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Spinal astrocyte-neuron lactate shuttle contributes to the PACAP/PAC1 receptor-induced nociceptive behaviors

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Keywords: pain, pituitary adenylate cyclase-activating polypeptide, astrocyte-neuron lactate shuttle, protein kinase C, monocarboxylate transporter, glycogenolysis

Abbreviations used: artificial cerebrospinal fluid (ACSF), adenylate cyclase (AC), astrocyte-neuron lactate shuttle (ANLS), central nervous system (CNS), glial fibrillary acidic protein (GFAP), intrathecal (i.t.), maxadilan (Max), monocarboxylate transporter (MCT), pituitary adenylate cyclase-activating polypeptide (PACAP), protein kinase A (PKA), protein kinase C (PKC), phorbol 12-myristate 13-acetate (PMA), glycogen phosphorylase (PYGB), vasoactive intestinal polypeptide (VIP)

Abstract Previously, we showed that spinal pituitary adenylate cyclase-activating polypeptide (PACAP)/PAC1 receptor signaling triggers long-lasting pain behaviors through astroglial activation. Since astrocyte-neuron lactate shuttle (ANLS) could be essential for long-term synaptic facilitation, we aimed to elucidate a possible involvement of spinal ANLS in the development of the PACAP/PAC1 receptor-induced pain behaviors. A single intrathecal administration of PACAP induced short-term spontaneous aversive behaviors, followed by long-lasting mechanical allodynia. These pain behaviors were inhibited by DAB, an inhibitor of glycogenolysis, and this inhibition was reversed by simultaneous L-lactate application. In the cultured spinal astrocytes, the PACAP-evoked glycogenolysis and lactate secretion were inhibited by a protein kinase C (PKC) inhibitor, and the PKC inhibitor attenuated the PACAP-induced pain behaviors. Finally, an inhibitor for the monocarboxylate transporters blocked the lactate secretion from the spinal astrocytes and inhibited the PACAP-induced pain behaviors. These results suggested that PAC1 receptor-PKC-ANLS signaling is involved in the PACAP-induced pain behaviors.

Introduction

Transfer of lactate from astrocytes to neurons is activated when synaptic activity is increased, and this mechanism is now known as the astrocyte-neuron lactate shuttle (ANLS), that could account for the coupling between synaptic activity and energy delivery (*Magistretti and Allaman, 2015*). Since lactate secretion from astrocytes is mainly originated from glycogen via glycogenolysis rather than from glycolysis (*Dringen et al., 1993*), inhibition of glycogenolysis or lactate transport in rat hippocampus significantly attenuated the working memory and long-term fear memory (*Newman et al., 2011; Suzuki et al., 2011*). In addition, a brain-specific glycogen synthase knockout mouse showed a significant deficiency in the acquisition with an associative learning task and concomitantly, activity-dependent changes in hippocampal synaptic strength (*Duran et al., 2013*). It is also reported that ANLS in amygdala is critical for the reconsolidation of cocaine memory in rats (*Boury-Jamot et al., 2015; Zhang et al., 2015*). At the cellular level, lactate may potentiate NMDA receptor-mediated currents in cultured neurons and neurons in the nucleus of the solitary tract (*Nagase et al., 2014; Yang et al., 2014*). These findings suggested that ANLS could contribute to many forms of neuronal plasticity in the CNS, including plastic changes in the spinal nociceptive transmission. However, it is still unclear what kinds of

65 neurotransmitters evoke the ANLS activation during any forms of neuronal plasticity.

66

67 Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated
68 from ovine hypothalamic extracts based on its ability to stimulate adenylate cyclase in
69 rat anterior pituitary cell cultures (*Miyata et al., 1989; Miyata et al., 1990*). In normal
70 state, PACAP specific receptor, PAC1 receptor, is particularly abundant in central
71 nervous system (CNS) including spinal dorsal horn (*Dickinson et al., 1999; Jongsma et*
72 *al., 2000; Sakashita et al., 2001; Vaudry et al., 2009; Yokai et al., 2016*), where
73 PACAP-immunoreactive fibers are also considerably localized (*Moller et al., 1993;*
74 *Dun et al., 1996a; Dun et al., 1996b; Narita et al., 1996*), and PACAP
75 mRNA/immunoreactivity in rat dorsal root ganglia is markedly upregulated in
76 peripheral nerve injury or inflammation (*Zhang et al., 1995; Zhang et al., 1998;*
77 *Jongsma et al., 2003; Mabuchi et al., 2004*). These observations coupled with other
78 lines of evidence propose that PACAP/PAC1 receptor system could play an important
79 role in the modulation of spinal nociceptive transmission.

80

81 We have previously demonstrated in mice that a single intrathecal (i.t.) injection of
82 PACAP or a PAC1 receptor specific agonist, maxadilan (Max) (*Moro and Lerner, 1997*),

induced spontaneous aversive behaviors, such as licking, biting, and scratching directed toward the caudal part of the body for more than 30 min (*Shimizu et al., 2004; Ohnou et al., 2016*), and the aversive behaviors were followed by a induction of the mechanical allodynia, which lasted for more than 84 days (*Yokai et al., 2016*). However, vasoactive intestinal polypeptide (VIP), which share VIP/PACAP (VPAC) 1 or VPAC2 receptors with PACAP, failed to induce these nociceptive behaviors (*Ohnou et al., 2016; Yokai et al., 2016*). 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 (*Ohnou et al., 2016; Yokai et al., 2016*). These results suggested critical role of PAC1 receptor in 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-aminoadipate, an astroglial toxin, attenuated the induction of both aversive behaviors and mechanical allodynia. Interestingly, L-α-aminoadipate were still effective to reverse the mechanical allodynia even if it was i.t.- administered 84 days after spinal PAC1 receptor stimulation (*Yokai et al., 2016*). These results suggest that long-lasting spinal astocytic activation underlies the PACAP/PAC1 receptor-induced aversive behaviors and mechanical allodynia. However, how spinal astrocytes

101 contribute to these nociceptive behaviors is still unknown.

102

103 Glycogen is the single largest energy reserve in the brain, and is predominantly
104 localized in astrocytes (*Cataldo and Broadwell, 1986*). Among the neurotransmitters
105 and bioactive substances examined up until today, PACAP is suggested to have potent
106 glycogenolytic activity with an EC_{50} value of 0.08 nM (*Magistretti et al., 1998*). To the
107 best of our knowledge, PACAP has the highest efficacy to induce glycogenolysis.
108 However, it is not known whether PACAP is involved in the spinal ANLS. Therefore,
109 we aimed to examine possible involvement of PACAP/PAC1 receptor system in the
110 spinal ANLS, and also test whether the spinal ANLS contributes to the PACAP/PAC1
111 receptor-induced nociceptive behaviors.

112

113 ## Results

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

116 We first examined whether spinal ANLS played important role in the PACAP/PAC1
117 receptor-induced nociceptive behaviors in mice. After a single i.t. administration of Max
118 (50 pmol) or PACAP (100 pmol), aversive behaviors such as licking and biting

119 gradually appeared within 0 ~ 5 min, reached to the plateau around 15 ~ 20 min, and
120 maintained at least for 30 min. The co-injection of DAB, a glycogen phosphorylase
121 (PYGB) inhibitor, dose-dependently (1 ~ 100 pmol) attenuated the development of the
122 Max- or PACAP- induced aversive behaviors (***Figure 1A, C***). In addition, a single i.t.
123 administration of Max or PACAP markedly decreased mechanical threshold (induction
124 of mechanical allodynia) from day 1 (after the cessation of the aversive behaviors), and
125 this decrease persisted at least for 21 days after the administration. The co-injection of
126 DAB with Max or PACAP also dose-dependently attenuated the induction of
127 mechanical allodynia by Max or PACAP (***Figure 1B, D***).
128
129 **Suppression of the PAC1 receptor-evoked nociceptive behaviors by DAB was**
130 **reversed by i.t. co-injection of L-lactate**
131 Because lactate secretion from astrocytes was mainly derived from glycogen store
132 (***Dringen et al., 1993***), we then asked whether the inhibition of the PAC1
133 receptor-induced nociceptive behaviors by DAB could be reversed by the
134 co-administration of exogenous L-lactate. I.t. injection of L-lactate (1 nmol) in
135 combination with Max (50 pmol) + DAB (10 pmol) significantly reversed the
136 anti-aversive and anti-allodynic effects of DAB on the PAC1 receptor-induced

137 nociceptive behaviors (*Figure 2A, B*). Similarly, i.t. supplementation of L-lactate (1
138 nmol) also blocked the inhibitory effects of DAB (100 pmol) on the PACAP-induced
139 aversive behaviors and mechanical allodynia (*Figure 2C, D*). These results suggested
140 that spinal ANLS has critical role in the PACAP/PAC1 receptor-induced nociceptive
141 behaviors. However, L-lactate (10 nmol) alone did not show any pain related behaviors
142 (data not shown).
143
144 **Possible involvement of PKC in the PACAP/PAC1 receptor-evoked glycogenolysis**
145 **in cultured spinal cord astrocytes**
146 We prepared cultured astrocytes from newborn mice spinal cords, and confirmed that
147 most cells were suggested to be astrocytes, < 1% cells were oligodendrocytes, and no
148 neurons or microglial cells were detected, when visualized with immunocytochemistry
149 for specific markers of individual cells (*Figure 3 - figure supplement 1*). In order to
150 identify the receptor subtypes involved in the PACAP-evoked glycogenolysis in the
151 spinal astrocytes, we exposed PACAP, VIP or Max and determined glycogen amount in
152 the cells. Exposure of PACAP (0.0001 ~ 10 nM) or Max (0.1 ~ 10 nM)
153 dose-dependently decreased glycogen amount (*Figure 3A, B*), but VIP failed to
154 decrease the glycogen amount even at the concentration of 10 nM (*Figure 3C*),

155 suggesting that PAC1 receptor is primarily involved in the glycogenolysis. Interestingly,
156 this pharmacological characteristic of glycogenolysis was very similar to that of the
157 PACAP-induced nociceptive behaviors in mice reported previously (***Ohnou et al., 2016; Yokai et al., 2016***). This PACAP-induced glycogenolysis was completely
159 abolished by the DAB (1 mM) (***Figure 3D***), indicating that the glycogenolysis by
160 PACAP would be mediated by PYGB. Since PAC1 receptor can couple with both G_s
161 and G_q proteins, leading to the activation of the protein kinase A (PKA) and protein
162 kinase C (PKC), respectively (***Deutsch and Sun, 1992***), we co-applied GF109203X as a
163 PKC inhibitor, H89 or Rp-8-Br-cAMPS as a PKA inhibitor, or Sq22.536 as an adenylate
164 cyclase (AC) inhibitor with PACAP. We found that GF109203X (5 μM) significantly
165 inhibited the PACAP-evoked glycogenolysis (***Figure 3E***), but neither the PKA
166 inhibitors (***Figure 3F***), nor the AC inhibitor (***Figure 3 - figure supplement 2A***) affected
167 the glycogenolysis. Although these results may contradict previous reports that
168 cAMP/PKA signaling pathway stimulated glycogenolysis in chick brain or cultured
169 mouse cortical astrocytes (***Sorg and Magistretti, 1991; Gibbs, 2016***), forskolin, an AC
170 activator, significantly stimulated glycogenolysis in cultured spinal astrocytes (***Figure 3 – figure supplement 2B***). These results suggested that PAC1 receptor might not be
172 coupled with the G_s protein/PKA pathway, and support the significance of the G_q

173 protein/PKC pathway for the PACAP/PAC1 receptor-induced glycogenolysis in our
174 cultured spinal cord astrocytes.
175
176 **PKC is crucial for the PACAP/PAC1 receptor-evoked lactate secretion in the**
177 **cultured spinal astrocytes**
178 To investigate whether lactate secretion from astrocytes is increased by the
179 PACAP/PAC1 receptor/PKC pathway, we measured the lactate amounts in the
180 supernatants from the cultured spinal astrocytes after drug treatment. PACAP (1 nM)
181 significantly increased lactate amounts in the supernatant in both 0 ~ 60 min (early
182 phase) and 60 ~ 120 min (late phase) after the exposure (***Figure 4A, B***). PMA (100 nM),
183 a PKC activator, significantly elevated lactate secretion in the late phase (***Figure 4C, D***),
184 while forskolin (5 μM) failed to enhance lactate secretion (***Figure 4E, F***). In addition,
185 GF109203X (5 μM) significantly inhibited the PACAP-activated lactate secretion in
186 both phases (***Figure 4G, H***), suggesting the importance of PKC, but not PKA, in the
187 PACAP-activated lactate secretion. Furthermore, the inhibition of glycogenolysis by
188 DAB significantly decreased the lactate amount in the supernatant from the astrocytes
189 particularly in the late phase (***Figure 4I, J***), indicating that glycogenolysis is also
190 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 (***Figure 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

Finally, we asked whether lactate transport participated in the PACAP/PAC1 receptor-induced nociceptive behaviors. L-lactate is one of the substrates of monocarboxylate transporters (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

209 exhibit a distinct regional and cellular distribution in rodent brain. At the cellular level,
210 MCT1 is known to be expressed by endothelial cells of microvessels, by
211 ependymocytes as well as by astrocytes. MCT4 expression appears to be specific for
212 astrocytes. By contrast, the predominant neuronal MCT is suggested to be MCT2
213 (***Pierre and Pellerin, 2005***). It is reported that AR-C155858 potently inhibits MCT1 and
214 MCT2, but not MCT4 (***Ovens et al., 2010***). In our cultured spinal astrocytes,
215 AR-C155858 (1 μM) significantly inhibited lactate secretion evoked by PACAP at both
216 phases (***Figure 6A, B***). Simultaneous i.t. injection of AR-C155858 (1 nmol) with
217 PACAP significantly inhibited the development of the aversive behaviors (***Figure 6C***).
218 Intriguingly, i.t. administration of AR-C155858 transiently alleviated the mechanical
219 allodynia at 7 days after i.t. injection of PACAP (***Figure 6D***), suggesting that spinal
220 ANLS activation evoked by a single i.t. injection of PACAP may persist at least for 7
221 days.
222

223 **Discussion**

224 In this study, we have further characterized the nociceptive behaviors induced by the
225 spinal PAC1 receptor activation and made the following findings: 1) PACAP could be
226 an endogenous inducer for spinal ANLS activation; 2) spinal ANLS activation by

227 PACAP/PAC1 receptor signaling contributed to nociceptive behaviors such as aversive
228 behaviors and mechanical allodynia; 3) PKC activation (possibly in the spinal
229 astrocytes) played an important role in the PACAP/PAC1 receptor-evoked spinal ANLS;
230 and 4) spinal ANLS activity underling the generation of the nociceptive behaviors
231 persisted at least for 7 days after the PAC1 receptor activation by a single i.t. injection
232 of PACAP.
233
234 **PACAP/PAC1 receptor-evoked ANLS activation is crucial for the nociceptive**
235 **behaviors**
236 In the present study, we showed that the simultaneous i.t. injection of DAB, the inhibitor
237 of glycogenolysis, blocked the development of aversive behaviors and mechanical
238 allodynia evoked by Max or PACAP, and this inhibition by DAB was reversed by the
239 combinational injection of L-lactate. These results suggested that the ANLS activation
240 may have significant role in the nociceptive behaviors by PACAP/PAC1 receptor
241 activation.
242
243 Chronic pain may need maladaptive synaptic plasticity in neural circuits (***Sandkuhler,***
244 ***2007; Kuner and Flor, 2016***). An in vivo electrophysiological study showed that DAB

245 inhibited the long-term potentiation by tetanus stimulus in hippocampal Schaffer
246 collateral-area CA1, and this impairment by DAB was rescued with the exogenous
247 L-lactate (*Suzuki et al., 2011*). Furthermore, L-lactate alone was shown to potentiate
248 AMPA receptor- or NMDA receptor-mediated inward currents, increase intracellular
249 calcium, and also upregulate the markers for neuronal activation (*Suzuki et al., 2011;*
250 *Nagase et al., 2014; Yang et al., 2014*). Thus, PACAP/PAC1 receptor-evoked spinal
251 ANLS might potentiate AMPA/NMDA receptors-mediated responses and induce
252 long-term potentiation, which might lead to aversive behaviors and long-lasting
253 mechanical allodynia.
254
255 Regarding the initiation and maintenance of inflammatory and neuropathic pain,
256 recent progresses also focus on the critical role of astrocytes in the spinal cord and brain
257 (*Ji et al., 2014; Grace et al., 2014*). Previously, we showed that i.t. injection of Max or
258 PACAP induced rapid and prolonged upregulation of spinal GFAP expression level in
259 parallel with the aversive behaviors and long-lasting mechanical allodynia, and
260 simultaneous application of L-α-aminoadipate, an astrocyte toxin, with Max or PACAP
261 markedly suppressed the aversive behaviors and the mechanical allodynia (*Ohnou et al.,*
262 *2016; Yokai et al., 2016*). Interestingly, it is suggested that glycogen metabolism is

associated with maturation of astrocytes, in which the expressional levels of GFAP and glycogen synthase or phosphorylase are increased in parallel (*Brunet et al., 2010*). In accordance with this notion, our present study revealed that ANLS activation indeed contributed to both development and maintenance of PACAP/PAC1 receptor-evoked nociceptive behaviors, since i.t. injection of AR-C155858 could prevent the development of the PACAP-induced aversive behaviors and ameliorate the mechanical allodynia 7 days after PACAP injection. 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.

PACAP/PAC1 receptor/PKC signaling pathway played an important role in spinal glycogenolysis

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} currents (*Baron et al., 2001*), indicating

281 selective coupling of PKC with PAC1 receptor. Indeed, in our present study,
282 PACAP-evoked glycogenolysis was significantly inhibited by the PKC inhibitor,
283 GF109203X, but not by the PKA inhibitors. These results suggested that PAC1
284 receptor/PKC signaling pathway is important for the glycogenolysis activation.
285
286 Previous reports which observed glycogenolysis in chick brain or cultured mouse
287 cortical astrocytes suggested that cAMP/PKA signaling pathway phosphorylated
288 glycogen phosphorylase and evoked glycogenolysis (*Sorg and Magistretti, 1991; Gibbs,*
289 *2016*). But in our cultured spinal astrocytes, both PKA inhibitors and AC inhibitor failed
290 to prevent the PACAP-evoked glycogenolysis. Although the reason why
291 PACAP-evoked glycogenolysis is not sensitive to the PKA inhibitors is currently
292 unknown, this may be due to the absence of the coupling of PACAP/PAC1 receptor to
293 G_s protein in the spinal astrocytes, because both PKA and adenylate cyclase inhibitor
294 failed to inhibit the PACAP-evoked glycogenolysis, and forskolin, a direct AC activator,
295 successfully evoked glycogenolysis. But further study is required to verify this
296 possibility.
297
298 It is previously suggested that VIP neurons exert their glycogenolytic action in the

299 neocortical region (*Magistretti and Allaman, 2015*), but in our cultured spinal
300 astrocytes, VIP failed to evoke glycogenolysis at the concentration up to 10 nM. This
301 discrepancy might be due to the regional difference, because in our unpublished results
302 with using cultured cortical astrocytes, VIP (EC_{50} = 0.43 nM) as well as PACAP (EC_{50} =
303 0.0084 nM) could evoke glycogenolysis. In addition, cortical astrocytes express VPAC2
304 receptors more abundantly than PAC1 receptor (our unpublished observation). These
305 results support the notion of the regional difference of astrocytic function, and the
306 glycogenolysis of the spinal astrocytes might be primarily mediated by PACAP
307 signaling, while that in the cortical astrocytes might be mediated by both PACAP and
308 VIP signaling.
309
310 The reason why DAB treatment with PACAP increased glycogen level more than
311 vehicle control might be due to the constitutive activity of PYGB. However, further
312 research is also necessary to elucidate the basal activity of PYGB in the spinal
313 astrocytes.
314
315 **PACAP/PAC1 receptor/PKC signaling-evoked glycogenolysis played a critical role**
316 **in the lactate secretion**

317 PACAP and PMA significantly enhanced lactate secretion. In addition, GF109203X and
318 DAB significantly inhibited the PACAP-enhanced lactate secretion. These results
319 proposed that PKC and a downstream glycogenolysis were important in the
320 PACAP-enhanced lactate secretion. It is previously reported that the PKC activation
321 resulted in an increase in the levels of MCT1 and MCT4 and the release of lactate in an
322 in vitro skeletal muscle cells (RD cells), while the PKA activation resulted in a
323 significant decrease in MCT1 expression in the RD cells as well as endothelial cells
324 (*Narumi et al., 2010; Narumi et al., 2012; Smith et al., 2012*). Although the PKA
325 activation by dbcAMP (cAMP analogue) or isoproterenol (selective β agonist) was
326 shown to significantly decrease glycogen level in astrocytes/neurons mixed culture, the
327 secretion of lactate, contrary to expectation, was unchanged or decreased (*Tarczyluc et*
328 *al., 2013*). Consistent with these reports, our preliminary observation using cultured
329 cortical astrocytes, forskolin significantly attenuated the lactate secretion, even though
330 significant glycogenolysis was observed (unpublished observation). These results
331 indicate that increased lactate secretion by PKC might involve the activation of MCT1
332 and MCT4, and in some cases, PKA might counteract PKC-induced action on lactate
333 secretion. Since forskolin treatment did not stimulate lactate secretion in the spinal
334 astrocytes, PACAP/PAC1 receptor/PKC signaling, but not PKA signaling, would be

335 involved in the lactate secretion in our present experimental condition.

336

337 It may be noteworthy that DAB significantly inhibited the lactate secretion in the late
338 phase after PACAP exposure, but failed to inhibit in the early phase. Our results may be
339 consistent with a previous report that DAB significantly attenuated the lactate secretion
340 during 60 ~ 180 min after the hypoglycemia on the astrocytes/neurons mixed culture,
341 while equivalent amount of lactate was secreted irrespective of the presence of DAB
342 during 0 ~ 60 min, even DAB completely inhibited glycogenolysis at both timings
343 (*Tarczyluk et al., 2013*). Thus, these observations may indicate that the secreted lactate
344 in the late phase is originated from glycogenolysis, and the secretion in the early phase
345 may be partly derived from the readily releasable pool of lactate in the spinal astrocytes.

346

347 **PACAP/PAC1 receptor-induced PKC activation is important for the nociceptive**
348 **behaviors**

349 We found that developments of the PACAP/PAC1 receptor-induced aversive behaviors
350 and mechanical allodynia were significantly attenuated by the PKC inhibition with
351 GF109203X. Although many lines of evidence strongly suggested the significance of
352 PKC in inflammatory or neuropathic pain (*Vel´azquez et al., 2007*), it is still unknown

353 what kinds of PKC isozymes actually contribute to the PACAP/PAC1 receptor-evoked
354 ANLS activation, or in which cell types the PKC was activated. PKC has several
355 isozymes and, western blotting showed that phospho-PKC-δ, -θ and -ζ levels were
356 significantly increased in ipsilateral spinal cord after spinal nerve injury, but expression
357 levels of phospho-PKC-α and -β were not significantly changed (*Gosselin et al., 2013*).
358 Further double labeling immunofluorescence showed that phospho-PKC-δ co-localized
359 with astrocytic markers and phospho-PKC-θ and -ζ co-localized with neuronal markers
360 in spinal cord (*Gosselin et al., 2013*). Another research group reported that the i.t.
361 injection of a PKC-δ inhibitor significantly attenuated paclitaxel-induced mechanical
362 allodynia in mice (*He and Wang, 2015*). These results suggested that PKC-δ might be
363 an astrocytic PKC isoform and contribute to the pain behaviors observed in this study.
364 Interestingly, it is indicated that PMA-induced stimulation of MCT4 expression can be
365 mediated through a novel PKC isozyme, especially PKC-δ, and rottlerin, a selective
366 PKC-δ inhibitor, cancelled PMA-induced MCT4 protein expression in the RD cells
367 (*Narumi et al., 2012*). Further study is needed to elucidate the contribution of PKC
368 isozymes in the PACAP/PAC1 receptor-induced spinal ANLS and nociceptive
369 behaviors.
370

371 It may be worth noting our previous data here that i.t. injection of the PKA inhibitor,
372 Rp-8-Br-cAMPS, could ameliorate the PACAP-induced aversive behaviors (***Ohnou et***
373 ***al., 2016***). Currently it may be difficult to precisely reconcile with the present
374 observation that the PKA inhibitor failed to prevent PACAP-evoked glycogenolysis in
375 the spinal astrocytes, but we could speculate that neuronal PKA signaling would also
376 contribute to the expression of the pain behaviors. However, further extensive studies
377 are necessary to uncover the interaction of PKC and PKA signaling in the
378 PACAP/PAC1 receptor-induced pain behaviors.
379
380 **MCTs had an important role in PACAP/PAC1 receptor-induced nociceptive**
381 **behaviors**
382 In cultured spinal astrocytes, AR-C155858 potently inhibited PACAP-evoked lactate
383 secretion. In the behavioral study, the simultaneous i.t. injection of AR-C155858
384 significantly attenuated the development of the PACAP-induced aversive behaviors. In
385 addition, AR-C155858 also transiently reversed the PACAP-induced mechanical
386 allodynia 7 days after the PACAP injection. These observations suggested the important
387 contribution of lactate transportation via MCTs to the induction as well as the
388 maintenance of the nociceptive behaviors evoked by the spinal PACAP/PAC1 receptor

389 signaling. The significance of MCTs on the synaptic plasticity was previously reported.
390 Hippocampal MCT1 expression but not MCT2 or MCT4 expression was significantly
391 upregulated upon inhibitory avoidance training in rats, and blocking the function of
392 MCT1, MCT2 or MCT4 by the antisense oligonucleotides impaired long-term memory
393 (***Suzuki et al., 2011***). Moreover, in the cocaine-induced conditioned place preference
394 test, re-exposure to a cocaine-associated context significantly increased the expression
395 of MCT1 and MCT2, and disrupting their expression led to the impairment of
396 reconsolidation (***Zhang et al., 2015***). At the cellular level, L-lactate-induced increased
397 expressions of immediately early genes such as *Arc*, *c-fos* and *Zif268* were blocked in
398 the presence of a MCT inhibitor, UK5099 (***Yang et al., 2014***). To our knowledge, the
399 present study is the first report establishing the significance of MCTs in nociceptive
400 behaviors.
401
402 In summary, we have shown that the interaction between spinal dorsal horn neurons
403 and astrocytes evoked by PACAP/PAC1 receptor-induced ANLS activation is critically
404 involved in the development and maintenance of the nociceptive behaviors. The
405 signaling pathway linking between PAC1 receptor activation and ANLS might be at
406 least partially mediated by PKC pathway. Although verifying the possible involvement

of PACAP/PAC1 receptor signaling in human pain as well as animal pain models requires further rigorous studies, targeting spinal ANLS may provide a new opportunity to treat intractable chronic pain.

Materials and methods

Key resources table

Reagent type	Designation	Source	Identifiers
Drug	Maxadilan	Dr. M Tajima, Shiseido	
Drug	DAB	Wako	040-24941
Drug	PACAP	Peptide Institute	4221-v
Drug	L-lactate	Sigma-Aldrich	71718
Drug	VIP	Peptide Institute	4110-v
Drug	GF109203X	Wako	079-03811
Drug	H89	Seikagaku Co.	120836
Drug	Rp-8-Br-cAMPS	Sigma-Aldrich	A165
Drug	PMA	Wako	545-00261
Drug	Forskolin	Merck Millipore	344281
Drug	AR-C155858	Merck Millipore	533436

Drug	Sq22.536	Enzo Biochem Inc.	BML-CN140
Antibody	mouse anti-GFAP	Merck Millipore	MAB360
Antibody	mouse anti-MAP2	Sigma-Aldrich	M-4403
Antibody	rabbit anti-Iba1	Wako	019-19741
Antibody	mouse anti-CNPase	Abcam	ab6319

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 ± 1°C) and humidity (55 ± 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 no. MD15082), 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.

443 **Intrathecal (i.t.) injection and behavioral observation**

444 I.t. injection was given in a volume of 5 μl by percutaneous puncture through an
445 intervertebral space at the level of the fifth or sixth lumbar vertebra, according to a
446 previously reported procedure (*Yokai et al., 2016*). An investigator, who was unaware of
447 the drug treatment, performed all of the behavioral experiments.

448

449 Aversive behavior was evaluated according to our previous report (*Ohnou et al.,*
450 *2016*). Before i.t. injection, mice were placed and habituated in a glass cylinder (φ14 ×
451 18 cm) with a filter paper at the bottom over 20 min. Immediately after i.t. injection, the
452 mice were placed again in the same glass cylinder and the number of pain behaviors
453 consisting of licking, biting and scratching directed toward the caudal part of the body
454 was counted every 1 min. The cumulative number of events was pooled over 5 min bins
455 of observation, and analyzed.

456

457 The assessment of mechanical thresholds was also carried out according to the
458 previously described methods (*Yokai et al., 2016*). Briefly, mechanical sensitivity was
459 evaluated with calibrated von Frey hairs (Stoelting, Wood Dale, IL) by measuring the
460 tactile stimulus producing a 50% likelihood of hind paw withdrawal response (50%

461 gram threshold), which was determined using the up-down paradigm (*Chaplan et al.,*

462 *1994*).

463

464 **Drugs**

465 PACAP (38 amino acid form) and VIP were purchased from Peptide Institute Inc.

466 (Osaka, Japan). Maxadilan was kindly donated by Dr. M Tajima (Shiseido, Japan).

467 1,4-dideoxy-1,4-imino-d-arabinitol (DAB), GF109203X and phorbol 12-myristate

468 13-acetate (PMA) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka,

469 Japan). Forskolin and AR-C155858 was from Merck Millipore (Darmstadt, Germany).

470 H89 was from Seikagaku Co. (Tokyo, Japan). L-lactate and Rp-8-Br-cAMPS were from

471 Sigma-Aldrich Co. LLC (St. Louis, MO). Sq22.536 was from Enzo Biochem Inc.

472 (Farmingdale, NY). These drugs were made up as concentrated stock solution in MilliQ

473 water, 0.1 M acetic acid or dimethyl sulfoxide, aliquoted, and stored at –30°C and

474 diluted just before use. Aliquot of drugs were diluted to the desired concentration in

475 artificial cerebrospinal fluid (ACSF: NaCl 138 mM, KCl 3 mM, $CaCl_2$ 1.25 mM, $MgCl_2$

476 1 mM, D-glucose 1 mM) immediately prior to use. The doses for i.t. injection of

477 PACAP38 (100 pmol) and maxadilan (50 pmol) we chose in this study were determined

478 according to our previous reports (*Shimizu et al., 2004; Ohnou et al., 2016; Yokai et al.,*

479 *2016*).

480

481 **Cultured spinal cord astrocytes**

482 Spinal cords from postnatal day 1 and 2 mice were dispersed by 0.25% trypsin and
483 subsequently plated on culture flasks which were previously coated with 0.75 ug/mL
484 poly-l-lysine (Sigma-Aldrich, St. Louis, MO). Cells were cultured in Dulbecco's
485 modified Eagle medium (Nacalai Tesque, Osaka, Japan) supplemented with 10% fetal
486 bovine serum (Hyclone, South Logan, UT) until cell density reached to confluent. After
487 reaching to confluent, culture flasks were shaken at 240 rpm for 6 hrs to remove
488 microglia or oligodendrocyte precursor cells. Remaining astrocytes on culture flasks
489 were then detached and subsequently plated on the appropriate culture dishes at the
490 density of 1×10^4 cells/cm^2, and used when the cell number reached to the confluent
491 again, which usually took 3 days. All of the cultured astrocytes used in the current
492 experiments was passaged by 3 or 4 times.

493

494 **Glycogen assay**

495 Cultured spinal astrocytes were exposed to PACAP, VIP, Max or forskolin for 1 hr,
496 washed in the ice-cold phosphate buffered saline 3 times, and harvested in ice-cold

497 MilliQ water by scratching. Samples were then boiled for 10 min, sonicated, and
498 centrifuged. Glycogen amount in the supernatant was measured by the Glycogen Assay
499 Kit (BioVision Inc., San Francisco, CA) according to the manufacturer's instruction.
500 The exposure of DAB, H89, Rp-8-Br-cAMPS, Sq22.536 or GF109203X was started 30
501 min before PACAP exposure. The amount of glycogen was normalized by the amount
502 of the protein contained in the samples, which was measured by the Bradford's assay kit
503 (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.
504
505 **Lactate assay**
506 Culture medium was replaced to Krebs-Ringer buffer (NaCl 135 mM, KCl 5 mM,
507 $CaCl_2$ 1 mM, $MgSO_4$ 1 mM, KH_2PO_4 0.4 mM, D-glucose 5.5 mM, HEPES 20 mM),
508 and the astrocytes were habituated in this buffer for 2 hrs. The cultured spinal astrocytes
509 were then exposed to PACAP, PMA or forskolin for 60 min, and conditioned buffer was
510 harvested (0 ~ 60 min). Subsequently, flesh Krebs-Ringer buffer with drugs was added
511 on the cultured astrocytes again, and harvested the conditioned buffer for another 60
512 min (60 ~ 120 min). The time course was determined according to the previous paper
513 which suggested DAB inhibited lactate secretion in 60 ~ 180 min after the treatment,
514 but not in 0 ~ 60 min (*Tarczyluk et al., 2013*). 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.

Statistical analysis

Experimental data are expressed as mean ± SEM. For mechanical threshold analyses, we employed the Mann-Whitney U-test for single comparisons or the Friedman test followed by the Steel 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.

Acknowledgements

The authors thank all the staff members of the Institute of Laboratory Animal Science Reserch Support Center, Kagoshima University.

Additional information

Funding

Funder	Grant reference number	Author
JSPS Kakenhi	17K08310	Yuki Kambe
Yamada Research Grant		Yuki Kambe
JSPS Kakenhi	16K15339	Takashi Kurihara
JSPS Kakenhi	17K08599	Atsuro Miyata

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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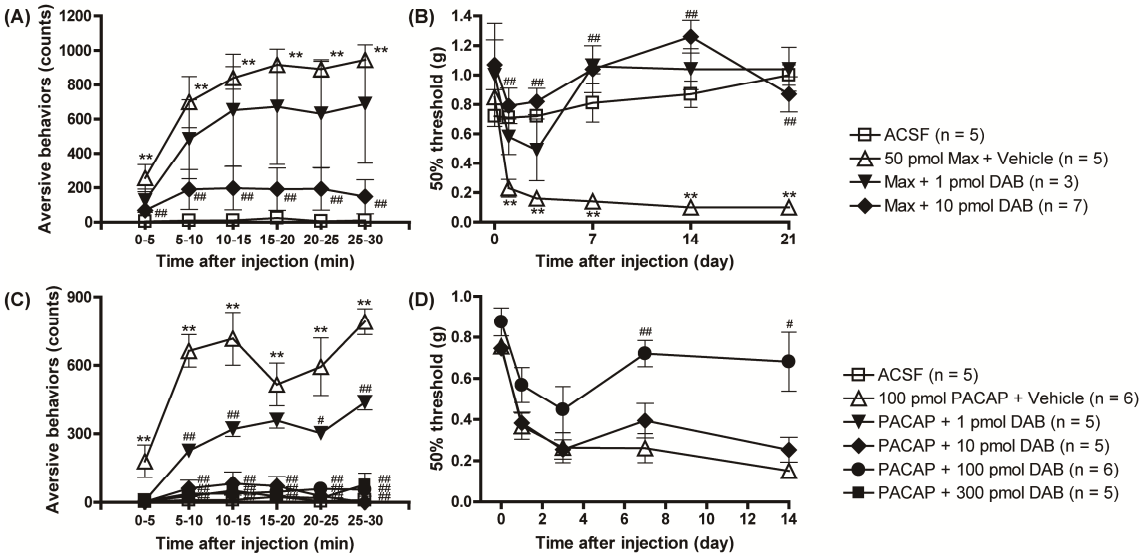


Figure 1. A critical role of glycogenolysis in PACAP/PAC1 receptor-evoked nociceptive behaviors.

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).

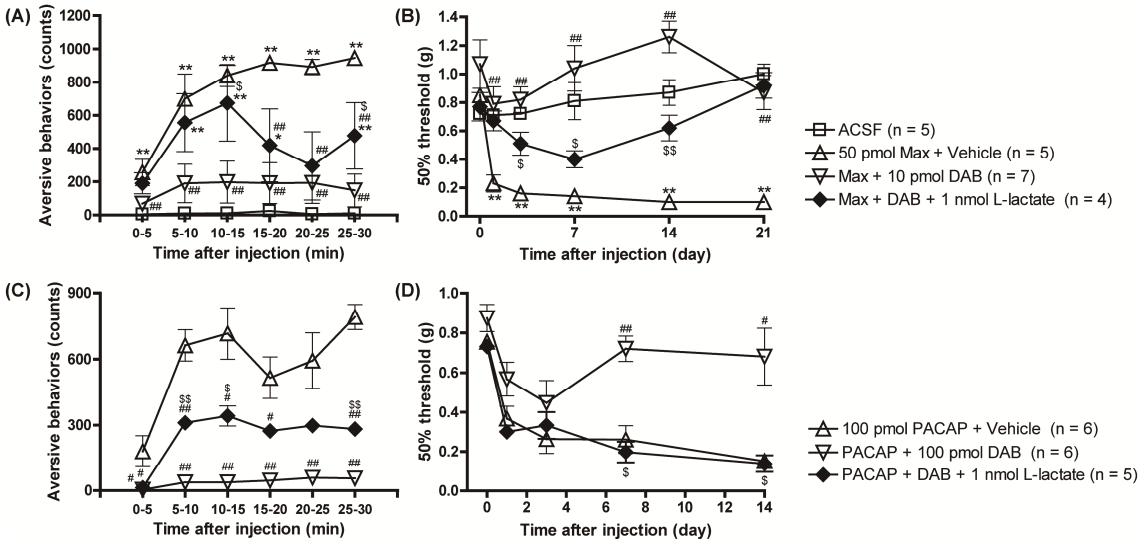


Figure 2. Simultaneous injection of L-lactate reversed the inhibitory effects of DAB on the PAC1 receptor-induced nociceptive behaviors.

I.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).

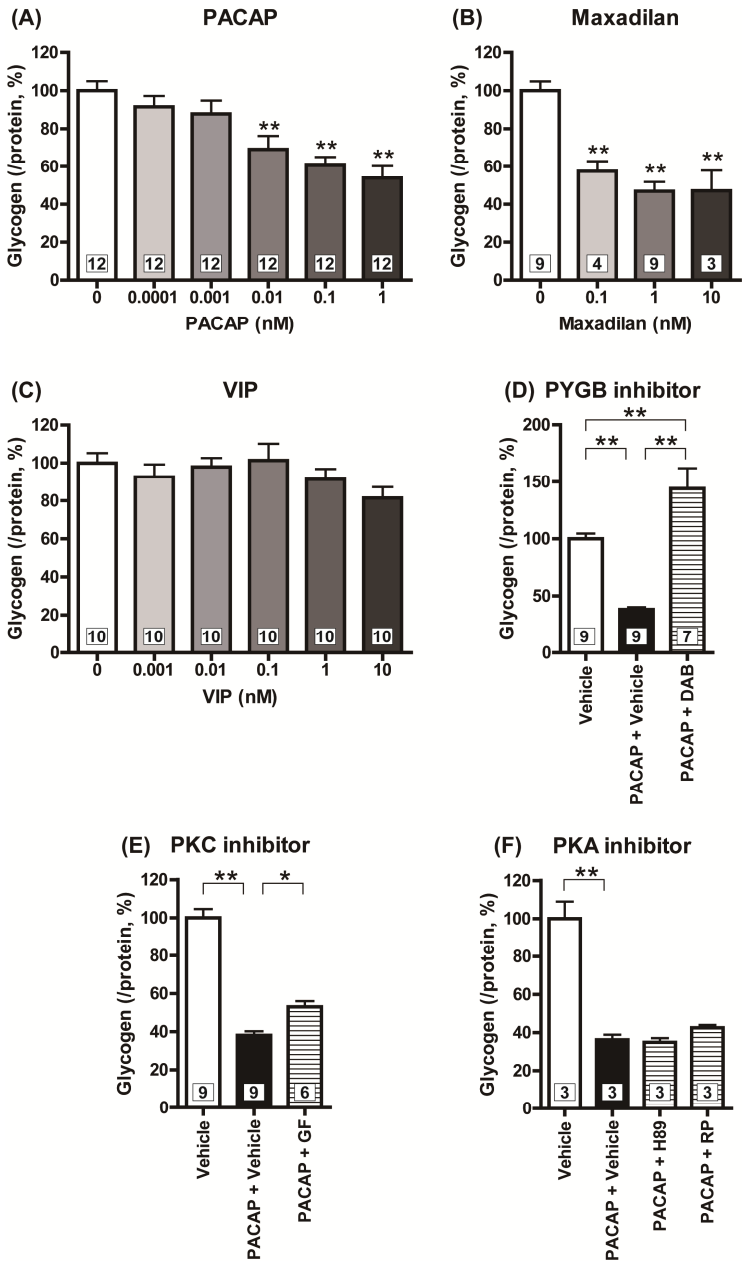
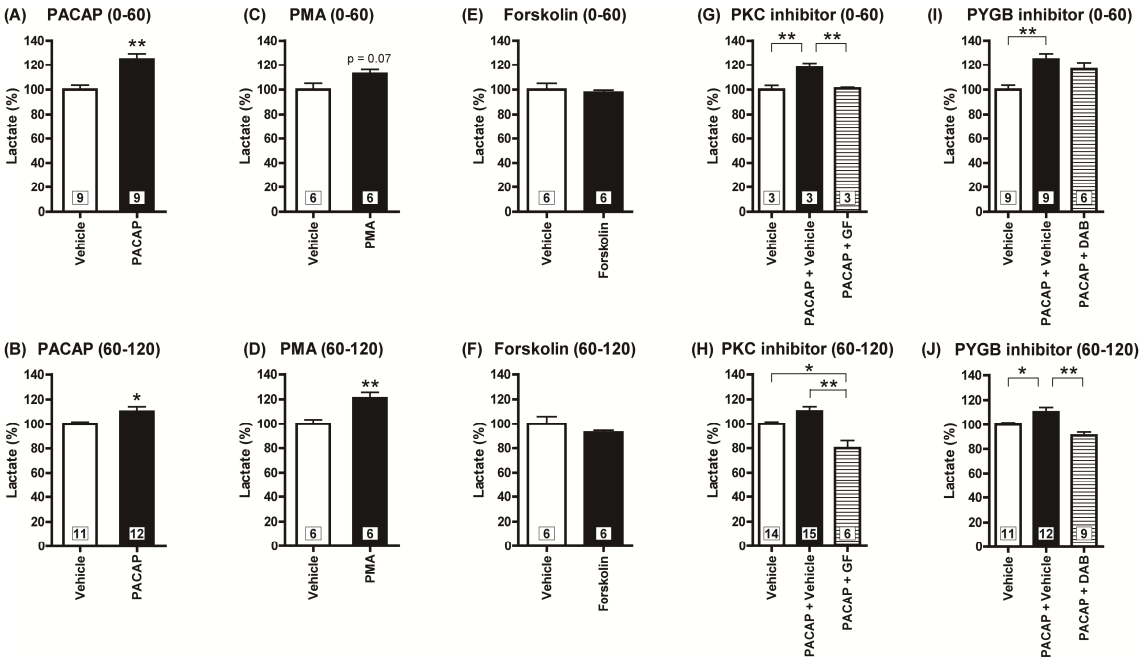


Figure 3. PACAP/PAC1 receptor/PKC signaling pathway induced glycogenolysis in cultured spinal cord astrocytes.

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

773 measured 60 min after the exposure. Cultured astrocytes were pre-incubated with DAB
774 (1 mM, **D**), GF109203X (GF, 5 μM, **E**), H89 (10 μM, **F**), or Rp-8Br-cAMPS (RP, 100
775 μM, **F**) for 30 min prior to PACAP (1 nM) exposure, and glycogen amounts contained
776 in the astrocytes were measured 60 min after the PACAP exposure. *P<0.05 and
777 **P<0.01 when compared with 0 nM (Vehicle) data in (A) ~ (C), and compared groups
778 are indicated above in (D) ~ (F). Statistical significance was evaluated by the Dunnett
779 test for (A) ~ (C), and Tukey test for (D) ~ (F). Exact sample sizes are indicated in the
780 graphs.



781

782

783 **Figure 4.** A crucial role of PACAP/PAC1 receptor/PKC-induced glycogenolysis in the

784 lactate secretion from cultured spinal astrocytes.

785 After the treatment with PACAP (1 nM) (**A, B**), PMA (100 nM) (**C, D**) or forskolin (5

786 μM) (**E, F**), the supernatants from the cultured spinal astrocytes were differentially

787 harvested during 0 ~ 60 min (**A, C, E**) or 60 ~ 120 min (**B, D, F**), and lactate amounts

788 were measured. Cultured astrocytes were pre-incubated with GF109203X (GF, 5 μM, **G,**

789 **H**) or DAB (1 mM, **I, J**) for 30 min prior to PACAP exposure, and then exposed to

790 PACAP (1 nM) for 120 min. Culture supernatants were differentially harvested during 0

791 ~ 60 min (**G, I**) or 60 ~ 120 min (**H, J**) after the PACAP addition, and lactate amounts

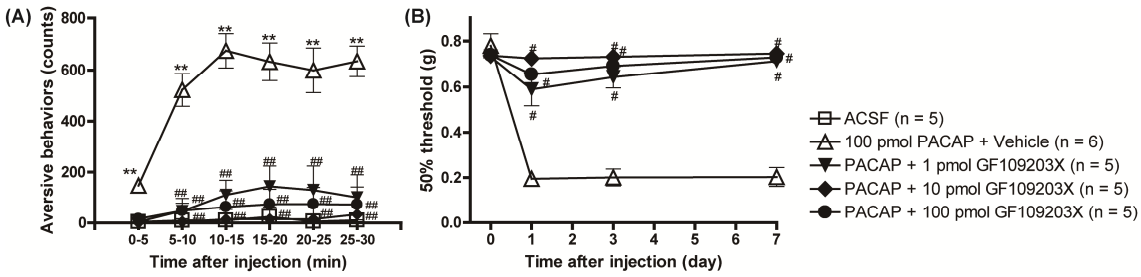
792 were measured. *P<0.05 and **P<0.01 when compared with vehicle data in (A) ~ (F),

793 and compared groups are indicated above in (G) ~ (J). Statistical significance was

794 evaluated by the Student t-test for (A) ~ (F), and Tukey test for (G) ~ (J). Exact sample

795 sizes are indicated in the graphs.

796

797 

798

799 **Figure 5.** The importance of PKC in the PACAP/PAC1 receptor-induced nociceptive

800 behaviors.

801 Simultaneous i.t. injection of GF109203X (1 ~ 100 pmol) with PACAP (100 pmol)

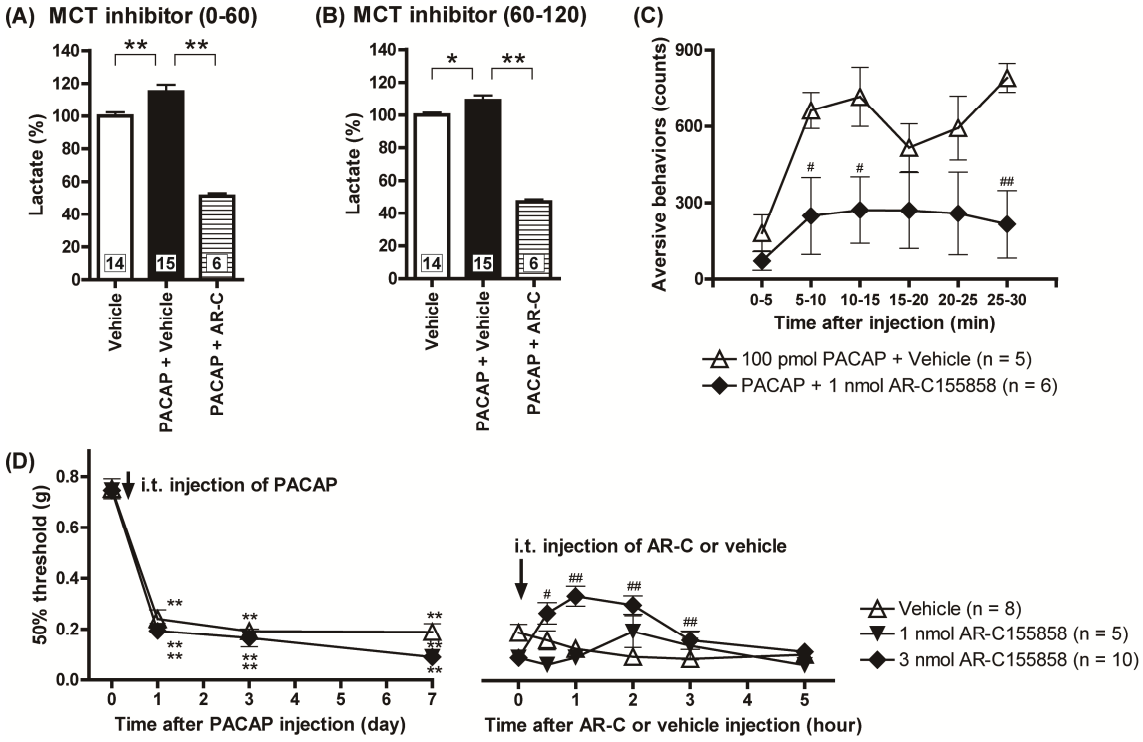
802 dose-dependently reduced the PACAP-induced aversive behaviors (**A**) and mechanical

803 allodynia (**B**). *P<0.05 and **P<0.01 when compared with ACSF data. $^{\#}$P<0.05 and

804 $^{\#\#}$P<0.01 when compared with PACAP + Vehicle data. Statistical significance was

805 evaluated by the Tukey test for (A), and Steel test for (B).

806



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808

809 **Figure 6.** Spinal MCTs play a crucial role in both induction and maintenance of the

810 PACAP/PAC1 receptor-induced nociceptive behaviors.

811 Cultured spinal astrocytes were pre-incubated with AR-C155858 (AR-C, 1 μM) 30 min

812 prior to PACAP exposure, and then exposed to PACAP (1 nM) for 120 min. The culture

813 supernatants were differentially harvested during 0 ~ 60 min (**A**) or 60 ~ 120 min (**B**)

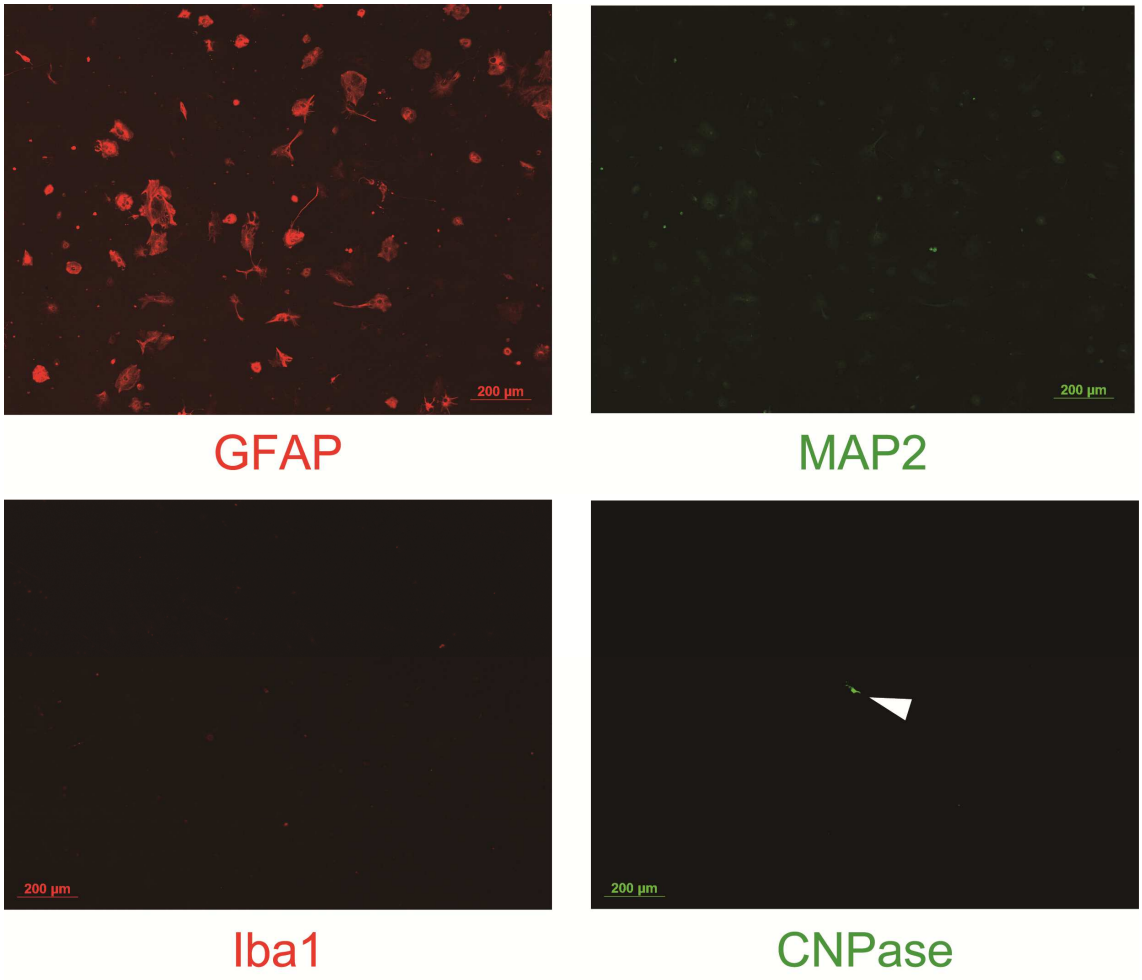
814 after the PACAP addition, and lactate amounts in the culture supernatants were

815 measured. *P<0.05 and **P<0.01 (compared groups are indicated above). Exact sample

816 sizes are indicated in the graphs. (**C**) I.t. co-administration of AR-C155858 (1 nmol)

817 with PACAP reduced the expression of the aversive behaviors. *P<0.05 and **P<0.01

818 when compared with PACAP + Vehicle data. (**D**) Transient reversal of the
819 PACAP-evoked mechanical allodynia by intrathecal AR-C155858. AR-C155858 was
820 injected 7 days after intrathecal PACAP. **P<0.01 when compared with pre-PACAP
821 data. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 when compared with vehicle-treated control. Statistical
822 significance was evaluated by the Tukey test for (A) and (B), Student t-test for (C), and
823 Steel test for (D).
824



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826

827 **Figure 3 - figure supplement 1.** Representative immunofluorescent images of the

828 cultured spinal astrocytes. The purity of astrocytes was examined by staining with

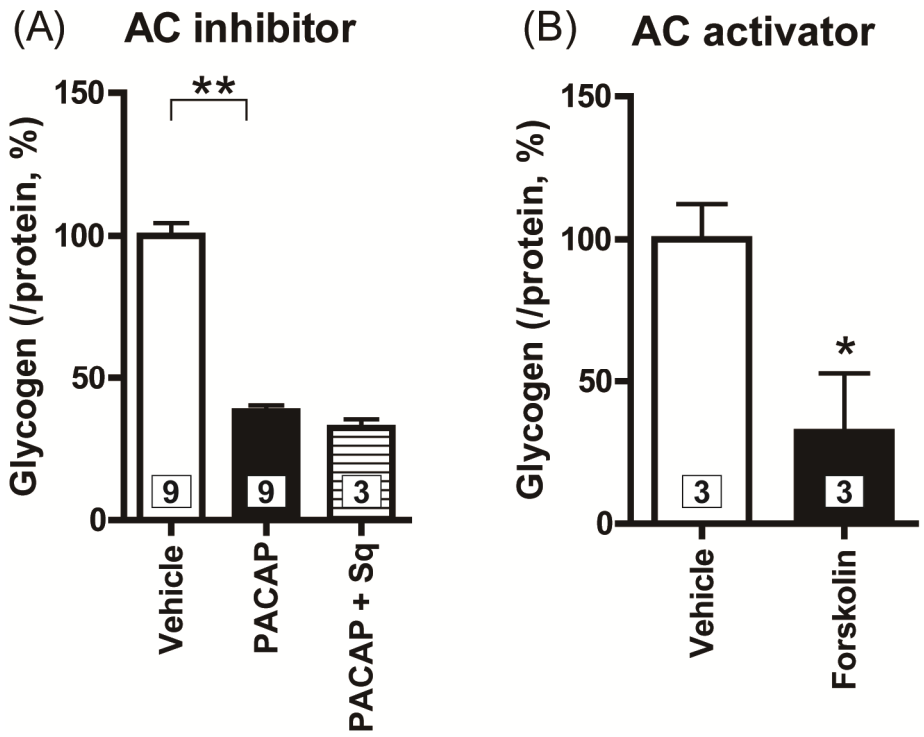
829 primary antibodies against GFAP (an astrocyte marker), MAP2 (a neuron marker), Iba 1

830 (a microglia marker) and MBP (an oligodendrocyte marker). Each marker was

831 visualized by an appropriate fluorescent dye-conjugated secondary antibody. Arrowhead

832 represented an incidentally contaminated oligodendrocyte. Bar = 200 μm.

833



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835

836 **Figure 3 – figure supplement 2.** PAC1 receptor might not be coupled with the G_s

837 protein/PKA pathway in the cultured spinal astrocytes.

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

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

840 were measured 60 min after the PACAP exposure (**A**). Cultured spinal astrocytes were

841 exposed to forskolin (5 μM), and glycogen amounts contained in the astrocytes were

842 measured 60 min after the exposure (**B**). *P<0.05 and **P<0.01 when compared with

843 Vehicle. Statistical significance was evaluated by the Dunnett test for (A) and Student

844 t-test for (B). Exact sample sizes are indicated in the graphs.