Cytohesin-2 mediates group I metabotropic glutamate receptor-dependent mechanical allodynia through the activation of ADP ribosylation factor 6 in the spinal cord

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ARTICLE INFO

Keywords:
- Chronic pain
- Dorsal horn
- mGluR5
- Arf6
- Small GTPase
- Membrane trafficking

ABSTRACT

Group I metabotropic glutamate receptors (mGluRs), mGluR1 and mGluR5, in the spinal cord are implicated in nociceptive transmission and plasticity through G protein-mediated second messenger cascades leading to the activation of various protein kinases such as extracellular signal-regulated kinase (ERK). In this study, we demonstrated that cytohesin-2, a guanine nucleotide exchange factor for ADP ribosylation factors (Arfs), is abundantly expressed in subsets of excitatory interneurons and projection neurons in the superficial dorsal horn. Cytohesin-2 is enriched in the perisynapse on the postsynaptic membrane of dorsal horn neurons and forms a protein complex with mGluR5 in the spinal cord. Central nervous system-specific cytohesin-2 conditional knockout mice exhibited reduced mechanical allodynia in inflammatory and neuropathic pain models. Pharmacological blockade of cytohesin catalytic activity with SecinH3 similarly reduced mechanical allodynia and inhibited the spinal activation of Arf6, but not Arf1, in both pain models. Furthermore, cytohesin-2 conditional knockout mice exhibited reduced mechanical allodynia and ERK1/2 activation following the pharmacological activation of spinal mGluR1/5 with 3,5-dihydroxyphenylglycine (DHPG). The present study suggests that cytohesin-2 is functionally associated with mGluR5 during the development of mechanical allodynia through the activation of Arf6 in spinal dorsal horn neurons.

1. Introduction

The spinal cord dorsal horn is the first relay center where dorsal horn neurons form excitatory synapses with axon terminals of primary sensory neurons carrying nociceptive information from peripheral tissues. Additionally, it is also the principle integration center where nociceptive information is actively processed and modulated through elaborate neural networks, depending on the strength and duration of nociceptive stimuli (Peirs and Seal, 2016; Todd, 2010). Intense and persistent noxious stimuli caused by inflammation and nerve injury induce plastic changes in neurons and neural circuitry in the dorsal horn, leading to increased excitability of nociceptive transmission, a condition referred to as central sensitization (Ilaism et al., 2009; Latremoliere and Woolf, 2009; Woolf, 2011). It is clinically related to chronic pain with manifestations, such as spontaneous pain, augmented pain responses to noxious stimuli (hyperalgesia), and pain generated by low-threshold innocuous stimuli (allodynia).

In the spinal cord, glutamate mediates excitatory neurotransmission, synaptic plasticity, and central sensitization through two classes of receptors: ionotropic and metabotropic glutamate receptors (Bleakman et al., 2006; Liu and Salter, 2010). Ionotropic glutamate receptors mediate fast excitatory synaptic transmission, and are composed of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-type receptors (AMPARs), kainate-type receptors, and N-methyl-D-aspartate-type receptors (NMDARs). Metabotropic glutamate receptors (mGluRs) mediate slower excitatory synaptic transmission and are composed of group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and...
Among these, group I mGluRs are coupled to the Gq family heterotrimeric G protein, which stimulates phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, thereby leading to intracellular calcium mobilization and protein kinase C (PKC) activation. They are abundantly expressed in the spinal cord, with mGluR5 predominantly expressed in the superficial dorsal horn (laminae I and II) and mGluR1α in the deeper dorsal horn (laminae III – V) and the ventral horn (laminae VI – IX) (Alvarez et al., 2000). In dorsal horn neurons, mGluR5 is enriched preferentially in the marginal region of the postsynaptic density (PSD) of excitatory synapses, and are ideally positioned to respond only to persistent intense stimulation (Vidyunmazsky et al., 1994). Since it was first reported that intrathecal administration of a selective group I mGluR agonist, 3,5-dihydroxyphenylglycine (DHPG), induces spontaneous nociceptive behaviors (Fisher and Coderre, 1996), evidence has been first reported that intrathecal administration of a selective group I mGluR agonist, 3,5-dihydroxyphenylglycine (DHPG), induces spontaneous nociceptive behaviors (Fisher and Coderre, 1996), evidence has accumulated that group I mGluRs play a pivotal role in central sensitization in various types of inflammatory and neuropathic pain models by modulating ion channels such as NMDARs and K⁺ channels, and inducing gene transcription through multiple signaling pathways, including PKC, Src, and extracellular signal-regulated kinase 1/2 (ERK1/2) (Chicchio and Nicoletti, 2012; Guo et al., 2004; Hu et al., 2007; Karim et al., 2001; Kawasaki et al., 2004; Pereira and Goudet, 2019; Vincent et al., 2016; Vincent et al., 2017).

ADP ribosylation factors (ARFs) are small GTP-binding proteins that mediate membrane trafficking, actin cytoskeleton remodeling, and lipid metabolism/signaling (D’Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011; Gillingham and Munro, 2007). There are six mammalian ARF proteins, which are divided into three classes, based on their structural similarity: class I (Arf1, Arf2, and Arf3), class II (Arf4 and Arf5), and class III (Arf6). Both class I and II ARFs are localized primarily in the Golgi complex and have partially redundant functions in membrane trafficking within the Golgi complex. On the other hand, Arf6 is localized to the plasma membrane and endosomes, and regulates vesicular transport between the plasma membrane and endosomes, as well as the reorganization of the actin cytoskeleton beneath the plasma membrane. Like other small GTP-binding proteins, ARfs function as binary molecular switches by cycling between GTP-bound active and GDP-bound inactive states. The proper timing, duration and location of the activation of ARFs are tightly controlled by two regulatory proteins: guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP, and GTPase-activating proteins that stimulate GTP hydrolysis (Donaldson and Jackson, 2011; Gillingham and Munro, 2007; Sztul et al., 2019). All mammalian ARF-GEFs share a conserved catalytic domain of approximately 200 amino acids, known as the Sec7 domain, and are classified into seven subfamilies, i.e., the GBF1 (Golgi-specific brefeldin A-resistance factor 1), BIG (brefeldin A-inhibited ARFGEF), cytohesin, EFA6 (exchange factor for Arf6)/Psd (PH and Sec7 domain-containing protein), BRAG (brefeldin A-resistant ARF-GEF)/IQSEC (IQ and Sec7 domain-containing), and FBXO8 (F-box only protein 8) subfamilies, based on their structural similarity and sensitivity to brefeldin A, a fungal metabolite secreted by Eupenicillium brefeldihamus (Casanova, 2007; Cox et al., 2004; Gillingham and Munro, 2007).

Cytohesin-2, also known as ARNO (Arf nucleotide-binding site opener), is a member of the cytohesin Arf-GEF subfamily with guanine nucleotide exchange activity toward Arf1 and Arf6 in vitro (Chardin et al., 1996; Frank et al., 1998a; Kolanus, 2007). In the central nervous system (CNS), cytohesin-2 is abundantly expressed in developing and mature neurons (Ito et al., 2018), and is implicated in Arf6-dependent neuronal processes, including the formation of axons and dendrites of developing hippocampal neurons (Hernandez-Dievie et al., 2002; Hernandez-Devie et al., 2004), and the pathfinding of commissural axons in the developing spinal cord during midline crossing (Kinosita-Kawada et al., 2019). Cytohesin-2 has previously been shown to form a protein complex with group I mGluRs in the rat brain through the interaction with tamalin, also known as GRASP (GRP1-associated scaffold protein), a PDZ (postsynaptic density protein-95/discs large/zonula occludens-1) domain-containing scaffolding protein enriched in the PSD fraction (Kitano et al., 2002), suggesting the possible functional relationship between cytohesin-2 and mGluR1/5. Although a recent discovery of SecinH3 (Hafner et al., 2006), a cell-permeable selective inhibitor of the cytohesin family, has revealed the functional involvement of cytohesins in various pathological processes related to neurological disorders, such as amyotrophic lateral sclerosis (Hu et al., 2019; Zhai et al., 2015) and Alzheimer’s disease (Yan et al., 2016), it remains unknown whether cytohesin-2 has a functional involvement in chronic pain.

In this study, we investigated the functional role of cytohesin-2 in the spinal cord, with special attention to its relationship with mGluR5. We first demonstrated that cytohesin-2 is expressed in subsets of excitatory interneurons and projection neurons in the dorsal horn, and forms a protein complex with mGluR5 in the spinal cord. We then demonstrated that CNS-specific cytohesin-2 conditional knockout (cKO) mice reduced mechanical allodynia in inflammatory and neuropathic pain models of mice. Pharmacological blockade of cytohesin catalytic activity with SecinH3 in the spinal cord reduced mechanical allodynia and activation of Arf6, but not Arf1, in both pain models. Furthermore, cytohesin-2 cKO mice exhibited marked reduction in mechanical allodynia and ERK1/2 activation after stimulation of spinal mGluR1/5 with DHPG. The present findings provide the first evidence for the involvement of the cytohesin-2-Arf6 pathway in inflammatory and neuropathic pain in spinal dorsal horn neurons.

2. Materials and methods

2.1. Animals

All experimental protocols involving animals were approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine. All efforts were made to reduce the number of animals used. To generate the CNS-specific cytohesin-2 cKO mice, we prepared the floxed cytohesin-2 mouse line carrying two loxP sites flanking the exons 6 and 7 (ICYTH2) (Torii et al., 2015) and the transgenic mouse line carrying Cre recombinase under the control of the nestin promoter (nestin-Cre, Stock No. 003771, Jackson Laboratory, Bar Harbor, ME). The founder mice (Cre/–/ICYTH2/ICYTH2) were crossed to homozygous ICYTH2 mice (–/–, ICYTH2/ICYTH2) to produce cKO (Cre/–/ICYTH2/ICYTH2) and control (–/–, ICYTH2/ICYTH2) littermates. For evaluation of the floxed cytohesin-2 allele, the PCR primers used were 5’-TCAGGAAATGTCTCTCAAATAAGA-3’ (sense) and 5’-AATCTCTGGTCAATCGGTATC-3’ (antisense). Floxed and wild-type cytohesin-2 alleles displayed ~390 bps and ~240 bps, respectively. The PCR primers used to identify the cre transgene were 5’-TTGGGTCATTACCGCGTGCATGAC-3’ (sense) and 5’-GCCGCGATT-GATAGCTGCGGTG-3’ (antisense). The PCR product was ~750 bps. Ninety male wild-type C57BL/6 mice (CLEA Japan) in the 8th to 12th postnatal weeks were used for pain models, eight for immunofluorescent staining, six for immunoelectron microscopic analysis, and six for immunoprecipitation assay. For analyses using cytohesin-2 cKO and control mice, 40 male mice of each genotype were used for pain models, 15 for DHPG models, 6 for immunoblots, and 4 for immunoperoxidase analyses. Eight male nestin-Cre mice were included for pain models to exclude the possibility about differences in nociceptive behaviors between wild-type and nestin-Cre mice (Supplementary Fig. S2). Mice (5 per cage) were housed and maintained in the air-conditioned room (about 20–24 °C) with a 12 h light-dark cycle with free access to food and water. Prior to the experiments, mice were anesthetized with sodium pentobarbital (50 mg/kg of body weight, i.p.) or inhalation of 2.5% isoflurane and assessed the anesthesia level by pedal reflex in order to minimize animals’ suffering. Mice were killed by decapitation under deep anesthesia with sodium pentobarbital (100 mg/kg of body weight, i.p.) or the inhalation anesthesia with 4–5% isoflurane for the sampling of fresh brains and spinal cords.
### Table 1
Antibodies used in the present study.

| Antibody | Immunogen | Species and dilution | Source |
|----------|-----------|----------------------|--------|
| Cytosolin-2 (N7) | Recombinant protein corresponding to the N-terminal 70 amino acids (1-70) of mouse cytosolin-2 | rabbit polyclonal, 1.0 μg/mL (IB, IHC, IF, IEM) | This study |
| Cytosolin-2 (C2) | Recombinant protein corresponding to the C-terminal amino acids (387-400) of mouse cytosolin-2 | guinea pig polyclonal, 1.0 μg/mL (IB) | Ito et al., 2018 |
| Arf6 | Synthetic peptide corresponding to the 10 amino acids (166-175) of mouse Arf6 | guinea pig and rabbit polyclonal, 1.0 μg/mL (IB, IF) | Hara et al., 2016 |
| Arf1 | Recombinant protein corresponding to the 162 amino acids (1-162) of human Arf1 | rabbit polyclonal, 1.0 μg/mL (IB) | GeneTex |
| Calretinin | Recombinant protein corresponding to the 271 amino acids (1-271) of human calretinin | mouse monoclonal, 0.5 μg/mL (IF) | Proteintech, 66496-1-lg |
| Substance P | C-terminal region of substance P | rat monoclonal, 0.5 μg/mL (IF) | Santa Cruz, sc-21715, NCI/34HL |
| NeuN | Purified neuronal cell nuclei from mouse brain | mouse monoclonal, 0.5 μg/mL (IF) | Millipore |
| NK1R | Recombinant protein corresponding to the amino acids (325-407) of human NK1R | mouse monoclonal, 0.5 μg/mL (IF) | Santa Cruz, sc-365091, D-11 |
| Calbindin | Recombinant protein corresponding to the full-length of mouse calbindin | goat polyclonal, 0.5 μg/mL (IF) | Frontier institute, Calbindin-Go-Af1040 |
| PKCy | Recombinant protein corresponding to the 14 amino acids (684-697) of mouse PKCy | goat polyclonal, 0.5 μg/mL (IF) | Frontier institute, PKCy-Go-Af840 |
| Pax2 | Recombinant protein corresponding to the 135 amino acids (229-363) of human Pax2 | goat polyclonal, 0.5 μg/mL (IF) | R&D systems, AF3364 |
| Homer1b/c | Recombinant protein corresponding to the C-terminal 215 amino acids of mouse homer1c | guinea pig polyclonal, 0.5 μg/mL (IB, IF) | Frontier institute, Homer1-Rb-Af1000 |
| Homer1 | Recombinant protein corresponding to the N-terminal 175 amino acids of mouse homer1 | rabbit polyclonal, 0.5 μg/mL (IF) | Frontier institute, Homer1-Rb-Af4800 |
| VGluT2 | Recombinant protein corresponding to the C-terminal 23 amino acids (559-582) of mouse VGluT2 | goat polyclonal, 0.5 μg/mL (IF) | Frontier institute, VGluT2-Go-Af1040 |
| EEA1 | Recombinant protein corresponding to the amino acids (3-281) of human EEA1 | mouse monoclonal, 0.5 μg/mL (IF) | BD Biosciences, 610457 |
| Syntaxin13 | Recombinant protein corresponding to the 243 amino acids (8-250) of mouse syntaxin13 | guinea pig and rabbit polyclonal, 0.5 μg/mL (IF) | Hara et al., 2013 |
| LAMP2 | Synthetic peptide corresponding to the human LAMP2 | goat polyclonal, 0.5 μg/mL (IF) | Santa Cruz, sc-365091, D-11 |
| mGluR5 | Recombinant protein corresponding to the 28 amino acids (1144-1171) of mouse mGluR5 | rabbit and goat polyclonal, 1.0 μg/mL (IB, IF, IEM) | Frontier institute, mGluR5-Rb-Af300 |
| Gaq/11/14 | Recombinant protein corresponding to the C-terminal 300 amino acids (60-359) of human Gq11/14 | mouse monoclonal, 0.5 μg/mL (IF, IEM) | Santa Cruz, sc-365906, G-7 |
| Olig2 | Recombinant protein corresponding to the N-terminal 323 amino acids (1-323) of human Olig2 | goat polyclonal, 0.5 μg/mL (IF) | R&D systems, AF2418 |
| GFAP | Recombinant protein corresponding to the 65 amino acids (335-400) of mouse GFAP | goat polyclonal, 0.5 μg/mL (IF) | Frontier institute, GFAP-Go-Af1000 |
| Iba1 | Synthetic peptide corresponding to the C-terminal 13 amino acids (135-147) of human Iba1 | goat polyclonal, 1.0 μg/mL (IF) | Abcam, Ab5076 |
| MBP | Full-length of human MBP | rabbit polyclonal, 0.5 μg/mL (IHC) | Millipore |
| ERK1/2 | Synthetic peptide corresponding to the C-terminal region of human ERK1/2 | rabbit monoclonal, 1:500 (IB) | Cell Signaling Technology, #4695 |
| Phospho-ERK1/2 | Synthetic phosphopeptide corresponding to residues around pThr202/pTyr204 of human ERK1/2 | rabbit monoclonal, 1:500 (IB) | Sigma-Aldrich, T9026 #DM1A |
| α-tubulin | Recombinant protein corresponding to the C-terminal 5 amino acids (426-430) of α-tubulin | mouse monoclonal, 0.02 μg/mL (IB) | Sigma-Aldrich, #A9285 |
| HA | Synthetic peptide: YPYDVPDYA (HA-tag) | rabbit polyclonal, 0.2 μg/mL (IB) | Millipore, MBL 561-5 |

IB, immunoblotting; IEM, immunoelectron microscopy; IF, immunofluorescence; IHC, immunohistochemistry.
2.2. Antibodies and antigens

The antibodies used in the present study are summarized in Table 1. To prepare antibodies to produce antibodies against cytohesin-2 (N7) and Homer1b/c, the N-terminal region (amino acids 1–70) of mouse cytohesin-2 and C-terminal 215-amino acids (152–366) of mouse Homer1c, a region shared by Homer1b, were amplified by PCR using a mouse brain first-strand cDNA library and the following PCR primers, in which a Sall restriction enzyme site (underlined) and stop codon (small letters) were added to the 5′ end of forward and reverse primers, respectively: 5′-GTCGACCATGAGACGGTTAGCTACGACC-3′ and 5′-taCTTTCCGECATTGCTACCTCGC-3′ for cytohesin-2 (1–70); 5′-GTCGACGGCAATGTCAGGTTGAAGC-3′ and 5′-taCTTGTCATTCCAGATGCCTGGC-3′ for Homer1c (152–366). To prepare antigens for pre-absorption test in the post-embedding immunoelectron microscopy analysis, we also amplified cDNA fragments encoding C-terminal 28-amino acids of mouse mGlur5 and C-terminal 300-amino acids of human Gria1 from mouse brain and HeLa cell first-strand cDNA libraries, respectively, by PCR with the following primers: 5′-GTCGACCTCGAATGTCGCCGCTCAG-3′ and 5′-taCAAGGATGAAGCATC-3′ for mGlur5 (1144–1171); 5′-GTCGACGGCGCATCATCA-3′ and 5′-taGACGAGGTTGACTC-3′ for Gia1 (60–359). PCR products were subcloned into a pGEM-Teasy vector (Promega, Madison, WI). The inserts were then digested with SalI and NotI restriction enzymes and subcloned into the SalI and NotI sites of pGEX-4T-2 (Promega) and a modified pMAL-2c vector (New England BioLabs, Beverly, MA) that carries SalI and NotI restriction enzyme sites in the same reading frame as pGEX-4T-2. The antigens were bacterially expressed as fusion proteins of glutathione S-transferase (GST) or maltose-binding protein (MBP) in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside at 20 °C overnight. GST- and MBP-fusion proteins were purified with glutathione-Sepharose 4B (GE Healthcare, Piscataway, NJ) and amylose-resin (New England Biolabs), respectively. The GST-cytohesin-2 (1–70) and GST-Homer1c (152–366) fusion proteins were emulsified with Freund’s adjuvant and injected subcutaneously into guinea pigs or rabbits five times at 2-week intervals, and specific antibodies were purified from antisera by respective MBP-fusion proteins coupled to CNBr-activated Sepharose 4B beads (GE Healthcare). The specificity of anti-cytohesin-2 (N7) antibody was characterized by immunoblotting, in which a single band of 45-kDa detected by this antibody in lysates of the spinal cord and brain from control mice was markedly attenuated in those from cytohesin-2 cKO mice, consistent with the results obtained with the previously characterized anti-cytohesin-2 antibody (C2) raised against the C-terminal region (Ito et al., 2018) (Fig. 4A). The novel anti-Homer1b/c antibody detected a single 43-kDa immunoreactive band in the lysates of the mouse brain and HeLa cells transfected with pCMV-HA vector carrying Homer1c, but not in the naïve HeLa cell lysate (Supplementary Fig. S1A). Furthermore, double immunofluorescence revealed that it produced almost the same labeling pattern as the previously characterized anti-Homer1 antibody (Gutierrez-Mecinas et al., 2016) (Supplementary Fig. S1B, C).

2.3. Immunostaining

Under deep anesthesia, mice were transcardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) and 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M PB for immunohistochemistry at light and electron microscopic levels, respectively. Brains, lumbar spinal cords, and dorsal root ganglia were post-fixed with the same fixative for 3 h. For immunofluorescence, floating sections were made at a thickness of 50 μm on a vibrating microtome (VT1000, Leica, Nussloch, Germany). Sections were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 30 min, incubated with 5% donkey serum in PBS for 30 min, and with an anti-cytohesin-2 antibody (N7, 1.0 μg/mL, rabbit) overnight. For triple immunofluorescence staining, floating sections were incubated with anti-cytohesin-2 (N7, 1.0 μg/mL, rabbit) and anti-substance P (0.5 μg/mL, rat, Santa Cruz, Dallas, TX), and anti-calretinin (0.5 μg/mL, mouse, Proteintech, Rosemont, IL) antibodies. For double immunofluorescence staining, sections were incubated with the following combinations of primary antibodies: anti-cytohesin-2 (N7, 1.0 μg/mL, rabbit) and anti-NeuN (0.5 μg/mL, mouse, Millipore, Burlington, MA), anti-neurokinin 1 receptor (NKIR) (0.5 μg/mL, mouse, Santa Cruz), anti-calbindin (0.5 μg/mL, goat, Frontier institute, Ishikari, Japan), anti-calretinin (0.5 μg/mL, mouse, Proteintech), anti-PKCγ (0.5 μg/mL, goat, Frontier institute), anti-Pax2 (0.5 μg/mL, goat, R&D systems, Minneapolis, MN), anti-Homer1b/c (0.5 μg/mL, guinea pig), anti-Homer1 (0.5 μg/mL, rabbit, Frontier institute), anti-vesicular glutamate transporter 2 (VGlut2) (0.5 μg/mL, goat, Frontier institute), anti-early endosomal antigen 1 (EEA1) (0.5 μg/mL, mouse, BD Biosciences, Franklin Lakes, NJ), anti-syntaxin 13 (0.5 μg/mL, guinea pig) (syntaxin 12, Hara et al., 2013), anti-lysosome-associated membrane glycoprotein 2 (LAMP2) (0.5 μg/mL, goat, Santa Cruz), anti-Arfa (1.0 μg/mL, guinea pig) (Hara et al., 2016), anti-Mangs (1.0 μg/mL, goat, Frontier institute), or anti-Gia1/11/14 (0.5 μg/mL, mouse, Santa Cruz). The immunoreaction was visualized using species-specific secondary antibodies conjugated with Alexa488, Alexa594, or Alexa647 (0.5 μg/mL for each; A-21206, A-21203, A-21247, Invitrogen, Waltham, MA; 705-585-147, 706-585-148, Jackson ImmunoResearch, West Grove, PA, USA). Sections were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, 10236276001, Roche, Mannheim, Germany) and examined using a confocal laser scanning microscope (LSM 710, Carl Zeiss, Oberkochen, Germany). For immunoperoxidase staining, brains and spinal cords were embedded in paraffin and cut at a thickness of 5 μm using a sliding microtome (REMT-710, Yamato, Asaka, Japan). Dorsal root ganglia immersed in 30% sucrose in 0.1 M PB were cut at a thickness of 20 μm with a cryostat (CM 3050 s, Leica). Sections were treated with 5% donkey serum in PBS, followed by incubation with the anti-cytohesin-2 antibody (1.0 μg/mL, rabbit) or myelin basic protein (MBP) (0.5 μg/mL, rabbit, Millipore) overnight, and with peroxidase-conjugated secondary antibody (Histofine, MAX-PO(R), Nichirei, Tokyo, Japan) for 2 h. The immunoreaction was visualized in substrate solution containing 3,3′-diaminobenzidine and hydrogen peroxide (K3468, DAKO, Carpinteria, CA). For pre-embedding immunoelectron microscopy, free-floating sections were incubated with rabbit anti-cytohesin-2 antibody (N7, 1.0 μg/mL, rabbit) overnight and subsequently with nanogold-conjugated anti-rabbit IgG (1:100, Nanoprobe, Yaphank, NY) at room temperature for 2 h. Immunogold particles were intensified using a HQ Silver Enhancement kit (Nanoprobe). The sections were post-fixed with 2% osmium tetoxide, dehydrated, and embedded in epoxy resin. Ultrathin sections were made at a thickness of 70 nm on an ultra-microtome (Ultracut, Leica), contrasted with 2% uranyl acetate, and examined using an electron microscope (H-7650, Hitachi, Tokyo, Japan).

Post-embedding immunogold electron microscopy was performed as described previously (Fukaya et al., 2003) with minor modifications. Microtublar sections of the spinal cord (400 μm in thickness) were cryoprotected with 30% sucrose in 0.1 M PB and frozen rapidly with liquid propane in a Leica EM CPC unit. Frozen sections were immersed in 0.5% uranyl acetate in methanol at −90 °C in a Leica AFS freeze-substitution unit, infiltrated at −45 °C with Lowicryl HM-20 resin (Lowi, Waldkirch, Germany), and polymerized with UV light. Ultrathin sections on nickel grids were treated successively with 1% human serum albumin (Wako, Osaka, Japan)/0.1% Tween 20 in Tris-buffered saline (TBS, pH 7.5) for 1 h, primary antibodies (5 μg/mL for each) in HTBST overnight, and 10-nm colloidal gold-conjugated antibodies (1:100, British Bio Cell International, Cardiff, UK) in HTBST for 2 h. Finally, sections were stained with 2% uranyl acetate solution for 15 min and 1% lead citrate solution for 1 min, and examined using an electron microscope (H-7650, Hitachi). For quantitative analysis, the lateral distribution of cytohesin-2, mGlur5, and Gqα11/14 at axo-dendritic asymmetric synapses in synaptic glomeruli of laminae I–II of the spinal
dorsal horn were evaluated by measurement of the length from the center of gold particles to the edge of PSD on electron microscopic images using Image J software (NIH). The quantitative data of the post-embedding immunogold analysis were obtained from more than 180 synapses from 3 mice for each genotype. For antigen pre-absorption test, ultrathin sections were treated with anti-mGluR5 and anti-Goα/11/14 antibodies (5 μg/mL) in HTBST supplemented with the GST-fusion proteins of mGluR5 (1144-1171) and Ga (60-359) (20 μg/mL), respectively (Supplementary Fig. S3).

2.4. Immunoprecipitation

Immunoprecipitation was performed as described previously (Fukaya et al., 2020). The dorsal half of the mouse lumbar spinal cord was homogenized in a buffer consisting of 10 mM Tris-HCl (pH 7.4), 320 mM sucrose, 10 mM EDTA, 10 mM EGTA, and a cocktail of protease inhibitors (11,697,498,001, Roche). After the nuclear and mitochondrial fractions were removed, the supernatants were incubated with rabbit anti-cytohesin-2 (N7) IgG- or rabbit anti-mGluR5 IgG-conjugated magnetic beads (Dynabeads, DB10004, Thermo Fisher, Waltham, MA). The immunoprecipitates were washed extensively with the binding buffer. The immunoprecipitates and lysates were subjected to immunoblotting with antibodies against cytohesin-2 (C2, guinea pig) or mGluR5 (goat).

2.5. Immunoblotting

The lumbar spinal cord of mice was homogenized in a buffer consisting of 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.0), 0.4 mM phenylmethylsulfonyl fluoride, and a cocktail of protease inhibitors (11,697,498,001, Roche). After the nuclear and mitochondrial fractions were removed, the samples (P2 fraction, 200 μg) were lysed with a binding buffer consisting of 50 mM Tris-HCl (pH 7.4) and 1% Triton X-100 for 1 h at 4 °C, and immunoprecipitated with rabbit anti-cytohesin-2 (N7) IgG- or rabbit anti-mGluR5 IgG-conjugated magnetic beads (Dynabeads, DB10004, Thermo Fisher, Waltham, MA). The immunoprecipitates were washed extensively with the binding buffer. The immunoprecipitates and lysates were subjected to immunoblotting with antibodies against cytohesin-2 (C2, guinea pig) or mGluR5 (goat).

2.6. Arf activation assay

Arf activation assay was performed as described previously (Nakayama and Takatsu, 2005). Under deep anesthesia, the freshly dissected dorsal half of the lumbar spinal cord for each condition was homogenized with the lysis buffer containing 25 mM HEPES, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, and 2% glycerol. After centrifugation for 5 min at 1000 g, the supernatants were incubated with GST-Golgi-localized, γ-adaptin ear-containing, Arf-binding protein 1 (GGA1) fusion protein immobilized on glutathione-Sepharose 4B (GE Healthcare) for 1 h at 4 °C to bind active GTP-bound forms of Arf1 and Arf6. The beads were washed 4 times with the lysis buffer. The beads were lysed with SDS-PAGE sampling buffer containing 0.1 M DTT, and incubated for 15 min at 65 °C. The samples were electrophoretically separated on a 12.5% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween 20, membranes were incubated with anti-Arf1 IgG (1.0 μg/mL, rabbit, GeneTex) or anti-Arf6 IgG (1.0 μg/mL, rabbit) overnight and then with a horseradish peroxidase-linked secondary antibody (1: 10,000, Thermo Fisher) for 2 h. Immunoreactive bands were visualized using a chemiluminescent detection kit (ECL-PLUS) and an image analyzer (ImageQuant LAS4000, GE Healthcare). ImageJ (NIH) was used to measure the densities of immunoreactive bands. Each immunoreactive intensity of GFP-Arf1 and GFP-Arf6 was normalized by that of total Arf1 and Arf6, respectively.

2.7. Pain models

For an inflammatory pain model, inflammation was induced by a subcutaneous injection of 10 μL of complete Freund’s adjuvant (CFA; Sigma-Aldrich) into the right hind paw under anesthesia with isoflurane (2–3%). For the control, mice were injected with sterile saline. For a neuropathic pain model, chronic constriction nerve injury (CCI) model was performed as described previously (Bennett and Xie, 1988; Seltzer et al., 1990). Briefly, under anesthesia with isoflurane, mice had skin incision just below the right hip bone, parallel to the sciatic nerve. The right sciatic nerve was exposed by separating the fasciae of biceps femoris and glutaeus maximus muscles. The injury was performed by the three loose ligations of one-half of sciatic nerve. The nerve was tied loosely with 6–0 silk at 1-mm spacing, until a brief twitch in the respective hind limb was observed.

2.8. Intrathecal administration

Mice were anesthetized with inhalation of 2–3% isoflurane and the skin of the lower back was incised to visualize the lumbar region. Intrathecal injection of the cytosol inhibitor SecinH3 (2849, Tocris Bioscience, Minneapolis, MN) or group 1 mGluR agonist DHPG was performed by puncture between L5 and L6 vertebrae, which was confirmed to be successful by the observation that it evoked a tail-flick response. Control mice were injected with 10 μL of the artificial cerebrospinal fluid (ACSF). After the administration of SecinH3, mice were subjected to inflammatory or CCI pain models.

2.9. Behavioral analysis

Mechanical pain sensitivity was assessed by measuring the withdrawal threshold of the paw in response to mechanical stimuli using von Frey filaments (TACTILE TEST AESTHESIO, Muromachi Kikai, Tokyo). Mice were placed in a plastic cage with a mesh floor, and allowed to habituate over 20 min. The filaments were applied perpendicularly to the midplantar surface of the hind paw for 5 s. The response to the filament was considered to be positive when mice immediately withdrew or licked the hind paw. Each filament was applied 10 times at an interval of 30 s. Measurement was started from the smallest filament in ascending order, and the smallest filament eliciting response over six times was considered as the threshold stimulus. The strength of the von Frey stimuli ranged from 0.008 to 2.0 g. To evaluate the motor performance, a simple accelerating rotarod test was applied using a rotarod apparatus (O-hara, Tokyo, Japan) programmed to accelerate from 4 rpm to 40 rpm over 2 min and then remain constant speed at 40 rpm for 1 min. Three male mice of each genotype were placed on the rod rotating at 4 rpm and underwent three trials with a 30-min interval. The latency until mice fell off the rod was recorded.
2.10. Colocalization assay

To evaluate the colocalization between cytohesin-2 and synaptic or endosomal markers in double immunofluorescence, the colocalization coefficient, which represents the proportion of pixels colabeled with two fluorophores in all pixels labeled with a fluorophore, was measured using ZEN software (Manders split coefficient, Carl Zeiss). Colocalization coefficient data were obtained from more than 1000 μm² of neuropil region for each combination.

2.11. Statistical analysis

For quantitative comparison, Student’s t-test was used for comparisons between the two evaluations within each group. Statistical significance was set at p < 0.05. For multiple comparisons, the one-factor analysis of variance (ANOVA) with Tukey-Kramer post hoc test and two-factor repeated measures ANOVA with post hoc threshold analyses, respectively. Data are presented as mean ± SD or SEM. Statistical analyses were performed using the StatView software 5.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Perisynaptic and endolysosomal localization of cytohesin-2 in dorsal horn neurons

To examine the expression of cytohesin-2 at the protein level in the spinal cord, we first performed immunohistochemical analyses using a specific antibody against cytohesin-2 (N7) raised in this study. In the lumbar spinal cord of wild-type adult male mice, the immunofluorescence for cytohesin-2 was distributed throughout the gray matter, especially enriched in the dorsal horn (Fig. 1A), which was completely abolished in cytohesin-2 cKO mice (Fig. 1B). Triple staining of the dorsal horn revealed that cytohesin-2 was concentrated particularly in laminae I and II, which were marked by substance P and calretinin, respectively (Fig. 1C).

Since the dorsal horn is composed of neurochemically distinct subsets of projection neurons and interneurons, and glial cells (Todd, 2010; Todd, 2017), we characterized the cell types expressing cytohesin-2 by double immunofluorescence labeling with cytohesin-2 and various cell-type markers for neurons (NeuN), projection neurons and excitatory interneurons in lamina I (NKIR), excitatory interneurons (calbindin, calretinin, and PKCy), inhibitory interneurons (Pax2), or glial cells (GFAP for astrocytes, Iba1 for microglia, and Olig2 for oligodendrocytes). In laminae I and II, nearly all cytohesin-2-positive cells (97.9%) were immunoreactive for NeuN, a neuronal marker (Fig. 1D), whereas cytohesin-2-expressing cells were rarely co-labeled by glial markers for astrocytes, microglia, or oligodendrocytes (data not shown). NKIR-expressing neurons were largely immunoreactive for cytohesin-2 in lamina I (Fig. 1F, G; 76.0%), and excitatory interneurons marked by calbindin, calretinin, or PKCy were also immunoreactive for cytohesin-2 (Fig. 1H–J; calbindin, 77.8%; calretinin, 85.7%; PKCy, 72.7%). In contrast, inhibitory interneurons labeled by Pax2 were rarely immunoreactive for cytohesin-2 (Fig. 1K; 5.2%). These findings suggested that cytohesin-2 is expressed in subsets of excitatory interneurons and probably in projection neurons in the superficial dorsal horn.

Since the immunofluorescence for cytohesin-2 appeared as fine puncta at a higher magnification, we investigated which subcellular compartments and organelles cytohesin-2-immunoreactive puncta correspond to by double immunofluorescence labeling with synaptic and endosomal marker antibodies (Fig. 2A–F, Table 2). In the neuropil area, cytohesin-2-immunoreactive puncta markedly overlapped with those for Homer1b/c, a marker for excitatory postsynapses (Fig. 2A), whereas cytohesin-2-immunoreactive puncta were closely apposed to or partially overlapped with VGlut2, corresponding to the majority of presynaptic terminals of glutamatergic dorsal horn neurons (Todd et al., 2003) (Fig. 2B). In the soma and proximal neuropils, cytohesin-2-immunoreactive puncta were partially overlapped or closely associated with EEA1 (Fig. 2C), syntaxin13 (Fig. 2D), and LAMP2 (Fig. 2E), markers of early endosomes, recycling endosomes, and late endosomes/lysosomes, respectively. In addition, cytohesin-2 was frequently colocalized with Arf6 in punctate structures of various sizes in soma and neuropils (Fig. 2F), consistent with previous findings that cytohesin-2 functions as a GEF for Arf6 in hippocampal neurons (Hernandez-Devize et al., 2007; Hernandez-Devize et al., 2004) and N1E-115 neuroblastoma cell line (Torii et al., 2012; Torii et al., 2014; Yamauchi et al., 2009). Quantification of the degree of colocalization of cytohesin-2 with various markers was summarized in Table 2. To be noted, some NeuN-positive dorsal horn neurons showed nuclear labeling for cytohesin-2 (Fig. 1E), which was completely attenuated in cytohesin-2 cKO mice (Fig. 1B), suggesting that cytohesin-2 may function within the nucleus, as is the case for other Arf-GEFs such as BIG1 and BRAG2 (Padilla et al., 2004; Dunphy et al., 2007).

We further examined the subcellular localization of cytohesin-2 in dorsal horn neurons at the ultrastructural level by immunoelectron microscopy. In pre-embedding immunoelectron microscopy, silver-intensified immunoreactive metal particles for cytohesin-2 were distributed in various subcellular compartments, including cell bodies, axons, presynaptic terminals, and dendritic shafts. In soma and dendritic shafts, immunoreactive metal particles were frequently associated with vesicular or tubular membrane structures and with the plasma membrane (Fig. 2G). Quantitative analysis revealed that the density of immunoreactive particles was higher in somatodendritic compartments than in axonal compartments, with the highest density in the dendritic area adjacent to synapses (Fig. 2H). Post-embedding immunoelectron microscopy of axo-dendritic asymmetric synapses of glomeruli in the superficial dorsal horn revealed that immunogold particles for cytohesin-2 were distributed on the postsynaptic membrane with preferential accumulation around the edge of the PSD (Fig. 2I). In contrast, the immunolabeling was markedly attenuated in the same compartment of cytohesin-2 cKO mice (Fig. 2J, K; gold particles/μm², mean ± SD; control: 6.268 ± 1.022; cytohesin-2-cKO: 0.520 ± 0.229, p = 0.00068, t-test, n = 3 mice for each), suggesting the specificity of the immunolabeling. A quantitative analysis of the lateral distribution of cytohesin-2 on the postsynaptic membrane revealed that cytohesin-2 was distributed most densely within 60 nm around the edge of the PSD, and gradually decreased with increasing distance from the PSD, suggesting the preferential perisynaptic localization of cytohesin-2 at excitatory post-synapses (Fig. 2L).

3.2. Cytohesin-2 forms a protein complex with mGluR5 in the spinal cord

Among group I mGluRs, mGluR5 is expressed predominantly in the superficial dorsal horn and localizes to the perisynapse of asymmetric synaptic terminals of dorsal horn neurons (Alvarez et al., 2000; Vidnyanszky et al., 1994). Furthermore, mGluR1/5 was shown to form a protein complex with cytohesin-2 through its interaction with tamalin, a synaptic scaffolding protein, in the rat brain (Kitano et al., 2002). Indeed, double immunofluorescence staining of the dorsal horn revealed a high degree of colocalization of immunoreactive puncta for cytohesin-2 with those for mGluR5 and Gog/11/14, the cognate Gs protein for mGluR5 (Fig. 2A, B, Table 2). In the immunoprecipitation assay (Fig. 3C), the anti-mGluR5 antibody immunoprecipitated cytohesin-2 from the P2 fraction of the dorsal half of lumbar cord, but the reverse immunoprecipitation with the anti-cytohesin-2 antibody failed to detect mGluR5. These findings suggest that a subpopulation of cytohesin-2 forms a protein complex with mGluR5 in the spinal cord, consistent with the anatomical findings that cytohesin-2 exhibited wide subcellular distribution in dorsal horn neurons.
Fig. 1. Expression of cytohesin-2 in the spinal cord of adult mice. (A, B) Immunofluorescence staining of the lumbar spinal cord of wild-type (A) and cytohesin-2 conditional knockout (B) mice with anti-cytohesin-2 (N7) antibody. Nuclear staining with DAPI (blue) is included in the images. Note that the immunolabeling for cytohesin-2 observed in the lumbar spinal cord of the wild-type mouse was completely attenuated in that of the cytohesin-2 conditional knockout mouse. (C) Triple immunofluorescence of the dorsal horn with antibodies against cytohesin-2 (green, C1), calretinin (red, C2), and substance P (SP) (blue, C2), showing the prominent immunolabeling for cytohesin-2 in laminae I and II. (D–K) Double immunofluorescence staining of the dorsal horn with antibodies against cytohesin-2 (D1–K1) and NeuN (D2, E2), NK1R (F2, G2), calbindin (H2), calretinin (I2), PKCγ (J2), or Pax2 (K2). Note the expression of cytohesin-2 in subsets of excitatory interneurons and projection neurons, but not in Pax2-positive inhibitory interneurons. Arrowheads point to neurons co-immunolabeled with cytohesin-2 and markers. Arrows in E indicate nuclear immunolabeling for cytohesin-2 in some NeuN-positive neurons. CC, central canal; DH, dorsal horn; VH, ventral horn. Scale bars: 500 μm in (A, B); 100 μm in (C–K). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. Cytohesin-2 cKO mice show reduced mechanical allodynia in inflammatory and neuropathic pain models

Group I mGluRs are implicated in nociceptive transmission and plasticity in the spinal cord dorsal horn (Chiechio and Nicoletti, 2012; Luo et al., 2014; Neugebauer, 2002). We thus examined the functional involvement of cytohesin-2 in inflammatory and neuropathic pain models using cytohesin-2-deficient mice. The conditional deletion of cytohesin-2 in Schwann cells has previously been shown to affect the myelination of the peripheral nervous system (PNS) (Torii et al., 2015). To avoid the effect of cytohesin-2 deletion on the PNS, we generated CNS-specific cytohesin-2 cKO mice by crossing floxed cytohesin-2 knock-in mice with nestin-Cre transgenic mice.

Immunoblotting using anti-cytohesin-2 antibodies raised against its divergent N-terminal or C-terminal region showed a marked reduction in the 45-kDa immunoreactive band for cytohesin-2 in total lysates of the spinal cord and brain from cytohesin-2 cKO mice, without any truncated products, compared with those from wild-type control mice (Fig. 4A). A faint residual immunoreactive band for cytohesin-2 in the spinal lysate from cytohesin-2 cKO mice was likely to reflect the expression of cytohesin-2 in the spinal cord dorsal horn, showing the highest density of immunolabeling for cytohesin-2 in the superficial dorsal horn, showing the highest density of immunolabeling for cytohesin-2 in the perisynaptic region. Scale bars: 10 μm in (A–F); 500 nm in (G, I, J). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Subcellular localization of cytohesin-2 in dorsal horn neurons. (A–F) Double immunofluorescence labeling of the dorsal horn with antibodies against cytohesin-2 (A1–F1) and Homer1b/c (A2), VGluT2 (B2), EEA1 (C2), syntaxin13 (D2), LAMP2 (E2), or Arf6 (F2). Merged images in C2–F2 include nuclear staining with DAPI (blue). Note the partial overlapping of cytohesin-2-immunoreactive puncta with Arf6 and markers for excitatory postsynapses, endosomes, or lysosomes. (G) Pre-embedding immunoelectron microscopy showing the association of immunoreactive metal particles for cytohesin-2 with membrane vesicles and tubules (arrows) in the dendritic shaft (Dn). (H) Quantification of the density of immunoreactive metal particles for cytohesin-2 in neuronal subcellular compartments, showing the highest density of immunolabeling for cytohesin-2 in the dendritic area adjacent to excitatory synapses (Dn(Sy)). Ax, axonal shaft; CB, cell body; Dn(Sh), dendritic shaft region without synapses; DT, nerve terminal. (I, J) Post-embedding immunoelectron microscopy of the postsynaptic dendritic membrane of asymmetric synapses in glomeruli of control (I) and cytohesin-2 conditional knockout (cKO) (J) mice using anti-cytohesin-2 antibody. Arrowheads point to immunolabeling for cytohesin-2 along the postsynaptic membrane. (K) Quantification of the labeling density for cytohesin-2 in dorsal horn neurons of control and cytohesin-2 cKO mice (gold particles/μm², mean ± SD; control: 6.268 ± 1.022; cytohesin-2-cKO: 0.520 ± 0.229, *p = 0.00068, t-test, n = 3 mice for each). Note significant attenuation of immunolabeling in cytohesin-2 cKO mice. (L) Quantification of the lateral distribution of immunogold particles for cytohesin-2 from the edge of the postsynaptic density (PSD) along the postsynaptic membrane of asymmetric synapses of glomeruli in the superficial dorsal horn, showing the highest density of immunolabeling for cytohesin-2 in the perisynaptic region. Mi, mitochondria. Scale bars: 10 μm in (A–F3); 500 nm in (G, I, J). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Table 2
Colocalization assay for Cytohesin-2 with synaptic and endosomal markers in the spinal dorsal horn.

|                    | Colocalization coefficient |
|--------------------|---------------------------|
| Cytohesin-2 vs. Homer1b/c | 0.143 ± 0.048             |
| Cytohesin-2 colocalized with Homer1b/c | 0.231 ± 0.022             |
| Homer1b/c colocalized with cytohesin-2 | 0.041 ± 0.038             |
| VGlut2 colocalized with VGlut2 | 0.098 ± 0.028             |
| Cytohesin-2 colocalized with VGlut2 | 0.573 ± 0.152             |
| mGluR8 colocalized with cytohesin-2 | 0.179 ± 0.046             |
| Cytohesin-2 colocalized with mGluR8 | 0.262 ± 0.077             |
| Gaq11/14 colocalized with cytohesin-2 | 0.337 ± 0.043             |
| Cytohesin-2 vs. EEA1 | 0.121 ± 0.046             |
| Cytohesin-2 colocalized with EEA1 | 0.361 ± 0.090             |
| Cytohesin-2 vs. syntaxin13 | 0.213 ± 0.110             |
| Syntaxin13 colocalized with cytohesin-2 | 0.371 ± 0.133             |
| Cytohesin-2 vs. LAMP2 | 0.034 ± 0.020             |
| LAMP2 colocalized with cytohesin-2 | 0.402 ± 0.062             |
| Cytohesin-2 vs. Arf6 | 0.088 ± 0.065             |
| Arf6 colocalized with cytohesin-2 | 0.507 ± 0.091             |

Data are presented as mean ± SD.

The spinal dorsal horn.

Values are presented as mean ± SEM; Control-Ipsi: 0.34 ± 0.05 g, cKO-Ipsi: 0.057 ± 0.035 g, p < 0.001; day 2, Control-Ipsi: 0.09 ± 0.03 g, cKO-Ipsi: 0.27 ± 0.07 g, p < 0.05; day 4, Control-Ipsi: 0.10 ± 0.03 g, cKO-Ipsi: 0.18 ± 0.05 g, p < 0.05; day 7, Control-Ipsi: 0.19 ± 0.05 g, cKO-Ipsi: 0.34 ± 0.08 g, p < 0.05, n = 19 mice for each group). The mechanical nociceptive thresholds tended to be higher in cKO mice than in control mice at 4 and 7 days post-operation, although they did not reach statistical significance. To exclude the potential effects of the nestin-Cre line on nociceptive behaviors, we confirmed that there were no significant differences in the nociceptive responses following CFA injection orCCI between nestin-Cre and wild-type mice (Supplementary Fig. S2).

3.4. Arf1 and Arf6 are activated in the dorsal horn in inflammatory and neuropathic pain models

Since cytohesin-2 possesses guanine nucleotide exchange activity toward Arf1 and Arf6 in vitro (Frank et al., 1998b), we examined the effect of inflammatory and neuropathic pain on the activation of Arf1 and Arf6 in the spinal dorsal horn by a pull-down activation assay, using GST-GGA1 fusion proteins that can bind specifically to GTP-bound Arfs (Nakayama and Takatsu, 2005). Pull-down precipitates and total lysates of the dorsal spinal cord at various time points following the CFA injection orCCI were immunoblotted with antibodies against Arf1 and Arf6. GTP-Arf6 was significantly increased twofold at 2 h, peaked at 12 h, and returned to the control level at 24 h after CFA injection (Fig. 5C, D; one-way ANOVA, F(4, 15) = 3.32, p = 0.039, followed by Tukey-Kramer post hoc test, mean ± SEM; 0 h, 1.00 ± 0.17, n = 6 mice; 2 h, 2.02 ± 0.35, n = 3, p = 0.009; 6 h, 2.54 ± 0.24, n = 3, p = 0.001; 12 h, 2.61 ± 0.78, n = 6, p = 0.036; 24 h, 1.44 ± 0.29, n = 3, p = 0.096), whereas the GTP-Arf1 level was significantly increased at 6 h and remained higher at least until 24 h after the CFA injection compared with the control level (Fig. 5C, D; one-way ANOVA, F(4, 10) = 4.05, p = 0.033, followed by Tukey-Kramer post hoc test, mean ± SEM; 0 h, 1.00 ± 0.02; 2 h, 1.19 ± 0.08, p = 0.032; 6 h, 3.03 ± 0.90, p = 0.045; 12 h, 2.88 ± 0.38, p = 0.004; 24 h, 2.29 ± 0.33, p = 0.009, n = 3 mice for each group). In the CCI model, a significant increase in GTP-Ar6 was detectable only at 12 h after CCI (Fig. 5D, F; one-way ANOVA, F(4, 10) = 3.931, p = 0.036, followed by Tukey-Kramer post hoc test, mean ± SEM; 0 h, 1.00 ± 0.24; 2 h, 0.67 ± 0.09, p = 0.130; 6 h, 1.33 ± 0.07, p = 0.131; 12 h, 2.74 ± 0.37, p = 0.009; 24 h, 1.92 ± 0.87, p = 0.180, n = 3 mice for each group), whereas the GTP-Arf1 level was significantly higher than the control level at 2 h, 6 h, and 12 h (Fig. 5D, H; one-way ANOVA, F(4, 10) = 4.307, p = 0.027, followed by Tukey-Kramer post hoc test, mean ± SEM; 0 h, 1.00 ± 0.23; 2 h, 2.47 ± 0.46, p = 0.046; 6 h, 2.11 ± 0.25, p = 0.033; 12 h, 3.86 ± 0.17, p = 0.001; 24 h, 4.41 ± 1.70, p = 0.119, n = 3 mice for each group). These findings suggested that both Arf1 and Arf6 are activated in the dorsal horn in inflammatory and neuropathic pain models.

3.5. SecinH3 reduces mechanical allodynia and spinal Arf6 activation in pain models

To examine whether the catalytic activity of cytohesins in the spinal cord is required for mechanical allodynia in inflammatory and neuropathic pain models, SecinH3 was intrathecally administered to wild-type mice 2 h before CFA injection orCCI. Pretreatment with SecinH3 at two different doses (0.1 or 1 μg/g body weight) significantly increased the ipsilateral paw-withdrawal threshold to mechanical stimuli at 2 days after CFA injection, as compared to the control, and tended to be
effective until 7 days post-injection, although then no longer statistically significant (Fig. 6A, Table 3; two-way repeated measures ANOVA, F(3,12) = 3.536, p = 0.043, followed by Tukey-Kramer post hoc test, mean ± SEM; CFA injection, Arf6, one-way ANOVA, F(2,12) = 3.965, p = 0.047, followed by Tukey-Kramer post hoc test, mean = 0.041; CFA injection, Arf1, one-way ANOVA, F(2, 9) = 5.826, p = 0.024, followed by Tukey-Kramer post hoc test, control: 1.00 ± 0.17, n = 3; CFA: 3.06 ± 0.78, p = 0.031, n = 3; CFA + SecinH3: 2.58 ± 0.38, p = 0.010, n = 3) or CCI (Fig. 6D, F, H; Arf6, one-way ANOVA, F(2, 6) = 5.956, p = 0.031, followed by Tukey-Kramer post hoc test, mean ± SEM; control, 1.00 ± 0.37; CCI, 2.47 ± 0.51, p = 0.024; CCI + SecinH3, 0.98 ± 0.51, p = 0.480; Arf6, one-way ANOVA, F(2, 6) = 8.514, p = 0.018, followed by Tukey-Kramer post hoc test, control, 1.00 ± 0.31; CCI, 2.05 ± 0.16, p = 0.041; CCI + SecinH3, 2.61 ± 0.33, p = 0.025, n = 3 mice for each group). Together, these findings suggested that SecinH3 has an anti-allodynic effect by inhibiting the catalytic activity of cytohesins toward Arf6 in the spinal cord.

3.6. Cytohesin-2 cKO mice exhibit reduced mechanical allodynia induced by pharmacological activation of spinal mGluR1/5

Pharmacological activation of spinal mGluR1/5 by intrathecal administration of DHPG, a selective agonist of group 1 mGluRs, induces spontaneous nociceptive behaviors, such as licking of the flanks, hindpaws, and tail, as well as thermal and mechanical hypersensitivity (Adwanikar et al., 2004; Fisher and Coderre, 1996; Fisher and Coderre, 1998). To provide evidence for the functional linkage between cytohesin-2 and mGluR1/5 during chronic pain, we compared the effect of DHPG on nociceptive responses between control and cKO mice.

**Fig. 3.** Cytohesin-2 forms a protein complex with mGluR5 in the spinal cord. (A, B) Double immunofluorescence labeling of the superficial dorsal horn with antibodies against cytohesin-2 (A1, A2), and mGluR5 (A2) or Gaq/11/14 (B3), showing the partial overlapping of cytohesin-2-immunoreactive puncta with mGluR5 and Gaq/11/14. (C) Immunoprecipitation analysis. The P2 fraction of the dorsal spinal cord of adult mice was subjected to immunoprecipitation with control, anti-cytohesin-2, or anti-mGluR5 IgG. The lysate (input) and immunoprecipitates were subjected to immunoblotting with anti-cytohesin-2 or anti-mGluR5 IgG. Scale bars: 10 μm.
Fig. 4. Characterization of central nervous system-specific cytohesin-2 conditional knockout mice. (A) Immunoblotting. Total lysates of the spinal cord and brain from control (Cont) and conditional knockout (cKO) mice were subjected to immunoblotting with antibodies against N- and C-terminal regions of cytohesin-2, and α-tubulin. Note the marked attenuation of a 45-kDa immunoreactive band for cytohesin-2 in the spinal and brain lysates from cKO mice. (B, C, D) Immunoperoxidase staining of sagittal sections of the brain (B) and transverse sections of the spinal cord (C, D) from the two genotypes with anti-cytohesin-2 IgG, showing the marked attenuation of immunolabeling for cytohesin-2 in cKO mice. (E, F) Nissl staining of transverse sections of the spinal cord from control (E) and cKO (F) mice. (G–J) Immunoperoxidase staining for cytohesin-2 of the dorsal root ganglion (DRG) of control (G, H) and cKO (I, J) mice. Note the comparable expression of cytohesin-2 in DRG neurons between the two genotypes. (K–N) Immunoperoxidase staining of the spinal cord (K, M) and dorsal root (L, N) of control (K, L) and cKO (M, N) mice for MBP. The boxed regions of the dorsal root in (K) and (M) are magnified in (L) and (N), respectively. Cb, cerebellum; Cx, cerebral cortex; DH, dorsal horn; DR, dorsal root; Mb, midbrain; MO, medulla oblongata; OB, olfactory bulb; Th, thalamus; VH, ventral horn. Scale bars: 1 mm in (B); 200 μm in (C–G, I); 100 μm in (K, M); 20 μm in (H, J); 5 μm in (L, N).
Fig. 5. Reduced mechanical allodynia in cytohesin-2 conditional knockout mice and the activation of Arf1 and Arf6 in inflammatory and neuropathic pain models. (A, B) Time-course changes in paw-withdrawal thresholds following complete Freund’s adjuvant (CFA) injection and chronic constriction injury of the sciatic nerve (CCI). Note significantly higher withdrawal thresholds of the ipsilateral paw of conditional knockout (cKO) mice compared with those of the control mice at 2, 4, and 7 days after CFA injection (A, control-Ipsi vs cytohesin-2 cKO-Ipsi, two-way repeated measures ANOVA, $F(3,48) = 3.799, p = 0.015$, with Tukey-Kramer post hoc test), and at 2 days after CCI (B, control-Ipsi vs cytohesin-2 cKO-Ipsi, two-way repeated measures ANOVA, $F(3,117) = 2.953, p = 0.035$, with Tukey-Kramer post hoc test), respectively. * $p < 0.05$ vs control-Ipsi in A and B. (C–H) Pull-down Arf activation assay. (C, D) Representative immunoblots of Arf6 and Arf1 activation assay in inflammatory (C) and CCI (D) models. The lysates of dorsal spinal cords at various time points following the CFA injection or CCI were subjected to pull-down assay with GST-GGA1 fusion protein, and the total lysates (input) and precipitates (pull-down) were immunoblotted with antibodies against Arf6 and Arf1. (E–H) Quantification of the activation of Arf6 (E, F) and Arf1 (G, H) at various time points following CFA injection (E, G) and CCI (F, H) (E, one-way ANOVA, $F(4,15) = 3.324, p = 0.039$; F, one-way ANOVA, $F(4,10) = 3.931, p = 0.036$; G, one-way ANOVA, $F(4,10) = 4.053, p = 0.033$; H, one-way ANOVA, $F(4,10) = 4.307, p = 0.027$, with Tukey-Kramer post hoc test). * $p < 0.05$ vs 0 h in E–H. The activation level of Arf6 or Arf1 at each time point was normalized by that at 0 h.
level of ERK1/2 by approximately 30% after 1 h (Fig. 7B, C; control: 0.153 ± 0.081, t-test, n = 6 mice for each group), suggesting that the functional linkage between mGluR1/5 and ERK1/2 was impaired in cytohesin-2 cKO mice. It should be noted that the protein level of mGluR5 in the spinal cord was comparable between the two genotypes before and after DHPG stimulation (Fig. 7B, D; control: mean ± SEM; vehicle, 1.000 ± 0.216; DHPG, 1.056 ± 0.153, p = 0.837, t-test, n = 6 mice for each group; cytohesin-2 cKO: mean ± SEM; vehicle, 1.054 ± 0.171; DHPG, 0.888 ± 0.151, p = 0.481, n = 6 mice for each group), excluding the possibility that altered ERK1/2 activation could be attributed to differences in spinal mGluR5 levels between genotypes.

### 3.8. Cytohesin-2 cKO mice exhibit altered subsynaptic localization of mGluR5

Synaptic distribution of mGluR1/5 is regulated by various mechanisms including their interactions with scaffolding proteins and the actin cytoskeleton, and membrane trafficking (Scheethals and MacGillavry, 2018). In addition, cytohesin-2 regulates membrane trafficking and actin cytoskeleton organization through the activation of Arf6 (Turner and Brown, 2001), and tamalin regulates the distribution and trafficking of mGluR1/5 (Kitano et al., 2002; Pandey et al., 2020). Therefore, we compared the subcellular localization of mGluR5 and Gq/11/14 on the postsynaptic membrane in the synaptic glomeruli in the superficial dorsal horn, where nociceptive primary afferents terminate (Ribeiro-da-Silva et al., 1985), between wild-type and cytohesin-2 cKO mice, by means of post-embedding immunoelectron microscopy. A quantitative analysis of the lateral distribution of immunogold particles for mGluR5 along the postsynaptic dendritic membrane in synaptic glomeruli revealed that the frequency of mGluR5-immunoreactive particles associated with postsynaptic membrane peaked within 60 nm outside of the edge of the PSD, although immunoreactive particles for mGluR5 were widely distributed in the postsynaptic membrane domains including the PSD, and perisynaptic and extrasynaptic regions (Fig. 8A, E). In contrast, in cytohesin-2 cKO mice, the proportion of mGluR5-immunoreactive particles was significantly decreased in the perisynaptic region, and increased reciprocally in the PSD, as compared with the control (Fig. 8B, F; J; PSD: mean ± SEM; control, 24.253 ± 2.834%; cytohesin-2 cKO, 44.265 ± 0.860%; p = 0.002, t-test; perisynapse: control, 43.641 ± 4.686%; cytohesin-2 cKO, 27.406 ± 0.565%; p = 0.026; extrasynapse: control, 32.106 ± 3.775%; cytohesin-2 cKO, 28.326 ± 0.297, p = 0.375, n = 3 mice for each genotype, 435 particles for control, 448 particles for cytohesin-2 cKO), although there were no statistically significant differences in the total number of mGluR5-immunoreactive particles associated with the plasma membrane, or the frequency of mGluR5-immunoreactive particles in the extrasynaptic region between the two genotypes (Fig. 8I, J; mGluR5 density, number/μm, mean ± SEM; control: 1.061 ± 0.127; cytohesin-2 cKO: 0.946 ± 0.117, p = 0.543, t-test, n = 3 mice for each genotype). On the other hand, immunogold labeling for Gq/11/14 were distributed most abundantly in the perisynaptic region within 120 nm from the edge of the PSD, and there were no significant differences in the lateral distribution profile of Gq/11/14 on the postsynaptic membrane between control and cytohesin-2 cKO mice (Fig. 8C, D, G, H; PSD: mean ± SEM; control, 21.020 ± 1.243%; cytohesin-2 cKO, 21.136 ± 1.221%, p = 0.950, t-test; perisynapse: control, 37.068 ± 2.003; cytohesin-2 cKO, 40.299 ± 1.856, p = 0.302; extrasynapse: control, 41.906 ± 3.244; cytohesin-2 cKO, 38.563 ± 3.035, p = 0.494, n = 3 mice for each genotype, 390 particles for control, 444 particles for cytohesin-2 cKO). These findings suggested that the spatial coupling between mGluR5 and Gq/11/14 protein at the postsynapse is disrupted in cytohesin-2 cKO mice. Finally, the specificity of the synaptic immunolabeling of mGluR5 and Gq/11/14 was confirmed by the pre-absorption test, in which immunogold signals on the synaptic membrane were significantly attenuated by the incubation of the primary antibodies with their respective antigens (Supplementary Fig. S3; gold particles/μm, mean ± SEM; anti-mGluR5: 1.786 ± 0.350; anti-cytohesin-2 cKO: 0.217 ± 0.108, p = 0.00176, t-test, n = 3 mice for each group; anti-Gq/11/14: 1.065 ± 0.274; anti-Gq/11/14 + antigen: 0.269 ± 0.050, p = 0.00853, t-test, n = 3).

### 4. Discussion

Accumulating evidence suggests that disturbances in Arf-mediated membrane trafficking and actin cytoskeleton remodeling in neurons are associated with various neurodevelopmental and neurological disorders, including periventricular heterotopia (Sheen et al., 2004), intellectual disability with seizure and autism spectrum disorder (Falace et al., 2010; Levy et al., 2019; Shoubridge et al., 2010a; Shoubridge et al., 2010b).
et al., 2010b), amyotrophic lateral sclerosis (Sivadasan et al., 2016; Zhai et al., 2015), and Alzheimer’s disease (Sannerud et al., 2011; Tang et al., 2015). In this study, we provided the first evidence for the functional involvement of the cytohesin-2–Arf6 pathway in inflammatory and neuropathic pain.

One of the major findings is that conditional deletion of cytohesin-2 in the CNS reduced mechanical allodynia in CFA inflammation and CCI pain models. Torii et al. (2015) previously reported that conditional deletion of cytohesin-2 in Schwann cells reduced myelination in the sciatic nerve. However, the present immunohistochemical analyses revealed that there were no detectable differences in the immunoreactive intensity of MBP and number of myelinated fibers in the dorsal root of spinal nerves or white matter of the spinal cord between control and CNS-specific cytohesin-2 cKO mice, excluding the possibility that a disturbance in impulse transmission caused by impaired myelination affected the anti-allodynic phenotype. We also demonstrated that
cytohesin-2 was expressed in dorsal root ganglion neurons at a comparative level between the two genotypes. Thus, the anti-allodynic phenotype observed in the mutant mice was likely to be caused by the lack of cytohesin-2 in the CNS, but not in the PNS. Furthermore, intrathecal administration of SecinH3 similarly reduced mechanical allodynia, indicating that the cytohesin catalytic activity in the spinal cord is required for the development of mechanical allodynia. Although SecinH3 broadly inhibits the cytohesin family members (Hafner et al., 2006), the phenotypic similarity between mice lacking cytohesin-2 in the CNS and those administered intrathecally with SecinH3 suggests that cytohesin-2 in the spinal cord is responsible for the development of mechanical allodynia. Finally, the Arf pull-down activation assay demonstrated that, although both Arf6 and Arf1 were activated in both pain models, intrathecal SecinH3 administration blocked the activation of Arf6, but not Arf1, in the dorsal spinal cord. Interestingly, the activation of Arf6 in the dorsal spinal cord was peaked at 12 h after CFA injection or surgery, the genetic deletion of cytohesin-2 or pharmacological inhibition of cytohesins had a relatively long-lasting anti-allodynic effect. Together, it is reasonable to conclude that the cytohesin-2 mediates the initiation and development of mechanical allodynia through the activation of Arf6 in the dorsal horn. However, it should be mentioned that chronic pain can develop as a consequence of enhanced excitability in any neural circuits or nuclei that relay and process somatosensory information, including brain stem nuclei, periaqueductal gray, thalamus, somatosensory cortex, cingulate cortex, hippocampus and amygdala, as well as the spinal cord and PNS (Apkarian et al., 2005; Basbaum et al., 2009; Kuner, 2010). Since cytohesin-2 is widely expressed in the CNS (Ito et al., 2018), we cannot completely exclude the possibility that reduced mechanical allodynia observed in cKO mice may be attributed to the lack of cytohesin-2 in other brain regions in addition to the spinal cord.

Mechanical allodynia is considered to be a pathological condition in which the balance between excitatory and inhibitory neural circuits to projection neurons in the dorsal horn is disturbed, thereby allowing innocuous low-threshold mechanical inputs to gain access to and activate nociceptive projection neurons via polysynaptic pathways (Peirs et al., 2020; Peirs and Seal, 2016; Todd, 2010). Concerning cell types or neural circuits in which cytohesin-2 mediates mechanical allodynia in
Fig. 8. Cytohesin-2 conditional knockout mice exhibit altered subsynaptic localization of mGluRS, but not Gαq/11/14. (A–D) Representative micrographs of post-embedding immunoelectron microscopy of asymmetric synapses of glomeruli in the superficial dorsal horn from control (A, C) and conditional knockout (cKO) (B, D) mice for mGluRS (A, B) and Gαq/11/14 (C, D). Arrowheads indicate immunogold particles on the postsynaptic membrane. NT, nerve terminal. (E–H) Histograms of the lateral distribution of immunogold particles for mGluRS (E, F) and Gαq/11/14 (G, H) from the edge of the postsynaptic density (PSD) along the postsynaptic membrane of asymmetric synapses of glomeruli in the superficial dorsal horn from control (E, G) and cytohesin-2 cKO (F, H) mice. (I) Quantification of the localization of immunogolds for mGluRS to the plasma membrane (control vs cKO, \( p = 0.543 \), t-test). (J, K) Quantification of the proportion of immunogolds for mGluRS (J) and Gαq/11/14 (K) in the PSD, perisynaptic, and extrasynaptic regions. Note the reciprocal changes in the proportion of mGluRS between the PSD and perisynaptic region in cKO mice compared with the control (J, PSD: control vs cKO, \( p = 0.002 \), t-test; perisynapse: control vs cKO, \( p = 0.026 \); extrasynapse: control vs cKO, \( p = 0.375 \)), without any differences in the subsynaptic distribution pattern of Gαq/11/14 between the two genotypes (K, PSD: control vs cKO, \( p = 0.950 \), t-test; perisynapse: control vs cKO, \( p = 0.302 \); extrasynapse: control vs cKO, \( p = 0.494 \)). Scale bars: 500 nm.
the spinal cord, we demonstrated that cytohesin-2 was expressed abundantly in dorsal horn neurons, particularly in laminae I and II. By double immunofluorescence, we further found that cytohesin-2 was expressed primarily in a subset of excitatory interneurons labeled by PKCγ, calbindin, calretinin, or NK1R, and probably in NK1R-immunoreactive projection neurons in the superficial dorsal horn. Recent evidence has indicated that excitatory interneurons in the dorsal horn, including neurons transiently expressing VGLUT3, and neurons expressing PKCγ or calretinin, form an essential neural pathway for mechanical allodynia (Peirs et al., 2020; Peirs and Seal, 2016). Therefore, it is plausible that cytohesin-2 mediates mechanical allodynia in an excitatory neural circuit in the dorsal horn. Since dorsal horn neurons are highly diverse in their neurochemical, electrophysiological, and morphological properties (Peirs et al., 2020; Todd, 2017), it is difficult to define a unique functional neural population by any one of these markers such as neuropeptides, receptors, and intracellular signaling molecules. Thus, further studies using multiple lines of evidence obtained by immunoprecipitation, immunofluorescence, and immunoelectron microscopy in the present study suggested that cytohesin-2 forms a protein complex with mGluR5 in the perisynaptic region, and is ideally positioned to mediate mGluR5-dependent nociceptor sensitization. Indeed, mechanical allodynia induced by intrathecal DHGTP administration was significantly reduced in cytohesin-2 cKO mice. Furthermore, cytohesin-2 cKO mice exhibited reduced DHGTP-induced ERK1/2 activation in the spinal cord. The ERK1/2 pathway is the major signaling pathway downstream of mGluR1/5 which is coupled to the actin cytoskeleton through its direct interaction with F-actin (Piattelli et al., 2001; Bredt et al., 2001), and is implicated in chronic pain through the regulation of ion channels and gene transcription by phosphorylating a serine residue at position 392 within the C-terminal region and/or by limiting the entry of mGluR5 into the PSD. It was recently shown to mediate activity-dependent trafficking of tamalin to the postsynapse, consequently reducing the molecular crowding of the PSD or increasing the number of slots in the PSD, and leading to the displacement of mGluR5 into the PSD. In addition, the actin cytoskeleton may also regulate the perisynaptic accumulation of mGluR5 by anchoring mGluR1/5 to the perisynaptic region and/or by limiting the entry of mGluR5 into the PSD. It was previously shown that polymerized actin is highly concentrated and dynamic in the perisynaptic region (Frost et al., 2010), and that mGluR5 is coupled to the actin cytoskeleton through its direct interaction with F-actin-binding proteins, such as α-actinin-1 (Cabello et al., 2007) and Filamin-A (Enz, 2002). Since cytohesin-2 regulates various Arf6-dependent actin-based cellular processes, such as cell morphology (Frank et al., 1998b), migration (Sanyt and Casanova, 2001), and neurite outgrowth (Hernandez-Dieviez et al., 2004; Torii et al., 2012; Torii et al., 2014; Yamauchi et al., 2009), the disturbance in the Arf6-dependent actin cytoskeleton dynamics caused by the lack of cytohesin-2 in the postsynapse could also account for the shift of mGluR5 into the PSD from the perisynaptic region.

The mechanism for the activation of cytohesin-2 during chronic pain is also an important issue for future research. The catalytic activity of the cytohesin family is subject to complex regulation by intramolecular autoinhibition and phosphorylation. The catalytic activity of cytohesin-2 was reported to be autoinhibited by the intramolecular interaction of the linker region between the Sec7 and PH domain and a C-terminal amphilic helix region containing a polybasic motif with the Sec7 domain in a pseudosubstrate mechanism, which is released by the phosphorylation of a serine residue at position 392 within the C-terminal polybasic region by PKC (DiNitto et al., 2007; Frank et al., 1998b). In addition, the Src family tyrosine kinase Fyn was shown to activate cytohesin-2 through phosphorylation of a tyrosine residue at position 392 in the C-terminal region of cytohesin-1, which is conserved in cytohesin-2, in Schwann cells during the myelin formation (Yamauchi et al., 2012). Interestingly, the PKC–Src pathway is a critical signaling cascade for central sensitization, which is activated by postsynaptic mGluR1/5 stimulation and leads to an increase in the channel activity of NMDARs through the phosphorylation of NMDARs, particularly the GluN2B subunit, in dorsal horn neurons (Guo et al., 2004). Therefore, it is attractive to speculate that cytohesin-2 may be activated by PKC and/
or Fyn downstream of mGluR5 in response to nociceptive stimuli, leading to the activation of the Arf6 pathway in dorsal horn neurons.

Finally, in actual clinical settings, it is frequently difficult to distinguish the primary cause of chronic pain clearly between neuropathy and inflammation. It is noteworthy that intrathecal SecinH3 administration had anti-nociceptive effects on both inflammatory and neuropathic mechanical allodynia. Therefore, manipulation of the cytohesin-2–Arf6 pathway could be an attractive target for chronic pain, with a broad therapeutic window. However, considering the fundamental roles of Arf-dependent cellular processes, general blockage of the cytohesin-2–Arf6 pathway with SecinH3 is unlikely to be clinically applicable. Indeed, mice fed with SecinH3 have been reported to develop hepatic insulin resistance associated with the preclinical stages of type 2 diabetes (Hafner et al., 2006). Further investigations of the cytohesin-2–Arf6 signaling pathway in nociception may reveal new opportunities for developing novel therapeutic strategies for chronic pain.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2021.105466.

Author contributions
A.I., M.F., and H.S. designed the study, performed experiments, analyzed data, and drafted the paper. T.S. and Y.H. supervised part of the immunoprecipitation and immunohistochemical analyses; H.O. supervised the behavioral analyses; J.Y. generated and provided the cytohesin-2 cKO mice.

Declaration of competing interest
The authors declare no conflict of interest.

Acknowledgements
This work was supported by JSPS KAKENHI Grant No. 20K07250 (H.S.) and No. 20K22962 (A.I.), the Science Research Promotion Fund (H.S.), The Nakatomi Foundation (M.F.), Takeda Science Foundation (M.F.), and Kitasato University Research Grant for Young Researchers (T.S.). The authors thank Dr. Masahiko Watanabe (Hokkaido University) for providing antibodies and antigens, and Ms. Noriko Nemoto (the Bioimaging Center, Kitasato University) for excellent technical support in electron microscopic analyses.

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