Endogenous oxytocin exerts anti-nociceptive and anti-inflammatory effects in rats

Haruki Nishimura1,2,10, Mitsuhiro Yoshimura1,3,10,11✉, Makiko Shimizu1, Kenya Sanada1, Satomi Sonoda4, Kazuaki Nishimura1, Kazuhiko Baba1,2, Naofumi Ikeda1,2, Yasuhiro Motojima2, Takashi Maruyama1, Yuki Nonaka1, Ryoko Baba5, Tatsushi Onaka6, Takaumi Horishita7, Hiroyuki Morimoto5, Yasuhiro Yoshida8, Makoto Kawasaki2, Akinori Sakai2, Masafumi Muratani9, Becky Conway-Campbell3, Stafford Lightman3 & Yoichi Ueta1,11✉

Oxytocin is involved in pain transmission, although the detailed mechanism is not fully understood. Here, we generate a transgenic rat line that expresses human muscarinic acetylcholine receptors (hM3Dq) and mCherry in oxytocin neurons. We report that clozapine-N-oxide (CNO) treatment of our oxytocin-hM3Dq-mCherry rats exclusively activates oxytocin neurons within the supraoptic and paraventricular nuclei, leading to activation of neurons in the locus coeruleus (LC) and dorsal raphe nucleus (DR), and differential gene expression in GABA-ergic neurons in the L5 spinal dorsal horn. Hyperalgesia, which is robustly exacerbated in experimental pain models, is significantly attenuated after CNO injection. The analgesic effects of CNO are ablated by co-treatment with oxytocin receptor antagonist. Endogenous oxytocin also exerts anti-inflammatory effects via activation of the hypothalamus-pituitary-adrenal axis. Moreover, inhibition of mast cell degranulation is found to be involved in the response. Taken together, our results suggest that oxytocin may exert anti-nociceptive and anti-inflammatory effects via both neuronal and humoral pathways.
Oxytocin (OT), a neuropeptide synthesized in the hypothalamic supraoptic (SON) and paraventricular nuclei (PVN) and secreted from the posterior pituitary (PP) into the systemic circulation, elicits diverse actions in the peripheral and central nervous system (CNS). Different OT populations have been identified as magnocellular and parvocellular OT neurons. Magnocellular OT neurons in the SON and PVN (mPVN) affect adjacent neurons via somato-dendritic release and/or axonal projections to the PP. On the other hand, parvocellular OT neurons, distributed throughout the dorsal PVN (dPVN), send projections to the CNS, notably including the spinal dorsal horn. OT modulates social and sexual behaviors as well as sensory and autonomic functions through these neurosecretory systems, playing an important role in complex social interactions, such as maternal behavior, partnership, and social bonding. Malfunction of the OT system is believed to be responsible for the impaired social behavior associated with autism, social anxiety, stress disorder, and schizophrenia.

Additionally, many ambitious studies have suggested a role for OT in pain modulation and anti-nociceptive effects. Analgesic effects of OT have also been hypothesized to be mediated by vasopressin V1a receptor (V1aR). Different OT-ergic pathways are assumed to be involved in its anti-nociceptive action; one is the descending pain inhibitory system in the CNS and the other is via an indirect effect on the dorsal root ganglia (DRG) in the periphery. OT amplifies gamma amino butyric acid (GABA)-ergic inhibition, which hampers noxious signal transduction, at superficial and deep layers of spinal dorsal horn neurons. OT also modulates hypothalamus–pituitary–adrenal (HPA) axis activity under stressful conditions. In addition, OT is involved in the modulation of immune and inflammatory processes, which also might contribute to its analgesic effects. To date, we have demonstrated that OT is involved in the pain modulation using various experimental pain models in rats. Elucidation of the direct effects of endogenous OT on pain pathways, however, has been particularly challenging due to technical limitations regarding the lack of specificity in OT neurons.

To overcome the aforementioned technical challenges, chemogenetics, also known as designer receptors exclusively activated by designer drugs (DREADDs) and optogenetics have been applied to OT neurons. Using these techniques, Eliava et al. demonstrated that selective parvocellular OT activation inhibited sensory processing via wide dynamic range (WDR) neurons, suggesting that selective parvocellular OT activation inhibited pain pathways. Using these techniques, Eliava et al. demonstrated that the OT-hM3Dq-mCherry transgenic rat line was functioning in the transgenic rats. FIHc for Fos was performed at 120 min after s.c. injection of Saline or CNO (1 mg kg⁻¹) (n = 5–6 rats, each), and the number of mCherry neurons co-expressed with Fos-ir (neurons with red fluorescence was detected in the SON and PVN of WT rats (Supplementary Fig. 1a, b). As expected, serum OT and VP both remained unchanged after s.c. injection of either Saline or CNO in wild-type (WT) rats (Supplementary Fig. 1a–c), indicating that the effect was specific to CNO injection of OT-hM3Dq-mCherry transgenic rats. The transgene is not expressed in WT rats, and consistent with this, no mCherry fluorescence was detected in the SON and PVN of WT rats (Supplementary Fig. 1a, b). As expected, serum OT and VP both remained unchanged after s.c. CNO injection of WT rats (Supplementary Fig. 1d, e).

hM3Dq-mCherry was found to be localized in axons as well as in cell bodies of the OT neurons (Fig. 2f). In addition, mCherry was observed in the PP (Supplementary Fig. 2a). These results suggested that hM3Dq might be expressed in the axons and terminals as well as in the cell body of the OT neurons and may secrete OT via somato-dendritic release.

Both serum OT concentration and oxytocin (OXT) gene expression were assessed to investigate the dynamics of endogenous OT production after s.c. CNO injection. Serum OT concentration, analyzed by radioimmunoassay (RIA), was
significantly elevated at 30, 60, 120, and 180 min after s.c. CNO injection (1 mg kg\(^{-1}\)) in comparison to Saline (\(n = 6-7\) rats in each group at each time point) (Fig. 2g). OXT gene expression in the SON, mPVN, and dPVN, analyzed by in situ hybridization histochemistry (ISH) (Fig. 2h), was also significantly increased at 120 and 180 min after s.c. CNO injection (1 mg kg\(^{-1}\)) (\(n = 12\) slices from 6 rats in each group at each time point) (Fig. 2i). These results indicated that OT production, as well as OT secretion, was increased by chemogenetic activation of OT neurons.

On the other hand, s.c. CNO injection of OT-hM3Dq-mCherry transgenic rats did not affect gene expression of arginine vasopressin (AVP) in the hypothalamus nor did it affect serum VP concentration (Supplementary Fig. 2b–d), indicating that
CNO did not appear to affect adjacent VP neurons in the transgenic rats.

OT altered nociceptive behavior and activated noradrenergic neurons in the LC. The von Frey and hot plate tests were carried out to assess the effect of endogenous OT on mechanical/heat sensitivities in naïve transgenic rats. Both tests were carried out at 0, 30, 60, 120 and 180 min after the s.c. injection of Saline or CNO (1 mg kg$^{-1}$) (n = 11 rats, each). Withdrawal threshold, tested by von Frey filament, was significantly elevated at 30 min after s.c. injection of CNO compared to Saline (Fig. 3a). The latency of nocifensive behavior, evaluated by hot plate test, was markedly prolonged in rats after s.c. CNO injection compared to s.c. Saline-injected controls (Fig. 3b). Mechanical/heat sensitivities were not altered in WT rats after the s.c. injection of CNO, indicating that CNO alone did not affect nociceptive behaviors (Supplementary Fig. 4a, b).

We hypothesized that the anti-nociceptive effects of endogenous OT did arise from the activation of the descending pain inhibitory system. Fos expression was analyzed in the locus coeruleus (LC) (Fig. 3c) and dorsal raphe nucleus (DR) (Fig. 4a), two nuclei that are involved in the descending pain inhibitory system. Tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) are the rate-limiting enzyme in the biosynthesis of noradrenaline and serotonin (5-hydroxytryptamine; 5-HT), respectively. TH is expressed in noradrenergic neurons in the LC, and TPH2 is expressed in serotonergic neurons localized within the dorsal and median raphe nuclei of the CNS. We analyzed TH-ir and TPH-ir neurons to evaluate the expression of noradrenergic and serotonergic neurons in the LC (Fig. 3d) and DR (Fig. 4b), respectively. The number of TH-ir neurons was comparable between Saline and CNO (1 mg kg$^{-1}$), whereas the percentage of TH-ir neurons co-expressed with Fos-ir was significantly increased after s.c. CNO injection (1 mg kg$^{-1}$) (n = 10–12 slices from 5 to 6 rats, each) (Fig. 3f). ISH analysis of TH (Fig. 3e) revealed that the gene expression of TH was significantly increased at 120 and 180 min after s.c. injection of CNO (1 mg kg$^{-1}$) compared to Saline (n = 12 slices from 6 rats, each) (Fig. 3g). Typical representative FIHC images of the LC after s.c. injection with Saline or CNO in the transgenic rats are shown (Supplementary Fig. 3a).

OT-activated serotonergic neurons in the DR and inhibitory interneurons in the spinal dorsal horn. The DR was divided into four following subregions; ventral (DRv), inter-fascicular (DRi), dorsal (DRd) and ventrolateral wings (DRvl) since each subnucleus exhibits differential neuroanatomical and distinct functional roles (Fig. 4b). The number of TPH-ir neurons, Fos-ir neurons, and TPH-ir neurons co-expressed with Fos-ir neurons were significantly increased after s.c. CNO injection (1 mg kg$^{-1}$) (n = 10–12 slices from 5 to 6 rats, each) (Fig. 4d). The gene expression of TPH2 (Fig. 4c) in each subregion was also significantly increased (n = 12 slices from 6 rats, each) (Fig. 4e), a finding compatible with our FIHC results. Typical representative FIHC images of the DR after treatment with Saline or CNO in the transgenic rats are shown (Supplementary Fig. 3b).

FIHC for Fos and PAX2, which is a transcription factors that is exclusively expressed in the inhibitory interneurons in the spinal cord, was carried out in the dorsal horn of lumbar segment 5 (L5) (Fig. 4f) in the spinal cord (Fig. 4g). The dorsal horn was divided into the superficial layer (laminae I–II) and deep layer (laminae III–VI), as differential roles have been proposed depending on the region. The number of PAX2-ir neurons, Fos-ir neurons, and co-expressed PAX2-ir/Fos-ir neurons in the superficial layer and deep layer were dramatically increased in CNO-injected transgenic rats (1 mg kg$^{-1}$) compared to Saline-injected transgenic rats (n = 10–12 slices from 5 to 6 rats, each) (Fig. 4h, i).

In the LC, DR, and spinal dorsal horn, most regions were not affected by the s.c. injection of CNO (1 mg kg$^{-1}$) in WT rats (Supplementary Fig. 4c–j). The number of TPH-ir neurons, however, was statistically increased after the s.c. injection of CNO in the DRv and DRvl of WT rats without altering the number of Fos-ir neurons (Supplementary Fig. 4g). This may be one of the off-target effects of CNO. CNO contains a similar structure of clozapine, an antipsychotic drug that may lead to an up-regulation of TPH. Although the concentration of CNO used in our study is widely used for chemogenetic activation, and should not have major off-target effects, it is possible that it could induce the small but significant increase in TPH-ir neurons we observed in WT rats after CNO injection. In addition, several kinds of physical stress can increase the expression of TPH. Of note, however, we did not see any significant increase of TPH-ir + Fos-ir neurons in the DR of WT rats (Supplementary Fig. 4g).

OT induced differential gene expression in GABAergic neurons of the dorsal horn. Using retrograde tracer, we have confirmed direct and indirect neuronal projections into the L5 dorsal horn from the dPVN, LC and DR (Supplementary Fig. 5a–f). In addition, using our OT-monomeric red fluorescent protein 1 (mRFP1) transgenic rats, axons of OT neurons were detected in the LC, DR and the dorsal horn of the L5 spinal cord (Supplementary Fig. 5g). The results indicate that OT-ergic projections might directly and indirectly modulate pain transmission in the spinal dorsal horn.

We speculated that functional gene expression pathways related to anti-nociception might be changed after OT activation, since abundant innervations from OTPVN neurons were observed in the spinal dorsal horn. We therefore employed RNAseq to investigate this hypothesis in an unbiased manner, rather than using a targeted candidate approach. The dorsal horn, divided into a superficial layer (laminae I–II) and deep layer (laminae III–VI), was micro-dissected and analyzed separately (n = 30 specimens from 3 rats, each) (Supplementary Fig. 6a). Consistent RNAseq mapping patterns throughout the full length
of known housekeeping genes, along with consistent read counts across the different samples indicated good data quality (Supplementary Fig. 6b–d).

In the laminae I–II, 254 genes were significantly differentially expressed (p value < 0.05, |fold change| > 2) after chemogenetic activation of endogenous OT. On the other hand, 191 genes were significantly differentially expressed (p value < 0.05, |fold change| > 2) in the laminae III–VI (Fig. 5a–d). Interestingly, many of these genes have previously been shown to be predominantly expressed in GABA-ergic neurons. It is suggested that endogenous OT might directly and/or indirectly inhibit local afferent sensory transmission. Gene ontology (GO) enrichment analysis was performed for interpreting the functional categories of these altered genes in the dorsal horn (Fig. 5e, 5f, 5g, 5h, 5i).
f). GO terms that were related to “inflammatory response” or “immune response” were enriched after the chemogenetic activation of OT in the spinal dorsal horn. It therefore now appears that endogenous OT might also modulate the spinal inflammation and/or immune response, resulting in suppressing sensory transmission. Raw RNA sequencing data are available at Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information (NCBI) (accession number GSE210528).

OT alleviated hyperalgesia in a neuropathic pain model via neuronal pathway. We employed the Seltzer model which is developed by partial ligation of the right sciatic nerve as a neuropathic pain model (Fig. 2a). This model enables us to evaluate the effect of OT on neuropathic pain since hypersensitivities against mechanical and heat stimulation develop rapidly, and continue for a substantial amount of time. Withdrawal threshold (mechanical sensitivity) and the latency of nocifensive behavior (heat sensitivity to 52.5 °C) were promptly decreased after the Seltzer surgery (Supplementary Fig. 7a, b). In addition, we have confirmed the effects of CNO (1 mg kg⁻¹) in Sham-operated animals at post-operative day 10. The results were consistent with naïve transgenic rats (Supplementary Fig. 7c, d). To exclude unwanted effects of surgical intervention, the experiment was performed at 10 days after the surgery. Mechanical/heat sensitivities were evaluated for 3 h after administering Saline or CNO (1 mg kg⁻¹) (Fig. 6b). Withdrawal threshold was significantly elevated at 0.5, 1, and 2 h after the s.c. injection of CNO (Fig. 6d),

Fig. 2 OT-hM3Dq-mCherry was functioning in the transgenic rats. Endogenous mCherry, Fos-ir and merged images of the SON (a) and PVN (b) at 120 min after the s.c. injection of Saline or CNO (1 mg kg⁻¹). c Percentage of Fos-ir neurons in mCherry neurons in the SON and PVN (n = 10–12 slices from 5 to 6 rats, each). **P < 0.01 vs. Saline. d The PVN was anatomically divided into magnocellular PVN (mPVN) and dorsal parvocellular PVN (dPVN). e Percentage of Fos-ir neurons in the mPVN and dPVN (n = 10–12 slices from 5 to 6 rats, each). **P < 0.01 vs. Saline. f Confocal images of endogenous mCherry (red), OT-ir (blue), Fos-ir (green) and merged images of a single OT⁺PVN neuron at 120 min after the s.c. injection of CNO. White arrow heads, dendrite of OT⁺PVN neuron. g The serum OT concentration at 0, 10, 30, 60, 120, and 180 min after the s.c. injection of Saline or CNO (n = 6–7 rats in each group at each time point). *P < 0.05; **P < 0.01 vs. Saline at the same time point. #P < 0.05; ##P < 0.01 vs. CNO at 0 min. h In situ hybridization (ISH) histochemistry of oxytocin (OXT) in the SON and PVN. i Gene expression of OXT in the SON, mPVN, and dPVN at 0, 30, 60, 120, and 180 min after the s.c. injection of Saline or CNO (n = 12 slices from 6 rats, each). **P < 0.01 vs. Saline at the same time point. ###P < 0.01 vs. CNO at 0 min. Scale bars in (a, b, d, h, 200 μm and 50 μm (in magnified images). Scale bars in (f), 10 μm. See also Supplementary Figs. 1, 2.
whilst the latency of nocifensive behavior was markedly prolonged at 1 h after the s.c. injection of CNO compared to Saline (n = 9 rats, each) (Fig. 6e).

Next, we used OTR antagonist to examine whether the antihyperalgesic effects of OT on neuropathic pain were ascribed to neuronal or humoral targets of OT (Fig. 6c). Rats were either treated with an intraperitoneal (i.p.) injection of OTR antagonist (L-371,257, dissolved in dimethyl sulfoxide (DMSO) [10 mg kg\(^{-1}\)]) or intrathecal (i.t.) injection of OTR antagonist (Atosiban, dissolved in saline [1 µg µL\(^{-1}\), 12 µg per rat]) or both (n = 9 rats, each). Since OT itself does not cross the blood brain barrier (BBB), the role of neuronal/humoral OT could be differentiated by using these antagonists. The dose of i.t. OTR antagonist was very small that the effects on periphery was negligible. Thereafter, all rats were
Fig. 4 OT-activated serotonergic neurons in the DR and inhibitory interneurons in the spinal dorsal horn. a Schematic illustration of the dorsal raphe nucleus (DR). b Tryptophan hydroxylase (TPH)-ir and Fos-ir in the DR at 120 min after the s.c. injection of CNO (1 mg kg⁻¹). DR were divided into ventral (DRv), inter-fascicular (DRi), dorsal (DRd) and ventrolateral “wings” (DRvl). cISH image of TPH2 in the DR. d TH-ir neurons, Fos-ir neurons and percentage of their co-expression in the DR (n = 10–12 slices from 5 to 6 rats, each). e Gene expression of TPH2 in the DR after the s.c. injection of Saline or CNO (n = 12 slices from 6 rats, each). f Schematic illustration of the lumbar segment 5 (L5) in the spinal cord. g PAX2-ir, Fos-ir and merged images in the L5 spinal cord at 120 min after the s.c. injection of CNO. h PAX2-ir neurons in the superficial layer (laminae I–II) and deeper layer (laminae III–VI) of the L5 (n = 10–12 slices from 5 to 6 rats, each). i Percentage of co-localization of Fos-ir and PAX2-ir neurons in the L5 (n = 10–12 slices from 5 to 6 rats, each). Scale bars, 200 μm and 50 μm (in magnified images). *P < 0.05; **P < 0.01 vs. Saline. #P < 0.01; ##P < 0.01 vs. CNO at 0 min. See also Supplementary Figs. 3, 4.

Fig. 5 OT induced differential gene expression in GABA-ergic neurons of the dorsal horn. Heat map of the altered genes in the laminae I–II (a) and laminae III–VI (b) of the spinal cord after the chemogenetic activation of endogenous OT by using RNAseq. The samples for the analysis were collected at 120 min after the s.c. injection of Saline or CNO (1 mg kg⁻¹). Scale represents normalized values subtracted by row mean divided by standard deviation. Volcano plot of the genes in the laminae I–II (c) and laminae III–VI (d) of the spinal cord. The genes that were significantly altered were depicted by orange color dots. Red-colored dots indicate subset of the genes that are predominantly expressed in GABA-ergic neurons. Gene ontology (GO) terms that were enriched by endogenous OT in the laminae I–II (e) and laminae III–VI (f) of the spinal cord. Raw RNA sequencing data are available at Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information (NCBI) (accession number GSE210528). See also Supplementary Figs. 5, 6.
Fig. 6 OT alleviated hyperalgesia in a neuropathic pain model via neuronal pathway. a The Seltzer surgery was performed via dorsal approach. Experimental procedures administering CNO after the Seltzer surgery (b) and OT receptor (OTR) antagonist treatment (c). Results of the manual von Frey (d) and hot plate tests (e) after the s.c. injection of Saline or CNO (1 mg kg\(^{-1}\)) in the Seltzer model (n = 9 rats, each). Data are represented as mean ± SEM. **P < 0.01 vs. Saline at the same time point. #P < 0.05; ##P < 0.01 vs. CNO at 0 min. Results of the manual von Frey (f) and hot plate tests (g) after the s.c. injection of CNO (1 mg kg\(^{-1}\)) pretreatment with L-371,257 dissolved in DMSO [10 mg kg\(^{-1}\)] for OTR antagonist i.p., Atosiban dissolved in saline [1 μg μL\(^{-1}\)] for OTR antagonist i.t., and/or vehicle in the Seltzer model (n = 9 rats, each). Data are represented as mean ± SEM. **P < 0.01 vs. Vehicle i.p. + Vehicle i.t., #P < 0.05; ##P < 0.01 vs. OTR antagonist i.p. + Vehicle i.t. at the same time point. See also Supplementary Fig. 7.
subcutaneously injected with CNO (1 mg kg$^{-1}$). Mechanical/heat sensitivities were evaluated for 3 h. Vehicle i.p. and i.t. treatment did not affect both withdrawal threshold and the latency of nociceptive behavior, whilst combined i.p. plus i.t. injection of OTR antagonist ablated the effects of CNO (Fig. 6f, g), suggesting that endogenous OT was involved in the alteration of mechanical/heat sensitivities. Interestingly, the effects of CNO on withdrawal threshold and the latency of nociceptive behavior were not affected by i.p. injection of OTR antagonist, but was completely abolished by i.t. injection of OTR antagonist (Fig. 6f, g). These results suggested that the neuronal OT-ergic pathway plays a greater role in the inhibition of mechanical/heat transmissions, than the humoral OT pathway does, at least in the neuropathic pain model.

OT alleviated spontaneous nociceptive behaviors and hyperalgesia in inflammatory pain models via the humoral pathway. We also examined the effect of endogenous OT on the spontaneous nociceptive behaviors using the formalin test model$^{28}$ (Fig. 7a). Strikingly, the right foot pad swelling that resulted from s.c. injection of 5% formalin (100 µL) was significantly attenuated by pretreatment with CNO (1 mg kg$^{-1}$) (n = 11 rats, each) (Fig. 7c, d). Total licking time (analyzed at 5 min intervals for 60 min) was significantly decreased in CNO compared to Saline (n = 5–6 rats, each) (Fig. 7e). This decrease was detected in both the 1st and 2nd phase (Fig. 7f).

As with the Seltzer model, OTR antagonist was administered (Fig. 7b). Rats were either treated with i.p. injection of OTR antagonist (L-371,257 [10 mg kg$^{-1}$]), i.t. injection of OTR antagonist (Atosiban [1 µg µL$^{-1}$, 12 µg per rat]) or both (n = 6 rats, each). Thereafter, all rats were subcutaneously injected with CNO (1 mg kg$^{-1}$). Total licking time (analyzed at 5 min intervals for 60 min) was then measured. As observed in the neuropathic pain model, vehicle i.p. plus i.t. treatment did not affect licking time, whereas combined i.p. plus i.t. injection of OTR antagonist ablated the effect of CNO (Fig. 7g). Surprisingly, the effects of CNO on licking time were not affected by i.t. injection of OTR antagonist, but was ablated by i.p. injection of OTR antagonist (Fig. 7g, h). These findings were in contrast to the results observed in the neuropathic pain model. Furthermore, the effects of CNO on right foot pad swelling was also abolished by the i.p. injection of OTR antagonist (Fig. 7i). The results demonstrated that humoral OT might play a greater role in the reduction of spontaneous nociceptive behaviors and inflammations.

We also tested the effects of endogenous OT on carrageenan knee arthritis model which is one of the classical inflammatory pain models$^{39}$. Knee inflammation, along with mechanical/heat hypersensitivities, developed rapidly after the intraarticular (i.a.) injection of carrageenan (0.1 mL of 3% λ-carrageenan) and lasted for at least 24 h (Supplementary Fig. 8a–c). After the development of knee arthritis at 3 h after the i.a. injection of carrageenan, either Saline or CNO (1 mg kg$^{-1}$) was subcutaneously injected, then, mechanical/heat sensitivities and knee diameter were measured for 24 h. As with the formalin model, significant attenuation of mechanical/heat hypersensitivities and alleviation of knee swelling were observed after CNO treatment (Supplementary Fig. 8d–g).

OT exerted an anti-inflammatory response by inhibiting mast cell degranulation. The results from the inflammatory pain model experiment led us to speculate that, peripheral OT, rather than central OT, may be more relevant to the anti-inflammatory response, especially for the reduction of local swelling. Peripheral OT may exert an anti-nociceptive response by inhibiting local inflammatory responses both directly and indirectly. Histological analysis of right foot pad revealed that the thickness of the subcutaneous tissue was significantly attenuated by pretreatment with CNO (1 mg kg$^{-1}$) (n = 3–5 rats, each) (Fig. 8a, c). In toluidine blue staining, the morphology of the granules in the mast cells appeared distinctly different in the CNO-pretreated group compared to the Saline-pretreated group. Therefore, chemogenic activation of OT appeared to inhibit degranulation from the subcutaneous mast cells (n = 5 rats, each) (Fig. 8b, d), potentially explaining why peripheral OT suppressed local inflammation. Using a transmission electron microscope (TEM), abundant granules were seen in the mast cells of CNO-pretreated group, whilst more degranulated mast cells were observed in Saline-pretreated group after the injection of formalin (Fig. 8e).

For further confirmation, a mast cell stabilizer (Disodium cromoglicate (DSCG)) dissolved in saline [50 mg mL$^{-1}$] was used and compared with CNO (1 mg kg$^{-1}$). At 30 min prior to the test, Saline, CNO (1 mg kg$^{-1}$), or DSCG (50 mg kg$^{-1}$) was s.c. injected, then 5% formalin (100 µL) was injected into the right hind paw. Strikingly, the effects of DSCG on the formalin test were comparable to CNO (Supplementary Fig. 9a–c). These results might strengthen the mast cell hypothesis.

OT affected hypothalamus–pituitary–adrenal (HPA) axis under pathological condition. Since significant attenuation of inflammation was observed after pretreatment with CNO in the formalin test, we speculated that the HPA axis, which plays an important role in the anti-inflammatory response, might be modified by endogenous OT. However, no alteration was detected for either gene expression of corticotropin-releasing hormone (CRH) or pro-opiomelanocortin (POMC) after s.c. CNO injection of transgenic rats (n = 12 slices from 6 rats, each) (Fig. 9a–c). We further confirmed a lack of increase in Fos-ir in CRH-ir neurons after the chemogenic activation of OT (n = 12 slices from 6 rats, each) (Fig. 9d, e). In addition, the serum concentration of adrenocorticotropic hormone (ACTH) (Fig. 9f) and corticosterone (CORT) (Fig. 9g) remained unchanged after chemogenic activation of OT in naïve transgenic rat (n = 6 rats in each group at each time point). Therefore, it seems that the HPA axis may not play a role in the anti-inflammatory response induced by OT under naïve condition.

We also measured serum OT, CRH, adrenocorticotropic hormone (ACTH), and CORT under the pathological condition, as we speculated that OT might exert greater effects on the HPA axis in the stressful context of physical trauma and/or injury. Immediately after the 5% formalin injection (100 µL) into the right hind paw, Saline or CNO (1 mg kg$^{-1}$) was s.c. injected, then blood concentrations were measured by RIA or ELISA (n = 5 rats in each group at each time point). OT elevation after the s.c. injection of CNO (1 mg kg$^{-1}$) was confirmed (Fig. 9h). Although serum CRH and ACTH were not altered, CORT was significantly increased at 1 h after the s.c. injection of CNO (1 mg kg$^{-1}$) (Fig. 9i–k). In addition, restraint stress was performed to explore the modulative effects of OT on HPA axis in different transgenic rats. Although restraint stress itself caused significant elevation of OT, an additional effect of CNO on serum OT was also observed (Supplementary Fig. 10). However, no alteration of HPA axis was observed after acute restraint stress (Supplementary Fig. 10), indicating that endogenous OT may play differential roles in modulating the HPA axis, depending on the type or duration of stress.

A model describing the putative mechanism of endogenous OT on pain modulation, supported by our finding, is illustrated schematically (Fig. 10).

Discussion We have generated a transgenic rat line that expresses hM3Dq and mCherry specifically in OT neurons in the SON and PVN.
Endogenous OT was involved in pain transmission via both neuronal and humoral pathways. OT might exert anti-nociceptive, anti-hyperalgesic, and anti-inflammatory effects, indicating that this fascinating nonapeptide could be one of the potential therapeutic candidates for various pain- and inflammatory-related diseases.

We primarily focused on the LC, DR, and spinal dorsal horn as they play the crucial roles in pain transmission, although OT also acts on other parts of brain such as the periaqueductal gray, rostral ventromedial medulla, parabrachial nucleus, and amygdala. OT neurons activate GABA-ergic interneurons and may suppress pain signals. Indeed, OT receptors are expressed in the LC and DR\(^{30}\). Using optogenetic technique, it has been shown that OT is released into the LC from hypothalamic PVN OT fibers, activating noradrenergic neurons by co-release of OT and
glutamate. Noradrenergic neurons in the LC and serotonergic neurons in the DR are important transitional nuclei for the descending pain inhibitory system. The administration of local anesthetic directly into the LC contributed to alleviated neuropathic pain. Abundant serotonergic neurons and other neuro-transmitters and/or neuro-modulators containing neurons are expressed in the DR. Their direct, or indirect via the nucleus raphe magnus, descending projections modulate the responses caused by noxious stimulation of the spinal dorsal horn neurons. On the other hand, their ascending projections directly modulate the responses of pain sensitive neurons in the thalamus. Arcuate nucleus of the hypothalamus may also be involved in analgesic effects. Interestingly, Fos expression in TPH positive neurons was the most significantly increased in the lateral wing of the DR which is a stress-sensitive region. Serotonergic neurons in this area contribute to adoptive response to stress. Also, TPH positive neurons located in the lateral wing area participate in modulating pain signals. It is thus likely that endogenous OT may stimulate these neurons, resulting in an altered nociceptive threshold. In the present study, CNO induced a transient analgesia in naïve and sham Seltzer model rats; a different finding to the observation by Eliava et al. and Iwasaki et al. (bioRxiv2022. https://doi.org/10.1101/2022.02.23.481531). Presumably, the different outcome between these studies is methodological, with our approach targeting a larger population of OT neurons rather than selectively targeting the neuronal circuit to the spinal cord.

The neuronal networks from dPVN, LC and DR to L5 spinal dorsal horn was confirmed using a retrograde tracer, consistent with results from previous studies. OT innervation was more prominent between L4 and L6 in the superficial layers (laminae I–II). Given that OT is possibly involved in modulating pain processing, the neuronal networks is convincing. Of note, many genes that were expressed in GABA-ergic neurons were altered by the chemogenic activation of OT in the spinal dorsal horn. It is still unclear whether the inflammatory response or immune response directly affected mechanical/heat threshold. However, in the Seltzer model, inflammatory or immune modification by endogenous OT might alleviate hyperalgesia since inflammation of the spinal cord is the main pathology of the neuropathic pain. Also, the results indicated that OT may be involved in remodeling of the injured spinal cord. OT activates nerve growth factor and IGF-1 which play important roles in the healing process of nerve injury. In addition, OT administration resulted in accelerating the recovery from sciatic nerve injury in rats. The results of the OTR antagonist intervention on inflammatory pain models may also support this hypothesis.

The activation of endogenous OT alleviated both neuropathic and inflammatory pain including spontaneous nociceptive behaviors. Strikingly, intrathecallly administered OTR antagonist (atosiban) abolished the effects of OT in neuropathic pain model, whilst intraperitoneallly administered OTR antagonist (L-371,257) ablated the effects of OT in nociceptive pain model. These results suggest that the main site of action of OT is different depending on the type of pain. It should be noted that atosiban is a biased agonist of OTR-Gi pathway rather than an OTR antagonist. Atosiban has been shown to eventually inhibit the function of the neurons or cells that express OTRs. We chose to use atosiban as an OTR antagonist for pragmatic reasons since it has been widely used as an OTR antagonist. Previous studies have reported the analgesic effects of OT on neuropathic pain and nociceptive pain in rodents. In humans, however, it is still controversial. The reason for the discrepancy is probably due to OT’s short half-life, poor BBB penetration, and lack of specificity to OTRs since OT has similar affinities with V1aR and the Transient Receptor Potential Vanilloid type-1 (TRPV1). Previous studies have reported that analgesia induced by systemic OT treatment is mediated by the V1aR and TRPV1. Other kinds of social behavior such as social interactions induced by OT is also mediated by V1aR. Recently, a OTR agonist with greater specificity and longer half-life could induce a long-lasting reduction in inflammatory pain-induced hyperalgesia symptoms. For the use of OT as an analgesic drug in the clinic, these types of OTR agonists are promising and warrant further investigation.

Although beyond the scope of this study, DRG might be also affected by endogenous OT as OTRs are expressed in afferent neurons of the DRG. OTRs are expressed predominantly in non-peptidergic C-fiber cell bodies in the DRG indicating that humoral OT can penetrate DRG and directly act at the peripheral level of pain structures. It is speculated that direct action of OT on DRG, as well as on anti-inflammatory action, might contribute to its peripheral analgesic effects.

Chemogenic activation of OT alleviated the swelling of ipsilateral foot pad after formalin injection by inhibiting mast cell degranulation. Previous study has reported that cardiac ischemia/
Fig. 8 OT exerted an anti-inflammatory response by inhibiting mast cell degranulation. Morphology of hematoxylin and eosin (HE)-stained (a) and toluidine blue (TB)-stained (b) hind paw after the s.c. injection of saline or 5% formalin (100 µL) into the hind paw. Scale bar in (A), 50 µm. Scale bar in (B), 10 µm and 5 µm (in magnified image). c The subcutaneous tissue thickness measured using the HE-stained hind paw slices (n = 6-10 slices from 3 to 5 rats, each). **P < 0.01 vs. Saline s.c. + Saline s.c. ##P < 0.01 vs. Saline s.c. + Formalin s.c. d Number of mast cells in subcutaneous tissue either granulated or degranulated was manually counted (n = 10 slices from 5 rats, each). **P < 0.01 vs. Saline s.c. + Formalin s.c. e Granulated and degranulated mast cell captured by transmission electron microscope (TEM) after formalin injection. N, nucleus. Red arrow heads indicate the granules that were degranulated from a mast cell. Scale bars, 5 µm. See also Supplementary Fig. 9.
Reperfusion injuries were attenuated by inhibition of the degranulation of cardiac mast cells by pretreatment with OT. Peterson et al. have examined the effect of OT on carrageenan-induced inflammation in rat hind paws. They demonstrated that OT administration reduced the edema of the paw with reduced activity of myeloperoxidase. They also suggested that OT’s anti-inflammatory effect of OT was comparable to the effect of the glucocorticoid. Although OTRs are expressed in mast cells, they are also expressed in the fibroblasts that exist closely to mast cells. Peripheral OT might therefore inhibit degranulation of mast cells by modifying the function of the fibroblasts. Previous studies have shown that OT might modulate the HPA axis. OT’s effect on the HPA axis probably reflects axonal transport and local regulation of transcription factors in the...
neural lobe in the PP\(^5\). Indeed, we have confirmed that the HPA axis was not affected under naive condition but was affected after formalin injection. Neumann has reported the effects of OT on HPA axis under physical and psychological stress\(^5\). OTPVN neurons exert a tonic inhibition on ACTH secretion possibly via inhibiting CRH neuronal activity under basal state. On the other hand, under the stressed condition, OTPVN upregulates HPA axis via activating CRH neurons. However, further studies are needed to clarify the effects of OT on the HPA axis as there are still many unknowns.

In the present study, we have succeeded in generating a transgenic rat line, expressing excitatory DREADDs specifically in OT neurons. The development of chemogenetics and optogenetics has revolutionized the field, especially for investigating the role of specific peptides in behavioral changes by enabling activation of specific neurons. Virus-mediated transfection, also known as transduction, has been well-established and commonly used to insert foreign genes into targeted neurons, rather than just selected populations of cells. However, this cannot be achieved by the virus-mediated transfection. The anti-inflammatory effects of OT are region-specific and may be mediated by OT neurons, rather than just selected populations of cells.

### Methods

**Animals and ethics approval.** Non-transgenic and heterozygous transgenic Wistar (Crl:WI, Japan Charles River, Yokohama, Japan) rats were bred and group-housed (n = 3 per cage) under normal laboratory conditions (temperature, 24 ± 1.0°C; light/dark cycle, lights on at 22.00 h; humidity, 55 ± 5%) with access to food and water at least for 2 weeks. All rats used in the experiments were acclimatized in the reversed light condition for at least 2 weeks. All experiments were performed in strict accordance with guidelines on the use and care of laboratory animals as set out by the Physiological Society of Japan and approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health (approval No. AE21-006).

**Conducts for microinjection.** A chimeric OT-hM3Dq-mCherry BAC clone transgene construct was purified for microinjections. The hM3Dq-mCherry sequence from the hM3Dq-mCherry cassette (Plasmid #44361, Addgene, Cambridge, MA, USA) was used for the transgene\(^5\). Next, SV40 poly A sequence was cloned into the hM3Dq-mCherry sequence. Finally, an hM3Dq-mCherry-SV40 poly A cassette was introduced into the rat OT gene in place of the genomic start codon. Hence, hM3Dq-mCherry should be specifically expressed under the OT promoter in the transgenic rat. Three transgenic founder male rats were identified by Southern blot analysis using genomic tail DNA with a 32P-labeled mCherry probe.

**Test substances.** CNO (Sigma-Aldrich Japan Co. LLC., Tokyo, Japan) was dissolved in saline (Otsuka Pharmaceutical Co. LTD., Tokyo, Japan)\(^5\). OTR

### Fig. 10 The putative mechanism of endogenous OT on pain modulation. A model describing the putative mechanism of endogenous OT on pain modulation is illustrated schematically. Central OT may exert anti-nociceptive effects both directly and indirectly via descending inhibitory system. On the other hand, peripheral OT may alleviate inflammatory response by inhibiting degranulation from mast cells and by modulating HPA axis under pathological condition, resulting in hindering pain transmission. Created with BioRender.com.
antagonist Atosiban (Sigma-Aldrich Japan Co. Ltd., Tokyo, Japan) was dissolved in saline and i.v. 371.257 (Tocris Bioscience, Bristol, UK) was dissolved in DMF143,79. The CRH (Tocris Bioscience Co., Ltd., Tokyo, Japan) was dissolved in saline and used as a mast cell stabilizer65.

Fluorescent immunohistochemistry. The rats were deeply anesthetized with i.p. injection of three types of mixed anesthetic agents (in combination with 0.3 mg kg−1 of medetomidine, 4.0 mg kg−1 of midazolam, and 5.0 mg kg−1 of butorphanol). They were transcardially perfused with 0.1 M phosphate buffer (pH 7.4) containing heparin (1000 U E−1), followed by 4% paraformaldehyde in 0.1 M PB. The brains were carefully removed, and a small block that included the hypothalamus was isolated. The blocks were post fixed with 4% paraformaldehyde in 0.1 MPB for 4 h at 4 °C. Then, the tissue was cryoprotected in 20%(−w/v) sucrose in 0.1 MPB for 8 h at 4 °C. Fixed tissue was cut into 30 µm using a microtome (REM-700; Yamato Kohki Industrial Co., Ltd., Saitama, Japan). The observed nuclei were identified according to the coordinate that given in the rat brain atlas66. Information of the primary (Supplementary Table 1) and secondary antibodies (Supplementary Table 2) used in the present study are summarized. Sections cut by a microtome were rinsed twice with 0.1 M phosphate-buffered saline (PBS) and washed in 0.1 M PBS (pH 7.6) containing 0.3% Triton X-100 (PBST). They were incubated in a primary antibody solution for 48 h at 4 °C. After being washed twice in 0.1 M PBST, the floating sections were incubated in a secondary antibody solution for 2 h at room temperature. They were then washed twice in PBST and 10 min mounted on a slide glass and cover-slipped using vectashield (Vector Laboratories Co. Ltd., CA, USA). Images scanned by a confocal laser scanning microscopy were reconstructed by using imaging software (ZEN 3.2, blue edition) provided with the LSM880 laser scanning microscope (Carl Zeiss Co. Ltd. Oberkochen, Germany). In addition, images scanned by all-In-one microscopy (BZ-800, Keyence, Osaka, Japan) were used to analyze the percentages of Fos-ir induction.

Co-localization of mDM3q-mCherry, OT, VP, CRH, and Fos. Each captured image by a confocal laser scanning microscopy was printed onto a paper in an expanded size. Subsequently, the printed papers were blinded and endogenous mCherry positive neurons and each protein-ir neurons were manually counted at least two researchers to avoid skewing the results. We counted two cross sections (four nuclei including right and left) of each nucleus and the results were averaged. To prevent double-counting, we checked the cross mark on the printed paper every (four nuclei including right and left) of each nucleus and the results were averaged. We counted two cross sections (four nuclei including right and left) of each nucleus and the results were averaged. To prevent double-counting, we checked the cross mark on the printed paper every (four nuclei including right and left) of each nucleus and the results were averaged.

Sequencing RNaseq analysis. FASTQ files were imported to CLC Genomics Workbench (CLC GW, v10.1.1, Qiagen, Hilden, Germany). Sequence reads were mapped to rat reference genome (Rattus norvegicus) using bowtie2. blasted sequences were visualized by exporting BAM files, converting into bedgraph files, and uploading to UCSC Genome Browser ([https://genome.ucsc.edu/]). Box plot and principal component (PCA) plot were produced using raw read counts in CLC GW. To identify differentially expressed genes, total read counts were analyzed by Empirical Analysis tool in DGE tool in CLC GW. Analyses were performed using an Illumina NextSeq500 (Illumina, Inc., San Diego, CA, USA) with a high-output kit in paired-end reads (v2, 2 × 36), according to the manufacturer’s instructions. Raw RNA sequencing data are available at Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information (NCBI) (accession number GSE120538).

RNAseq data analysis. Quality control of reads was performed using an Illumina NextSeq500 (Illumina, Inc., San Diego, CA, USA) with a high-output kit in paired-end reads (v2, 2 × 36), according to the manufacturer’s instructions. Raw RNA sequencing data are available at Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information (NCBI) (accession number GSE120538).
defined as the commencement of licking their formalin-injected limb in Saline-treated rats. Formalin-induced pain evokes three main behavioral responses: licking, tonic flexion and paw jerk. They were recorded for 60 min after the formalin injection. The total time of licking the injected hind paw (at 5 min interval for 60 min) were analyzed. Pain induced by formalin in rodents has two phases which reflect different pathological processes. After the formalin injection, animals show early or acute painful responses (0–7 min) which imitate the direct activation of nociceptors, then, attenuation or quiescent of nociceptive responses in an interphase is observed, followed by a long-lasting period of nociceptive behaviors which might last for more than 45 min. According to the previous study,27 we defined the 1st and 2nd phase as 0–10 and 10–60 min, respectively. The results were blinded and analyzed randomly in duplicate by at least two researchers to avoid bias.

### Carrageenan knee arthritis model
The rats were anesthetized with inhalation of sevoflurane for 2–3 min in a glass chamber. An i.a. injection of 0.1 ml of 3% λ-carrageenan (Sigma, St. Louis, MO, USA) which was dissolved in 0.9% NaCl was administered into the right hind knee joint using 25-gauge injection needles.

### Measurement of joint swelling
To assess joint swelling induced by carrageenan i.a. injections, the diameters of the right and left knee joints were measured using digital calipers before the i.a. injection and at 3, 6, and 12 h after the i.a. injection on the right knee. The difference between the lateral and medial collateral ligament was defined as the knee joint diameter.24 Averaged diameter for each group at each time point was calculated and analyzed.

### Seltzer model
After deeply anesthetized with i.p. injection of three types of mixed anesthetic agents (in combination with 0.3 mg kg⁻¹ of medomidine, 4.0 mg kg⁻¹ of midazolam, and 5.0 mg kg⁻¹ of butorphanol), the one-third to half diameter of the right sciatric nerve was ligated with 6-0 silk suture after exposure. The rats that showed drop-foot were omitted from the analysis.

### Pathological examination after formalin test
Right feet of rats, which were used for the formalin test, were amputated, and kept in 4% formalin. Then, fixed feet were cut into 6-μm sections and stained with hematoxylin and eosin (HE) and toluidine blue (TB) to assess morphological changes.

### Transmission electron microscope observation
Specimens were cut into 6 µm sections and stained with hematoxylin and eosin (HE) and toluidine blue (TB) to assess morphological changes. Semithin sections were dehydrated and embedded in epoxy resin. Semithin sections were stained with uranyl acetate and lead citrate, and observed under transmission electron microscope. Observation of the rat sciatic nerve was performed using a JEM-1011 Transmission Electron Microscope (JEOL, Japan).

### Measurement of serum CRH, ACTH, and CORT (ELISA)
Plasma concentrations of CRH (YK131 Mouse/Rat CRF-HS ELISA kit, Yanahara Institute Inc., Fujinomiya, Japan), ACTH (ACTH Rat, Mouse EIA kit, PHOENIX PHARMACEUTICALS, INC., CA, USA), and CORT (Corticosterone ELISA kit, Cayman Chem., MI, USA) were analyzed by ELISA. Procedures were implemented by a protocol attached in each ELISA kit. All results were duplicated, and the averaged value in each sample was calculated.

### Restraint stress
The transgenic rats were pretreated with Saline or CNO (1 mg kg⁻¹) at 30 min prior to the restraint stress. Restraint stress was induced by taping all 4 limbs of the rat to metal mounts attached to a wooden board.25 The immobilization lasted for 30 min as an acute stress stimulus. Rats were killed by decapitation without being anesthetized to collect the trunk blood immediately after the end of the immobilization.

### Statistics and reproducibility
The mean ± standard error of the mean (SEM) was calculated from the results. All data were analyzed by student t test, one-way ANOVA, or two-way ANOVA followed by a Bonferroni-type adjustment for multiple comparisons using R software. Statistical significance was set at P < 0.05.

### Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability
The materials, including OT-HM3Dq-mCherry transgenic rats, are available from the corresponding authors, upon reasonable request. The data that support the findings of this study are available from the corresponding authors, upon reasonable request. Raw RNA sequencing data are available at Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information (NCBI) (accession number GSE210528).

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**Competing interests**
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**Correspondence** and requests for materials should be addressed to Mitsuhiro Yoshimura or Yoichi Ueta.

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