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Duloxetine deteriorates prefrontal noradrenergic pain facilitation, but reduces locus coeruleus activity to restore endogenous analgesia in chronic neuropathic pain state

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Chronic neuropathic pain increases basal noradrenaline (NA) concentrations in the spinal cord and brain. Spinal NA further increased by duloxetine (DLX) alleviates neuropathic pain, however, the roles of basal and DLX induced NA in brain regions have not been well investigated. α2-adrenoceptor antagonist, atipamezole, to the medial prefrontal cortex (mPFC) produced anti-allodynia at 6 weeks following spinal nerve ligation (SNL6W) but produced no effects in the earlier phase of SNL animals (SNL2W). The anti-allodynia effect of intraperitoneal DLX is attenuated in SNL6W, and the combination of intraperitoneal DLX and atipamezole to the mPFC enhanced the anti-allodynia in SNL6W. Intrathecal atipamezole in SNL6W reduced DLX analgesia. Microdialysis experiments revealed that DLX increased NA in the mPFC and spinal cord in the same manner in both SNL6W and SNL2W. These results suggested that the basal mPFC NA maintains neuropathic pain in SNL6W. The spinal NA increased by DLX produces analgesia, but NA in mPFC may counteract the action of spinal NA. In the locus coeruleus (LC), DLX increased NA around LC and decreased the pERK immunoreactivity to restore noxious stimuli induced analgesia. These results suggest that reduced LC activity may lead to the restoration of stimulus responsive activity.

Keywords Duloxetine, Noradrenaline, Locus coeruleus, Prefrontal cortex, Endogenous analgesia

Noradrenergic neurons project widely from the LC to the central nervous system (CNS), where the NA regulates brain functions such as attention and cognition[1]. Under normal conditions, the LC responds to painful stimuli and is involved in endogenous analgesia by increasing the release of NA in the spinal dorsal horn. However, neuropathic pain causes abnormal sustained excitation of LC to reduce stimulus responsiveness, which results in attenuated endogenous analgesia[2]. It is also reported that sustained excitation of the LC increases NA concentrations at the projection site including spinal cord and mPFC[3–5]. The mPFC is responsible for the symptoms of neuropathic pain observed at the late phase following peripheral nerve injury, such as impaired attention related behavior[5]. In addition, increased NA in the mPFC has been reported to be involved in the maintenance of pain in the trigeminal neuropathic pain model[6].

DLX is one of the first-line drugs in the treatment of neuropathic pain and is a serotonin and noradrenaline reuptake inhibitor that increases NA concentrations in the synaptic cleft by inhibiting NA transporters in CNS[7]. Spinal NA increased by DLX suppresses the pain signals by substituting for the descending noradrenergic pain inhibitory system[3,8]. It is reported that the intraperitoneal DLX can increase spinal NA to produce analgesia even in the neuropathic pain model at the late phase, however, it is attenuated overtime[9]. The previous study concluded that the reduction of spinal cholinergic analgesia contributes to the attenuation of the analgesic effects of DLX. However, the effects of brain NA, which already increased following nerve injury and the further influenced by DLX is not examined.

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It is also reported that DLX restores endogenous analgesia, which is diminished in the chronic pain animals[10,11]. Our previous studies have demonstrated that DLX improves attenuated endogenous analgesia in a neuropathic pain model at the late phase following nerve injury by increasing spinal NA content[10]. However, we could not reveal the effects of DLX on the neuronal activity in the noradrenergic LC neurons. Some previous studies reported that DLX decreases LC activity[11,12]. We hypothesized that DLX reduces abnormal LC activity induced by nerve injury to restore responsive ability to novel stimuli.

As mentioned, the neuropathic animals in the early phase of the segmental spinal nerve injury model (SNL2W) and late phase animals (SNL6W) show obviously different attention and pain related behaviors, probably due to elevated baseline noradrenaline concentration in the PFC and the LC dysregulation. We set the following three purposes for this study. One is to investigate the roles of mPFC NA in pain processing in SNL6W by comparing with SNL2W. We observed the changes of allodynia after injection of noradrenergic antagonists to bilateral mPFC. The second aim was to investigate the role of mPFC NA, already increased by nerve injury, on allodynia when further increased by DLX. We have microdialysis experiments in the mPFC to measure NA of SNL6W and SNL2W following intraperitoneal DLX and measured paw withdrawal threshold following the combination of DLX and noradrenergic antagonists to mPFC. The third purpose is to investigate the effects of DLX on the LC neuronal activity and the endogenous analgesia, and how DLX restores endogenous analgesia. To examine this, we also conducted microdialysis around LC, immunohistochemistry in the noradrenergic neurons in LC using neuronal activity marker, and measured noxious stimuli induced analgesia (NSIA) as an indicator of endogenous analgesia. We also conducted an experiment using a designer receptor exclusively activated by designer drugs (DREADD) to suppress the LC activity to examine whether it can reproduce the restoration of NSIA in chronic neuropathic pain animals.

Results

The alterations of the roles of the mPFC NA between SNL2W and SNL6W rats

To investigate whether the duration of neuropathic pain alters the roles of mPFC NA in analgesia between the SNL2W and SNL6W rats, α2- or α1-adrenoceptor antagonists were administered to the bilateral mPFC. In male SNL2W rats, administration of atipamezole or benoxathian did not alter withdrawal thresholds (Fig. 1A). However, in male SNL6W rats, administration of atipamezole significantly increased the withdrawal threshold at the 15 min compared to pre value (pre vs. 15 min: 5.0 ± 1.5 vs. 8.8 ± 6.3 g, $p < 0.05$, Fig. 1B). The values returned to the same as before administration at 30 min after atipamezole administration. No change was observed in the benoxathian and vehicle group. The same changes are observed in female SNL6W following atipamezole injection (pre vs. 15 min: 3.3 ± 1.3 vs. 7.4 ± 4.7 g, $p < 0.05$, Fig. 1C). No change was observed in benoxathian and vehicle group of female SNL6 W either.

Anti-allodynic effects of DLX in SNL2W and SNL6W rats

We examined the anti-allodynic effects of DLX administered intraperitoneally in male SNL2W and SNL6W rats. The AUC (paw withdrawal threshold × time after administration) was calculated (Fig. 2A). Two-way ANOVA showed the significant main effects of DLX ($F_{1,28}$=33.38, $p < 0.0001$, partial η2 = 0.54). However, there were no main effects of SNL surgery ($F_{1,28}$=2.24, $p = 0.14$, partial η2 = 0.07) nor main effects for the interaction ($F_{1,28}$=3.98, $p = 0.056$, partial η2 = 0.12). The post hoc test suggests that AUC was significantly increased at a dose of DLX 10 mg/kg in the SNL2W rats compared with vehicle treated SNL2W (11.54 ± 2.30 vs. 36.40 ± 16.27 g×hour, $p < 0.0001$). In SNL6W rats, there was no significant difference in AUC between the DLX and vehicle ($p = 0.030$).

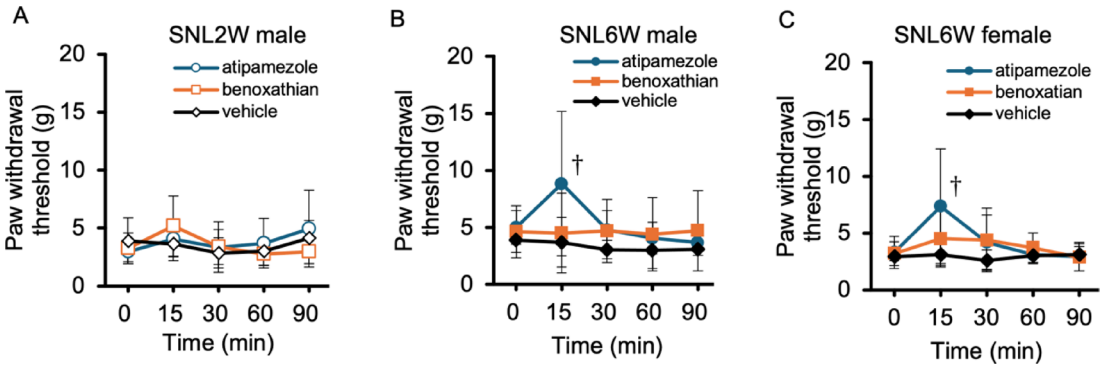

Fig. 1. Effects of adrenoceptor antagonists administration to bilateral mPFC on the allodynia in SNL2W and SNL6W rats. Atipamezole (α2-adrenoceptor antagonist) or benoxathian (α1-adrenoceptor antagonist) was administered to the mPFC and the withdrawal threshold was assessed using the von Frey test over time (baseline 0, 15, 30, 60, and 90 min). In SNL2W rats, the drugs did not change the withdrawal threshold (**A**). However, atipamezole increased the withdrawal threshold in SNL6W rats (**B**). The same changes are observed in female SNL6W following atipamezole injection (C). The data are expressed as the mean ± SD, $n = 8$ each, †: $p < 0.05$ versus vehicle. Two-way repeated ANOVA followed by the Student's t-test with Bonferroni's correction for multiple comparisons. SNL; spinal nerve ligation, mPFC; medial prefrontal cortex.

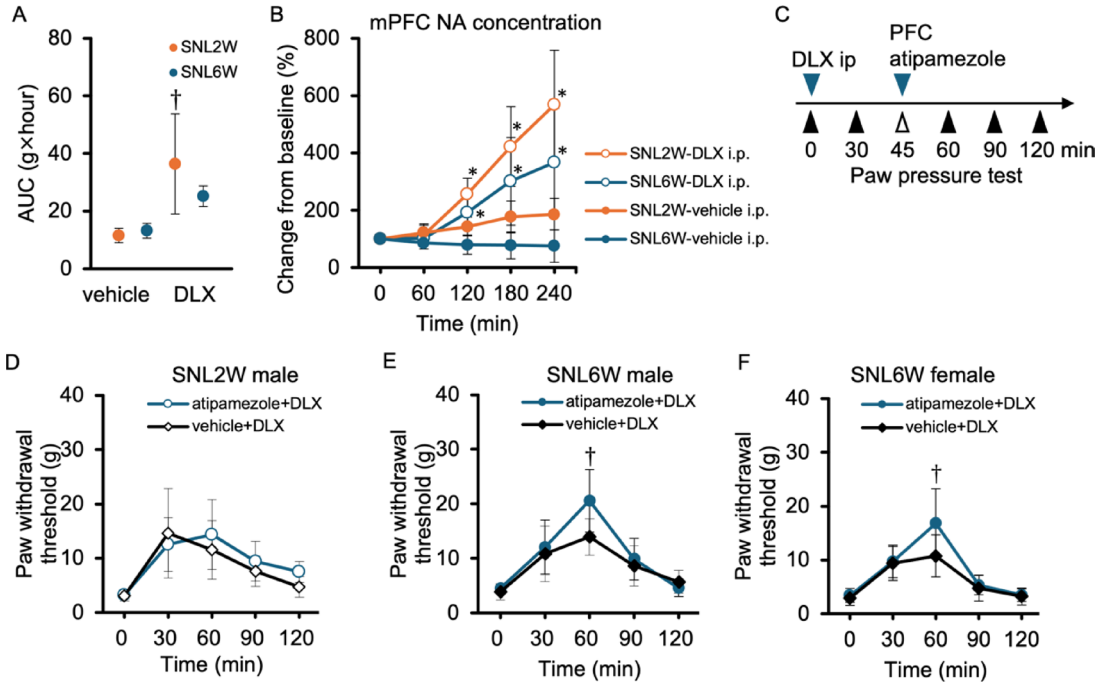

Fig. 2. The role of mPFC NA in analgesic effects of DLX alters over time following nerve injury. Intraperitoneal DLX (10 mg/kg) analgesia is attenuated in SNL6W (**A**). However, the changes in mPFC NA concentration following intraperitoneal DLX are not different between SNL2W and SNL6W (**B**). The time course of intra-mPFC injection of the α2-adrenoceptor antagonist, atipamezole combined with intraperitoneal DLX (10 mg/kg) is shown in (**C**). The combination did not change the anti-allodynic effects in SNL2W (**D**). However, the combination increased the DLX analgesia in SNL6W (E). The same changes are observed in female SNL6W (**F**). The data are expressed as the mean ± SD. $n = 8$ each (**A**, **D**, **E**) $n = 6$ each (**B**, **F**). *: $p < 0.05$ versus time 0, †: $p < 0.05$ versus corresponding control. #: $p < 0.05$ versus SNL6W rats. Two-way repeated ANOVA followed by the Student's t-test with Bonferroni's correction for multiple comparisons. SNL, spinal nerve ligation; mPFC, medial prefrontal cortex; NA, noradrenaline; DLX, duloxetine.

Vehicle used in this experiment did not alter the withdrawal threshold compared with non-treated SNL animals. Therefore, this suggest that the analgesic effect of DLX was attenuated in SNL6W compared to SNL2W rats.

DLX administration increases the NA concentration in the mPFC

We performed microdialysis to investigate the changes in NA concentration at the mPFC following DLX administration in SNL2W and SNL6W rats. Two-way ANOVA showed the significant main effects of DLX ($F_{3,19}$=11.37, p= 0.0001, partial η2 =0.64), and main effects of time after DLX injection ($F_{4,76}$=50.15, p< 0.0001, partial η2 =0.73), and main effects of interaction ($F_{12,76}$=14.08, p< 0.0001, partial η2 =0.68). The NA concentration in the mPFC was significantly increased after 120 to 240 min of DLX administration compared with that before administration in both SNL2W and SNL6W rats (Fig. 2B), and there was no difference between the groups.

Antagonizing the NA action in the mPFC enhances the analgesic effect of DLX

Furthermore, to investigate the effects of NA in mPFC on DLX analgesia, atipamezole, and DLX were administered in sequence so that both peak effects overlapped (Fig. 2C). Two-way ANOVA showed the significant main effects of time after drugs (F4,46 = 36.3, p< 0.0001, partial η2 = 0.72), and main group × time interaction (F4,56 = 2.77, p= 0.036, partial η2 =0.16), but no main effects of group (F1,14 = 3.58, p= 0.079, partial η2 =0.20). These analyses suggest that there was no significant difference in the intraperitoneal DLX with mPFC atipamezole group compared to the intraperitoneal DLX + mPFC vehicle group in male SNL2W rats (Fig. 2D), however, in male SNL6W rats, mPFC atipamezole resulted in an increased withdrawal threshold (20.6 ± 5.7 vs. 13.9 ± 3.3 g, p< 0.05, Fig. 2E). The same changes are observed in female SNL6W following intraperitoneal DLX + mPFC atipamezole (16.8 ± 5.9 vs. 10.8 ± 3.6 g, p< 0.05, Fig. 2F). Two-way ANOVA results were as follows: main effects of time after drugs (F4,40 = 45.27, p< 0.0001, partial η2 = 0.82), main group × time interaction (F4,40 = 3.39, p= 0.017, partial η2 =0.25), main effects of group (F1,10 = 1.54, p= 0.24, partial η2 =0.13). These results indicated NA in mPFC of SNL6W is involved in the maintenance of neuropathic pain in both sexes. Moreover, NA in the mPFC may contribute towards attenuating the analgesic effect of DLX at the spinal cord dorsal horn.

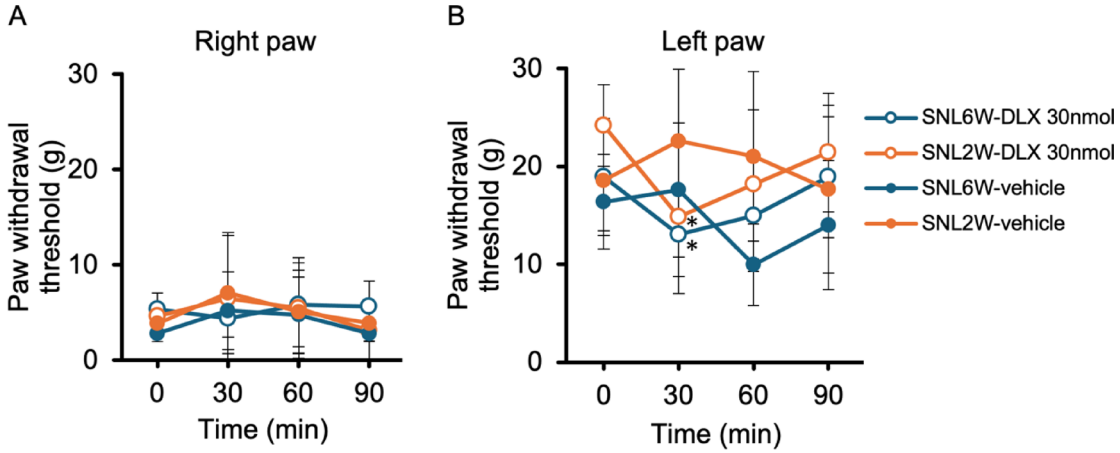

Fig. 3. The roles of mPFC noradrenaline increased by local DLX injection. Local injection of DLX in bilateral mPFC (30 nmol) did not alter the withdrawal threshold in the nerve injured paw in both SNL2W and SNL6W rats (**A**). Bilateral mPFC DLX decreased the threshold in the un-injured paw of both groups (**B**). The data are expressed as the mean ± SD. n = 11,12,7,8. *: $p < 0.05$ versus time 0. Two-way repeated ANOVA followed by the Student's t-test with Bonferroni's correction for multiple comparisons. SNL; spinal nerve ligation, DLX; duloxetine.

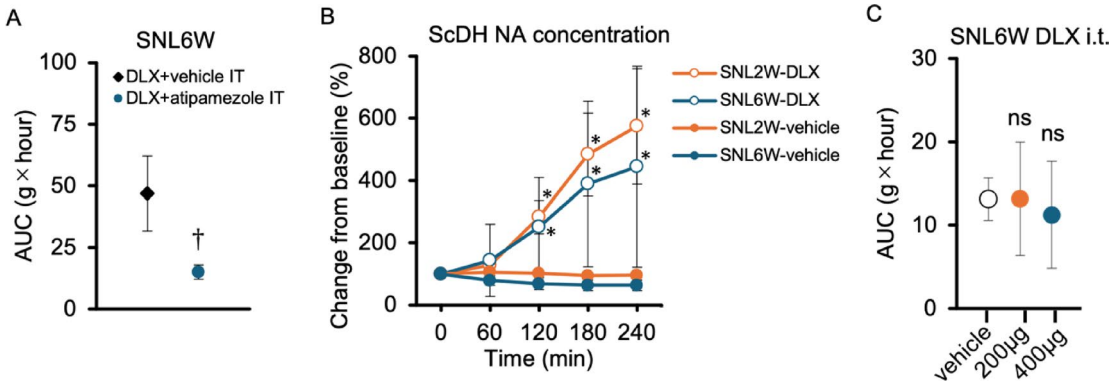

Fig. 4. DLX analgesia is mediated by spinal α2 adrenoceptor. (**A**) Intraperitoneal DLX analgesia is diminished by intrathecal α2 adrenoceptor antagonist, atipamezole, in SNL6W rats. Atipamezole (20 μg/5μL) or vehicle was intrathecally injected through a catheter 15 min before DLX administration (30 mg/kg). The AUC was calculated from the withdrawal threshold value over time (baseline 0, 30, 60, 90, 120, 180 min) following intraperitoneal DLX administration. Changes in NA concentration in the ScDH following local DLX administration (2 μM) by reverse microdialysis are shown in (**B**). DLX administration resulted in a significant increase in NA concentration in both SNL2W and SNL6W rats. (**C**) However, intrathecal DLX did not produce anti-allodynic effects in SNL6W. The data are expressed as the mean ± SD. n = 8 each (**A**), n = 6 each (**B**), n = 8,9,6 (**C**). *: $p < 0.05$ versus time 0, †: $p < 0.05$ versus corresponding control. ns: not significant vs. vehicle. SNL; spinal nerve ligation, ScDH; spinal cord dorsal horn, NA; noradrenaline, DLX; duloxetine, AUC, area under the curve.

The roles of mPFC NA increased by local DLX injection

We investigated whether DLX administration to mPFC contributes to analgesia or pain. SNL2W and SNL6W rats were bilaterally administered vehicle or 30 nmol/0.5 μL of DLX. Paw withdrawal thresholds were measured by von Frey test at 30, 60 and 90 min after drug administration. There were no significant differences in the nerve injured paw in both SNL2W and SNL6W rats (Fig. 3A). However, paw withdrawal thresholds in the uninjured paw significant transient decreased in both SNL2W and SNL6W rats (SNL2W: 22.1 ± 6.9 vs. 14.9 ± 7.8 g, SNL6W: 17.6 ± 6.9 vs. 12.9 ± 4.5 g, $p < 0.05$ respectively, Fig. 3B). Two-way ANOVA results were as follows: main effects of DLX ($F_{3,33} = 3.38$, $p = 0.038$, partial $\eta 2 = 0.23$), main effects of time ($F_{3,39} = 2.53$, $p = 0.06$, partial $\eta 2 = 0.07$), main group × time interaction ($F_{9,99} = 3.05$, $p = 0.0029$, partial $\eta 2 = 0.21$).

We investigated the analgesic effects of NA in the spinal cord dorsal horn, which are increased by systemic DLX administration, in SNL6W rats. Atipamezole or vehicle was intrathecally administered, before intraperitoneal DLX (30 mg/kg). The AUC of mechanical hypersensitivity was significantly reduced in the atipamezole compared to the vehicle (46.9 ± 15.2 vs. 15.0 ± 2.9 g×hour, $p < 0.05$, Fig. 4A). Therefore, the analgesic effect of DLX was

inhibited by α2 adrenoceptor antagonist administered intrathecally. In SNL6W rats, NA, which is increased by DLX intraperitoneal administration, contributes to analgesia via α2 adrenoceptor in the spinal cord dorsal horn.

To confirm the analgesic effects of DLX are mediated by spinal NA, we performed microdialysis to investigate the changes in NA concentration at the spinal dorsal horn following local DLX perfusion in SNL2W and SNL6W rats. Two-way ANOVA results were as follows: main effects of time after drugs ($F_{4,100} = 31.55$, $p < 0.0001$, partial $\eta 2 = 0.46$), main effects of group ($F_{3,25} = 13.41$, $p < 0.001$, partial $\eta 2 = 0.50$), main group × time interaction ($F_{12,100} = 13.46$, $p < 0.0001$, partial $\eta 2 = 0.52$). These results suggest that the local perfusion of DLX (2 μM) increased NA concentration in the spinal dorsal horn in both SNL2W and SNL6W rats. The NA concentration was significantly increased after 120 to 240 min of DLX administration compared with that before administration (Fig. 4B). The changes from the baseline value are not different between the SNL2W and SNL6W.

We investigated the effects of intrathecal DLX in SNL6W rats. Rats were intrathecally administered vehicle or 200–400 μg of DLX, and paw withdrawal thresholds were measured by von Frey test at 30, 60, and 90 min after drug administration. There were no significant differences in analgesic effects in all groups (Fig. 4C).

Effect of DLX on the LC

We examined the effect of DLX administration on the LC, which is the NA initiating nucleus. First, we performed in vivo microdialysis to investigate the changes in NA concentration around the LC following intraperitoneal DLX administration in SNL2W and SNL6W rats. Two-way ANOVA results were as follows: main effects of time ($F_{4,80} = 15.46$, $p < 0.0001$, partial $\eta 2 = 0.43$), main effects of group ($F_{3,20} = 6.42$, $p = 0.0032$, partial $\eta 2 = 0.49$), effects of group x time interaction ($F_{12,80} = 5.85$, $p < 0.0001$, partial $\eta 2 = 0.47$). The NA concentration in the LC was also significantly increased after 120 min of DLX administration compared with that before administration in both rats (Fig. 5A). The changing ratio is not different between the SNL2W and SNL6W.

Then, to investigate the effects of DLX on the LC neuronal activity, immunohistochemical staining was performed using an anti-pERK antibody as an indicator of the activity of the LC neurons (Fig. 5B). One hour after DLX administration (10 mg/kg), perfusion fixation was performed and brain tissue containing LC was sectioned. Two-way ANOVA showed the significant main effects of DLX ($F_{1,14}$=59.99, $p < 0.01$, partial $\eta 2 = 0.8108$) and the main effects of SNL surgery ($F_{2,28}$=2.96, $p < 0.10$, partial $\eta 2 = 0.1443$) and main effects for the interaction ($F_{2,47}$=3.49, $p < 0.05$, partial $\eta 2 = 0.4996$). The pERK-positive rate was significantly reduced in all groups after DLX treatment compared to vehicle (naive: 34.0 ± 26.9 vs. 1.3 ± 3.9%, SNL2W: 53.4 ± 20.4 vs. 2.2 ± 2.3%, SNL6W: 21.6 ± 13.4 vs. 3.6 ± 4.0%, $p < 0.05$ respectively, Fig. 5C). These results suggest that DLX suppresses LC activity, while increasing NA in the axon terminals projecting from the LC through reuptake inhibition.

To examine the significance of reduced LC activity induced by DLX, we evaluated the effect of DLX administration on NSIA in SNL6W. Rats were administered 10 mg/kg of DLX intraperitoneally, and 24 h later capsaicin was injected subcutaneously into the left forepaw, and the withdrawal threshold at the left hind paw was measured by the paw pressure test. Two-way ANOVA showed the significant main effects of the drug ($F_{1,10}$=30.08, $p < 0.01$, partial $\eta 2 = 0.7505$) and the main effects of time after capsaicin ($F_{4,40}$=12.75, $p < 0.01$, partial $\eta 2 = 0.5605$) and main effects for the interaction ($F_{4,40}$=11.42, $p < 0.01$, partial $\eta 2 = 0.5331$). These results suggest that the vehicle treated SNL6W showed no elevation of withdrawal threshold, which suggests significant attenuation of NSIA as previously reported[2]. However, the withdrawal threshold was significantly increased in the DLX group compared to the vehicle, at 30, 60, and 90 min after capsaicin administration (Fig. 5D). The similar effects of DLX on NSIA was observed in female (Fig. 5E). This suggests DLX could restore endogenous analgesia.

We also performed immunohistochemical staining of $\alpha 2_A$ adrenoceptor and NET in the LC to investigate how NA, which is increased by DLX administration, suppresses the LC. Rats were randomly divided into three groups (naive, SNL2W, and SNL6W), performed perfusion fixation, and brain tissue containing LC was sectioned. Immunohistochemical staining was performed using anti-$\alpha 2_A$ adrenoceptor, anti-NET, and anti-DβH antibody. Representative images are shown (Fig. 5F). Area fraction of $\alpha 2_A$ adrenoceptor-positive in the region of interest is 12.1 ± 4.1%, 21.6 ± 6.3%, 14.8 ± 3.6%, respectively (Fig. 5G). One-way ANOVA showed significant main effects of group ($F_{2,22}$=6.94, $p < 0.01$, $R^2 = 0.8330$). Multiple comparisons analysis showed that the $\alpha 2_A$ adrenoceptor-positive area of SNL2 W was significantly more than naive ($p < 0.05$). The area fraction of NET-positive in the range of interest was shown (Fig. 5H). One-way ANOVA showed significant main effects of the group ($F_{2,22}$=5.89, $p < 0.01$, $R^2 = 0.7673$). Multiple comparisons analysis suggested that SNL6W had significantly less area than naive (SNL6W: 31.6 ± 12.3% vs. naive: 58.7 ± 15.6%, $p < 0.05$).

Restoration of NSIA by chemogenetic inhibition of LC in SNL6W rats. To confirm the reduction of abnormal LC activity is required for the restoration of NSIA which is attenuated in SNL6W, we used inhibitory (Gi) DREADD to suppress the LC neuronal activity. One hour After intraperitoneal administration of CNO (1 mg/kg), withdrawal thresholds of the left paw (uninjured paw) were measured as baseline. The baseline threshold is decreased compared to before CNO administration in Gi-DREADD treated SNL6W (pre; 140.3 ± 12.5 g, 60 min; 94.3 ± 22.7 g), but no change in the control-AAV treated group (pre; 142.9 ± 12.5 g, 60 min; 141.2 ± 17.0 g). Subsequently, capsaicin/saline was injected and the NSIA was measured as previously described (Fig. 6A). Two-way repeated ANOVA showed significant main effects of group (Gi or control, $F_{3,16}$=8.74, $p < 0.01$, partial $\eta 2 = 0.6209$) and main effects of time after capsaicin administration ($F_{4,15}$=2.60, $p < 0.05$, partial $\eta 2 = 0.1397$), but the effects of the interaction ($F_{12,99}$=1.75, $p < 0.10$, partial $\eta 2 = 0.2468$) was not significant. The post hoc test showed withdrawal threshold change was increased at time points of 30, 60, 90, and 120 min in the Gi-DREADD with capsaicin group (Fig. 6B). Rats treated with control-AAV showed no change even after capsaicin injection in the same manner as SNL6W without AAV treatment. These results suggest that inhibition of LC neuronal activity by inhibitory DREADD produces NSIA in SNL6W with a reduced baseline withdrawal threshold.

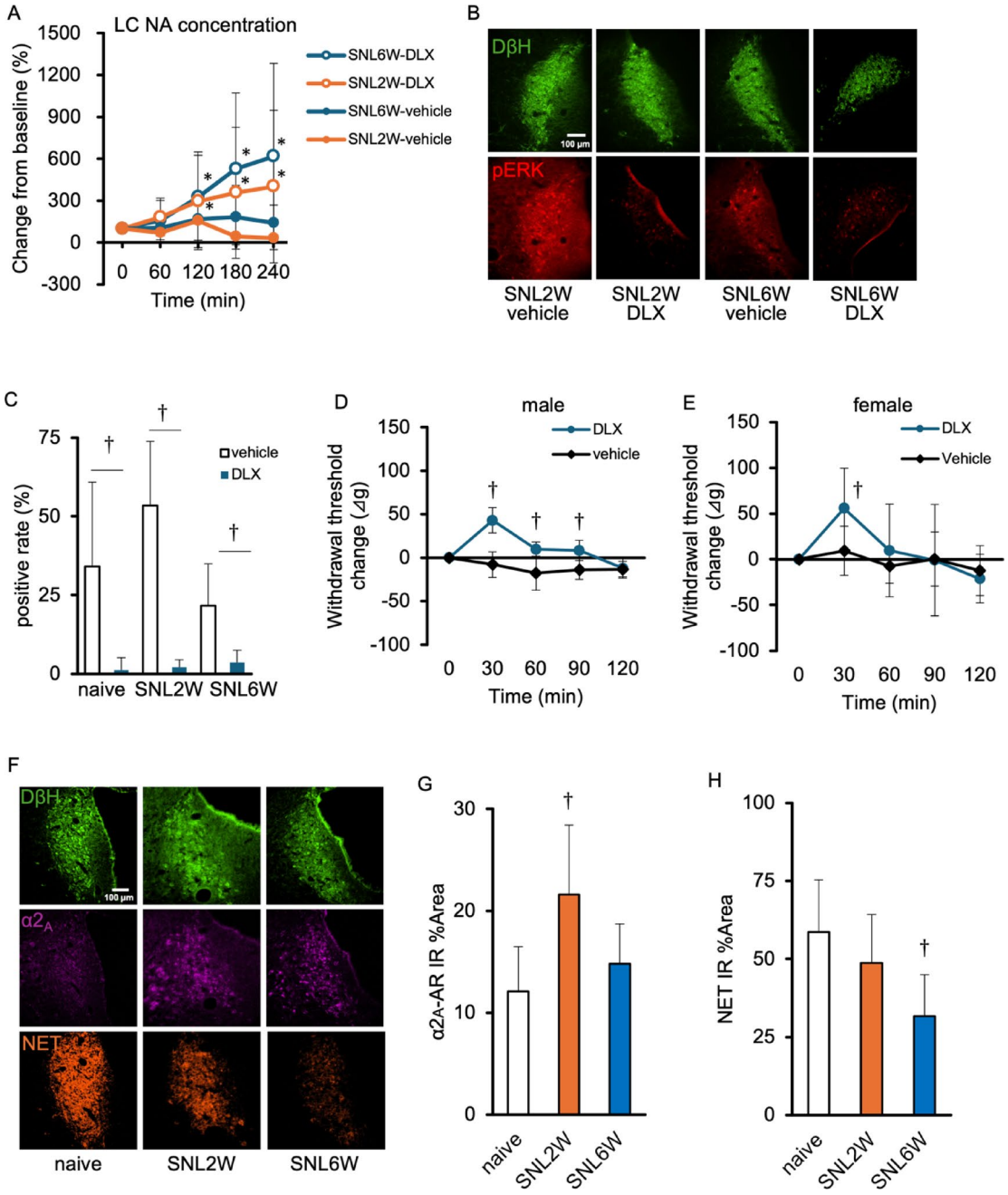

Fig. 5. DLX decreases the neuronal activity of LC to restore NSIA. (**A**) Changes in mPFC NA concentration following intraperitoneal DLX (10 mg/kg) are not different between SNL2W and SNL6W. (**B**) Immunohistochemical staining of pERK-positive cells in the LC of SNL2W and SNL6W rats are shown. (**C**) DLX (10 mg/kg) decreased pERK-positive rate in SNL2W and SNL6W rats. (**D**) DLX (10 mg/kg) or vehicle was administered 24 h before the NSIA measurement. Capsaicin was injected subcutaneously at the left forepaw and changes in the left hind paw withdrawal threshold were examined with paw pressure test over time (baseline 0, 30, 60, 90, and 120 min) in male SNL6W rats. Intraperitoneal DLX restored noxious stimuli induced analgesia (NSIA), which is diminished in SNL6W rats. (**E**) The similar effects of DLX on NSIA was observed in female. (**F**) Representative immunohistochemical staining results of α2A-AR and NET in the LC. Area fraction of α2A-AR-IR area (**G**) and NET-IR area (**H**) of naive, SNL2W, and SNL6W rats are shown. The data are expressed as the mean ± SD. $n = 6$ (**A**), $n = 8$ each 3–4 slices per rat (**C**), $n = 6$ (**D**), $n = 8$ (**E**), $n = 8,8,7$ (**G**,**H**). †: $p < 0.05$ versus naive. One-way ANOVA followed by the Student's t-test with Bonferroni's correction for multiple comparisons. The scale bar: 100 μm. LC; locus coeruleus. NA; noradrenaline, SNL; spinal nerve ligation. DLX; duloxetine, α2A-AR; α2A adrenoceptor, DβH; dopamine beta hydroxylase, NET; noradrenaline transporter, IR; immunoreactivity.

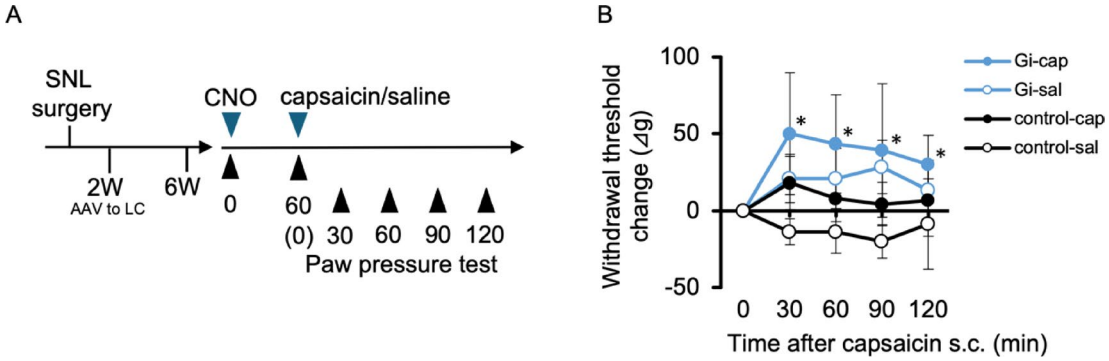

Fig. 6. Restoration of noxious stimulus induced analgesia (NSIA) by chemogenetic inhibition of LC in SNL6W rats. (**A**) The course of the experiment. Inhibitory DREADD containing AAV (Gi) or control AAV (control) containing only mCherry were injected in the bilateral LC of SNL animal approximately 4 weeks before the test day. Capsaicin was injected subcutaneously at the left forepaw 1 h after the intraperitoneal CNO (clozapine-N-oxide, 1 mg/kg i.p.) administration, and changes in paw withdrawal threshold were examined with paw pressure test over time (baseline 0, 30, 60, 90, and 120 min). The differences in the withdrawal threshold following capsaicin injection (Δg) were shown in (**B**). The withdrawal threshold of Gi-DREADD-treated group (Gi-cap) increased compared with the value of before capsaicin injection. This indicates that the inhibition of LC activity can produce capsaicin induced NSIA like effects. The data are expressed as the mean ± SD. $n = 6$ rats for each group. * $p < 0.05$ versus time 0. Two-way repeated ANOVA followed by the Student's t-test with Bonferroni's correction for multiple comparisons. SNL; spinal nerve ligation, 2W; 2week after SNL surgery, 6W; 6week after SNL surgery, AAV; adeno-associated virus, DREADD; designer receptor exclusively activated by designer drugs, AAV; adeno associated virus, CNO; clozapine N oxide, LC; locus coeruleus.

Discussion

DLX is the first line drug for neuropathic pain, and it is reported that the analgesia is mediated by the spinal NA increased by reuptake inhibition. In this study, we investigated the effects of DLX in the mPFC, where NA levels gradually increase due to abnormal neural excitation in the LC over time after peripheral nerve injury[5]. Our present results suggest that increased NA in mPFC maintains neuropathic pain in the late phase (6 weeks: SNL6W), but not at the early phase (2 weeks: SNL2W) in both sexes. There are differences in behavior and effects of some analgesics for neuropathic pain between the two phases, and one possible explanation is thought to be the increase in noradrenaline levels in the brain and spinal cord and sustained excitation of the LC noradrenergic neurons[2,5]. The acute DLX analgesia is still mediated by the spinal NA in the SNL6W, however, the mPFC NA counteracts the analgesic effects. Therefore, the analgesic effects of acute systemic DLX for SNL6W are attenuated compared with the SNL2W. We also examined the effects of DLX on the LC. Abnormal excitation of LC also results in attenuated noradrenergic endogenous analgesia at the late phase, as previously reported[13]. Systemic DLX restored noradrenergic endogenous analgesia by inhibitory effects on LC neuronal activity through $\alpha 2_A$ adrenoceptor and NET.

A previous study has reported that NA concentration in mPFC increases over time following nerve injury due to the abnormal neuronal activity of LC and subsequent impairment of attention behavior[5] at the late phase of neuropathic pain (SNL6W). In this study, administration of α2 adrenoceptor antagonist, atipamezole, into the mPFC produced transient analgesia not at the early phase (SNL2W) but at the late phase of neuropathic pain (SNL6W) (Fig. 1B). This suggests the elevated basal NA in mPFC contributes to the maintenance of pain at the late phase following SNL surgery. A previous study reported that administering an α1 adrenoceptor antagonist to the mPFC showed analgesia in a rat model of trigeminal neuralgia[6]. In our study, administration of α1 adrenoceptor antagonist, benoxathian, did not produce analgesia. This may be because of the difference in the pain model and the duration after the nerve injury.

Our microdialysis study showed that the DLX increases NA in mPFC in the same manner in both SNL6W and SNL2W (Fig. 2B). However, the acute analgesic effect of intraperitoneal DLX is attenuated in the SNL6W compared with in the SNL2W (Fig. 2A). Moreover, a combination of intraperitoneal DLX and atipamezole injection to the mPFC increased paw withdrawal thresholds only in SNL6W (Fig. 2D). These results suggest that mPFC NA in the SNL6W not only maintains neuropathic pain but also counteracts DLX analgesia, that results in attenuated analgesic effects.

In the late phase of the neuropathic pain model (SNL6W), attenuation of DLX analgesia was reported[9]. Kato et al. have shown that there is no difference in the spinal NA concentration after intraperitoneal DLX, and that the reduction of spinal cholinergic neurons in SNL6W is a possible mechanism of the attenuation[9]. Our present findings make it clearer that DLX produces its analgesic effect primarily by increasing spinal NA and acetylcholine. Besides this spinal mechanism, our results suggest that DLX produces analgesia mainly by increasing spinal NA and acetylcholine.

We also examined the direct local DLX administration to mPFC could reproduce analgesia or pain. Rats were bilaterally administered vehicle or 30 nmol/0.5 μL of DLX at SNL2W and SNL6W. Paw withdrawal thresholds were measured by von Frey test at 30, 60, and 90 min after drug administration. We could not observe analgesia

in the nerve injured paw (Fig. 3A), however, observed the transient decrease of withdrawal threshold in the uninjured paw in both SNL2W and SNL6W (Fig. 3B). These could partially explain that DLX directly increases NA in mPFC, resulted in prefrontal pain facilitation. However, although the presence of NA-mediated functional changes in the mPFC neurons is predicted, the detailed mechanisms for analgesic responses need to be further examined.

Our results showed the spinal NA increased by DLX produces analgesia, and mPFC NA counteracts it. We hypothesized that intrathecal DLX could produce analgesia. SNL6W rats were intrathecally administered vehicle or 200–400 μg of DLX, and their paw withdrawal thresholds were measured by the von Frey test at 30, 60, and 90 min after drug administration. However, we could not observe strong analgesia (Fig. 4C). Therefore, we perfused DLX through the microdialysis probe to investigate whether NA is increased in the spinal cord of SNL6W. DLX administration by reverse microdialysis increased spinal NA concentrations of SNL2W and SNL6W in the same manner (Fig. 4B). In addition, it is reported that intraperitoneal DLX can increase the spinal NA concentration in both animals[9]. In the present study, intraperitoneal DLX administration showed analgesia, which was antagonized by intrathecal atipamezole (Fig. 4A). A previous study suggests fast active transport mechanisms out of cerebrospinal fluid either back into the blood or into the spinal cord tissue[14]. Our results suggest that intrathecal DLX did not produce analgesia due to the low transferability from the cerebrospinal fluid to spinal cord tissue.

The LC projects NA innervation widely in the CNS[1]. It is involved in the modulation of brain and spinal cord[15–18]. Some previous studies suggest that chronic pain increases the neuronal activity of LC to increase NA in the brain and spinal cord, which may lead to altered brain function and pain modulation[5,19]. In our previous study, SNL6W showed elevated pCREB expression compared to the naive animals and repeated DLX treatment did not alter the LC activity evaluated by the pCREB expression[10]. On the other hand, DLX treatment restored endogenous analgesia[11]. We concluded accumulation of NA in the spinal cord might contribute to the restoration. In the present study, DLX treatment reduced pERK expression in the noradrenergic LC neurons of SNL6W, and the restoration of endogenous analgesia was also observed (Fig. 5B-D). It is also reported that DLX reduces neuronal activity analyzed by electrophysiology[12]. Our results cannot reveal the mechanisms of how DLX restores the LC activity, however, our microdialysis experiments suggest that DLX can increase NA around LC probably by NA transporter inhibition (Fig. 5H). The noradrenergic LC neurons may have recurrent axons to release NA to themselves, and the LC neurons may be inhibited by the NA probably via α2 adrenoceptor. Our previous study suggests DLX or amitriptyline, which show noradrenaline reuptake inhibition, activate BDNF-trkB signaling to restore endogenous analgesia in SNL6W[20]. Further experiments are needed to explore how DLX directly or indirectly alters LC activity.

As a result, LC neurons can be activated by another stimulation. Our DREADD experiments also support the benefits of reduction of noradrenergic LC neuronal activity in SNL6W in terms of restoration of endogenous analgesia. Inhibitory DREADD treatment in noradrenergic LC neurons showed a decrease in withdrawal threshold (numerical data only). However, could restore endogenous analgesia, which is diminished in SNL6W. Impairment of endogenous analgesia is reported in various types of chronic pain, therefore DLX can be beneficial to chronic pain patients[21].

Based on the present experiments, prescribing DLX makes sense for chronic pain patients because DLX can reduce abnormal LC activity to restore endogenous analgesia, which is often attenuated in chronic pain patients[21]. However, in the chronic phase of neuropathic pain, the acute analgesic effect of DLX is mainly mediated by spinal NA and the mPFC NA maintains the pain. DLX increases both spinal and mPFC NA, therefore, to maximize the effects of DLX, DLX should be delivered to the spinal dorsal horn. However, intrathecal DLX did not produce analgesia in this study, while spinal direct perfusion of DLX increased NA. This suggests that DLX has a unique pharmacological profile with low transferability to the spinal tissue. Although inhibition of LC may lead to the suppression of endogenous analgesia and result in transient enhancement of pain, DLX has an analgesic effect that occurs independently of LC activity due to its increased NA in the spinal cord and therefore is considered to remain an important analgesic option for patients with chronic pain.

Limitations

We acknowledge our present study has some limitations. We did not conduct dose-response analysis in experiments using atipamezole to ensure that the results are not due to the supraphysiological dose. We did not examine the roles of serotonin either. We have reported serotonergic pain facilitation[22]. The similar mechanism in the PFC may coexist with the present results. Furthermore, the experiments to explore the roles of PFC subregions should be conducted since the mPFC is not a homogeneous structure[23]. We also need further examinations to explore how PFC NA or DLX affects pain modulation, because mPFC may activate descending pain inhibition directly or indirectly by communicating to ACC or PAG.

Conclusion

DLX increases NA in both the mPFC and spinal cord by reuptake inhibition at the axon terminals, besides DLX inhibits the abnormal excitation of the LC caused by neuropathic pain probably by autoinhibition. Sustained increases in NA in the mPFC in the late phase of neuropathic pain are responsible for pain potentiation. The spinal NA contributes to the analgesic effects, but mPFC NA counteracts it. The inhibition of LC lead to the improvement of stimulus responsiveness of LC to restore endogenous analgesia even in chronic neuropathic pain.

Methods

Animals

This experiment was approved by the Gunma University Animal Care and Experimentation Committee (Maebashi, Japan. No.20–029 and 22–001). All experiments were conducted in accordance with the guidelines of Animal Research and the study adhered to the ARRIVE guidelines[24] ensuring compliance with ethical standards for animal research.

Male and female Sprague-Dawley (SD) rats (180–220 g, SLC, Shizuoka, Japan) or male DβH-Cre rats (180–220 g, W-Tg(DβH-tTA,-cre)2_7 Fusa) were used in the experiments. The W-Tg(DβH-tTA, -cre)2_7 Fusa rat was developed by Drs. F. Kato and Y. Takahashi (Jikei University) with support from the C.B.S.N project conducted by Drs. K. Kobayashi (Fukushima Medical University) and Y. Yanagawa (Gunma University), using a DβH-tTA-2 A-Cre-BAC construct developed by Dr. R. Kaneko (Gunma University). Animals were kept in a room with a temperature of 24 ± 0.5 ℃, and a 12-hour cycle (8:00–20:00) of light and dark. Food and water were provided ad libitum. Rats with indwelling catheters or cannulas were housed alone in cages.

A total of 224 SD rats and 24 DβH-Cre rats were used. To reduce the number of rats used in the experiment as much as possible, rats that had been behaviorally experimented with at 2 weeks following SNL were still used at 6 weeks. Of these, rats that died ($n = 12$) or were paralyzed ($n = 2$) in the lower limb due to surgery, or had abnormal cannulation or catheter position ($n = 8$) were excluded from the analysis.

Viral vectors and injection

The AAV vectors which contain inhibitory DREADD receptor or control used in this study were purchased from Addgene (pAAV-hSyn-DIO-hM4D(Gi)-mCherry, #44362, pAAV-hSyn-DIO-mCherry, #50459). The vectors were suspended in phosphate buffered saline and stored at − 80 ℃ until injected. The rats were fixed to a stereotaxic frame under isoflurane anesthesia as previously described. The skull just above the bilateral LC (AP:−9.8 mm, ML:±1.5 mm from bregma, DV:5.5–6.0 mm) was drilled, AAV vector (0.3 µl) was then infused to LC with Hamilton neuros syringe (65469-01, Nevada, USA) for 10 min using micro injector (IMS-30, Narishige, Japan) attached to the stereotaxic frame, and allowed to diffuse for 5 min before the syringe was withdrawn. The injection procedure was repeated for the contralateral LC. Four weeks after surgery was designated as the recovery period.

SNL procedure

A neuropathic pain model was created in which the right L5 nerve of rats was ligated and transected[22,25]. In brief, rats were first inducted into anesthesia with isoflurane 5% and maintained at 1.5–2%. A skin incision was made to expose and remove the right L6 transverse process to identify the right L5 nerve; the nerve was ligated with 5–0 silk and the distal portion was cut. The fascia and skin were sutured, and the rats were allowed to awaken from anesthesia to confirm that there were no problems with lower extremity movement. Behavioral experiments were conducted 2 and 6 weeks after SNL surgery.

PFC cannulation

While maintaining anesthesia with isoflurane 1.5–2%, the rat was fixed to stereotaxic frames with non-ruptured ear bars (KOPF, Tujunga, CA). The posterior ear and head incision sites were locally anesthetized with 1% xylocaine 0.2 mL, and 1 mg/kg butorphanol was injected subcutaneously for intraoperative analgesia. The double guide cannula (C235G-0.5, 4 mm, Plastics One, P1 Technologies, USA) was implanted into mPFC located 3.0 mm rostral from bregma, ± 0.5 mm lateral, and 3.0–4.0 mm depth according to a rat brain atlas[5,26]. The cannula was fixed using dental resin (Tokuyama dental, JAPAN). One week after surgery was designated as the recovery period. After the behavioral experiments were completed, methylene blue (0.5 µL/side) was injected through the injection cannula to verify the cannula location. The rats were decapitated following 150 mg/kg of pentobarbital, and the brain was removed to check the catheter tip location. Animals in which the tip was not at the mPFC were excluded from the analysis.

Intrathecal catheterization

To administer drugs to the spinal cord dorsal horn, a catheter was placed intrathecally, as previously described[27]. Animals were anesthetized with isoflurane and their heads were secured in a stereotaxic frame. The occipital bone was exposed, and the cisternal membrane was incised to insert a 32-gauge polyurethane tube (0041, ReCathCO LLC, Allison Park, PA, USA; Intramedic) directed 7.5 cm caudally. The muscle layer and skin were sutured for closure. Animals exhibiting abnormal hind limb movement upon awakening from anesthesia were excluded from the experiment. A recovery period of one week was allowed. Following the completion of behavioral experiments, rats showing reversible paralysis in the hind limbs upon administration of 5% lidocaine (0.5 mg/10 µL) through the catheter were considered a correctly positioned catheter.

Drugs

Duloxetine hydrochloride (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in saline containing 50%(v/v) Dimethyl Sulfoxide (DMSO) and administered intraperitoneally at doses of 10 mg/kg or 30 mg/kg. Atipamezole (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), an α2-adrenoceptor antagonist, and benoxathian (Sigma-Aldrich, Co., St. Louis, USA), an α1-adrenoceptor antagonist, were dissolved in saline. For intrathecal administration, atipamezole was dissolved in 20 µg/5 µL and administered slowly using a 25 µL Hamilton's syringe through a pre-placed intrathecal catheter 15 min before intraperitoneal administration of DLX[28]. Drug administration to the mPFC was conducted by inserting an injection cannula (C235I-0.5, + 0.1 mm, Plastics One, P1 Technologies, USA) through the guide cannula previously placed in the mPFC. Atipamezole and benoxathian were each administered at concentrations of 10 µg/0.5 µL/site and 5 µg/0.5 µL/site,

respectively, using a 10 μL Hamilton's syringe and a micro syringe pump (EiCOM ESP-64)[29]. Under anesthesia with isoflurane, the drug was administered at a rate of 0.1 μL/min, allowing time for the drug diffusion for 1 min after completion of administration.

Behavioral tests

The experimenter performed behavioral tests without knowing which drug was administered to each animal.

Von Frey filament test

The experiment was conducted following the previously described protocol[30]. Mechanical hypersensitivity of the right hind paw was assessed using von Frey filaments (0.6–26 g, Stoelting, Wood Dale, IL, USA). During daylight hours, rats were placed in individual transparent acrylic boxes (10 × 20 × 24 cm) with a mesh floor for at least 30 min of acclimatization. The right hind paw was applied to the von Frey filament in a slight flexion for 5 s. When the rat exhibited behaviors, such as lifting or avoiding the paw, it was considered a positive response, and then a lighter filament was used. The up-down method was used to determine the 50% withdrawal threshold. A cutoff value of 6 g was set, and rats with baseline values exceeding this threshold were excluded from the experiments.

Noxious stimulus induced analgesia (NSIA)

The paw pressure test was conducted to assess NSIA in rats, according to the previously described[31,32]. While holding the rat manually, pressure applied to the left hind paw was gradually increased using an Analgesymeter (37215, Ugo Basile, Comerio, Italy), and the mechanical withdrawal threshold was measured. The recorded result was based on the pressure at which the rat withdrew its paw or vocalized, and the average of two measurements was adopted. To avoid tissue damage, the maximum pressure was set at 250 g. Three days of pre-experimental training were conducted. On the day before the experiment, following the habituation period, rats were administered either DLX (10 mg/kg) or normal saline (1 mL/kg) via intraperitoneal injection. 24 h after DLX administration, the rats were briefly anesthetized with isoflurane and administered capsaicin (150 μg/50 μL) subcutaneously into the left forepaw. The withdrawal threshold of the left hind paw was measured every 30 min post-administration. Inhibitory DREADD- or control-AAV injected DβH-Cre rats received the same procedure without DLX/saline injection, but clozapine-N-oxide (CNO, 1 mg/kg, intraperitoneally, Enzo Life Sciences, NY) injection 1 h prior to the capsaicin injection.

Immunohistochemistry

Rats were given DLX 10 mg/kg intraperitoneally, 1 h later anesthesia was induced with isoflurane 5%, and sodium pentobarbital 100 mg/kg was administered intraperitoneally. A cannula was inserted through the apex and irrigated with cooled 0.01 M phosphate-buffered saline (PBS) containing 1% sodium nitrile, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and placed in PFA at 4 ℃ for 1 day for fixation. They were then placed in 30% sucrose at 4 ℃ for 5 days for cryoprotection. The brains containing the LC were excised, encapsulated with compound (Tissue Tek®), and stored at −80 ℃. The sections were cut out at a thickness of 20 μm using a cryostat (LEICA CM1900).

Immunostaining was performed by the floating method. Sections were first washed three times in 0.01 M PBS and blocked in PBS containing 1.5% Normal Donkey Serum (NDS) and 0.1% Triton at room temperature for 1.5 hours Sections were then incubated in primary antibodies diluted in PBS containing NDS and Triton at 4 ℃. They were incubated for 2 days for pERK and 1 day for $\alpha2_A$ adrenoceptor and noreadrenaline transporter (NET), respectively. Primary antibodies were mouse anti-dopamine-β-hydroxylase (DβH) antibody (1:1000, MAB308, Millipore), sheep anti-DβH) antibody (1:1000, ab19353, abcam), rabbit anti-pERK antibody (1:600, Cat#4370, Cell Signaling technology), rabbit anti-$\alpha2_A$ adrenoceptor antibody (1:1000, RA14110, Neuromics) and mouse anti-NET antibody (1:1000, NET05-2, Mab Technologies). Secondary antibodies were Cy2 conjugated Donkey anti-mouse IgG (1:200, 715-225-151, Jackson Immuno Research Laboratories), Cy3 conjugated Donkey anti-mouse IgG (1:600, 715-165-151, Jackson Immuno Research Laboratories), Alexa Fluor 488-conjugated Donkey anti-sheep IgG (1:200, 713-545-147, Jackson Immuno Research Laboratories), Alexa Fluor 647-conjugated Donkey anti-rabbit IgG (1:500, 711-605-152, Jackson Immuno Research Laboratories), and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:20,000, D21490, Thermo Fisher Scientific). Sections were incubated in secondary antibodies diluted in PBS containing NDS and Triton for three hours at room temperature. After washing with PBS, they were attached to glass slides, dehydrated with ethanol and permeabilized with xylene, covered with DPX mounting medium (06522, Sigma Aldrich).

Three to four sections containing LC were selected from one rat, and eight rats in each group were analyzed. Fluorescence signals were captured using a fluorescence microscope (eclipse Ni-E, Nikon Co., Tokyo, Japan). Image data were analyzed with ImageJ and cellpose[33,34]. First, The DβH-immunoreactive area was defined as the LC area. Artificial intelligence-based segmentation software, cellpose, was used to produce the DAPI mask. Using the DAPI mask, the mean gray value of the pERK was measured using ImageJ. pERK-positive cells were then identified using a determined threshold. The pERK-positive ratio was determined by dividing the number of pERK positive cells among all DAPI positive-LC cells. For the $\alpha2_A$ adrenoceptor and NET analysis, we set the appropriate thresholds, and the area above the threshold value within the range of interest (500 × 500 pixels) was calculated using ImageJ.

Microdialysis

In vivo Microdialysis (mPFC, LC)

One or five weeks after SNL surgery, rats were anesthetized with 1–2% isoflurane and placed securely in a stereotaxic frame. A sterile steel-guided cannula (CXG-4 or CXG-6, EICOM CO., Kyoto, Japan) was implanted into the right mPFC or right LC as described previously[5,35]. The coordinates for placement of the tip of the guide

cannula for mPFC were 3.0 mm posterior and 0.5 mm lateral to bregma, and 3.0 mm ventral from the surface of the dura mater, for LC, 9.8 mm posterior and 1.6 mm lateral to bregma, and 5.5 mm ventral from the surface of the dura mater according to the rat brain atlas[26]. Rats were allowed to recover for 1 week, and anesthesia was induced with 2% isoflurane and then maintained with 1.5% isoflurane during the study to produce the same experimental condition as spinal microdialysis as described below. A heating blanket was used to maintain the rectal temperature at 37.0 ± 0.5 ℃. A microdialysis probe (CX-4-02 or CX-I-6-01, outer diameter = 0.22 mm, inner diameter = 0.20 mm, membrane length = 2–1 mm; Eicom Co.) was inserted through the guided cannula into the mPFC or LC 1 h before the experiment and perfused with Ringer's solution (1.0 mL/min). After collecting a 60-minute baseline sample, 10 mg/kg of DLX was injected intraperitoneally. Samples were collected every 60 min.

Concentrations of NA in the microdialysates were measured by high-performance liquid chromatography systems with electrochemical detection (HTEC-500, Eicom Co.).

In vivo spinal Microdialysis

Microdialysis on the spinal dorsal horn was performed according to a method described in our previous study[36]. In brief, SNL2 W and SNL6 W rats were anesthetized with 2.0% isoflurane, and anesthesia was maintained during the measurements using 1.5% isoflurane in 100% oxygen. The rectal temperature of the animals was maintained at 37.0 ± 0.5 ℃ using a heating blanket. Saline was infused into the rats at a rate of 1 mL/h through a cannulated tail vein using an infusion pump system (Fusion 400, Chemyx, Stafford, TX, USA). The L4-L6 spinal cord was exposed by a T13-L1 laminectomy. Microdialysis probes (CX-I-8–01, Eicom Co., Kyoto, Japan) were inserted into the right spinal dorsal horn, which was perfused with Ringer's solution (147 mmol/L NaCl, 4 mmol/L KCl, 2.3 mmol/L CaCl2) at a rate of 1 µL/min. After 60 min of constant perfusion, dialysates were collected at 60 min intervals. After the collection of a dialysate for the baseline sample, 2 µM DLX solution dissolved by the Ringer's solution was perfused through the microdialysis probe. The concentration of the DLX is based on the previous paper[37]. NA concentrations were measured 240 min following the perfusion using separate high-pressure liquid chromatographic systems with electrochemical detection (HTEC-500, Eicom Co)[2,36]. The column used for the measurement of NA concentrations was EICOMPAK CAX (3.0 × 150 mm, Eicom Co.). The changes in the concentrations of NA following DLX local perfusion were determined as a percentage of the baseline concentration (100%). After the microdialysis experiment, the rats were humanely killed by an intraperitoneal injection of pentobarbital (150 mg/kg).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using R (version 4.4.0). Statistical analysis was performed using repeated two-way analysis of variance (ANOVA), with multiple comparisons using the Bonferroni test as a post hoc analysis. The result of the area under the curve (AUC) was analyzed by non-repeated two-way ANOVA or Student's t-test. The result of Immunohistochemistry was analyzed by one-way ANOVA, with multiple comparisons using the Bonferroni test as a post hoc analysis. A value of $p < 0.05$ was considered a statistically significant difference.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

Y.A. Designed and conducted the study, analyzed the data, and wrote the original draft.T.S., X.H., Y.H., and T.H. Designed and conducted the study, analyzed the data, and wrote the review and editing.H.O. amd S.S. Designed the study and wrote the review and editing.

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Declarations

Competing interests

The authors declare no competing interests.

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