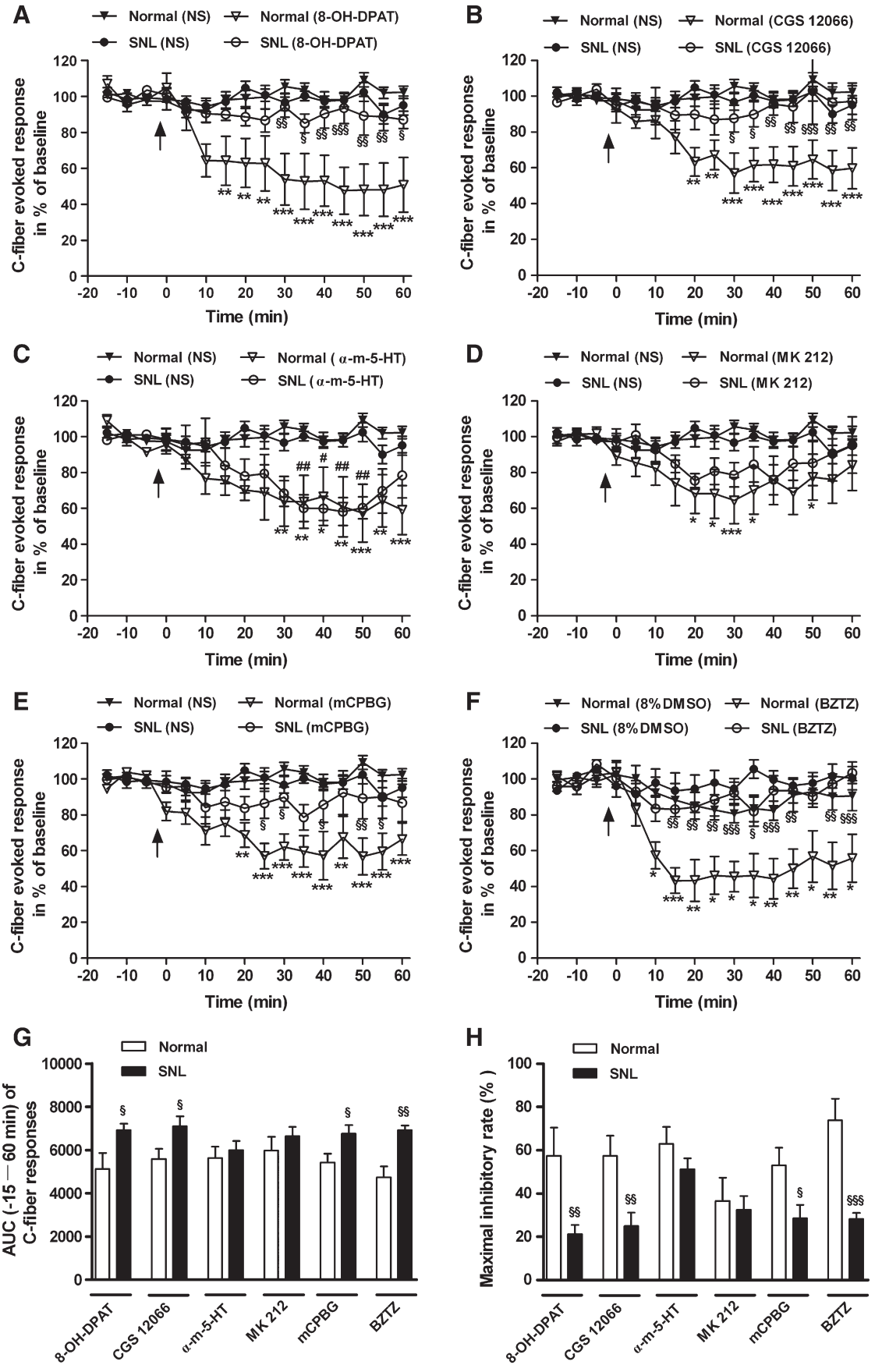
It is well accepted that the descending inhibitory 5-HT pathways play important roles in governing the sensitivity of dorsal horn neurons as well as the pain transmission in the spinal cord in normal conditions (Braz and Basbaum, 2008; Heinricher et al., 2009; Jeong et al., 2004; Sommer, 2006; You et al., 2005). Dysfunction of these descending inhibitory pathways produces hypersensitivity to pain and even lowers the pain threshold so that normally nonnoxious stimuli become painful (Nakae et al., 2008b; Stahl and Briley, 2004). However, whether this dysfunction takes place in the descending inhibitory 5-HT pathways in rats with neuropathic pain still remains largely unclear. In the present study, we found that the inhibitory effects of 5-HT on the C-fiber responses of WDR neurons decreased significantly in SNL rats as compared with those in normal rats, suggesting that a dysfunction of the inhibitory 5-HT system occurred after SNL. Our present results confirmed and extended previous findings that 5-HT was active against pain in normal rats (Bardin et al., 2000a; Crisp et al., 1991; Liu et al., 2007; Solomon and Gebhart, 1988) as well as in neuropathic pain rats (Bardin et al., 2000b; Eaton et al., 1997). Its effects, however, were less marked and occurred only at doses that were 100- to 1000-fold higher in neuropathic rats than those producing antinociception in normal rats (Bardin et al., 2000b). The low potency and efficacy of 5-HT to produce antinociceptive effects in neuropathic pain models suggest that a loss or dysfunction in descending inhibitory 5-HT pathways may happen in these pathological conditions (Laporte et al., 1995; Sounvoravong et al., 2004).

To further determine which subtypes of 5-HT receptors were involved in the dysfunction of 5-HT after SNL, we examined the effects of various 5-HT receptor agonists on the C-fiber responses of WDR neurons in normal and SNL rats. We found that all applied 5-HT receptor agonists including 1A, 1B, 2A, 2C, 3, and 4 receptor agonists significantly inhibited the C-fiber responses of WDR neurons in normal rats, whereas only 5-HT_{2A} receptor agonist exerted the inhibitory effects on the C-fiber responses in SNL rats. The inhibitory effects of 5-HT 1A, 1B, 3, and 4 receptor agonists decreased significantly in SNL rats as compared with those in normal rats; the effective doses of 5-HT 1A and 3 receptor agonists on the C-fiber responses were up to 10-fold higher in SNL rats than those in normal rats (data not shown). These data suggest that the inhibitory effects of multiple 5-HT receptors agonists, including 5-HT 1A, 1B, 3, 4, and probably 2C receptor subtypes, on the C-fiber responses of WDR neurons decreased following SNL. Our present results are consistent with previous reports that dorsal rhizotomy produces selective lesion of the descending serotonergic system on 5-HT_{1A}, 5-HT_{1B}, 5-HT_3, and 5-HT_4 receptors in the rat spinal cord (Laporte et al., 1995) and that infraorbital nerve injury causes a significant decrease in the RNA editing efficiency of 5-HT_{2C} receptor in the rat cervical spinal cord (Nakae et al., 2008a). As a consequence, various agonists of 5-HT 1A, 1B, 2, 3, and 4 receptor subtypes exert antinociceptive effects in normal rats (Bardin et al., 2000a; Crisp et al., 1991; Jeong et al., 2004; You et al., 2005), whereas only 5-HT 1A and 2 receptor agonists have antiallodynic effects in neuropathic rats (Deseure et al., 2003; Honda et al., 2006; Obata et al., 2001; Van Steenwinckel et al., 2008; Wei and Pertovaara, 2006). Taken together, these data provide direct evidence that a decrease in the inhibitory effects of 5-HT and its multiple receptors agonists on the C-fiber responses of WDR neurons appears to occur following spinal nerve injury.

3.2. *Decrease in 5-HT receptor antagonists on the 5-HT-induced inhibition of C-fiber responses of WDR neurons following spinal nerve injury*

In agreement with the decrease in 5-HT receptor agonists on the C-fiber responses of WDR neurons after SNL, our present study also found that in normal rats, most antagonists of 5-HT receptor subtypes including 1B, 2C, 3, and 4 receptor antagonists significantly antagonized the 5-HT-induced inhibition on the C-fiber responses of WDR neurons, whereas in SNL rats, at the same doses as used in normal rats, only 5-HT_{2A} receptor antagonist could antagonize the 5-HT-induced inhibition. At two-fold higher doses as used in normal rats, all of the receptor antagonists except 5-HT_{2C} and 5-HT_3 receptor antagonists still showed significant antagonistic effects on

Fig. 3 – Decrease in the inhibitory effects of 5-HT receptors agonists on the C-fiber responses of WDR neurons in SNL rats. (A)–(F) Effects of spinal application of 5-HT receptor agonists including 5-HT_{1A} receptors agonist 8-OH-DPAT at 5 μg (A), 5-HT_{1B} receptor agonist CGS 12066 at 50 μg (B), 5-HT_{2A} receptor agonist α-m-5-HT at 3 μg (C), 5-HT_{2C} receptor agonist MK 212 at 10 μg (D), 5-HT_3 receptor agonist mCPBG at 10 μg (E), or 5-HT_4 receptor agonist BZTZ at 30 μg (F), on the C-fiber responses of WDR neurons in normal and SNL rats. Note that all applied 5-HT receptor agonists significantly inhibited the C-fiber responses in normal rats, whereas only 5-HT_{2A} receptor agonist α-m-5-HT inhibited the C-fiber responses in SNL rats. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ as compared with NS or vehicle control in normal rats; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ as compared with NS control in SNL rats; $^{\S}P<0.05$, $^{\S\S}P<0.01$, and $^{\S\S\S}P<0.001$, compared between normal and SNL group, two-way ANOVA followed by Bonferroni *post hoc* test, $n=5$–8. (G) AUC of 5-HT receptor agonists on the C-fiber responses in normal and SNL rats. (H) The maximal inhibitory rate of 5-HT receptor agonists on the C-fiber responses in normal and SNL rats. Note that the inhibitory effects of 5-HT 1A, 1B, 3, and 4 receptor agonists on the C-fiber responses decreased significantly in SNL rats as compared with those in normal rats. $^{\S}P<0.05$, $^{\S\S}P<0.01$, and $^{\S\S\S}P<0.001$, compared between normal and SNL groups, two-tailed unpaired Student's *t*-test, $n=5$–8. Data are expressed as mean ± SEM. Arrow, application of NS, vehicle, or agonist.


A
Normal (NS)
Normal (8-OH-DPAT)
SNL (NS)
SNL (8-OH-DPAT)
C-fiber evoked response in % of baseline
Time (min)
B
Normal (NS)
Normal (CGS 12066)
SNL (NS)
SNL (CGS 12066)
C-fiber evoked response in % of baseline
Time (min)
C
Normal (NS)
Normal (α-m-5-HT)
SNL (NS)
SNL (α-m-5-HT)
C-fiber evoked response in % of baseline
Time (min)
D
Normal (NS)
Normal (MK 212)
SNL (NS)
SNL (MK 212)
C-fiber evoked response in % of baseline
Time (min)
E
Normal (NS)
Normal (mCPBG)
SNL (NS)
SNL (mCPBG)
C-fiber evoked response in % of baseline
Time (min)
F
Normal (8% DMSO)
Normal (BZTZ)
SNL (8% DMSO)
SNL (BZTZ)
C-fiber evoked response in % of baseline
Time (min)
G
Normal
SNL
AUC (-15 — 60 min) of C-fiber responses
8-OH-DPAT
CGS 12066
α-m-5-HT
MK 212
mCPBG
BZTZ
H
Normal
SNL
Maximal inhibitory rate (%)
8-OH-DPAT
CGS 12066
α-m-5-HT
MK 212
mCPBG
BZTZ

the 5-HT-induced inhibition in SNL rats. These results further confirmed the dysfunction of multiple 5-HT receptor subtypes following spinal nerve injury, in which 5-HT 1B, 2C, 3, and 4 receptor subtypes were likely to be involved. However, we could not conclude whether the 5-HT_{1A} receptor was involved in mediating the decrease in descending inhibitory 5-HT

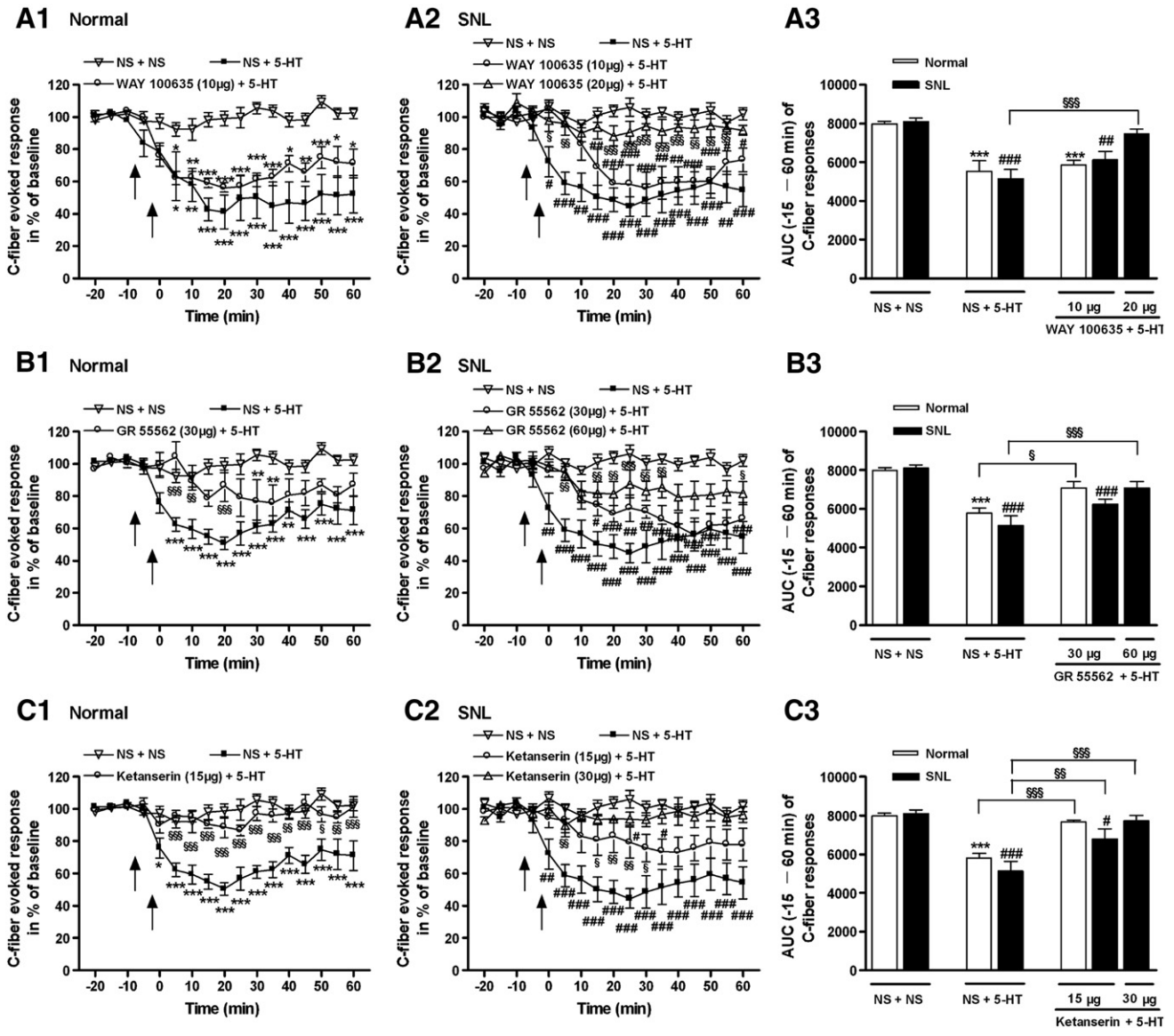


Fig. 4 – Effects of 5-HT receptor antagonists on the 5-HT-induced inhibition of C-fiber responses of WDR neurons in normal and SNL rats. (A1)–(A3) 5-HT_{1A} receptor antagonist WAY 100635 at 10 μg did not affect the 5-HT-induced inhibition on the C-fiber responses either in normal rats (A1) or in SNL rats (A2), the areas under the time–course curve (AUC) of WAY 100635 on the 5-HT-induced inhibition of C-fiber responses in normal and SNL rats were summarized in (A3). (B1)–(B3) 5-HT_{1B} receptor antagonist GR 55562 at 30 μg significantly antagonized the 5-HT-induced inhibition on the C-fiber responses in normal rats (B1) but not in SNL rats (B2), AUC of GR 55562 on the 5-HT-induced inhibition of C-fiber responses were summarized in (B3). (C1)–(C3) 5-HT_{2A} receptor antagonist ketanserin at 15 μg could significantly antagonize the 5-HT-induced inhibition of C-fiber responses both in normal rats (C1) and in SNL rats (C2). AUC of ketanserin on the 5-HT-induced inhibition was summarized in (C3). (D1)–(D3) 5-HT_{2C} receptor antagonist RS 102221 at 30 μg; (E1)–(E3) 5-HT_3 receptor antagonist MDL 72222 at 15 μg; (F1)–(F3) 5-HT_4 receptor antagonist GR 113808 at 15 μg; note that these antagonists antagonized the 5-HT-induced inhibition only in normal rats but not in SNL rats. When at twofold higher doses as used in normal rats, all of the receptor antagonists except 5-HT_{2C} receptor antagonist RS 102221 and 5-HT_3 receptor antagonist MDL 72222 still showed significant antagonistic effects on 5-HT-induced inhibition of the C-fiber responses (A3)–(F3). $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ as compared with NS or vehicle control in normal rats; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.01$ as compared with NS or vehicle control in SNL rats; $^{\S}P<0.05$, $^{\S\S}P<0.01$, and $^{\S\S\S}P<0.001$, compared between normal and SNL group, one or two-way ANOVA followed by Bonferroni *post hoc* test, $n=5–8$. Data are expressed as mean ± SEM. First arrow, application of NS, vehicle, or antagonist; second arrow, application of NS, vehicle, or 5-HT (1.5 μg in normal rats and 10 μg in SNL rats, respectively).

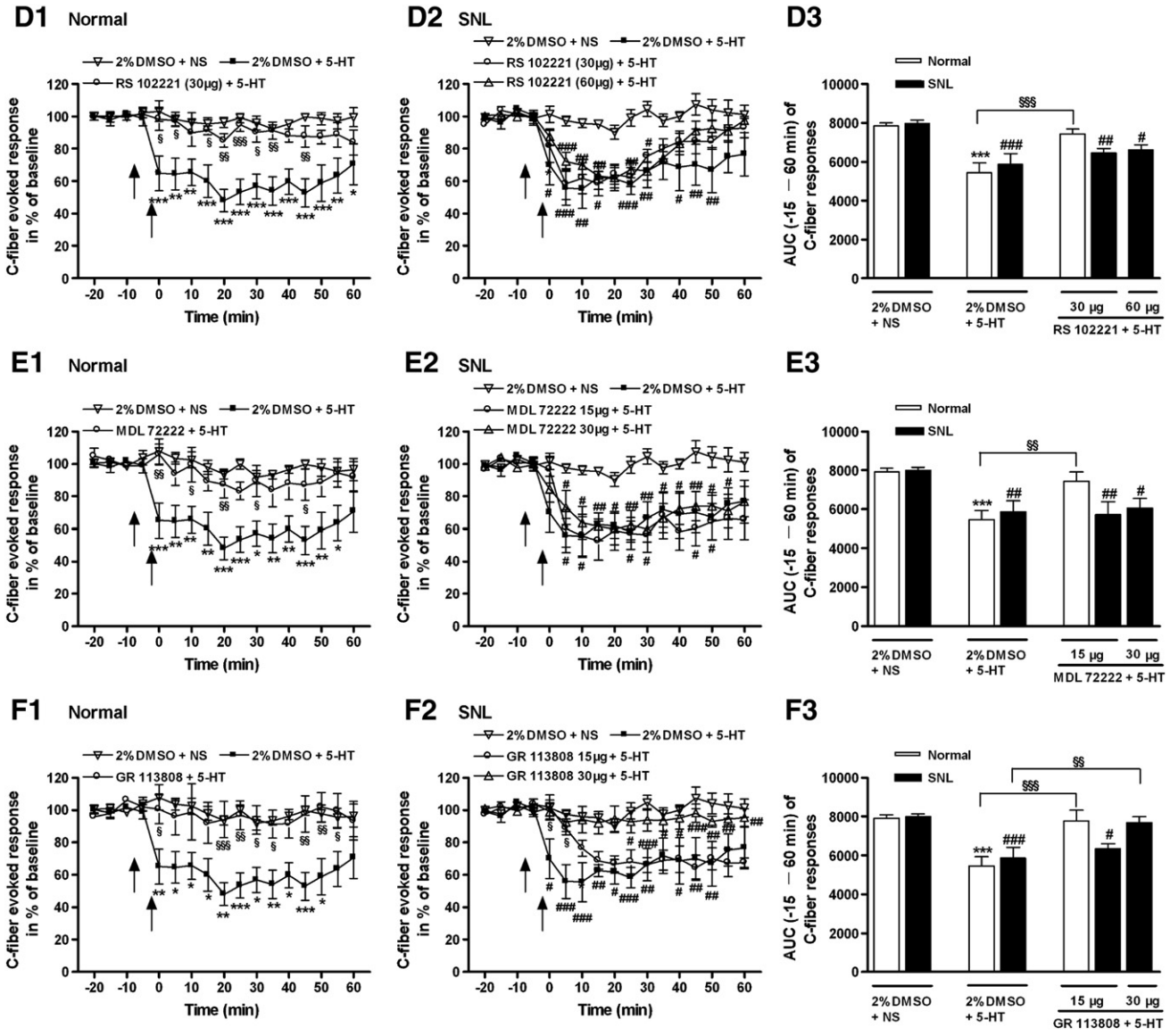


Fig. 4 (*continued*).

system after SNL, because the discrepancy was observed on the effects of its agonist and antagonist. We observed the inhibitory effect of 5-HT_{1A} receptor agonist (8-OH-DPAT) on the C-responses of WDR neurons in normal rats, and proved a decrease in the inhibitory effect of 8-OH-DPAT on the C-responses in SNL rats. However, we did not observe any antagonistic effect of 5-HT_{1A} antagonist (WAY 100635) on the 5-HT-induced inhibition either in normal rats or in SNL rats. This discrepancy may be related to the selectivity of the receptor for drugs. Unlike WAY 100635, which is a highly selective antagonist for 5-HT_{1A} receptor (Bardin et al., 2000a), 8-OH-DPAT is a low selective agonist for 5-HT_{1A} receptor. It has been reported that except for 5-HT_{1A} receptor, 8-OH-DPAT also shows a moderate affinity for 5-HT_7 receptor (Harte et al., 2005). Therefore, the inhibitory effect of 8-OH-DPAT on the C-responses was probably mediated through the 5-HT_7 rather than the 5-HT_{1A} receptor, or by interaction with the both receptors. Further studies will be necessary to determine the effects of 5-HT_7 receptor on the C-responses of WDR neurons in normal and SNL rats.

Together with the effects of agonists and antagonists of 5-HT, we found that only 5-HT_{2A} receptor was involved in mediating the inhibitory effects of 5-HT on the C-responses after SNL. This result is consistent with the previous reports that the 5-HT_{2A} receptor plays an essential role in spinal suppression of nerve injury-induced neuropathic pain by 5-HT (Obata et al., 2001; Thibault et al., 2008; Van Steenwinckel et al., 2008). In addition, it has been demonstrated that fluvoxamine, a selective serotonin reuptake inhibitor, exerts its antiallodynic effects on neuropathic pain via 5-HT_2 receptors (Honda et al., 2006). In SNL rats, 5-HT acting on 5-HT_{2A} receptors may cause the release of substance P, which in turn acts on tachykinin NK1 receptors to cause the release of acetylcholine (ACH). The release of ACH and the activation of muscarinic receptor in the spinal cord appear to be a mechanism underling the inhibitory effects of 5-HT in SNL rats (Obata et al., 2002).

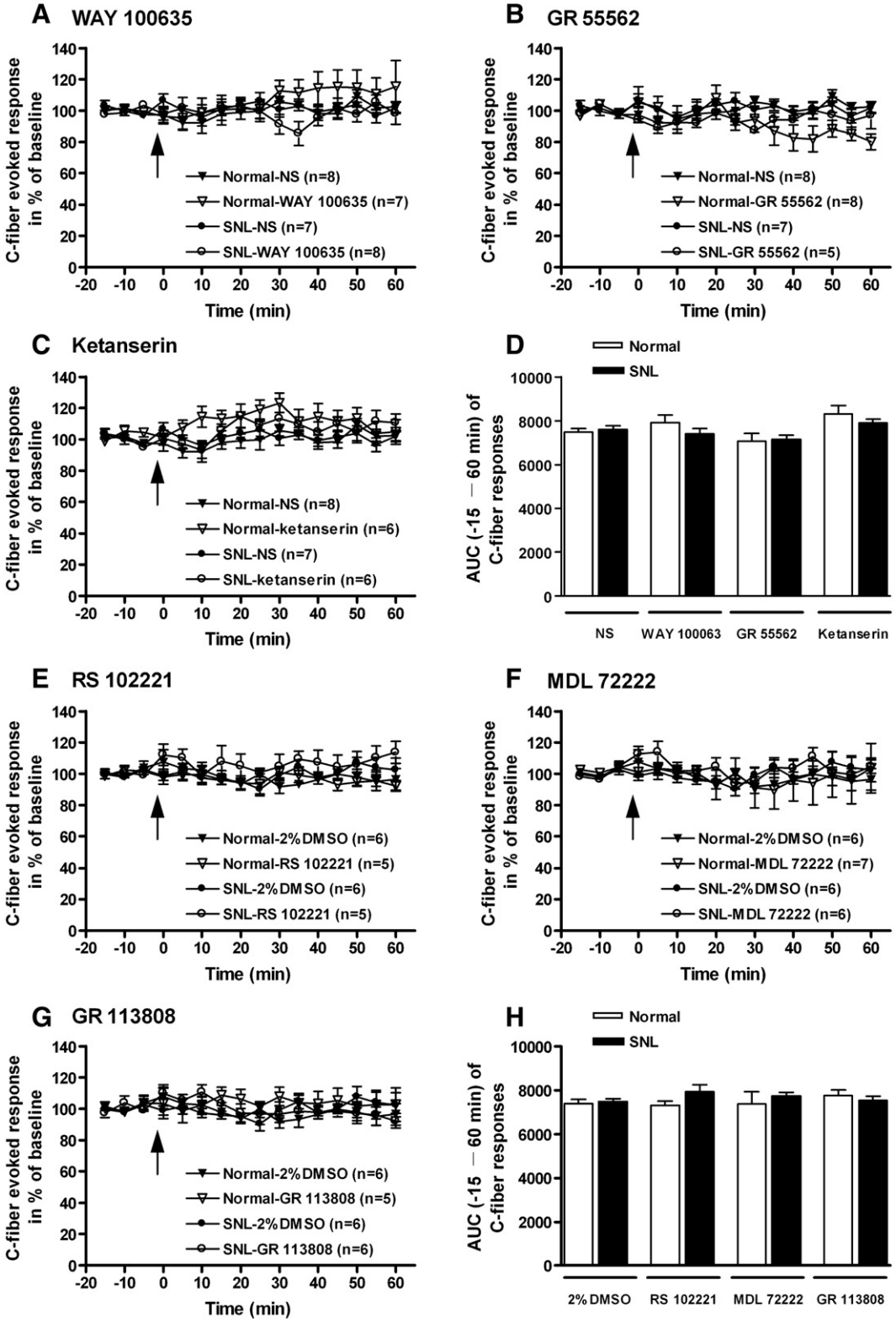


Fig. 5 – Effects of 5-HT receptor antagonist alone on the C-fiber responses of WDR neurons in normal and SNL rats. (A) 5-HT_{1A} receptor antagonist WAY 100635 (10 μg), (B) 5-HT_{1B} receptor antagonist GR 55562 (30 μg), (C) 5-HT_{2A} receptor antagonist ketanserin (15 μg), (D) AUC of the C-fiber responses in (A), (B), and (C); (E) 5-HT_{2C} receptor antagonist RS 102221 (30 μg), (F) 5-HT_3 receptor antagonist MDL 72222 (15 μg), (G) 5-HT_4 receptor antagonist GR 113808 (15 μg), (H) AUC of the C-fiber responses in (E), (F), and (G); note that none of the antagonist had any effect on the C-fiber responses either in normal rats or in SNL rats. One- or two-way ANOVA followed by Bonferroni *post hoc* test, $n = 5–8$. Data are expressed as mean ± SEM. Arrow, application of NS, vehicle, or antagonist.

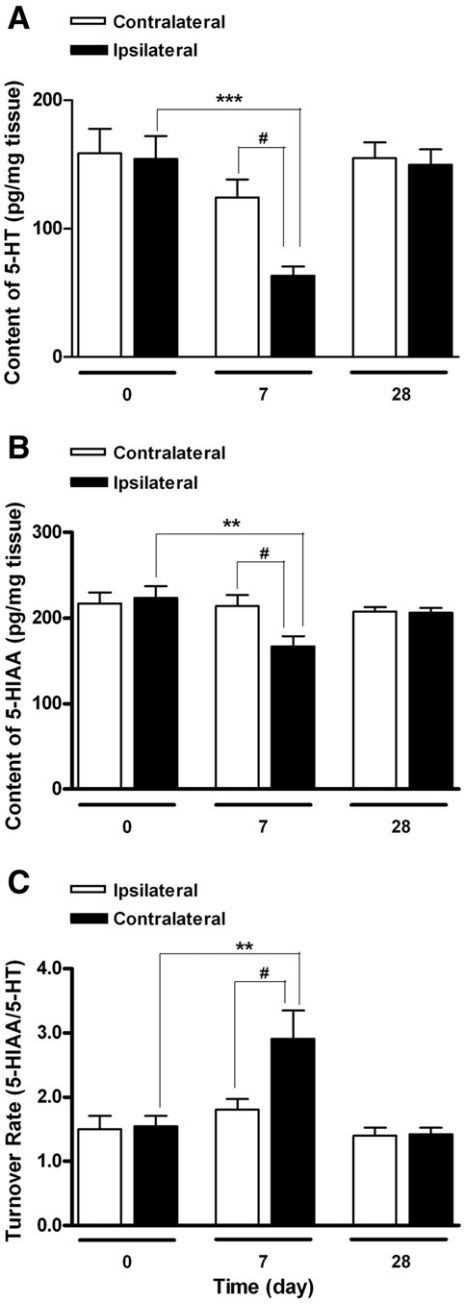


Fig. 6 – Decrease in the content of 5-HT and 5-HIAA in the spinal cord following SNL. (A) The 5-HT content. (B) The 5-HIAA content. (C) The turnover rate of 5-HT (5-HIAA/5-HT). Note that the content of 5-HT and 5-HIAA in the dorsal half of the lumbar spinal cord decreased significantly and the turnover rate of 5-HT was increased at day 7 after SNL. $^{**}P<0.01$, $^{***}P<0.001$, compared between before and after surgery; $^{\#}P<0.05$, compared between contralateral and ipsilateral spinal cord tissue, one ANOVA followed by Bonferroni *post hoc* test, $n=7$. Data are expressed as mean ± SEM.

Two reasons may explain the loss of most 5-HT receptor subtypes in mediating the inhibitory effects of 5-HT after SNL. First, decrease in the expression of 5-HT receptors and reduction of 5-HT affinity for binding to most 5-HT receptors following spinal nerve injury (Nakae et al., 2008b; Padayatti and Paulose, 1999; Thibault et al., 2008; Van Steenwinckel et al., 2008). Second, loss of inhibitory GABAergic neurons in rats with neuropathic pain (Eaton et al., 1998; Ibuki et al., 1997; Moore et al., 2002; Zeilhofer, 2008). For example, the decreases in 5-HT 1A, 1B, and 3 receptors binding and labeling in the rat spinal cord after dorsal rhizotomy (Laporte et al., 1995) and the reduction in the RNA editing efficiency of 5-HT_{2C} receptor in the rat cervical spinal cord following infraorbital nerve injury (Nakae et al., 2008a) probably cause the dysfunction of these receptor subtypes after nerve injury. In addition, it has been reported that the 5-HT_3 receptor is expressed in a subpopulation of GABAergic neurons in the mouse dorsal spinal cord (Huang et al., 2008). Application of 5-HT may activate GABAergic neurons via 5-HT_3 receptor and, therefore, evoke the release of GABA, which may in turn inhibit the C-fiber responses of WDR neurons in normal rats (Huang et al., 2008; Kawamata et al., 2003; Wang et al., 2008). As a consequence, the loss of inhibitory GABAergic neurons in the spinal cord may lead to the dysfunction of 5-HT_3 receptor in mediating the inhibitory effects of 5-HT following SNL. Similar reason may also be used to explain the dysfunction of 5-HT_4 receptor after SNL (Bianchi et al., 2002; Consolo et al., 1994).

3.3. *Decrease in the content of 5-HT and 5-HIAA in the spinal cord following spinal nerve injury*

In addition to the dysfunction of multiple 5-HT receptor subtypes on the C-fiber responses of WDR neurons following spinal nerve injury, our present study also found that the content of 5-HT and 5-HIAA in the dorsal half of the lumbar spinal cord decreased significantly and the turnover rate of 5-HT (5-HIAA/5-HT) increased after SNL. The increase in the 5-HT turnover was probably due to the reduction of 5-HT (e.g., decrease in release or increase in reuptake of 5-HT, or both) following nerve injury, which in turn accelerated the synthesis and release of 5-HT from the terminal of supraspinal serotonergic neurons. However, the net 5-HT content in the spinal cord was still decreased because of the loss or lesion of supraspinal serotonergic neurons following nerve injury (Eaton et al., 1997; Goettl et al., 2002; Hains et al., 2001a; Hains et al., 2002; Sounvoravong et al., 2004). These results are line with previous studies that the 5-HT level is markedly decreased in wide pain pathways including the cerebral cortex, the ventrobasal thalamus, the raphe magnus nucleus (NRM), and the spinal cord in multiple neuropathic pain models (Goettl et al., 2002; Hains et al., 2002; Sandrini et al., 1997; Sounvoravong et al., 2004). It has been reported that the predominant source of serotonergic input to the spinal cord arises most prominently from the NRM. Peripheral nerve injury probably induce plastic changes in structures and functions in the NRM, and decrease in the content of 5-HT in the spinal cord (Vogel et al., 2003). We cannot clarify the accurate relation between the decrease in the descending 5-HT content and the changes in spinal 5-HT receptors following nerve injury in our current study. Several lines of evidence implicate that they are two different plastic alterations that might take place independently after nerve injury (Goettl et al., 2002; Hains et al., 2002; Jeong et al., 2004; Laporte et al., 1995; Sounvoravong et al., 2004). Further studies

are needed to elucidate whether the changes in spinal 5-HT receptors are secondary to the decrease in the descending 5-HT or independent on it. Taken together, this study provides further confirmation to the notion that the descending inhibitory 5-HT system is likely to have undergone plastic changes such as decrease in the inhibitory effects on the C-fiber responses of WDR neurons after peripheral nerve injury (Heinricher et al., 2009; Ito et al., 2000; Nakae et al., 2008a; Sounvoravong et al., 2004; Yoshimura and Furue, 2006). Moreover, these results may also provide a reasonable explanation for the previous findings that the antinociceptive effects of 5-HT in neuropathic pain rats are much slight and differ from those observed in normal rats (Bardin et al., 2000b) and that the enhancement of serotonergic functions by i.t. injection of 5-HT, 5-HT reuptake inhibitors, or lumbar transplantation of serotonergic neurons, obviously alleviates the neuropathioc pain behaviors (Eaton et al., 1997; Hains et al., 2003; Honda et al., 2006; Horiuchi et al., 2003).

3.4. *Decrease in the descending inhibitory 5-HT system and its role in the development of neuropathic pain*

Apart from the descending inhibitory 5-HT system, it has been established that the descending facilitatory 5-HT pathways may also be involved in the development of neuropathic pain (Apkarian et al., 2009; Bee and Dickenson, 2007; Saade and Jabbur, 2008; Sanoja et al., 2008; Suzuki et al., 2004). It is clear that, in addition to intrinsic spinal mechanisms and peripheral inputs, the spinal excitability is influenced by activities from supraspinal sources, whether excitatory or inhibitory, and an overall balance of these events eventually govern the pain-related behavioral outcome (Heinricher et al., 2009). Under normal conditions, a balance is maintained between excitations and inhibitions. When this kind of equilibrium is disturbed after nerve injury, e.g., a decrease in inhibitory activity or an increase in excitatory activity or both, excitations are then excessively dominated, and consequently, hyperexcitability in the spinal nociceptive transmission neurons is produced. Therefore, such imbalance between pain inhibiting and facilitating outflows from the supraspinal sites may contribute to the development of central sensitization and, ultimately, to the nerve injury-induced neuropathic pain (Campbell and Meyer, 2006; Heinricher et al., 2009; Suzuki and Dickenson, 2005). In this study, we provide convincible evidence that a decrease in descending inhibitory 5-HT system appears to occur after SNL, and this kind of decrease may lead to the unbalance between excitations and inhibitions in the spinal cord, which in turn induce the central sensitization and neuropathic pain. However, since the functional changes in the descending serotonergic system was only assessed at a single time point in our present study, we cannot clarify whether the observed changes actually contribute to the abnormal pain behaviors in SNL rats. More time points will be added to examine the functional changes in the descending serotonergic system following SNL, and the correlation between the functional changes and abnormal pain behaviors will also be assessed in further studies.

In conclusion, our present study suggests that a decrease in the descending inhibitory 5-HT system, including 1B, 3, 4, and, probably, 1A or 2C receptor subtypes, appears to occur after spinal nerve injury. Moreover, the reduction of 5-HT content in the spinal cord dorsal horn may also be involved in the functional decrease in 5-HT-induced inhibition on the spinal nociception after spinal nerve lesion. Thus, this kind of decrease in the descending inhibitory 5-HT system is likely to play an important role in the development of neuropathic pain.

4. Experimental procedures

4.1. *Animals*

Male Sprague-Dawley rats weighing 220–250 g at the beginning of the experiment were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. The rats were housed in separated cages with free access to food and water. The room temperature was kept at 24±1 °C under natural light–dark cycle. All animal experimental procedures were conducted in accordance with the guidelines of the International Association for the Study of pain (Zimmermann, 1983) and were approved by the Animal Care and Use Committee of our university.

4.2. *Spinal nerve ligation (SNL)*

Under general anesthesia with chloral hydrate (0.3 g·kg^{-1}, i.p.), the left L5 spinal nerves distal to the dorsal root ganglia were tightly ligated with 4-0 silk sutures as described earlier by Kim and Chung (1992). In control animals, sham surgery with identical procedure except for ligation of the L5 spinal nerves was received. Animals were allowed to recover for 7 days before electrophysiological recording or high-performance liquid chromatography (HPLC) detection. Any rats exhibiting motor deficiency or lack of tactile allodynia were excluded from the study.

4.3. *Assessment of mechanical allodynia*

Mechanical allodynia, as a behavioral measure of neuropathic pain, was assessed by measuring 50% paw withdrawal threshold (PWT) as described in our previous reports (Qu et al., 2009; Xing et al., 2007). The 50% PWT in response to a series of von Frey filaments was determined by the up and down method (Chaplan et al., 1994) and was calculated as previously described (Dixon, 1980). The 50% PWT was obtained for all animals at day 1 before SNL surgery and day 7 after SNL surgery, respectively. Only those with significant mechanical allodynia, in which the 50% PWT in ipsilateral hindlimb was less than 4.0 g, were selected for further electrophysiological or HPLC studies.

4.4. *Electrophysiological studies*

4.4.1. *Surgery*

The rat was initially anesthetized with urethane (1.2–1.5 g·kg^{-1}, i.p.). The trachea was cannulated to allow mechanical ventilation with room air. A catheter was inserted into the right jugular vein for continuous infusion of Tyrode's solution [in mmol·L^{-1}: NaCl 137, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 1.0, $NaHCO_3$ 6.0, NaH_2PO_4 2.1, D-(+)-glucose 6.5; pH 7.4] at a rate of 1.0–

1.5 mL·h^{-1}. The rectal temperature was maintained at 36.5–37.5 °C via a feedback-controlled under-body heating pad. A pair of bipolar silver hook electrode was placed under the sciatic nerve immediately proximal to the trifurcation for electrical stimulation. The vertebral column was rigidly fixed in the frame with two clamps. The lumbar enlargement of the spinal cord was exposed by laminectomy at the vertebrae T13 and L1, and the dura covering lumbosacral spinal segments was carefully removed. A small well was built with 3% agar on the dorsal spinal cord at the recording segment to allow application of drugs or vehicles (Qu et al., 2009). The exposed spinal tissue was covered with warm (37 °C) saline solution.

After surgery, the animal was artificially ventilated with a small animal ventilator and paralyzed with curare (2.0 mg·kg^{-1}, i.v.), and continuous anesthesia and paralysis were maintained with urethane (0.10–0.15 g·kg^{-1}·h^{-1}) and curare (0.20 mg·kg^{-1}·h^{-1}) during the whole experiment. The depth of anesthesia was monitored by examination of pupillary size and reflexes. The physiological condition of the animal was monitored by recording the electrocardiogram (330–460 beats/min), end-expiratory CO_2 (3.5–4.5%), and rectal temperature (36.5–37.5 °C) and was maintained within the range indicated.

4.4.2. *Extracellular recording*

Single-unit extracellular recordings were made from the lumbar dorsal horn neurons within 1200 μm of the dorsal surface of the spinal cord with 2–5 MΩ parylene-coated tungsten microelectrodes (Friedrick Haer & CO., Bowdoinham, ME). The microelectrode was inserted perpendicularly into the dorsal horn from a point about midway between the midline and the medial edge of the dorsal root entry zone. During electrode advancement, electrical pulses (0.5 Hz, 0.3-ms pulse width, 0.4 mA) were applied to the ipsilateral sciatic nerve as search stimuli so that a neuron with no spontaneous firing could be identified. Once a single unit was identified, the receptive field and response characteristics were determined by a range of mechanical stimuli of varying intensities, including brushing or touching the skin with a cotton brush, light pressure with a probe, and pinching a fold of skin with toothed forceps. A neuron responding to innocuous tactile stimuli, light pressure, and noxious pinch in a graded manner was identified as a WDR neuron and was selected for further investigation (Qu et al., 2009). The recorded signals were amplified with an AC preamplifier, filtered with a passing bandwidth of 500–1000 Hz, displayed on an oscilloscope, and fed to a Pentium computer via a CED 1401 interface for offline analysis using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Spikes appearing 45–300 ms after stimulus were defined as C-fiber responses, i.e., responses in the WDR neurons evoked by C-fiber activation (Qu et al., 2009). Single-cell recording was ensured on the basis of amplitude and shape of the action potentials. In the present study, only C-fiber responses were examined and analyzed because the C-fiber responses of WDR neurons are usually regarded as related to nociception (Lu and Perl, 2007; Qu et al., 2009). All of the C-fiber responses values were expressed as percentage of the mean response value of three pre-drug consecutive trains of test stimuli. Cells showing variation of less than 20% were selected for further experiments.

4.4.3. *Measurement of drug effects*

The first part of the experiment was designed to investigate whether the inhibitory effects of 5-HT and its receptor subtypes agonists on the C-fiber responses of dorsal horn WDR neurons decreased following spinal nerve injury. In this experiment, a train of 10 stimuli (0.5 Hz, 0.5-ms pulse width, with a pulse current of 2×C-fiber response threshold) used as test stimulus was applied repeatedly to the sciatic nerve at 5-min interval, and poststimulus histograms from the responses of WDR neurons were constructed. After three stable control responses were recorded, different doses of 5-HT or various agonists of 5-HT receptor subtypes were applied topically to the dorsal surface of the spinal cord, and the post-drug responses evoked by the same test stimulus as above were measured at 5-min intervals for up to 60 min. 5-HT was topically applied in a 50-μL volume of solution, at the doses of 0.5, 1.5, and 5.0 μg in normal rats and, 1.0, 5.0, 10, and 100 μg in SNL rats, respectively. Various agonists of 5-HT receptor subtypes including 5-HT$_{1A}$ receptor agonist 8-OH-DPAT at the dose of 5 μg, 5-HT$_{1B}$ receptor agonist CGS 12066 at 50 μg, 5-HT$_{2A}$ receptor agonist α-m-5-HT at 3 μg, 5-HT$_{2C}$ receptor agonist MK 212 at 10 μg, 5-HT$_3$ receptor agonist mCPBG at 10 μg, and 5-HT$_4$ receptor agonist BZTZ at 30 μg were applied spinally in a volume of 50 μL in normal and SNL rats, respectively.

The second part of the experiment was designed to determine whether the antagonists of 5-HT receptor subtypes could block or attenuate the inhibitory effects of 5-HT on the C-fiber responses of WDR neurons and whether the inhibitory effects of the antagonists were different between normal and SNL rats. Various antagonists of 5-HT receptor subtypes including 5-HT$_{1A}$ receptor antagonist WAY 100635 (10 μg or 20 μg), 5-HT$_{1B}$ receptor antagonist GR 55562 (30 μg or 60 μg), 5-HT$_{2A}$ receptor antagonist ketanserin (15 μg or 30 μg), 5-HT$_{2C}$ receptor antagonist RS 102221 (30 μg or 60 μg), 5-HT$_3$ receptor antagonist MDL 72222 (15 μg or 30 μg), and 5-HT$_4$ receptor antagonist GR 113808 (15 μg or 30 μg) were spinally administrated 5 min before 5-HT (1.5 μg in normal rats and 10 μg in SNL rats, respectively) application.

4.4.4. *Chemical preparation and application*

5-Hydroxy-3-(2-aminoethyl) indole hydrochloride (5-HT) (Sigma-Aldrich, Saint Louis, MO) was used as a nonselective 5-HT receptor agonist. All other drugs were purchased from Tocris Cookson (Bristol, UK), unless otherwise stated. Agonists applied included 5-HT$_{1A}$ receptor agonist 8-OH-DPAT, 5-HT$_{1B}$ receptor agonist CGS 12066 (Sigma-Aldrich), 5-HT$_{2A}$ receptor agonist α-methyl-5-hydroxytryptamine maleate (α-*m*-5-HT), 5-HT$_{2C}$ receptor agonist MK 212, 5-HT$_3$ receptor agonist 1-(3-chlorophenyl) biguanide hydrochloride (mCPBG), and 5-HT$_4$ receptor agonist 2-[1-(4-piperonyl) piperazinyl]-benzothiazole (BZTZ). Antagonists used included 5-HT$_{1A}$ antagonist WAY 100635 (Sigma-Aldrich), 5-HT$_{1B}$ antagonist GR 55562, 5-HT$_{2A}$ antagonist ketanserin, 5-HT$_{2C}$ antagonist RS 102221, 5-HT$_3$ antagonist MDL 72222, and 5-HT$_4$ antagonist GR 113808 (Sigma-Aldrich). The doses of the agonists and antagonists were chosen according to our preliminary data and previous reports (Jeong et al., 2004; Liu et al., 2007; Obata et al.,

2001). RS 102221, MDL 72222, GR 113808, and CGS 12066 were dissolved in 2.0% dimethyl sulfoxide (DMSO), BZTZ in 8.0% DMSO, and other drugs were dissolved in normal saline (NS).

4.4.5. *Recording sites*

At the end of each experiment, the recording site was marked by electrolytic lesion (20.0 μA positive depolarizing DC current for 20 s), and the animal was subsequently euthanized with an overdose of pentobarbital sodium. The spinal cord was fixed in 4 °C 4% paraformaldehyde overnight, and sectioned into 20-μm-thick transverse sections on a cryostat and stained with cresyl violet. The recording site was then identified and plotted on a schematic representation of the lumbar spinal cord.

4.5. *High-performance liquid chromatography (HPLC)*

The contents of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were detected by using an HPLC apparatus with an electrochemical detector (Model 5600A Coul Array Detector System ESA, Brighton, MA). After being sacrificed by decapitation, the rat spinal cord was removed immediately in less than 2 min and was frozen by immersing in liquid nitrogen. The spinal cord was then allowed to warm to –20 °C and was divided into ipsilateral and contralateral to the side of the ligation in the lumbar dorsal half (L4–L6). The dissected tissue samples were stored at –80 °C until analysis (Omote et al., 1998). Tissues were homogenized in 200 μL of 400 mM ice-cold perchloric acid by ultrasonic disintegration over ice using an ultrasonic processor. The homogenate was placed in an ice bath for 60 min. Subsequently, the sample was centrifuged at 15,000 ×*g* for 20 min at 4 °C. The supernatant was transferred to a clean tube and measured for volume. One-half volume of a solution containing 20 mM potassium citrate, 300 mM potassium dihydrogen phosphate, and 2 mM EDTA.2Na was added and mixed in thoroughly to deposit perchloric acid. After incubating in an ice bath for 60 min, the mix was centrifuged at 15,000 ×*g* for 20 min at 4 °C. The supernatant was filtered through a 0.22-μm Millipore filter and then injected into the HPLC system for analysis. The mobile phase was 125 mM sodium citrate buffer containing 16% methanol, 0.1 mM EDTA.2Na, 1.2 mM 1-octanesulfonic acid sodium salt (Acros Organics, Morris Plains, NJ) adjusted to pH 4.3. The flow rate was set at 1.0 mL/min. Seven animals in each group were used.

4.6. *Statistical analysis*

All data were expressed as mean ± SEM. One- or two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test was used for multiple comparison. *F* values with their associated degrees of freedom (treatment, time, interaction, and residual) were expressed as $F_{(df \text{ of treatment, time, interaction/residual})}$ = *F* values (treatment, time, interaction) in two-way ANOVA, and $F_{(\text{df of treatment, residual})}$ = *F* values in one-way ANOVA. Two-tailed unpaired Student's *t*-test was used for the comparison of the mean values between two groups. Maximal inhibitory rate or area under the time–course curve (AUC) values during the analysis time was used to measure the summed effects of different drugs as described previously (Honda et al., 2006; Qu et al., 2009). Differences with $P<0.05$ were considered statistically significant.

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