Impaired neuronal macroautophagy in the prelimbic cortex contributes to comorbid anxiety-like behaviors in rats with chronic neuropathic pain

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Abstract

The medial prefrontal cortex (mPFC) underlies comorbid anxiety in chronic neuropathic pain. However, the molecular and neuronal mechanisms are not fully understood due to its functional heterogeneity. Here, we reported that impaired neuronal macroautophagy in the prelimbic cortical (PrL) subregion of the mPFC paralleled the occurrence of anxiety-like behaviors in rats with chronic spared nerve injury (SNI) and identified a causal links between PrL neuronal macroautophagy dysfunction and comorbid anxiety in neuropathic pain via specific manipulation of macroautophagy in different PrL neuronal subpopulations. Intriguingly, such macroautophagy impairment
was mainly observed in a c-Fos+ neuronal subpopulation in the PrL, which chemogenetic inactivation of this comorbid anxiety-related neuronal ensemble relieved pain-induced anxiety-like behaviors. Rescue macroautophagy impairment in this neuronal ensemble relieved chronic pain-associated anxiety and mechanical allodynia. Finally, we found rescuing neuronal macroautophagy in the PrL restored synaptic homeostasis at the molecular level. Taken together, our work provides novel insights into the role of PrL by differentiating its contribution in pain-induced comorbid anxiety from its modulation over general anxiety-like behaviors and reveals the role of neuronal macroautophagy underlying the comorbid anxiety-like behaviors.

**Keywords**

Chronic neuropathic pain; Anxiety-like behaviors; Comorbidity; Prelimbic cortex; Autophagy; Synaptic homeostasis.

**Introduction**

Pain is an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage [1]. Acute pain protects people from tissue damage, while chronic pain may lead to unnecessary suffering and frequently encompasses multiple behavioral dimensions. A large proportion of patients with chronic pain suffer from comorbid mood disorders, which in turn exacerbate the pain and make its management particularly unsatisfactory. Anxiety symptoms are among the most common comorbidities in chronic pain patients [2, 3], but both underlying mechanisms and effective treatments remain poorly explored [4]. Preclinical studies have reported depression- and anxiety-like behaviors in animal models of chronic pain [5–7], and start to reveal the contribution of maladaptive neuroplastic changes in pain-related brain areas, including the anterior cingulate cortex (ACC) [6, 8], insular cortex (IC) [9, 10], amygdala [11, 12], primary somatosensory cortex [13], hippocampus [14], bed nucleus of the stria terminalis (BNST) [15], and medial prefrontal cortex (mPFC) [14, 16, 17], in these affective comorbidities. The
mPFC plays key roles in memory [18], decision making [19], and anxiety [20–22], all of which are affected in the development of chronic pain. Morphological and functional reorganization [23], as well as imbalanced synaptic homeostasis have been identified in the prelimbic cortical (PrL) subregion of the mPFC in rodents under chronic pain conditions [24–26]. Lesion of the PrL attenuates CFA-induced hyperalgesia and anxiety-like behaviors in rats with chronic inflammatory pain [27], whereas optogenetically manipulating GABAergic interneurons are able to modulate both sensory and emotional responses to neuropathic pain [28]. These findings demonstrate a close link between pain-anxiety comorbidity and the PrL [27, 28].

Macroautophagy, an evolutionarily conserved process that delivers diverse cellular contents to lysosomes for degradation, is a broadly used homeostatic mechanism essential for brain physiology [29, 30]. Preferential vulnerability of neurons to macroautophagy loss addresses neuronal subtype-specific reliance on robust catabolism to maintain homeostasis and carry out their highly specialized functions [31]. Disruption in basal macroautophagy results in functional disturbances at the molecular, cellular and tissue levels, leading to physical and mental illnesses [32–35]. In peripheral nerves and the spinal cord, macroautophagy disruption in neurons and glial cells maintains nociceptive hypersensitivity in chronic neuropathic pain [36, 37]. In contrast, the roles of neuronal macroautophagy in pain-related brain areas, the PrL in particular, remain unclear. Indeed, long-term morphological and functional changes in PrL neurons, in parallel with protein accumulation and synaptic abnormalities [23–25], in the chronic phase of neuropathic pain give rise to the hypothesis that disrupted neuronal macroautophagy in the PrL may act as an important contributor to pain-anxiety comorbidity. However, neuronal ensembles even in the same brain region are not anatomically or functionally uniform but divide into distinct subpopulations[27, 38, 39]. Such heterogeneity raises difficulty in differentiating PrL contribution in pain-induced comorbid anxiety from its modulation over general anxiety-like behaviors.

In the present study, we identified impaired macroautophagy (hereafter termed
autophagy) in the PrL, comorbid anxiety-related neurons in particular, of rats with chronic neuropathic pain, which temporally paralleled and causally contributed to the occurrence of comorbid anxiety-like behaviors.

Materials and methods

Animals

Adult male Sprague–Dawley rats that weighed 220–250 g at the beginning of the experiments were provided by the Department of Laboratory Animal Sciences, Peking University Health Science Center (Beijing, China). Rats were housed in standard cages at 24 ± 1 °C, and maintained in a 12-h light-dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) with free access to food and water. All experiments were conducted in accordance with the guidelines of the International Association for the Study of Pain and approved by the Animal Care and Use Committee of Peking University Health Science Center. By the end of the experiment, animals were dissected for histological analysis.

Spared nerve injury (SNI) model of neuropathic pain

The SNI model was established in rats as previously described [40, 41]. In brief, the left common peroneal nerve and tibial nerve were tightly ligated with 5.0 silk sutures, and sectioned distal to the ligation with removal of 3 mm of the nerve stump. The sural nerve was kept intact. Only animals that developed mechanical allodynia were used. Sham surgery rats underwent all surgical procedures except for nerve injury. Baseline measurements were taken before the surgeries.

Assessment of mechanical alldynia

Rats were habituated for 20 min in a transparent plastic box on a metal mesh floor before testing. Von Frey filaments (0.41–15.1 g; North Coast, Gilroy, CA) were applied to the lateral plantar surface of the hind paws in the receptive field of the sural nerve for 2 – 3 s [41]. The 50% paw withdrawal threshold (PWT) was calculated by the “up and down” method [42]. Only rats with basal paw withdrawal threshold equal to 15.1 g were recruited for further experiments. Mechanical allodynia was measured at different time points after surgery. All behavioral tests were performed single-blindly.
Open field test for locomotor activity

The apparatus for the open field test was an opaque box (100 × 100 × 50 cm). The test was conducted in a quiet room with 50 lux illumination. Each rat was placed in the center of the field and its behaviors were videotaped for 10 min [43, 44]. The total distance traveled in the field was used to reflect motor function, whereas the avoidance of the central section of the open field could partially reflect heightened levels of anxiety-like behaviors. Percentage of time spent (C.Time) and distance traveled (C.Dis) in the central area (60 × 60 cm) and total distance traveled (T.Dis) in the field were analyzed by SMART software (version 2.5.21, Panlab, SMART Videotracking, Harvard Apparatus; RRID:SCR_002852). The apparatus was cleaned with 75% ethanol between sessions.

Elevated plus-maze for anxiety-like behaviors

The elevated plus-maze was constructed by black plexiglas, raised 70 cm above the floor, and consisted of two opposing enclosed arms with 40 cm high opaque walls and two opposing open arms of the same size (48 cm × 8 cm). The apparatus was placed in a 30 lux illuminated room, in which the rats were acclimatized for at least 30 min before the test. Each rat was placed in the center of the elevated plus-maze heading toward the same open arm, and videotaped in the following 10 min [43, 44]. The avoidance of open arms of the maze was used to indicate heightened levels of anxiety-like behaviors and total arm entries were used to reflect motor function. Percentage of time spent (O. Time) and numbers of entries (O. Entries) into open arms and total arm entries (T. Entries) were analyzed using the SMART software. The maze was cleaned with 75% ethanol between sessions.

Cannula implantation

Rats were anesthetized with 1% pentobarbital sodium (0.1 g/kg, i.p.) and positioned in a stereotaxic instrument (RWD Life Science, Shenzhen, China). A guide cannula (O.D. 0.48 mm/I.D. 0.34 mm, C.C 1.2 mm, RWD Life Science, Shenzhen, China) was stereotactically implanted 1.5 mm above PrL (AP: 2.9 mm; ML: ±0.6 mm;
The guide cannula was attached to the skull surface with four skull screws and dental acrylic. The matching cap (0.5 mm below the guide cannula, RWD Life Science, Shenzhen, China) was inserted into the guide cannula to prevent clogging. All rats were allowed to recover 1-week after implantation surgery before further experiments.

**Stereotactic microinjection of drug**

Chloroquine (CQ; #C6628, Sigma–Aldrich, USA) was dissolved in artificial cerebrospinal fluid (ACSF) prior to use. ACSF containing the following substances: 75 mM sucrose, 25 mM glucose, 87 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 0.5 mM CaCl2, 7 mM MgCl2, pH 7.35-7.4. Dissection The injection cannula (1.5 mm below the guide cannula, RWD, Shenzhen, China) was used for microinjection with a PE-10 tube connecting a microsyringe (RWD Life Science, Shenzhen, China). CQ (100 μM) and ACSF were injected into the PrL of either side over 2 min, respectively. The injection needle was held on for at least 2 min to allow drug diffusion [44]. The behavioral tests were performed at different time points after drug/vehicle injection. Rats with an incorrect guide cannula site (after slicing validation) were excluded from analysis.

**Stereotactic injection of adeno-associated viruses (AAV)**

AAV used *in vivo* was packaged and purchased from Vigene Biosciences (Jinan, China) or OBiO Technology (Shanghai, China). The AAV vectors used in this study are listed in Supplementary Table 2. Rats were anesthetized with 1% pentobarbital sodium (0.1 g/kg, *i.p.*) and positioned in a stereotactic instrument (RWD Life Science, Shenzhen, China). The scalp was shaved, a small sagittal incision was made to expose the skull, and a small hole was drilled in the skull above the requisite injection site. AAV virus solution was microinjected into the PrL (AP: 2.9 mm; ML: ±0.5 mm; DV: −3.0 mm) [45] with 0.5 μl/hole at a speed of 0.1 μl/min via a microinjection pump. The needle was kept on the site for 5 min to allow for virus diffusion and gradually withdrawn over 1 min to prevent possible leakage from the needle track. The incision
was sutured, and the rat was returned to its home cage for 1-week recovery before subsequent experiments.

**Chemogenetics**

For chemogenetics, AAV<sub>9</sub>-c-fos-rtTA-P2A-GFP and AAV<sub>9</sub>-TRE<sup>3G</sup>-hM4D(Gi)-mCherry were injected into the bilateral PrL. Three weeks after surgery, the rats were home caged and received Dox from food pellets for 3 days for activity-dependent labeling of PrL comorbid anxiety-related cells. The rats in each group were intraperitoneally given 0.5 mg/mL CNO (clozapine-N-oxide, 1 mg/kg) or an equal volume of sterile saline. Behavioral tests were performed 30 min after CNO injection.

**Immunofluorescence**

Rats were anesthetized with 1% pentobarbital sodium and intracardially perfused with 4% paraformaldehyde (PFA; in 0.1 M phosphate buffer, pH 7.4). Brains were postfixed with 4% PFA for 12 h and kept in 20% and 30% sucrose solutions in turn for dehydration. Forty-micrometer sections were sliced coronally using a cryostat microtome (model 1950, Leica). Free floating sections were washed in phosphate-buffered saline, blocked with a buffer containing 5% bull serum albumin and 0.3% Triton X-100 for 1 h, and incubated with primary antibodies at 4 °C overnight: mouse anti-NeuN (1:500, catalog #ab104224, Abcam), mouse anti-c-Fos (1:500, catalog #ab208942, Abcam), goat anti-EAAC1 (1:200, catalog #AB1520, Millipore), rabbit anti-Atg7 (1:500, catalog #ab133528, Abcam), and rabbit anti-LC3 (1:200, catalog #L7543, Sigma). Sections were then washed 3 times in phosphate-buffered saline and incubated with secondary antibody solution containing 4′,6-diamidino-2-phenylindole for 1.5 h at 37 °C: Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500, catalog #ab150113, Abcam), Alexa Fluor 568-conjugated goat anti-mouse IgG (1:500, catalog #A-11004, Invitrogen), Alexa Fluor 647-conjugated goat anti-mouse IgG (1:500, catalog #A-11004, Invitrogen), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500, catalog #A-21207, Invitrogen) and Alexa Fluor 488-conjugated donkey anti-goat IgG (1:500, catalog #A-11055, Invitrogen). Sections were washed 3 times and
mounted on microscope slides using the mounting medium (ZSGB-BIO, Beijing). Images were captured by a laser-scanning confocal microscope (model FV1000, Olympus) and visualized with Imaris 7.4.2 [46](Bitplane, Inc.).

**Western blot analysis**

Rat brains were extracted, embedded in optimum cutting temperature compound (0201 08926, Leica), and frozen in liquid nitrogen immediately. PrL tissues were extracted using No.9 puncture needles in a cryostat microtome according to the stereological location [47]. After being extracted from tissues by RIPA buffer (P1201-50, Applygen Technologies), an equivalent of 80 µg cytoplasmic protein was mixed with loading buffer containing 2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.25% bromophenol blue. Proteins were separated in 10% or 15% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (ISEQ00010, Merck Millipore). The membranes were blocked with 10% defatted milk at room temperature for 2 h, incubated with primary antibodies at 4 °C for 24 h: rabbit anti-Atg7 (1:1,000, catalog #ab133528, Abcam), rabbit anti-LC3B (1:1000, catalog #L7543, Sigma), rabbit anti-p62 (1:500, catalog #PM045, MBL), rabbit anti-LAMP1 Receptor 1 (1:1,000, catalog #ab24170, Abcam), rabbit anti-PSD95 (1:1000, catalog #ab18258, Abcam), mouse anti-Actin (1:1,000, catalog #TA-09, ZSGB-Bio), and mouse anti-GAPDH (1:1,000, catalog #TA-08, ZSGB-Bio), then washed in tris-buffered saline containing 0.1% Tween 20, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:1,000, catalog #zb-5307, ZSGB-Bio) or horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1,000, catalog #zb-2301, ZSGB-Bio) at room temperature for 1 h. Protein bands were detected using Western blotting luminol reagent (P1010, Applygen) and chemiluminescent imaging system (Tanon 4600, Tanon Science & Technology Co.). Blots were quantified with ImageJ software.

**Immunoprecipitation**

PrL tissues were extracted, and lysates were precleared by incubation with 30 mL protein G agarose beads on a rotator overnight at 4 °C. Precleared lysates were then...
incubated with beads that were conjugated with an antibody against LC3 (1:100, catalog# L8918, Sigma) overnight on a rotator at 4 °C. On the following day, the beads were washed in cold lysis buffer three times and then used for western blotting. IgG controls were used in parallel to ensure specificity.

Fluorescence-activated cell sorting

Six weeks after delivery of AAV9-CaMKII - Atg7-P2A-GFP or AAV9-CaMKII -GFP into the PrL, the rat was anesthetized with 1% pentobarbital sodium, and the brain was quickly extracted and sectioned on a vibratome on ice. Individual slices of interest were transferred to a small dish containing cold (4 °C) dissection media: 116 mM NaCl, 5.4 mM KCl, 26 mM NaHCO3, 1 mM NaH2PO4, 1.5 mM CaCl2, 1 mM MgSO4, 0.5 mM EDTA, 25 mM glucose, and 1 mM cysteine, bubbled with 95% O2 and 5% CO2. The PrL was dissected and treated with dissection media with papain (1 mg/mL; LS003119, Worthington) at 37 °C for 30 min. The tissues were dissociated into single cells by gentle trituration and filtered through a 70-μm cell strainer (F613462, BBI). Sorting was performed by a fluorescence-activated cell sorter (BD Biosciences) in the single-cell sorting mode to select neurons with high enhanced green fluorescent protein fluorescence for subsequent mass spectrometry analysis.

Mass spectrometry analysis

(1) Sample preparation

We used tissue protein extraction reagent (Thermo Scientific; 78510) to extract total protein from tissue samples and RIPA buffer (Thermo Scientific; 89990) for cell samples. After the concentration of the supernatant was measured, 50 μg of protein was precipitated. The supernatant was precipitated by trichloroacetic acid (TCA) solution at 4 °C for 4 h and centrifuged at 16,000× g for 30 min at 4 °C. After three washes with acetone, the precipitate was dried with a vacuum concentrator (Labconco, USA). The dried precipitate was resuspended in 40 μl 8 M urea in 500 mM Tris-HCl buffer (pH 8.5), incubated with 20 mM (2-carboxyethyl) phosphine hydrochloride (TCEP) (500 mM in 100 mM Tris/HCl pH 8.5) at room temperature for 20 min, and then alkylated
with 40 mM IAA in the dark for 30 min. The mixture was diluted with 200 µl of 100 mM Tris-HCl buffer (pH 8.5) to final concentration of 1.3 M urea, followed by digestion with 3 µg trypsin protease to a final concentration of 12.5 ng/µl at 37 °C for 16 h. Digestion was quenched by the addition of formic acid at a final concentration of 5%. The sample was desalted using a Monospin C18 column (GL Science, Tokyo, Japan). The peptides were redissolved in 50 µl Milli-Q water with 0.1 vol% formic acid. For data independent acquisition (DIA) experiments, iRT calibration peptides were spiked into the sample. Sample preparation for the spectral library. A total of 100 µg of protein was extracted from all samples.

(2) High-pH reversed-phase fraction

Approximately 100 µg mixed peptides were fractioned on chromatographic column (BEH C18, 300 A, 1.7 µm, 1 mm × 150 mm) coupled to a Waters Xevo™ ACQUITY UPLC (Waters, USA) within 80 min and concatenated into 62 fractions. The first fraction is mixed with the last fraction, and the rest is mixed with two fractions every 30-fraction sequentially. Finally, 31 fractions were obtained. All fractions were vacuum-centrifuged to dryness and reconstituted in 10 µl Milli-Q water with 0.1 vol% formic acid. iRT peptides were spiked before DDA analysis.

(3) Liquid chromatography

We employed a nanoElute liquid chromatography system (Bruker Daltonics). Peptides (200 ng of digest) were separated within 90 min at a flow rate of 300 nL/min on a 25 cm × 75 µm column with a laser-pulled electrospray emitter packed with 1.5 µm ReproSil-Pur 120 C18-AQ particles (Dr. Maisch). Mobile phases A and B were water and ACN with 0.1 vol% formic acid. The %B linearly increased from 2 to 22% within 70 min, followed by an increase to 37% within 8 min and a further increase to 95% within 5 min before the last 7 min 95% process.

(4) Mass spectrometry

All fraction samples were analyzed on a hybrid TIMS quadrupole time-of-flight mass spectrometer (Bruker timsTOF Pro) via a CaptiveSpray nanoelectrospray ion
source. The mass spectrometer was operated in data-dependent mode. We set the accumulation and ramp time to 100 ms each and recorded mass spectra in the range from m/z 100–1700 in positive electrospray mode. The ion mobility was scanned from 0.6 to 1.6 Vs/cm². The overall acquisition cycle of 1.16 s comprised one full TIMS-MS scan and 10 PASEF MS/MS scans. For data-independent acquisition, we defined quadrupole isolation windows as a function of the TIMS scan time to achieve seamless and synchronous ramps for all applied voltages. We defined up to 8 windows for single 100 ms TIMS scans according to the m/z-ion mobility plane. During scanning mode, the collision energy was ramped linearly as a function of the mobility from 59 eV at 1/K₀=1.6 Vs cm⁻² to 20 eV at 1/K₀=0.6 Vs cm⁻².

**Generation of spectral library and DIA data analysis**

Spectral libraries were generated with Spectronaut version 14.0 (Biognosys) using the default parameters. MS/MS spectra were matched against the rat UNIPROT database (only reviewed entries) (Rat 8160 entries, downloaded July 2021) All the parameters were default.

**Statistical analysis**

For proteomics data analysis, significantly different GO terms and EC numbers were calculated by t-tests adjusted using FDR (p < 0.05, FDR < 5%). The differentially expressed proteins between two groups were calculated by Student’s t-test (p < 0.05, fold-change > 2, FDR < 1% cutoff). ANOVA was used to analyze the significant differences between multiple groups (p < 0.05). The differentially expressed proteins were clustered using Mfuzz (version 2.46.0, https://www.bioconductor.org/packages/release/bioc/html/Mfuzz.html). PCA and cluster analyses based on Euclidean distance were performed using OmicsBean software.

The data were presented as mean ± SEM and analyzed using GraphPad Prism v9.0.0. Western blot data were analyzed by one- or two-way ANOVA followed by
Bonferroni’s post-hoc test. Animal behavioral data were analyzed by one- or two-way ANOVA followed by Bonferroni’s post-hoc test and paired or unpaired Student’s t-tests. For immunofluorescence analysis, data were analyzed using unpaired Student’s t-tests. Statistical significance was accepted at the level of $p < 0.05$ with asterisks in figures denoting $p$ values as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Results**

**Behavioral profile of SNI induced chronic neuropathic pain**

We first examined temporal profiles of pain behaviors in rats with spared nerve injury (SNI) of the sciatic nerves, a well-accepted model of neuropathic pain [40], and sham-operated rats. The SNI rats displayed an early onset and long-lasting mechanical allodynia (Fig. 1B), as previously described [43]. In addition to perceptual hypersensitivity, SNI rats developed anxiety-like behaviors, a common comorbidity of chronic neuropathic pain [48] in a late (SNI day 28) but not earlier (SNI day 14) stage, indicated by less time spent and less distance traveled in the central area of the open field (Figs. 1, C and E), as well as less time spent and fewer entries into the open arms of the elevated plus-maze (Fig. 1, D and F). In contrast, the locomotor activities of SNI rats remained unchanged, as indicated by a similar total distance traveled in the open field and similar total arm entries in the plus-maze (Fig. 1, C-F).

**Impaired neuronal autophagy in the PrL of SNI rats parallels the occurrence of comorbid anxiety-like behaviors**

We next conducted an unbiased, mass spectrometry-based, proteomic screening to compare protein expression between the PrL of SNI and sham-operated rats. We found that the expression of microtubule-associated protein light chain 3 (LC3) was significantly downregulated in the PrL of SNI rats on day 28 after SNI (0.86-fold of sham-control, Log$_2$FoldChange = -0.21, $p = 0.001$, Fig. 2A). Next, we carried out Western blot of PrL tissues on days 3, 7, 14, and 28 after modeling to quantify the following autophagy-related proteins: 1) LC3-II, an autophagosome marker, 2) SQSTM1/p62, an autophagy substrate marker and 3) LAMP1, a lysosome marker, to
assess whether autophagy processes were disrupted during the development of neuropathic pain. Western blot analysis revealed a significantly reduced level of autophagy in the PrL of SNI rats, indicated by a decrease in LC3-II, an increase in p62, and no downstream processes disrupted (no significant change in LAMP1) on day 28 but not earlier following SNI surgery (Fig. 2, B and C), which paralleled the occurrence of comorbid anxiety-like behaviors (Fig. 1, C and D).

Decreased LC3-II could be due to increased degradation or reduced formation. Chloroquine (CQ, an inhibitor of LC3-II degradation) blots were used to distinguish these possibilities [49, 50]. Inhibiting autophagic flux by blocking lysosomal acidification with CQ (100 μM, 4 h) significantly increased the level of LC3-II in both sham and SNI groups (Fig. 2, D and E). In contrast to CQ-treated sham group, CQ-treated SNI group displayed a reduced level of LC3-II, validating an autophagy impairment, resultant from reduced formation, in the PrL on day 28 after SNI (Fig. 2, D and E).

We further used double immunofluorescence staining with antibodies specific for autophagy proteins (LC3B) and neurons (NeuN) to assess the level of neuronal autophagy. Compared with that in the sham group, the number of LC3-II puncta in neuronal soma of SNI rats significantly decreased (Fig. 2, F and G), indicating impaired PrL neuronal autophagy in pain-anxiety comorbidity.

Together, these findings indicate that SNI induces long-lasting mechanical allodynia, and anxiety-like behaviors in the late stage which is accompanied by impaired neuronal autophagy in the PrL.

**Knockdown of autophagy related protein 7 (Atg7) in the PrL downregulates neuronal autophagy and induces early-onset anxiety-like behaviors in SNI rats**

The temporal concurrency of impaired autophagy and anxiety-like behaviors after SNI indicates possible causal links between these two processes. We hypothesized that artificial disruption of the autophagy process in PrL neurons, via neuron-specific downregulation of the critical autophagy-related gene Atg7 (Fig. 3A), would induce
early-onset pain-related anxiety behaviors after SNI. The expression of green fluorescent protein (GFP) was detected in PrL 3 weeks after AAV delivery (Fig. 3B). Western blot (Fig. 3C) and immunofluorescence staining of Atg7 and LC3 (Fig. 3D) confirmed reduced autophagy activity via adeno-associated viruses (AAV)-mediated knockdown of Atg7. Furthermore, GFP co-labeled with NeuN with high efficiency (Fig. 3, E and F). We found that downregulation of neuronal autophagy induced anxiety-like behaviors at a much earlier stage in rats with neuropathic pain (14 d after surgery) (Figs. 3, G-I), without affecting mechanical allodynia or baseline pain thresholds (Fig. 3J). Importantly, this anxiogenic effect was specific to rats with SNI, since rats with sham surgery did not develop anxiety-like behaviors after Atg7 knockdown.

These results suggest that impairment of neuronal autophagy in the PrL is sufficient to induce comorbid anxiety-like behaviors in neuropathic pain, but not general anxiety in normal rats.

**Overexpression of Atg7 in the PrL upregulates neuronal autophagy and attenuates comorbid anxiety-like behaviors in SNI rats**

To determine the necessity of autophagy impairment in comorbid anxiety and neuropathic pain, we next rescued neuronal autophagy deficits in the PrL via neuron-specific upregulation of Atg7 (Fig. 4, A-F). AAV-based rescue of neuronal autophagy impairment remarkably alleviated anxiety-like behaviors in rats with SNI (Fig. 4, G-I). Again, this anxiolytic effect was apparent only in SNI rats but not in normal rats. Intriguingly, mechanical allodynia was also relieved, but only during the specific phase characterized by anxiety-like behaviors (Fig. 4J). This was not surprising considering the substantial influence of negative affect on perceptual hypersensitivity under chronic pain conditions [51]. Together, these data demonstrate causal links between PrL neuronal autophagy dysfunction and comorbid anxiety in neuropathic pain.

**Upregulation of autophagy in comorbid anxiety-related PrL neurons exerts anxiolytic and analgesic effects**

The specific effect of autophagy manipulation on chronic pain-associated but not...
general anxiety raises the possibility that comorbid anxiety in chronic pain may be mediated by a specific neuronal ensemble in the PrL, which exhibits autophagy impairment. To test this hypothesis, we genetically labelled a subpopulation of c-Fos+ PrL neurons activated during the specific phase of neuropathic pain characterized by anxiety-like behaviors. This genetic system couples the promoter of c-fos, an immediate early gene frequently used as a biomarker of neuronal excitation, to the doxycycline (Dox) system for inducible gene expression [52, 53]. With this approach, the absence of Dox inhibited c-fos promoter-driven reverse tetracycline transactivator (rtTA) from binding to a tetracycline-responsive element (TRE) site, which subsequently prevented the expression of the artificially designed inhibitory Gi-coupled receptor hM4D(Gi) in labeled neurons. We found that chemogenetic inactivation of this neuronal subpopulation resulted in reduced anxiety-like behaviors in SNI rats, confirming its contributing role in chronic pain-induced anxiety (Fig. S1). We next performed double immunofluorescence staining with antibodies for LC3 and c-Fos to assess the level of autophagy in PrL c-Fos+ neurons. The staining revealed a significant decrease in the number of LC3-II puncta in c-Fos+ but not c-Fos− neurons (Fig. 5, A and B), indicating that PrL neuronal autophagy was selectively impaired in comorbid anxiety-related c-Fos+ neurons.

To rescue autophagy deficits in these c-Fos+ neurons, we injected AAV-c-Fos-rtTA-P2A-GFP with AAV-TRE3G-Atg7-P2A-mCherry in the bilateral PrL (Fig. 5C). In the presence of Dox, pain-comorbid anxiety elicited neuronal excitation selectively labeled c-Fos-expressing neurons with Atg7 and consequently enhanced autophagy activity. The expression of red fluorescent protein (RFP) was detected in PrL 3 weeks after AAV delivery (Fig. 5D). Immunofluorescence staining showed that most of the c-Fos+ neurons co-labeled with fluorescent proteins 3 weeks after SNI operation (Fig. 5, E and F), indicating that activity-dependent labeling with Atg7 recapitulated the induction of endogenous c-Fos in our system. Next, we tested the effect of subpopulation-specific upregulation of neuronal autophagy in the PrL on pain behaviors
(Fig. 5G). Upregulation of autophagy in PrL c-Fos$^+$ neurons exerted remarkable anxiolytic (Fig. 5, H and I) and antinociceptive effects (Fig. 5, J and K). Intriguingly, we also observed apparent relief of curling and lifting of hind paw ipsilateral to the operation, a common sign of rats with SNI indicative of pain (Fig. S2A).

We further investigated the properties of these neuronal ensembles by immunofluorescence staining. The labeled neuronal ensembles were mainly EAAC1$^+$ excitatory glutamatergic neurons (Fig. S2B). Consistent with previous results, specific upregulation of autophagy in excitatory glutamatergic neurons (Fig. S3), but bot in PV interneurons (Fig. S4), resulted in a relief of anxiety-like behaviors and mechanical allodynia in rats under chronic pain condition, indicating that excitatory glutamatergic neurons in the PrL participated in pain-related anxiety.

These findings suggest that neuronal autophagy impairment is not universal in the PrL, but mainly occurs in a specific c-Fos$^+$ pyramidal neurons crucial for comorbid anxiety in chronic neuropathic pain.

**Neuronal autophagy in the PrL modulates synaptic homeostasis**

Finally, we explored the molecular mechanisms underlying autophagy’s regulation on comorbid anxiety by combining fluorescence activated cell sorting (FACS) with mass spectrometry. Since sorting the specific c-Fos$^+$ neuronal subpopulation could not provide sufficient tissues for mass spectrometry analysis, AAV$_{9}$-CaMKII-Atg7-P2A-GFP and AAV$_{9}$-CaMKII-GFP transfected neurons in the PrL in SNI rats were sorted and sequenced by mass spectrometry to investigate the differentially expressed proteins (Fig. 6A). Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses were executed to reveal the functions and pathways of differentially expressed proteins. Compared with GFP-control group, we found 86 downregulated proteins in Atg7-upregulation group (Table S1), which were associated with glutamatergic synapses, GABAergic synapses, and chemical synaptic transmission (Fig. 6B and fig. S5). Among these, 17 downregulated proteins were involved in the component and function of synapse, including SH3 and multiple ankyrin
repeat domains 3 (Shank3), synaptosomal-associated protein 23 (Snap23), synaptotagmin-3 (Syt3) and Rab26 (Fig. 6C). We then performed immunoprecipitations of LC3 in PrL lysates and found that LC3 co-immunoprecipitated with synaptic scaffold protein Shank3 (Fig. 6D), indicating that Shank3 could be degraded by autophagy (autophagy substrates).

Since KEGG and GO analyses indicated the close relationship of these differentially expressed proteins with synaptic plasticity, we next examined the morphology of dendritic spines in the PrL with up- or down-regulation of general neuronal autophagy. We observed significantly decreased or increased densities of dendritic spines in the Atg7-upregulation (comparable density to the GFP group) or downregulation groups, respectively (Fig. 6, E and F). Furthermore, we assessed the postsynaptic density protein-95 (PSD-95), a marker for synaptic plasticity [54], to observe the molecular consequence of up- or down-regulation of neuronal autophagy in PrL. Consistent with the changes in dendritic spine density, upregulation of neuronal autophagy significantly decreased PSD-95 protein levels. Downregulation of neuronal autophagy had limited effects (Fig. 6, G and H), which might result from a floor effect, given the low level of autophagy at day 28 after SNI surgery.

These results indicate that PrL neuronal autophagy might, by turning over species in synaptic compartments such as scaffold protein Shank3, contribute to synaptic homeostasis and pain-related anxiety-like behaviors.

Discussion

**Comorbid anxiety and impaired neuronal autophagy in the PrL**

The biopsychosocial model of pain presents its physical symptoms as the denouement of a dynamic interaction between sensory, cognitive, affective, and social factors [55], all of which recruit higher brain structures including the mPFC [16]. Although pain and anxiety are widely known to interact with each other [56], only recent studies start to elucidate the pain-anxiety interaction at the network and synaptic
Structural and functional changes in the PrL have been reported in chronic pain [57, 58]. Concomitant imbalance of proteins and synaptic homeostasis may further implicate a disruption in neuronal autophagy and modulate chronic pain and associated anxiety [24–26]. In the present study, we identified disrupted neuronal autophagy in the PrL in a specific phase of neuropathic pain characterized by anxiety-like behaviors. This raises the possibility that PrL neuronal autophagy could be an important site for processing anxiety symptoms of pain. We assessed the anxiety-like behaviors with open field and elevated plus maze, two widely used paradigms for rodent anxiety-like behaviors [59]. Anxiety-like behaviors related to neuropathic pain in these tests develop approximately 3–4 weeks following induction of chronic pain [59], similar to our findings in the study.

Using AAV vectors driven by cell type–specific promoters, we observed that anxiety-like behaviors triggered by peripheral injury were induced at an earlier stage after the downregulation of neuronal autophagy. By contrast, elevating autophagy in PrL neurons produced anxiolytic effects. These effects were not observed in sham-operated animals but only in rats with nerve injury, suggesting that the anxiety-modulatory effect was not universal but specific to pain-associated comorbid anxiety. We also observed antinociceptive effects after upregulation of autophagy, which could result either directly from pain relief or indirectly from anxiolysis induced by autophagy restoration. We consider the latter mechanism more likely for two reasons. First, downregulation of neuronal autophagy did not change the paw withdrawal threshold of SNI rats. While this could result from a floor effect in the current paradigm, upregulation of autophagy did not attenuate mechanical allodynia until the phase characterized by anxiety, suggesting that the pain relief likely resulted indirectly from anxiolysis. Together, these data suggest that PrL neuronal autophagy deficits underlie comorbid anxiety in neuropathic pain.
Neuronal and molecular basis of autophagy impairment in neuropathic pain

The PrL is functionally heterogenous and encompasses multiple subpopulations recruited in different cognitive and behavioral processes. In the present study, we noticed that impaired neuronal autophagy was apparent mainly in comorbid anxiety-related neurons labelled by the cFos-rtTA-TRE system originally developed for memory research. A specific memory is thought to be encoded by a sparse population of neurons [53], and the same rationale could be applied to pain-anxiety comorbidity. We labelled a subpopulation of PrL neurons activated during the specific phase of neuropathic pain characterized by anxiety-like behaviors with hM4D(Gi) and demonstrated their role in comorbid anxiety with chemogenetic inactivation. Rescuing impaired neuronal autophagy in this sparse but specific ensemble of PrL neurons yields similar anxiolytic effects to manipulation on all or pyramidal neurons in the PrL.

Recent findings reinvigorate the question of how neuronal homeostasis is maintained by autophagy, leading to a notable conceptual shift in that selective degradation may be the dominant basal mode of autophagy, whereas bulk, non-selective degradation might be relegated to emergent needs [60]. Autophagy has been implicated in the regulation of synaptic morphology and function, by turning over species in both the presynaptic and postsynaptic compartments, from synaptic vesicle components to postsynaptic density components (for example, PSD-95, ARC, and glutamate and GABA receptors) in hippocampal and cortical neurons [61–66], thus contributes to specialized neuronal functions in a time- and space-dependent manner [64]. Consistent with these observations, GO and KEGG analyses reveal that upregulation of PrL neuronal autophagy acts an essential role in synaptic components and function, impaired neuronal autophagy might contribute to synaptic abnormalities during the development of neuropathic pain. Artificial upregulation of neuronal autophagy in SNI rats could promote degradation of synaptic proteins, relieve abnormal accumulation of damaged protein, restore the density of dendritic spines to normal level, thus maintain synaptic homeostasis in the direct or indirect pathway respectively and alleviate pain-
related behaviors.

Shank3 is a postsynaptic scaffold protein at glutamatergic synapses, that regulates synaptic development, function, and plasticity [67, 68]. Disruption of Shank3 in mouse model could result in synaptic defects and autistic-like behaviors (e.g., anxiety and social interaction deficits) [69]. Consistent with previous results showing the presence of Shank3 inside autophagosomes [61], we found that LC3 co-immunoprecipitated with Shank3 in PrL lysates, indicating that neuronal autophagy could regulate synaptic plasticity by autphagic degradation of postsynaptic scaffold proteins, consistent with the decreased density of dendritic spines in the Atg7-upregulation group. Although behavioral changes can be linked to synaptic changes, their effects can also be attributed to overall protein trafficking alterations in neurons, which may account for the breadth of features observed in GO analysis.

Relevance to potential treatments for pain concomitant mood disorders

Pain-induced anxiety is caused by multiple brain regions, such as the ACC [6, 8], amygdala [11, 12], and somatosensory cortex [13], which may undergo maladaptive neuroplastic changes under conditions of persistent pain, it is likely to be processed and encoded by different mechanisms at levels of neuronal network and molecular signaling in these regions. Thus, the role of autophagy in these areas may be implicated in the process of negative mood comorbidity by different mechanisms and merits further investigation, as well as its role in various types of neuronal cells given that microglia autophagy has been reported to be involved in the regulation of synapses and neurobehaviors [70, 71].

However, the mPFC may be an ideal target for therapy, given its easily accessible localization for electrical and magnetic modulation, abundant anatomical interconnection with other pain-modulatory areas [38, 72, 73], and particularly its importance in the prediction, modulation and amplification of chronic pain [74]. Insights into the imbalance of neuronal homeostasis induced by peripheral injuries are
prerequisites to understanding the neural basis of chronic pain and anxiety comorbidity in animal models and humans. Knowledge of the molecular mechanisms of neuronal autophagy in the mPFC may provide potential new targets for the treatment of pain and associated mood disorders, such as fear and anxiety.

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**Figure 1**

**A**
Observations of immunofluorescence staining for DAPI, c-Fos, LC3, and Merge in neuronal cells.

**B**
Graph showing the number of LC3-II puncta per μm² in the neuronal soma. 

**C**
Diagram illustrating the TRE^3G_4 Atg7 P2A mCherry construct.

**D**
Image of DAPI staining in different conditions.

**E**
Images of AAV^c-fos-rTA-P2A-GFP and AAV^TRE^3^G_4-Atg7 P2A-mCherry

**F**
Graph showing the percentage of mCherry positive cells.

**G**
Diagram showing the timeline for AAV administration and behavioral testing.

**H**
Graphs depicting open field test, C. Time (%), C. Dis (%), T. Dis (x10^6 cm²), and T. Entries.

**I**
Graphs showing elevated plus maze test, O. Time (%), O. Entries (%), and T. Entries.

**J**
Graph showing the 50% PWT (g) over time.

**K**
Graph showing the 50% PWT (g) before and after Dox treatment.

Legends:
- AAV^c-fos-rTA-P2A-GFP + AAV^TRE^3^G_4-Atg7 P2A-mCherry, Sham
- AAV^c-fos-rTA-P2A-GFP + AAV^TRE^3^G_4-Atg7 P2A-mCherry, SNI
- AAV^c-fos-rTA-P2A-GFP + AAV^TRE^3^G_4-Atg7 P2A-mCherry, Sham
- AAV^c-fos-rTA-P2A-GFP + AAV^TRE^3^G_4-Atg7 P2A-mCherry, SNI