Spinal astrocyte-neuron lactate shuttle contributes to the pituitary adenylate cyclase-activating polypeptide/PAC1 receptor-induced nociceptive behaviors in mice

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21

22 **Abstract**

23 We have previously showed that spinal pituitary adenylate cyclase-activating
24 polypeptide (PACAP)/PACAP type 1 (PAC1) receptor signaling triggered long-lasting
25 nociceptive behaviors through astroglial activation in mice. Since astrocyte-neuron
26 lactate shuttle (ANLS) could be essential for long-term synaptic facilitation, we aimed
27 to elucidate a possible involvement of spinal ANLS in the development of the
28 PACAP/PAC1 receptor-induced nociceptive behaviors. A single intrathecal
29 administration of PACAP induced short-term spontaneous aversive behaviors, followed
30 by long-lasting mechanical allodynia in mice. These nociceptive behaviors were
31 inhibited by 1,4-dideoxy-1,4-imino-d-arabinitol (DAB), an inhibitor of glycogenolysis,
32 and this inhibition was reversed by simultaneous L-lactate application. In the cultured
33 spinal astrocytes, the PACAP-evoked glycogenolysis and lactate secretion were
34 inhibited by DAB. In addition, a protein kinase C (PKC) inhibitor attenuated the
35 PACAP-induced nociceptive behaviors as well as the PACAP-evoked glycogenolysis
36 and lactate secretion. Finally, an inhibitor for the monocarboxylate transporters blocked
37 the lactate secretion from the spinal astrocytes and inhibited the PACAP- and spinal
38 nerve ligation-induced nociceptive behaviors. These results suggested that spinal PAC1
39 receptor-PKC-ANLS signaling contributed to the PACAP-induced nociceptive

40 behaviors. This signaling system could be involved in the peripheral nerve

41 injury-induced pain-like behaviors.

42

INTRODUCTION

Transfer of lactate from astrocytes to neurons is activated when synaptic activity is increased, and this mechanism is now known as astrocyte-neuron lactate shuttle (ANLS), that could account for the coupling between synaptic activity and energy delivery¹. Since lactate from astrocytes is originated from glucose and glycogen², inhibition of glycogenolysis or lactate transport in rat hippocampus significantly attenuates the working memory and long-term fear memory^{3, 4}. In addition, a brain-specific glycogen synthase knockout mouse showed a significant deficiency in the acquisition with an associative learning task and concomitantly, exhibited reduced activity-dependent changes in hippocampal synaptic strength⁵. It is also reported that ANLS in amygdala is critical for the reconsolidation of cocaine memory in rats^{6, 7}. At the cellular level, lactate was reported to potentiate NMDA receptor-mediated currents in cultured neurons and neurons in the nucleus of the solitary tract^{8, 9}. These findings suggested that ANLS could contribute to many forms of neuronal plasticity in the central nervous system (CNS), including plastic changes in the spinal nociceptive transmission. Indeed, mechanical allodynia evoked by pharmacogenetic activation of human M₃ muscarinic DREADDs (designer receptors exclusively activated by designer drugs) coupled to G_q on astrocytes is alleviated by the inhibition of the

monocarboxylate transporters (MCTs)¹⁰. However, it is still unclear what kinds of endogenous neurotransmitters evoke the ANLS activation in the spinal nociceptive transmission.

PACAP was originally isolated from ovine hypothalamic extracts based on its ability to stimulate adenylate cyclase in rat anterior pituitary cell cultures^{11, 12}. In normal state, PACAP specific receptor, PAC1 receptor, is particularly abundant in the CNS including spinal dorsal horn^{13, 14, 15, 16, 17}, where PACAP-immunoreactive fibers are also considerably localized^{18, 19, 20, 21}, and PACAP mRNA/immunoreactivity in rat dorsal root ganglia has been repeatedly shown to be markedly upregulated by peripheral nerve injury or inflammation^{22, 23, 24, 25}. These observations coupled with other lines of evidence propose that PACAP/PAC1 receptor system could play an important role in the modulation of spinal nociceptive transmission.

We have previously demonstrated in mice that a single intrathecal (i.t.) injection of PACAP or a PAC1 receptor specific agonist, maxadilan (Max)²⁶, induced spontaneous aversive behaviors, such as licking, biting, and scratching directed toward the caudal part of the body for more than 30 min^{27, 28}, and the aversive behaviors were followed by

an induction of the mechanical allodynia, which lasted for more than 84 days¹⁷.

However, vasoactive intestinal polypeptide (VIP), which share VIP/PACAP (VPAC) 1

or VPAC2 receptors with PACAP, failed to induce these nociceptive behaviors^{17, 28}.

Co-treatment of a PAC1 receptor antagonist, max.d.4 with PACAP, almost completely

inhibited the induction of both aversive behaviors and the mechanical allodynia^{17, 28}.

These results suggested a critical role of PAC1 receptor in the nociceptive transmission.

Immunohistochemical and immunoblotting studies revealed that spinal application of

PACAP or Max induced upregulation of an astrocyte marker, glial fibrillary acidic

protein (GFAP) at least for 84 days, and L- α -amino adipate, an astroglial toxin,

attenuated the induction of both aversive behaviors and mechanical allodynia.

Interestingly, L- α -amino adipate was still effective to reverse the mechanical allodynia

even if it was i.t.- administered 84 days after spinal PAC1 receptor stimulation¹⁷. These

results suggest that long-lasting spinal astocytic activation underlies the PACAP/PAC1

receptor-induced aversive behaviors and mechanical allodynia. However, how spinal

astrocytes contribute to these nociceptive behaviors is still unknown.

Glycogen is the single largest energy reserve in the CNS, and is predominantly

localized in astrocytes of the intermediate lobe of the pituitary gland²⁹. Among the

neurotransmitters and bioactive substances examined up until today, PACAP was suggested to have potent glycogenolytic activity with an EC_{50} value of 0.08 nM³⁰. To the best of our knowledge, PACAP had the highest efficacy to induce glycogenolysis in cultured astrocytes derived from the cerebral cortex. However, it is not known whether PACAP is involved in the spinal ANLS. Therefore, we aimed to examine possible involvement of PACAP/PAC1 receptor system in the spinal ANLS, and also test whether the spinal ANLS contributes to the PACAP/PAC1 receptor-induced nociceptive behaviors.

RESULTS

PACAP/PAC1 receptor-induced nociceptive behaviors were attenuated by the inhibition of glycogen phosphorylase with DAB

We first examined a possible involvement of spinal ANLS in the PACAP/PAC1 receptor-induced nociceptive behaviors in mice. After a single i.t. administration of Max (50 pmol) or PACAP (100 pmol), aversive behaviors such as licking and biting gradually appeared within 0 ~ 5 min, reached a plateau around 15 ~ 20 min, and was maintained at least for 30 min. The co-injection of DAB, a glycogen phosphorylase (PYGB) inhibitor, dose-dependently (1 ~ 100 pmol) attenuated the development of the

Max- or PACAP-induced aversive behaviors (Fig. 1a, c). In addition, a single i.t. administration of Max or PACAP markedly decreased mechanical threshold (induction of mechanical allodynia) from day 1 (after the cessation of the aversive behaviors), and this decrease persisted at least for 21 days after the administration. The co-injection of DAB with Max or PACAP also dose-dependently attenuated the induction of mechanical allodynia by Max or PACAP (Fig. 1b, d).

Suppression of the PAC1 receptor-evoked nociceptive behaviors by DAB was reversed by i.t. co-injection of L-lactate

We then asked whether the inhibition of the PAC1 receptor-induced nociceptive behaviors by DAB could be reversed by the co-administration of exogenous L-lactate. I.t. injection of L-lactate (1 nmol) in combination with Max (50 pmol) + DAB (100 pmol) significantly reversed the anti-aversive and anti-allodynic effects of DAB on the PAC1 receptor-induced nociceptive behaviors (Fig. 2a, b). Similarly, i.t. supplementation of L-lactate (1 nmol) also blocked the inhibitory effects of DAB (100 pmol) on the PACAP-induced aversive behaviors and mechanical allodynia (Fig. 2c, d). These results suggested that spinal ANLS has a critical role in the PACAP/PAC1 receptor-induced nociceptive behaviors.

133

134 **Possible involvement of PKC in the PACAP/PAC1 receptor-evoked glycogenolysis**
135 **in cultured spinal cord astrocytes**

136 We prepared cultured astrocytes from newborn mice spinal cords, and confirmed that
137 most cells were suggested to be astrocytes, < 1% cells were oligodendrocytes, and no
138 neurons or microglial cells were detected, when visualized with immunocytochemistry
139 for specific markers of individual cells (Fig. S1). In order to identify the receptor
140 subtypes involved in the PACAP-evoked glycogenolysis in the spinal astrocytes, we
141 exposed PACAP, VIP or Max and determined glycogen amount in the cells. Exposure of
142 PACAP (0.0001 ~ 10 nM) or Max (0.1 ~ 10 nM) dose-dependently decreased glycogen
143 amount (Fig. 3a, b), but VIP failed to decrease the glycogen amount even at the
144 concentration of 10 nM (Fig. 3c), suggesting that PAC1 receptor is primarily involved in
145 the glycogenolysis. Interestingly, this pharmacological characteristic of glycogenolysis
146 was very similar to that of the PACAP-induced nociceptive behaviors in mice reported
147 previously^{17, 28}. This PACAP-induced glycogenolysis was completely abolished by the
148 DAB (1 mM) (Fig. 3d), indicating that the glycogenolysis by PACAP would be
149 mediated by PYGB. Since PAC1 receptor can couple with both G_s and G_q proteins,
150 leading to the activation of the protein kinase A (PKA) and PKC, respectively (Deutsch

& Sun, 1992), we co-applied GF109203X as a PKC inhibitor, H89 or Rp-8-Br-cAMPS as a PKA inhibitor, or Sq22.536 as an adenylate cyclase (AC) inhibitor with PACAP. We found that GF109203X (5 μ M) significantly inhibited the PACAP-evoked glycogenolysis (Fig. 3e), but neither PKA inhibitors (Fig. 3f) nor the AC inhibitor (Fig. S2) affected the glycogenolysis. These results suggested that PAC1 receptor might not be coupled with the G_s protein/PKA pathway, and support the significance of the G_q protein/PKC pathway for the PACAP/PAC1 receptor-induced glycogenolysis in our cultured spinal cord astrocytes.

PKC is crucial for the PACAP/PAC1 receptor-evoked lactate secretion in the cultured spinal astrocytes

To investigate whether lactate secretion from astrocytes is increased by the PACAP/PAC1 receptor/PKC pathway, we measured the lactate amounts in the supernatants from the cultured spinal astrocytes after drug treatment. PACAP (1 nM) significantly increased lactate amounts in the supernatant in both 0 ~ 60 min (early phase) and 60 ~ 120 min (late phase) after the exposure (Fig. 4a, b). PMA (100 nM), a PKC activator, significantly elevated lactate secretion in the late phase (Fig. 4c, d), while forskolin (5 μ M), an AC activator, failed to enhance lactate secretion (Fig. 4e, f).

In addition, GF109203X (5 μ M) significantly inhibited the PACAP-activated lactate secretion in both phases (Fig. 4g, h), suggesting the importance of PKC, but not PKA, in the PACAP-activated lactate secretion. Furthermore, the inhibition of glycogenolysis by DAB significantly decreased the lactate amount in the supernatant from the astrocytes particularly in the late phase (Fig. 4i, j), indicating that glycogenolysis is also inevitable for the lactate secretion. These results pointed out that PACAP/PAC1 receptor/PKC pathway is a key signaling mechanism for the spinal ANLS activation.

Blockade of the PACAP/PAC1 receptor-induced nociceptive behaviors by the PKC inhibitor, GF109203X

In order to investigate whether PACAP/PAC1 receptor-induced nociceptive behaviors were also mediated through PKC pathway, effects of GF109203X was investigated. Both the aversive behaviors and the mechanical allodynia evoked by PACAP were dose-dependently inhibited by the simultaneous injection of GF109203X at a concentration range from 1 to 100 pmol (Fig. 5a, b), suggesting the importance of PKC activity in the PACAP/PAC1 receptor-induced nociceptive behaviors.

Pharmacological inhibition of monocarboxylate transporters attenuated the

PACAP/PAC1 receptor-induced nociceptive behaviors

We further asked whether lactate transport participated in the PACAP/PAC1 receptor-induced nociceptive behaviors. L-lactate is one of the substrates of MCTs, and MCTs are found in the brain where three isoforms — MCT1, MCT2 and MCT4 — have been described. Although the distribution patterns of these MCTs are not well-known in the spinal cord, each of these isoforms is suggested to exhibit a distinct regional and cellular distribution in rodent brain. At the cellular level, MCT1 is known to be expressed by endothelial cells of microvessels, by ependymocytes as well as by astrocytes. MCT4 expression appears to be specific for astrocytes. By contrast, the predominant neuronal MCT is suggested to be MCT2³¹. It is reported that AR-C155858 potently inhibits MCT1 and MCT2, but not MCT4 (Ovens et al., 2010). In our cultured spinal astrocytes, AR-C155858 (1 μ M) significantly inhibited lactate secretion evoked by PACAP at both phases (Fig. 6a, b). Simultaneous i.t. injection of AR-C155858 (1 nmol) with PACAP significantly inhibited the development of the aversive behaviors (Fig. 6c) and mechanical allodynia (Fig. 6d). Intriguingly, i.t. administration of AR-C155858 transiently alleviated the mechanical allodynia at 7 days after i.t. injection of PACAP (Fig. 6e).

Reversal of spinal nerve injury (SNL)-induced mechanical allodynia by the PYGB inhibitor, DAB, and MCT inhibitor, AR-C155858

Several lines of evidence indicate that spinal PACAP/PAC1 receptor could play an important role in the induction of peripheral nerve injury-induced neuropathic pain. For instance, development of neuropathic pain-like behaviors induced by SNL was shown to be abrogated in PACAP-deficient mice²⁵, and SNL-induced mechanical allodynia was ameliorated by the treatment with i.t. PACAP₆₋₃₈, a peptide antagonist of PAC1/VPAC2³². Recently, we developed several small-molecule PAC1 receptor selective antagonists^{33, 34} and showed that both i.t. and systemic (peroral) administration of these small-molecule antagonists significantly reversed SNL-induced mechanical allodynia³³. Thus, we finally tested the effects of DAB and AR-C155858 on SNL-induced mechanical allodynia. In this study, behavioral studies were performed with mice at 14 days after SNL (Fig. 7). The SNL induced marked mechanical hypersensitivity of the hindpaw ipsilateral to the ligation and i.t.-administration of DAB (100 pmol) significantly reversed the mechanical allodynia (Fig. 7a). Furthermore, i.t.-treatment of AR-C155858 (1 ~ 3 nmol) produced dose-dependent decreases in the mechanical allodynia (Fig. 7b). These results suggested that spinal ANLS activation might contribute to the pathogenic mechanism of peripheral nerve injury-induced

neuropathic pain.

DISCUSSION

In this study, we have further characterized the nociceptive behaviors induced by the spinal PAC1 receptor activation and made the following findings: 1) PACAP could be an endogenous inducer for spinal ANLS activation; 2) PAC1/PKC signaling played an important role in the PACAP-evoked spinal ANLS; 3) the spinal ANLS activation contributed to PACAP-evoked nociceptive behaviors such as aversive behaviors and mechanical allodynia; and 4) the spinal ANLS activity would be involved in the generation of SNL-induced mechanical allodynia.

In the present study, we showed that the simultaneous i.t. injection of DAB, the inhibitor of glycogenolysis, blocked the development of aversive behaviors and mechanical allodynia evoked by Max or PACAP, and this inhibition by DAB was reversed by the combinational injection of L-lactate. These results suggested that the ANLS activation may have a significant role in the nociceptive behaviors by PACAP/PAC1 receptor activation. Chronic pain may need maladaptive synaptic plasticity in neural circuits^{35, 36}. An in vivo electrophysiological study showed that DAB inhibited the long-term

241 potentiation by tetanus stimulus in hippocampal Schaffer collateral-area CA1, and this
242 impairment by DAB was rescued with the exogenous L-lactate⁴. Furthermore, L-lactate
243 alone was shown to potentiate AMPA receptor- or NMDA receptor-mediated inward
244 currents, increase intracellular calcium, and also upregulate the markers for neuronal
245 activation^{4, 8, 9}. Thus, PACAP/PAC1 receptor-evoked spinal ANLS might potentiate
246 AMPA/NMDA receptors-mediated responses and induce long-term potentiation, which
247 might lead to aversive behaviors and long-lasting mechanical allodynia. Regarding the
248 initiation and maintenance of inflammatory and neuropathic pain, recent progresses also
249 focus on the critical roles of astrocytes in the spinal cord and brain^{37, 38}. Previously, we
250 showed that i.t. injection of Max or PACAP induced rapid and prolonged upregulation
251 of spinal GFAP expression level in parallel with the aversive behaviors and long-lasting
252 mechanical allodynia, and simultaneous application of L- α -aminoadipate, an astrocyte
253 toxin, with Max or PACAP markedly suppressed the aversive behaviors and the
254 mechanical allodynia^{17, 28}. Interestingly, it is suggested that glycogen metabolism is
255 associated with maturation of astrocytes, in which the expressional levels of GFAP and
256 glycogen synthase or phosphorylase are increased in parallel³⁹. In accordance with this
257 notion, our present study revealed that ANLS activation indeed contributed to both
258 development and maintenance of PACAP/PAC1 receptor-evoked nociceptive behaviors,

since simultaneous i.t. injection of DAB and AR-C155858 could prevent the development of the PACAP-induced aversive behaviors and mechanical allodynia. Furthermore, i.t. administration of DAB and AR-C155858 at 7 or 14 days after i.t. PACAP injection or SNL surgery, respectively, ameliorate the evoked-mechanical allodynia. Although further study is needed to clarify the persistency of ANLS activation and its role in the long-lasting mechanical allodynia, these results suggested the therapeutic potential of the ANLS inhibitors for chronic pain treatments.

Glycogen contained in cultured spinal cord astrocytes was significantly decreased by the Max or PACAP, but not VIP, exposure. These results suggested that PAC1 receptor, but not VPAC1 nor VPAC2 receptor, was responsible for activating glycogenolysis. In rat atrial myocytes, a PKC inhibitor peptide reduced the PACAP-induced K_{ATP} currents without affecting the VIP-induced K_{ATP} currents⁴⁰, indicating selective coupling of PKC with PAC1 receptor. Indeed, in our present study, PACAP-evoked glycogenolysis was significantly inhibited by the PKC inhibitor, GF109203X. These results suggested that PAC1 receptor/PKC signaling pathway is important for the glycogenolysis activation. Previous reports which observed glycogenolysis in chick brain or cultured mouse cortical astrocytes suggested that cAMP/PKA signaling pathway phosphorylated

277 glycogen phosphorylase and evoked glycogenolysis^{41, 42}. But in our cultured spinal
278 astrocytes, both PKA inhibitors and AC inhibitor failed to prevent the PACAP-evoked
279 glycogenolysis. Although the reason why PACAP-evoked glycogenolysis is not
280 sensitive to the AC and PKA inhibitors is currently unknown, this may be due to the
281 absence of the coupling of PACAP/PAC1 receptor to G_s protein in the spinal astrocytes.
282 But further study is required to verify this possibility.

283

284 We found that developments of the PACAP/PAC1 receptor-induced aversive behaviors
285 and mechanical allodynia were significantly attenuated by the PKC inhibition with
286 GF109203X. In cultured astrocytes, PACAP and PMA significantly enhanced lactate
287 secretion. In addition, GF109203X and DAB significantly inhibited the
288 PACAP-enhanced lactate secretion. These results proposed that PKC and a downstream
289 glycogenolysis were important in the PACAP-enhanced lactate secretion and
290 nociceptive behaviors. Many lines of evidence strongly suggested the significance of
291 PKC in inflammatory and neuropathic pain⁴³. Double labeling immunofluorescence
292 showed that phospho-PKC- δ co-localized with astrocytic markers in spinal cord after
293 spinal nerve injury⁴⁴. These studies suggest that astrocytic PKC contributes to the
294 nociceptive behaviors. Although there are few evidence about the contribution of PKC

in astrocytic MCT activation, it is previously reported that the PKC activation resulted in an increase in the levels of MCT1 and MCT4 and the release of lactate in an in vitro skeletal muscle cells (RD cells), while the PKA activation resulted in a significant decrease in MCT1 expression in the RD cells as well as endothelial cells^{45, 46, 47}. Importantly, the mechanical allodynia by selective astrocytic G_q-PKC activation was ameliorated by pre-treatment with a MCT inhibitor, 4-CIN (α -cyano-4-hydroxycinnamate)¹⁰. These results indicate that PKC-induced lactate secretion through MCTs might be involved in the PACAP/PAC1 receptor-evoked nociceptive behaviors.

It may be worth noting our previous data here that i.t. injection of the PKA inhibitor, Rp-8-Br-cAMPS, could ameliorate the PACAP-induced aversive behaviors²⁸. Currently it may be difficult to precisely reconcile with the present observation that the PKA inhibitor failed to prevent PACAP-evoked glycogenolysis in the spinal astrocytes, but, for example, we could speculate that neuronal PKA signaling would also contribute to the expression of the nociceptive behaviors. However, further extensive studies are necessary to uncover the interaction of PKC and PKA signaling in the PACAP/PAC1 receptor-induced nociceptive behaviors.

313

314 It may be noteworthy that DAB significantly inhibited the lactate secretion in the late
315 phase after PACAP exposure, while the inhibitory effect was relatively weak in the early
316 phase. Our results may be consistent with a previous report that DAB significantly
317 attenuated the lactate secretion during 60 ~ 180 min after the hypoglycemia on the
318 astrocytes/neurons mixed culture, while equivalent amount of lactate was secreted
319 irrespective of the presence of DAB during 0 ~ 60 min, even DAB completely inhibited
320 glycogenolysis at both timings⁴⁸. Thus, these observations may indicate that the secreted
321 lactate in the late phase is originated from glycogenolysis, and the secretion in the early
322 phase may be partly derived from the readily releasable pool of lactate in the cultured
323 spinal astrocytes. However, the delayed in vitro inhibition of lactate secretion by DAB
324 is difficult to reconcile with the relatively rapid effect on the PACAP-induced aversive
325 behaviors, and further studies under the experimental setting close to in vivo condition
326 would be necessary in the future to answer the discrepancy.

327

328 In summary, the present study suggested that the interaction between spinal dorsal horn
329 neurons and astrocytes evoked by PACAP/PAC1 receptor-induced ANLS activation
330 could be critically involved in the development and maintenance of the nociceptive

behaviors. The signaling pathway linking PAC1 receptor activation and ANLS might be at least partially mediated by PKC pathway. Although verifying the possible involvement of PACAP/PAC1 receptor signaling in human pain requires further rigorous studies, targeting spinal ANLS may provide a new opportunity to treat intractable chronic pain.

MATERIALS AND METHODS

Animals

Male ddY mice (6 ~ 12 weeks old) or pregnant female ddY mice were purchased from Kyudo Co. Ltd. (Kumamoto, Japan) and housed under controlled temperature ($24 \pm 1^{\circ}\text{C}$) and humidity ($55 \pm 10\%$) with a 12-h light/dark cycle with food and water freely available.

Ethics

The animal experiments were approved by the Animal Care Committee of Kagoshima University (approval nos. MD15082 and MD18003), and were conducted in accordance with the ethical guidelines for the study of experimental pain in conscious animals of the International Association for the Study of Pain.

349

350 **I.t. injection and behavioral observation**

351 I.t. injection was given in a volume of 5 μ l by percutaneous puncture through an
352 intervertebral space at the level of the fifth or sixth lumbar vertebra, according to a
353 previously reported procedure¹⁷. An investigator, who was unaware of the drug
354 treatment, performed all of the behavioral experiments.

355

356 Aversive behavior was evaluated according to our previous report²⁸. Before i.t.
357 injection, mice were placed and habituated in a glass cylinder (ϕ 14 $\times$ 18 cm) with a
358 filter paper at the bottom for 20 min. Immediately after i.t. injection, the mice were
359 placed again in the same glass cylinder and the number of aversive behaviors consisting
360 of licking, biting and scratching directed toward the caudal part of the body was counted
361 every 1 min. The cumulative number of events was pooled for 5 min bins of observation
362 and analyzed.

363

364 The assessment of mechanical thresholds was also carried out according to the
365 previously described methods¹⁷. Briefly, mechanical sensitivity was evaluated with
366 calibrated von Frey hairs (Stoelting, Wood Dale, IL) by measuring the tactile stimulus

producing a 50% likelihood of hind paw withdrawal response (50% gram threshold), which was determined using the up-down paradigm⁴⁹.

Drugs

PACAP (38 amino acid form) and VIP were purchased from Peptide Institute Inc. (Osaka, Japan). Max was kindly donated by Dr. M Tajima (Shiseido, Japan). DAB, GF109203X and phorbol 12-myristate 13-acetate (PMA) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Forskolin and AR-C155858 were from Merck Millipore (Darmstadt, Germany). H89 was from Seikagaku Co. (Tokyo, Japan). L-lactate and Rp-8-Br-cAMPS were from Sigma-Aldrich Co. LLC (St. Louis, MO). Sq22.536 was from Enzo Biochem Inc. (Farmingdale, NY). These drugs were made up as concentrated stock solution in MilliQ water, 0.1 M acetic acid or dimethyl sulfoxide, aliquoted, and stored at –30°C and diluted just before use. The aliquot of drugs were diluted to the desired concentration in artificial cerebrospinal fluid (ACSF: NaCl 138 mM, KCl 3 mM, CaCl₂ 1.25 mM, MgCl₂ 1 mM, D-glucose 1 mM) immediately prior to use. The doses for i.t. injection of PACAP (100 pmol) and Max (50 pmol) we chose in this study were determined according to our previous reports^{17, 27, 28}.

Cultured spinal cord astrocytes

Spinal cords from postnatal day 1 and 2 mice were dispersed by 0.25% trypsin and subsequently plated on culture flasks which were previously coated with 0.75 µg/mL poly-L-lysine (Sigma-Aldrich, St. Louis, MO). Cells were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque, Osaka, Japan) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT) until cell density reaching to confluent. After reaching to confluent, culture flasks were shaken at 240 rpm for 6 h to remove microglia and oligodendrocyte precursor cells. Remained astrocytes on culture flasks were then detached and subsequently plated on the appropriate culture dishes at the density of 1×10^4 cells/cm² and used when the cell number reaching to the confluent again, which usually took 3 days. All the cultured astrocytes used in the current experiments were passaged by 3 or 4 times. All the cell-based experiments were carried out at 37°C.

Immunocytochemistry

Spinal cord astrocytes were plated on cover slips (Matsunami Glass, Tokyo, Japan). According to the reported protocols for immunocytochemistry⁵⁰, cells were fixed for 10 min with 4% paraformaldehyde, washed with PBS, and incubated in PBS containing 5% bovine serum albumin and 0.3% Triton X-100 for 60 min. Cells were then incubated

with antibodies specific for GFAP (Merck Millipore, AB5804, 1/1,000), microtubule associated protein-2 (MAP2, Sigma-Aldrich Co. LLC, M4403, AB_477193, 1/1,000), ionized calcium binding adaptor molecule-1 (Iba1, Wako Pure Chemical Industries, Ltd., 019-19741, AB_839504, 1/1,000) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase, Abcam, ab6319, AB_2082593, 1/1,000), followed by anti-mouse IgG and anti-rabbit IgG secondary antibodies coupled to CF488 and CF555 (Biotium, Hayward, CA, USA). Samples were mounted with CC/mount (Diagnostic BioSystems, Pleasanton, CA). The fluorescent signals were observed using a fluorescence microscope (BZ-X700: Keyence, Osaka, Japan).

Glycogen assay

Cultured spinal astrocytes were exposed to PACAP, VIP or Max for 1 h, and harvested by scratching. Immediately after harvest, samples were then boiled for 10 min, sonicated, and centrifuged. Glycogen amount in the supernatant was measured by the Glycogen Assay Kit (BioVision Inc., San Francisco, CA) according to the manufacturer's instruction. The exposure of DAB, H89, Rp-8-Br-cAMPS, Sq22.536 or GF109203X was started 30 min before PACAP exposure. The amount of glycogen was normalized by the amount of the protein contained in the samples, which was measured

by the Bradford's assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.

Lactate assay

Culture medium was replaced Krebs-Ringer buffer (NaCl 135 mM, KCl 5 mM, CaCl₂ 1 mM, MgSO₄ 1 mM, KH₂PO₄ 0.4 mM, D-glucose 5.5 mM, HEPES 20 mM). The astrocytes were then exposed to PACAP, PMA or forskolin for 60 min, and conditioned buffer was harvested (0 ~ 60 min, designated as "early phase"). Subsequently, fresh Krebs-Ringer buffer with the drugs was added on the cultured astrocytes again, and harvested the conditioned buffer for another 60 min (60 ~ 120 min, designated as "late phase"). The time course was determined according to the previous paper which suggested DAB inhibited lactate secretion in 60 ~ 180 min after the treatment, but not in 0 ~ 60 min⁴⁸. Lactate amount in the conditioned Krebs-Ringer buffer was measured by the L-lactate Assay Kit (BioVision Inc.) according to the manufacturer's instruction. The exposure of DAB or GF109203X was started 30 min before PACAP treatment.

Spinal nerve ligation (SNL) surgery

To produce peripheral neuropathic pain, SNL was carried out as described^{51, 52}. Briefly,

a midline incision was made in the skin of the back at the L4-S2 levels and the right paraspinal muscles were separated from the spinous processes, facet joints and transverse processes at the L4-S1 levels. The right L4-L5 transverse processes were removed, and the right L4 and L5 spinal nerves were ligated tightly with 8-0 silk thread.

Statistical analysis

Experimental data are expressed as mean $\pm$ SEM. For mechanical threshold analyses, we employed the Mann-Whitney U-test for single comparisons or the Friedman test followed by the Steel or Steel-Dwass test for multiple comparisons. For other analyses, single comparisons were made using the Student two-tailed unpaired *t*-test, and for multiple comparisons, one-way analysis of variance followed by the Dunnett or Tukey test was used. $P < 0.05$ was considered statistically significant.

COMPETING INTERESTS

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The raw source data for the main figures are included in Supplementary Data. Any other

data not included in the paper is available from the authors upon reasonable request.

The corresponding author, Dr. Takashi Kurihara will provide the requested data.

CODE AVAILABILITY STATEMENT

The authors declare no used code.

AUTHOR CONTRIBUTIONS

YK carried out experiments, performed statistical analysis, and drafted the manuscript.

MY carried out experiments and performed statistical analysis. TK conceived, participated in the design of the study, performed behavioral studies, and wrote the manuscript. AM and IT participated in the design of the study and reviewed the manuscript. All authors read and approved the final manuscript.

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673

FIGURE LEGENDS

Fig. 1 A critical role of glycogenolysis in PACAP/PAC1 receptor-evoked nociceptive behaviors. Simultaneous i.t. injection of DAB (1 or 10 pmol), a glycogenolysis inhibitor, with Max (50 pmol), blocked the induction of aversive behaviors (a) and mechanical allodynia (b). Simultaneous i.t. injection of DAB (1 ~ 300 pmol) with PACAP (100 pmol) prevented the induction of aversive behaviors (c) and mechanical allodynia (d). *P<0.05 and **P<0.01 when compared with ACSF data. #P<0.05 and ##P<0.01 when compared with Max + Vehicle in (a) and (b) or PACAP + Vehicle in (c) and (d). Statistical significance was evaluated by the Tukey test for (a) and (c), and Steel test for (b) and (d).

Fig. 2 Simultaneous injection of L-lactate reversed the inhibitory effects of DAB on the PAC1 receptor-induced nociceptive behaviors.

I.t. co-administration of L-lactate (1 nmol) with DAB (10 or 100 pmol) reinstated Max (a, b)- and PACAP (c, d)-induced aversive behaviors (a, c) and mechanical allodynia (b, d). *P<0.05 and **P<0.01 when compared with ACSF data. #P<0.05 and ##P<0.01 when compared with Max + Vehicle in (a) and (b) or PACAP + Vehicle in (c) and (d). \$P<0.05 and \$\$P<0.01 when compared with DAB-treated data. Statistical significance was evaluated by the Tukey test for (a) and (c), and Steel test for (b) and (d).

Fig. 3 PACAP/PAC1 receptor/PKC signaling pathway induced glycogenolysis in cultured spinal cord astrocytes.

Cultured astrocytes were exposed to PACAP (0.0001 ~ 1 nM) (**a**), Max (0.1 ~ 10 nM) (**b**) or VIP (0.001 ~ 10 nM) (**c**), and glycogen amounts contained in the astrocytes were measured 60 min after the exposure. Cultured astrocytes were pre-incubated with DAB (1 mM, **d**), GF109203X (GF, 5 μ M, **e**), H89 (10 μ M, **f**), or Rp-8Br-cAMPS (RP, 100 μ M, **f**) for 30 min prior to PACAP (1 nM) exposure, and glycogen amounts contained in the astrocytes were measured 60 min after the PACAP exposure. *P<0.05 and **P<0.01 when compared with 0 nM (Vehicle) data in (**a**) ~ (**c**), and compared groups are indicated above in (**d**) ~ (**f**). Statistical significance was evaluated by the Dunnett test for (**a**) ~ (**c**), and Tukey test for (**d**) ~ (**f**). Exact sample sizes are indicated in the graphs.

Fig. 4 A crucial role of PACAP/PAC1 receptor/PKC-induced glycogenolysis in the lactate secretion from cultured spinal astrocytes.

After the treatment with PACAP (1 nM) (a, b), PMA (100 nM) (c, d) or forskolin (5 μ M) (e, f), the supernatants from the cultured spinal astrocytes were differentially harvested during 0 ~ 60 min (a, c, e) or 60 ~ 120 min (b, d, f), and lactate amounts were measured. Cultured astrocytes were pre-incubated with GF109203X (GF, 5 μ M, g, h) or DAB (1 mM, i, j) for 30 min prior to PACAP exposure, and then exposed to PACAP (1 nM) for 120 min. Culture supernatants were differentially harvested during 0 ~ 60 min (g, i) or 60 ~ 120 min (h, j) after the PACAP addition, and lactate amounts were measured. * $P < 0.05$ and ** $P < 0.01$ when compared with vehicle data in (a) ~ (f), and compared groups are indicated above in (g) ~ (j). Statistical significance was evaluated

721 by the Student t-test for **(a)** ~ **(f)**, and Tukey test for **(g)** ~ **(j)**. Exact sample sizes are

722 indicated in the graphs.

723

Fig. 5 The importance of PKC in the PACAP/PAC1 receptor-induced nociceptive behaviors.

Simultaneous i.t. injection of GF109203X (1 ~ 100 pmol) with PACAP (100 pmol) dose-dependently reduced the PACAP-induced aversive behaviors (a) and mechanical allodynia (b). * $P < 0.05$ and ** $P < 0.01$ when compared with ACSF data. # $P < 0.05$ and ## $P < 0.01$ when compared with PACAP + Vehicle data. Statistical significance was evaluated by the Tukey test for (a), and Steel test for (b).

Fig. 6 Spinal MCTs contributed to both induction and maintenance of the PACAP/PAC1

receptor-induced nociceptive behaviors.

Cultured spinal astrocytes were pre-incubated with AR-C155858 (AR-C, 1 μ M) 30 min prior to PACAP exposure, and then exposed to PACAP (1 nM) for 120 min. The culture supernatants were differentially harvested during 0 ~ 60 min (**a**) or 60 ~ 120 min (**b**) after the PACAP addition, and lactate amounts in the culture supernatants were measured. * $P < 0.05$ and ** $P < 0.01$ (compared groups are indicated above). Exact sample sizes are indicated in the graphs. I.t. co-administration of AR-C155858 (1 nmol) with PACAP reduced the expression of the aversive behaviors (**c**) and mechanical allodynia (**d**). # $P < 0.05$ and ### $P < 0.01$ when compared with PACAP + Vehicle data. (**e**) Transient reversal of the PACAP-evoked mechanical allodynia by i.t. AR-C155858. AR-C155858 was injected 7 days after i.t. PACAP. ** $P < 0.01$ when compared with pre-PACAP data. # $P < 0.05$ and ### $P < 0.01$ when compared with vehicle-treated control. Statistical significance was evaluated by the Tukey test for (**a**) and (**b**), Student t-test for (**c**), Mann–Whitney U test for (**d**) and Steel test for (**e**).

Fig. 7 Requirement of spinal ANLS in the maintenance of SNL-induced mechanical allodynia.

(a) Transient reversal of SNL-evoked mechanical allodynia by i.t. DAB. DAB was injected 14 days after SNL surgery. * $P < 0.05$ and ** $P < 0.01$ when compared with Day 0 or time 0 data. # $P < 0.05$ and ## $P < 0.01$ when compared with vehicle-treated control.

(b) Significant anti-allodynic effects of i.t. AR-C155858 on SNL-induced neuropathic

757 pain model mice. AR-C155858 was injected 14 days after SNL surgery. *P<0.05 and
758 **P<0.01 when compared with Day 0 or time 0 data. #P<0.05 and ##P<0.01 when
759 compared with vehicle-treated control. +P<0.05 when compared with 1 nmol
760 AR-C155858-treated group. Statistical significance was evaluated by the
761 Mann–Whitney U test for comparison between pre- and post-SNL surgery, and
762 Steel-Dwass test for comparison between pre- and post-drug injection.

763

Fig. S1. Representative immunofluorescent images of the cultured spinal astrocytes. The purity of astrocytes was examined by staining with primary antibodies against GFAP (an astrocyte marker), MAP2 (a neuron marker), Iba 1 (a microglia marker) and CNPase (an oligodendrocyte marker). Each marker was visualized by an appropriate fluorescent dye-conjugated secondary antibody. Arrowhead represented an incidentally contaminated oligodendrocyte. Bar = 200 µm.

Fig. S2. PACAP/PAC1 receptor-evoked glycogenolysis might not be mediated by G_s

protein/PKA pathway in the cultured spinal astrocytes.

Cultured spinal astrocytes were pre-incubated with Sq22.536 (Sq, 100 μ M) for 30 min

prior to PACAP (1 nM) exposure, and glycogen amounts contained in the astrocytes

were measured 60 min after the PACAP exposure. ** $P < 0.01$ when compared with

Vehicle. Statistical significance was evaluated by the Dunnett test. Exact sample sizes

are indicated in the graphs.

Figures

Figure 1

A critical role of glycogenolysis in PACAP/PAC1 receptor-evoked nociceptive behaviors. Simultaneous i.t. injection of DAB (1 or 10 pmol), a glycogenolysis inhibitor, with Max (50 pmol), blocked the induction of aversive behaviors (a) and mechanical allodynia (b). Simultaneous i.t. injection of DAB (1 ~ 300 pmol) with PACAP (100 pmol) prevented the induction of aversive behaviors (c) and mechanical allodynia (d). * $P < 0.05$ and ** $P < 0.01$ when compared with ACSF data. # $P < 0.05$ and ## $P < 0.01$ when compared with Max + Vehicle in (a) and (b) or PACAP + Vehicle in (c) and (d). Statistical significance was evaluated by the Tukey test for (a) and (c), and Steel test for (b) and (d).

Figure 2

Simultaneous injection of L-lactate reversed the inhibitory effects of DAB on the PAC1 receptor-induced nociceptive behaviors. I.e. co-administration of L-lactate (1 nmol) with DAB (10 or 100 pmol) reinstated Max (a, b)- and PACAP (c, d)-induced aversive behaviors (a, c) and mechanical allodynia (b, d). * $P < 0.05$ and ** $P < 0.01$ when compared with ACSF data. # $P < 0.05$ and ## $P < 0.01$ when compared with Max + Vehicle in (a) and (b) or PACAP + Vehicle in (c) and (d). \$ $P < 0.05$ and \$\$ $P < 0.01$ when compared with DAB-treated data. Statistical significance was evaluated by the Tukey test for (a) and (c), and Steel test for (b) and (d).

Figure 3

PACAP/PAC1 receptor/PKC signaling pathway induced 697 glycogenolysis in cultured spinal cord astrocytes. Cultured astrocytes were exposed to PACAP (0.0001 ~ 1 nM) (a), Max (0.1 ~ 10 nM) (b) or VIP (0.001 ~ 10 nM) (c), and glycogen amounts contained in the astrocytes were measured 60 min after the exposure. Cultured astrocytes were pre-incubated with DAB (1 mM, d), GF109203X (GF, 5 μM, e), H89 (10 μM, f), or Rp-8Br-cAMPS (RP, 100 μM, f) for 30 min prior to PACAP (1 nM) exposure, and glycogen

amounts contained in the astrocytes were measured 60 min after the PACAP exposure. * $P < 0.05$ and ** $P < 0.01$ when compared with 0 nM (Vehicle) data in (a) ~ (c), and compared groups are indicated above in (d) ~ (f). Statistical significance was evaluated by the Dunnett test for (a) ~ (c), and Tukey test for (d) ~ (f). Exact sample sizes are indicated in the graphs.

Figure 4

A crucial role of PACAP/PAC1 receptor/PKC-induced glycogenolysis in the lactate secretion from cultured spinal astrocytes. After the treatment with PACAP (1 nM) (a, b), PMA (100 nM) (c, d) or forskolin (5 μ M) (e, f), the supernatants from the cultured spinal astrocytes were differentially harvested during 0 ~ 60 min (a, c, e) or 60 ~ 120 min (b, d, f), and lactate amounts were measured. Cultured astrocytes were pre-incubated with GF109203X (GF, 5 μ M, g, h) or DAB (1 mM, i, j) for 30 min prior to PACAP exposure, and then exposed to PACAP (1 nM) for 120 min. Culture supernatants were differentially harvested during 0 ~ 60 min (g, i) or 60 ~ 120 min (h, j) after the PACAP addition, and lactate amounts were measured. * $P < 0.05$ and ** $P < 0.01$ when compared with vehicle data in (a) ~ (f), and compared groups are indicated above in (g) ~ (j). Statistical significance was evaluated by the Student t-test for (a) ~ (f), and Tukey test for (g) ~ (j). Exact 721 sample sizes are indicated in the graphs.

Figure 5

The importance of PKC in the PACAP/PAC1 receptor-induced nociceptive behaviors. Simultaneous i.t. injection of GF109203X (1 ~ 100 pmol) with PACAP (100 pmol) dose-dependently reduced the PACAP-induced aversive behaviors (a) and mechanical allodynia (b). * $P < 0.05$ and ** $P < 0.01$ when compared with ACSF data. # $P < 0.05$ and ## $P < 0.01$ when compared with PACAP + Vehicle data. Statistical significance was evaluated by the Tukey test for (a), and Steel test for (b).

Figure 6

Spinal MCTs contributed to both induction and maintenance of the PACAP/PAC1 receptor-induced nociceptive 735 behaviors. Cultured spinal astrocytes were pre-incubated with AR-C155858 (AR-C, 1 μ M) 30 min prior to PACAP exposure, and then exposed to PACAP (1 nM) for 120 min. The culture supernatants were differentially harvested during 0 ~ 60 min (a) or 60 ~ 120 min (b) after the PACAP addition, and lactate amounts in the culture supernatants were measured. $*P < 0.05$ and $**P < 0.01$ (compared groups are indicated above). Exact sample sizes are indicated in the graphs. I.t. co-administration of AR-C155858 (1 nmol) with PACAP reduced the expression of the aversive behaviors (c) and mechanical allodynia (d). $\#P < 0.05$ and $##P < 0.01$ when compared with PACAP + Vehicle data. (e) Transient reversal of the PACAP-evoked mechanical allodynia by i.t. AR-C155858. AR-C155858 was

injected 7 days after i.t. PACAP. $**P<0.01$ when compared with pre-PACAP data. $\#P<0.05$ and $\#\#P<0.01$ when compared with vehicle-treated control. Statistical significance was evaluated by the Mann–Whitney U test for (d) and Steel test for (e).

Figure 7

Requirement of spinal ANLS in the maintenance of SNL-induced mechanical allodynia. (a) Transient reversal of SNL-evoked mechanical allodynia by i.t. DAB. DAB was injected 14 days after SNL surgery.

*P<0.05 and **P<0.01 when compared with Day 0 or time 0 data. #P<0.05 and ##P<0.01 when compared with vehicle-treated control. (b) Significant anti-allodynic effects of i.t. AR-C155858 on SNL-induced neuropathic pain model mice. AR-C155858 was injected 14 days after SNL surgery. *P<0.05 and **P<0.01 when compared with Day 0 or time 0 data. #P<0.05 and ##P<0.01 when compared with vehicle-treated control. +P<0.05 when compared with 1 nmol AR-C155858-treated group. Statistical significance was evaluated by the Mann–Whitney U test for comparison between pre- and post-SNL surgery, and Steel-Dwass test for comparison between pre- and post-drug injection.

Supplementary Files

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