Yin-and-yang bifurcation of opioidergic circuits for descending analgesia at the midbrain of the mouse

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In the descending analgesia pathway, opioids are known to disinhibit the projections from the periaqueductal gray (PAG) to the rostral ventromedial medulla (RVM), leading to suppression of pain signals at the spinal cord level. The locus coeruleus (LC) has been proposed to engage in the descending pathway through noradrenergic inputs to the spinal cord. Nevertheless, how the LC is integrated in the descending analgesia circuit has remained unknown. Here, we show that the opioidergic analgesia pathway is bifurcated in structure and function at the PAG. A knockout as well as a PAG-specific knockdown of phospholipase C β4 (PLCβ4), a signaling molecule for G protein-coupled receptors, enhanced swim stress-induced and morphine-induced analgesia in mice. Immunostaining after simultaneous retrograde labeling from the RVM and the LC revealed two mutually exclusive neuronal populations at the PAG, each projecting either to the LC or the RVM, with PLCβ4 expression only in the PAG-LC projecting cells that provide a direct synaptic input to LC-spinal cord (SC) projection neurons. The PAG-LC projection neurons in wild-type mice turned quiescent in response to opiates, but remained active in the PLCβ4 mutant, suggesting a possibility that an increased adrenergic function induced by the persistent PAG-LC activity underlies the enhanced opioid analgesia in the mutant. Indeed, the enhanced analgesia in the mutant was reversed by blocking α2-noradrenergic receptors. These findings indicate that opioids suppress descending analgesia through the PAG-LC pathway, while enhancing it through the PAG-RVM pathway, i.e., two distinct pathways with opposing effects on opioid analgesia. These results point to a therapeutic target in pain control.

descending analgesia pathway | opioid | periaqueductal gray | locus coeruleus | phopholipase C

Pain signals are processed by the ascending sensory circuit and modulated by the descending analgesic circuit. The periaqueductal gray (PAG) at the midbrain, a key region in the descending analgesia circuit, is rich in opioid receptors and generates endogenous opioid analgesic signals (1–6). These signals are relayed by the rostral ventromedial medulla (RVM) to the dorsal horn of the spinal cord (SC), where they suppress pain signals (5, 7). Swimming in warm water induces opioid-dependent, swim stress-induced analgesia (SSIA) through this circuit (8). Within the PAG, tonically active GABAergic interneurons inhibit output neurons that project to the RVM (9, 10). Endogenous opioids suppress this inhibitory influence of local GABAergic interneurons through mu opioid receptors (μORs), disinhibiting the antinociceptive drive of the neuronal outputs to the RVM to positively control descending analgesia (9, 10). Exogenous opioid analgesics, such as morphine, also act through these μORs (11, 12).

Phospholipase C (PLC), the enzyme responsible for calcium mobilization, represents a family of molecules that are coupled to μORs and affects protein kinase C (PKC) activation (13, 14). We have previously shown that the PLCβ isoform, PLCβ4, is required for pain sensory transmission at the thalamic level (15, 16). PLCβ4-PKC signaling is linked to T-type calcium channels and regulates the firing pattern of thalamocortical neurons to favor either tonic or burst firing, which efficiently sharpens the transition between open and closed gate status in pain sensory processing (15, 17, 18). In situ hybridization data show that PLCβ4 is abundantly expressed in the PAG (Allen Brain Atlas, mouse.brain-map.org), but its potential role in the descending pain control circuit has not been explored.

The locus coeruleus (LC) has recently been proposed to play a role in endogenous descending pain control through noradrenergic inputs, which act via α2-adrenoreceptors to inhibit both primary afferents and second-order projection neurons in the SC (19–28). The LC has an afferent connection from the PAG (29, 30), but how the LC is integrated in the descending analgesia circuits are unresolved.

In this study, motivated by the knowledge of its signaling interactions in the ascending pain pathway (15), we investigated the role of PLCβ4 in the descending pain system controlled by opioidergic signals. Our results demonstrate the existence of two opioidergic circuits that bifurcate at the PAG and exert opposing effects on descending analgesia.

Results

Opioid-Dependent Analgesia Is Enhanced in PAG-Specific Knockdown and PLCβ4-Null Mutant Mice. We previously reported that μOR-positive GABAergic neurons in the PAG mediate the opioidergic descending analgesia through a mechanism that is dependent on T-type calcium channels (10). In addition, neuronal burst firing, which is mediated by T-type calcium channels, is increased in PLCβ4-deleted neurons (15). We thus hypothesized that a deletion of PLCβ4 at the PAG might enhance opioidergic descending analgesia.

Significance

The midbrain periaqueductal gray (PAG) is a major site in the descending analgesia circuits for the endogenous, opioidergic pain control. Recently, the locus coeruleus (LC) has been proposed to play a role in endogenous pain control. Nevertheless, how the LC is integrated in the descending analgesia circuits remains elusive. Here, we provide evidence that the opioid circuit is bifurcated into two distinct pathways, each with an opposing effect on opioid analgesia at the PAG level: suppressing through the PAG-LC pathway while enhancing through the PAG-rostral ventromedial medulla pathway. Thus, the yin-and-yang model is proposed for the opioidergic descending analgesia. Moreover, our findings suggest an insight for a therapeutic target to control pain.

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To test this hypothesis, we initially examined baseline thermal sensitivity in both PLCβ4-knockout [PLCβ4($^{-/-}$)] mice and PAG-specific PLCβ4-knockdown (PLCβ4-shRNA) mice (Fig. 1*A*). PLCβ4 knockout significantly increased baseline thermal sensitivity ($t = 4.833$, $P = 0.0003$, unpaired two-tailed Student's t test; Fig. 1*B*), a result consistent with a previous study showing that PLCβ4($^{-/-}$) mice exhibit reduced nociception (15, 16). In contrast, PAG-specific knockdown of PLCβ4 did not significantly affect the baseline thermal sensitivity of the mouse ($t = 0.964$, $P = 0.366$, unpaired two-tailed Student's t test; Fig. 1*B*). We then assessed the performance of these mice in the opioid-dependent swim stress-induced analgesia (SSIA) assay. Interestingly, SSIA was significantly increased in both PLCβ4($^{-/-}$) ($t = 4.881$, $P = 0.0003$, unpaired two-tailed Student's t test; Fig. 1*C*) and PAG-specific PLCβ4-knockdown mice (Scrambled-shRNA vs. PLCβ4-shRNA, $t = 8.808$, $P < 0.001$, unpaired two-tailed Student's t test; Fig. 1*C*). In addition, we carried out the 2% formalin-induced pain test after SSIA in both wild-type and PAG-specific PLCβ4-knockdown group. Correspondingly, the PAG-specific PLCβ4-knockdown mice showed significantly reduced inflammatory pain response compared with the control group [$F_{1,228} = 21.88$, $P < 0.001$, repeated-measures (RM) ANOVA, *SI Appendix*, Fig. S1*A*]. Immunostaining of the brains after SSIA assays confirmed a reduction in PLCβ4 expression in the PAG of knockdown mice (*SI Appendix*, Fig. S2). These results indicate that PLCβ4 in the PAG negatively controls opioid-dependent analgesia without affecting basal thermal sensitization.

Next, we investigated exogenous opioid-induced analgesia in both wild-type and PLCβ4-defective mice after systemic injection (10 mg/kg) or PAG infusion (1 μg) of morphine through a cannula (*SI Appendix*, Fig. S3), following a previously described procedure (10). In wild-type mice, morphine induced a significant level of analgesia compared with saline-treated controls following both systemic injection ($F_{1,15} = 11.88$, $P = 0.0044$, RM ANOVA) and PAG infusion ($F_{1,32} = 26.57$, $P < 0.001$, RM ANOVA) (Fig. 1 *D* and *E*). Morphine also induced analgesia compared with saline in PLCβ4($^{-/-}$) mice, whether injected systemically ($F_{1,14} = 74.84$, $P < 0.001$, RM ANOVA) or incused in the PAG ($F_{1,31} = 69.14$, $P < 0.001$, RM ANOVA) (Fig. 1 *D* and *E*). Notably, the extent of the morphine-induced analgesia was significantly augmented in the mutant mice compared with wild-type mice following systemic injection ($t = 3.346$, $P = 0.0065$, unpaired two-tailed Student's t test; *SI Appendix*, Fig. S3*A*) or infusion into the PAG ($t = 5.533$, $P < 0.001$, unpaired two-tailed Student's t test; *SI Appendix*, Fig. S3*B*). These findings demonstrate that (*i*) the PAG is a major site for morphine-induced analgesia and (*ii*) PLCβ4 deficiency substantially enhances PAG-mediated, morphine-induced analgesia.

PLCβ4 Is Selectively Expressed in PAG→LC Circuits, but Not in PAG→RVM Circuits. The PAG has extensive connectivity with the brain network that includes not only the RVM (1, 2, 5, 31) but also the LC, which exerts an analgesic effect (19–22, 29, 30, 32). In an effort to define the relationship between PLCβ4 expression and PAG-RVM or PAG-LC projection neurons in the PAG, we injected the retrograde tracers, cholera toxin B (CTB) and fluorospheres, into the LC and RVM, respectively (*SI Appendix*, Fig. S5). Random fluorospheres labeling of PAG-RVM neurons was observed throughout the entire PAG region, consistent with a previous report (10), whereas retrograde labeling by CTB from the LC was predominantly observed in the lateral PAG and ventral PAG. Interestingly, there was no overlap of the two different fluorescent signals in the same cells, indicating that the two projection neurons constitute distinct cell populations.

To investigate the expression of PLCβ4 in these two neuronal populations, we preformed immunohistochemistry for PLCβ4 on retrograde-labeled sections of both PAG-LC and PAG-RVM projection neurons. Notably, PLCβ4 was expressed in PAG-LC projection neurons, which are located in the ventral region, but not in PAG-RVM projection neurons (Fig. 2 *A*, *B*, and *D* and *SI Appendix*, Fig. S6).

In addition, double immunofluorescence experiments performed using tissue from GAD67-GFP mice showed that the majority of PLCβ4 was detected in neurons that expressed CamKIIα (calcium/calmodulin-dependent protein kinase II), but not in neurons that expressed GAD67-GFP (*SI Appendix*, Fig. S7), indicating that PLCβ4 was primarily expressed in excitatory neurons at the PAG. Moreover, double immunostaining for

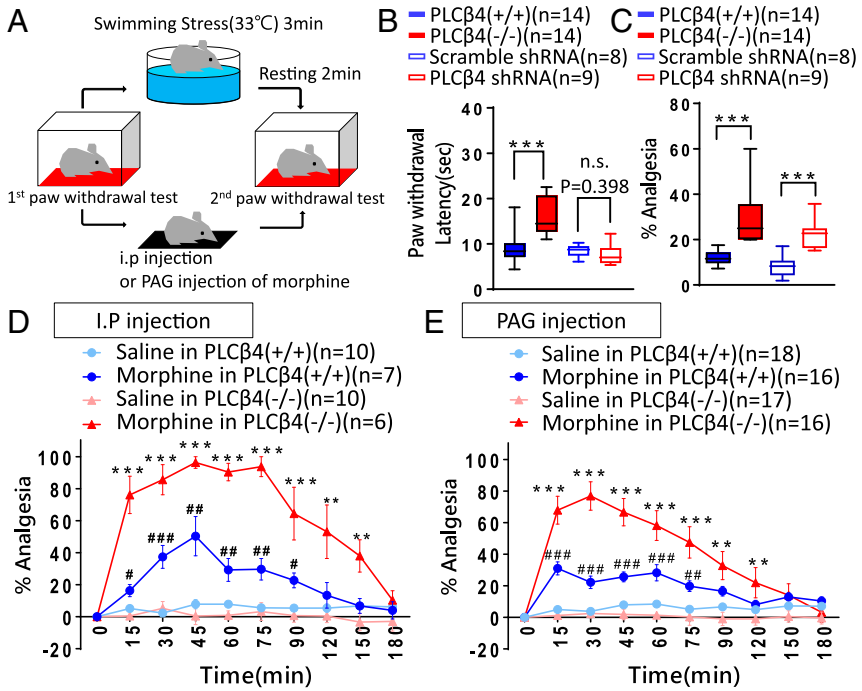

Fig. 1. Improved analgesia in PLCβ4($^{-/-}$) and PAG-specific PLCβ4-knockdown mice under endogenous and exogenous opioidergic conditions. (*A*) Schematic depiction of the endogenous opioid condition and the exogenous opioidergic condition paradigm used in this study. (*B*) Test of basal thermal sensitivity using a hotplate assay. (*C*) Comparison of the analgesic effect after the SSIA behavior test. (*D* and *E*) Time course of the effects of morphine-induced analgesia compared with saline control at the systemic and PAG level. Values are means ± SEM. (*,$^{\#}P < 0.05$; **,$^{\#\#}P < 0.01$; ***,$^{\#\#\#}P < 0.001$; n.s., not significant).

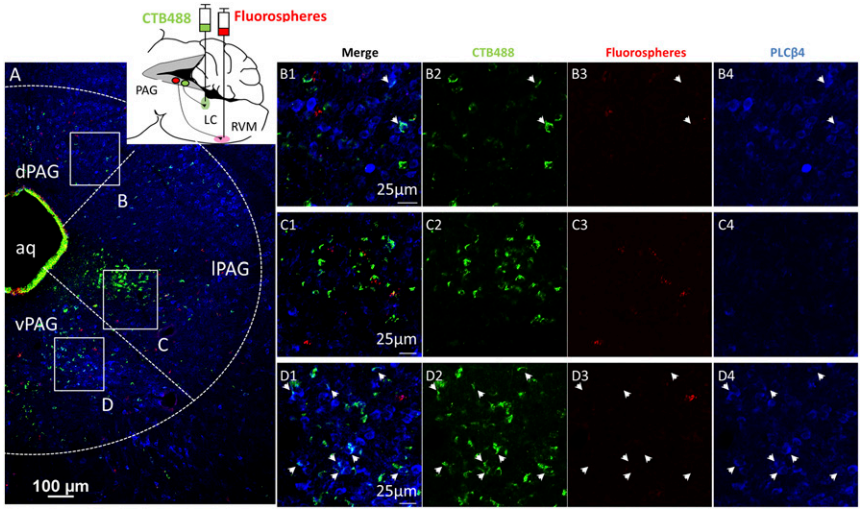

Fig. 2. PLCβ4 is expressed in ventral PAG-LC but not in PAG-RVM projection neurons. (*A*) Representative image of cells in the PAG retrogradely labeled with two different-color tracers. Schematic illustration of dual retrograde tracing from both the LC and RVM (*Inset*). (*B–D*) Zoomed view of the subcellular localization of CTB, fluorospheres, and PLCβ4 in the dorsal PAG (dPAG) (*B*), lateral PAG (lPAG) (*C*), and ventral PAG (vPAG) (*D*). Arrows indicate PLCβ4 immunoreactivity at LC retrogradely labeled neurons. (Scale bars: *A*, 100 μm; *B–D*, 25 μm.)

tyrosine hydroxylase (TH), a marker for the LC (33, 34), showed no PLCβ4 signal in LC neurons (*SI Appendix*, Fig. S8). These findings raised the possibility that, because it is selectively expressed in PAG-LC projections, PLCβ4 may modulate the LC-mediated analgesia circuit.

PLCβ4-Expressing PAG Neurons Make a Direct Synaptic Input to LC→SC Projection Neurons. Although it has been well known that LC neurons receive dense projection from the PAG (29, 30), and that LC neurons innervate the SC to modulate pain transmission (22), confirmation of the direct connectivity of these three brain regions has not been achieved. To address this issue, we used the cTRIO method (29) to examine whether PLCβ4-expressing PAG neurons make monosynaptic connections to LC-SC projection neurons (Fig. 3*A*). We injected a Cre-dependent CAV2 vector carrying the flippase (Flp) recombinase [CAV2-FLEX (loxP)-Flp] into the SC of TH-Cre mice. Because CAV2 infects axons and is retrogradely transported to cell bodies, this combination allows us to express Flp specifically in Cre-expressing LC-SC projection neurons. We then injected the Flp-activatable AAV vectors, AAV10-CAG-FLEX (Frt)-TVA-mCherry and AAV10-CAG-FLEX (Frt)-RG, into the LC to express TVA and rabies glycoprotein (RG) in these cells (Fig. 3*B*). Finally, we injected SADΔG-EGFP (EnvA) (35) in the same region (LC) to label upstream neurons that make direct synaptic contact with LC-SC projection neurons. As expected, we found that some PLCβ4-expressing PAG neurons expressed SADΔG-EGFP (Fig. 3*C*), indicating that PLCβ4-expressing PAG neurons have monosynaptic connectivity to LC neurons and suggesting that these PAG neurons directly regulate the LC-SC projection neurons.

Deletion of PLCβ4 Eliminates Opioid-Induced Suppression of Firing in PAG-LC Projection Neurons. To define the physiological mechanism underlying the enhanced opioid-mediated analgesia associated with a PLCβ4 deficiency, we explored the physiological response of PAG-LC projection neurons to opioids in brain slices. We focused on the caudal portion of the ventral region of the PAG because it has been shown that cells in this region are sensitive to morphine in vivo (36–38) and because retrogradely labeled PAG-LC projection neurons were confined to this region (Fig. 4*A*).

Patch-clamp recording showed no difference in the resting membrane potential of PAG-LC projection neurons between wild-type (−42.82 ± 2.99 mV) and PLCβ4($^{-/-}$) (−41.11 ± 1.2 mV) mice. Moreover, neither wild-type nor PLCβ4($^{-/-}$) PAG-LC projection neurons showed low-threshold spikes (LTSs) (Fig. 4 *B* and *D*). Next, we recorded the discharge activity of PAG-LC projection neurons at −35 mV for 1 min. The discharge activity of PAG-LC projection neurons under these conditions was about ∼4 Hz, and there was no significant difference in this activity between wild-type and PLCβ4($^{-/-}$) mice (Fig. 4 *C* and *E*). However, there was a significant difference in sensitivity to the endogenous opioid neurotransmitter, [met^5]-enkephalin (ME), between wild-type and PLCβ4-deficient neurons. ME is known to be released by stress in vivo and inhibits neurons mainly through μORs (4, 5, 39, 40). Wild-type PAG-LC projection neurons quickly responded to application of ME (30 μM) with a reduction in discharge activity and became progressively hyperpolarized (14.72 ± 2.84 mV) compared with untreated controls ($t = 4.389$, $P = 0.0070$, paired two-tailed Student's t test; Fig. 4 *C* and *F*). In contrast, PLCβ4($^{-/-}$) PAG-LC projection neurons exhibited no change in discharge activity or in the membrane potential in response to the ME treatment ($t = 0.197$, $P = 0.848$, paired two-tailed Student's t test; Fig. 4 *E* and *F*). Consistent with these results, treatment with the μOR agonist, [D-Ala(2),*N*-Me-Phe(4),Gly(5)-ol]-enkephaline (DAMGO), suppressed the neuronal activity of wild-type PAG-LC projection neurons but showed no effect on PLCβ4($^{-/-}$) PAG-LC projection neurons (*SI Appendix*, Fig. S9). To confirm that these results could not be attributed to a change in μORs caused by deletion of PLCβ4, we immunostained PAG-LC projection neurons for μORs. These experiments show that expression of μORs in PAG-LC projection neuron is not altered in PLCβ4($^{-/-}$) mice ($t = 0.748$, $P = 0.456$, unpaired two-tailed Student's t test; *SI Appendix*, Fig. S10). However, the response of the PAG-RVM projection neurons to the ME treatment was not changed in both groups (*SI Appendix*, Fig. S11). These findings suggest that PLCβ4 is required for the μOR-dependent signal transduction that mediates suppression of PAG-LC projection neurons. This, in turn, would lead to a decrease in the activity of downstream target noradrenergic neurons in the LC, resulting in a decrease in norepinephrine-dependent analgesia. In the absence of PLCβ4, noradrenergic neurons in the LC would remain active even in the presence of opioids, thus supporting norepinephrine-dependent analgesia. The net result is an enhancement in opioid-induced analgesia at the systems level in PLCβ4($^{-/-}$) mice compared with wild-type mice.

Enhanced Opioid-Dependent Analgesia in PLCβ4($^{-/-}$) Mice Is Reversed by Antagonism of α2-Adrenergic Receptors. RVM-SC projection neurons innervated the laminae I and II of the lumbar spinal cord (41). Also, the LC is the principal noradrenergic nucleus in the central nervous system and the main source of noradrenergic innervation to the spinal dorsal horn, forming a well-described

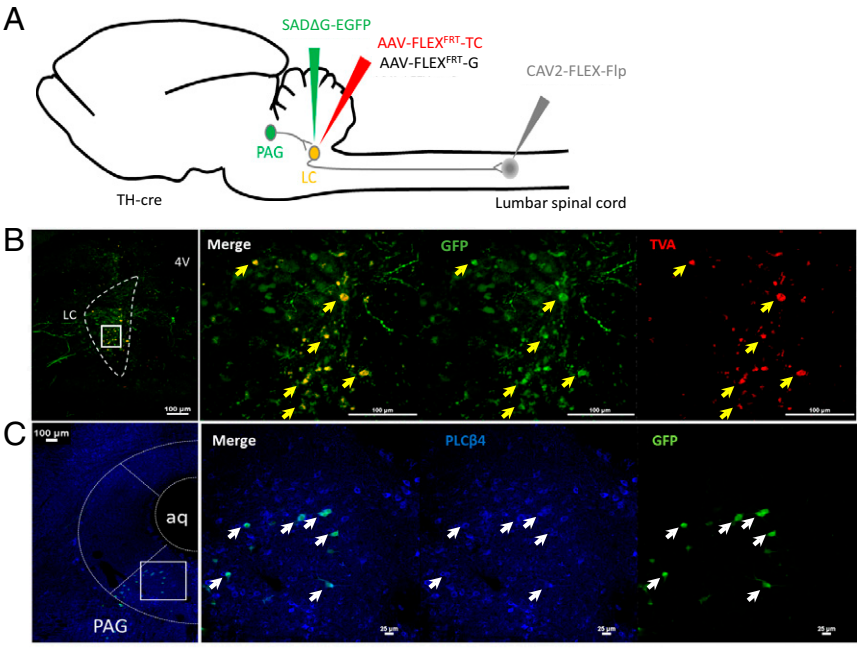

Fig. 3. Transsynaptic tracing of PAG → LC to LC → SC circuit by cTRIO. (*A*) Schematic depiction of experimental procedure. CAV2-FLEX (loxP)-Flp was injected into the SC of TH-Cre mice to express Flp in LC neurons that send projections to the PAG (day 1). Flp-dependent AAV carrying TVA, and RG were then injected into the LC (day 1). SADΔG-EGFP (EnvA) was injected into the same site (day 15). (*B*, *Left*) Distribution of the starter cells (TVA$^+$, SADΔG$^+$ cells) in the LC region. (*B*, *Right*) Magnifications of regions in white rectangles in images (yellow arrows). (*C*, *Left*) PLCβ4-expressing PAG neurons merged with the SADΔG+EGFP signals. (*C*, *Right*) Magnifications of regions in white rectangles in images (white arrows).

analgesic circuit (42, 43). We made the output of LC neurons projecting to the SC to express fluorescence in a Cre-dependent manner to substantiate that RVM and LC neurons engage SC in different pathways (*SI Appendix*, Fig. S12*A*). Axonal terminals from the LC were observed throughout the laminae I ∼ X of the lumbar spinal cord, which made us propose that the targeting of LC-SC neurons is different from that of the RVM-SC pathway (*SI Appendix*, Fig. S12*B*).

In the SC, norepinephrine suppresses pain signals through inhibitory actions on α2A-adrenoreceptors on central terminals of primary afferent nociceptors (presynaptic inhibition), as well as through direct α2-adrenergic actions on pain-relay neurons (postsynaptic inhibition) (19, 20, 23, 24, 27, 44). If the enhanced analgesic effect of locally or systemically infused morphine in PLCβ4($^{-/-}$) mice were a direct result of the continuous LC excitability cause by the persistent activity of PAG-LC projection neurons, blocking the noradrenergic inputs in this mutant should reverse the enhanced analgesic effect. First, we compared the c-Fos expression level in the LC between the wild-type and the PLCβ4($^{-/-}$) mice after the SSIA. As we expected, the PLCβ4($^{-/-}$) mice showed more c-Fos signals in the LC than the wild-type mice, implying hyperexcitability of the LC neurons in the mutant mice. ($t = 10.19$, $P < 0.001$, unpaired two-tailed Student's t test; *SI Appendix*, Fig. S13 *A* and *B*) Next, we used idazoxan, an α2-adrernergic receptor antagonist, that does not interfere with the morphine effect (45). i.p. administration of idazoxan (20 mg/kg) did not affect the basal thermal sensitivity in wild-type ($t = 0.103$, $P = 0.919$, unpaired two-tailed Student's t test) or PLCβ4($^{-/-}$)

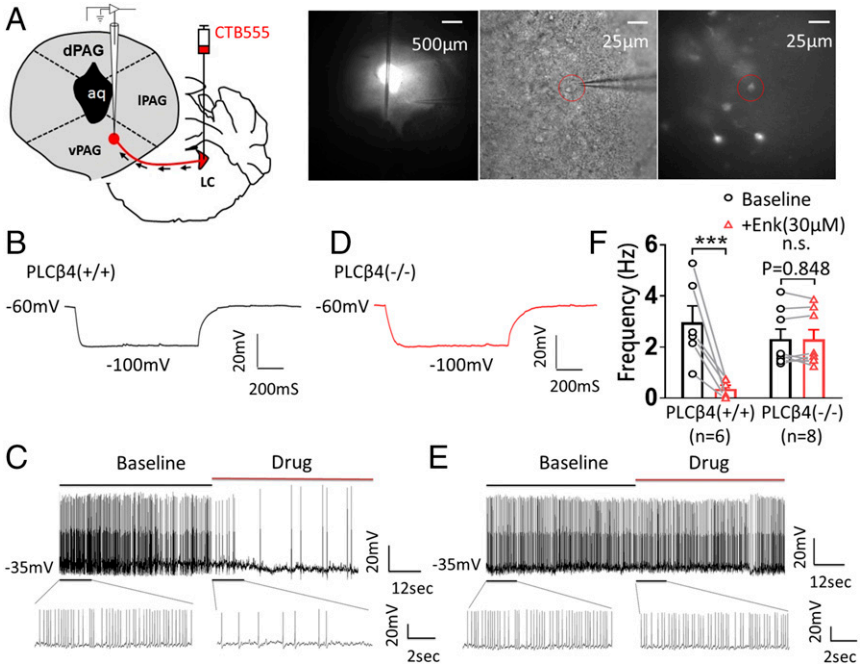

Fig. 4. Discharge activities at depolarized membrane potential in the LC-projecting neurons of the ventral PAG. (*A*, *Left*) Schematic depiction of retrograde labeling in the LC and patch-clamp recordings of cells in the PAG. (*A*, *Right* three images) DIC image (*Left* and *Middle*) and retrograde dye image of a patched neuron in the ventral PAG (vPAG; *Right*). Red circle indicates an example of PAG-LC projection neurons. (Scale bars: *A*, *Middle Left*, 500 μm; *A*, *Middle Right* and *Right*, 25 μm.) (*B* and *D*) Electrophysiological analysis of LTS in PAG-LC projection neurons from wild-type (*B*) and PLCβ4($^{-/-}$) (*D*) mice in response to hyperpolarizing current pulses. (*C* and *E*) Changes in firing patterns for two periods from PAG-LC projection neurons from wild-type (*C*) and PLCβ4($^{-/-}$) (*E*) mice. (*F*) Comparison of discharge activity before and after treatment with ME between in wild-type and PLCβ4($^{-/-}$) PAG-LC projection neurons. Values are means ± SEM. (***$P < 0.001$; n.s., not significant.)

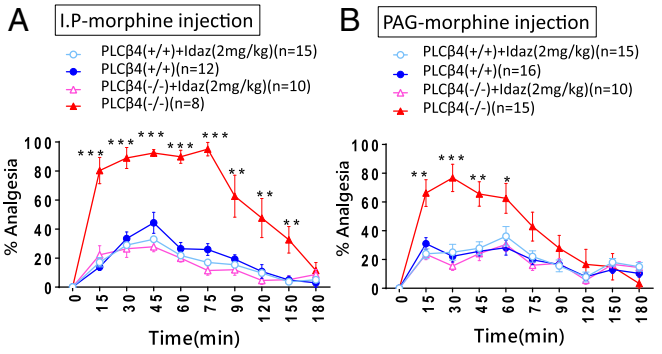

Fig. 5. The reversed opioidergic analgesia by antagonizing systemic α2-adrenergic receptors in PLCβ4 mice. (*A*) Time course of the analgesic effects of idazoxan and morphine in the wild-type and in the PLCβ4 mutant mice by systemic treatment. (*B*) Time course of the analgesic effects of i.p. injected idazoxan with the PAG-specific delivery of morphine in both the wild-type and the PLCβ4 mutant mice. Values are means ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

mice ($t = 0.137$, $P = 0.893$, unpaired two-tailed Student's t test) (*SI Appendix*, Fig. S14*A*). We subsequently infused morphine (10 mg/kg) in combination with a systemic injection of idazoxan to block α2-adrenergic receptor activation. As expected, idazoxan did not influence the analgesic effect of morphine in the wild-type mice, as measured by RM ANOVA ($F_{1,25} = 2.735$, $P = 0.1107$, Fig. 5*A*) or unpaired Student's t test ($t = 1.696$, $P = 0.102$, *SI Appendix*, Fig. S14*B*). Notably, in mutant mice, a systemic injection of idazoxan significantly reduced morphine-induced analgesia compared with morphine alone, resulting in a nearly complete reversal of the potentiated analgesia to wild-type levels ($F_{1,16} = 126.2$, $P < 0.001$, RM ANOVA, Fig. 5*A*; $t = 14.78$, $P < 0.001$, unpaired two-tailed Student's t test; *SI Appendix*, Fig. S14*B*). Next, we systemically injected idazoxan together with PAG-specific morphine infusion in both wild-type and PLCβ4-deficient mouse groups. Systemic idazoxan injection did not affect the analgesia induced by PAG-specific morphine infusion analgesia in wild-type mice ($F_{1,26} = 0.3768$, $P = 0.5447$, RM ANOVA, Fig. 5*B*; $t = 0.424$, $P = 0.674$, unpaired two-tailed Student's t test, *SI Appendix*, Fig. S14*C*). However, the increased PAG-specific morphine-induced analgesia observed in the PLCβ4($^{-/-}$) mice was returned to wild-type levels by i.p. injected idazoxan ($F_{1,23} = 7.678$, $P = 0.0109$, RM ANOVA, Fig. 4*B*; $t = 5.066$, $P < 0.001$, unpaired two-tailed Student's t test, *SI Appendix*, Fig. S14*C*).

Taken together, these results indicate that the persistent activity of LC neurons is responsible for the augmented opioid-induced analgesia in PLCβ4($^{-/-}$) mice.

Discussion

Yin-and-Yang PAG Bifurcation Model. Our data suggest that the stress-induced, opioid-dependent, descending analgesia circuits bifurcate at the PAG into two mutually exclusive pathways that exert opposite effects: The PAG-RVM projection enhances, and the PAG-LC projection suppresses, the descending analgesia (Fig. 6, *Left and Middle*).

The most investigated form of the physiological activation of opioid-linked pain-modulating circuits originates under the general framework of stress-induced analgesia (7, 46). The midbrain PAG was the first discovered descending pain-modulating site in the brain, and the PAG-RVM circuits project to the dorsal horn of the SC and the trigeminal nucleus caudalis, where pain-transmitting neurons are located. In the disinhibition model, opioids activate the PAG–RVM descending pathway via suppression of the inhibitory influence of local GABAergic interneurons, thereby enhancing the antinociceptive drive of the neuronal output to the SC (9, 10). Now the results in this paper reveal the complex organization of the PAG with regard to the control of the stress-induced opioid-dependent descending analgesia. Thus, opioid can both enhance and suppress the descending analgesia.

The Opioid Circuits in the PAG, a Safeguard Against the Adverse Effect of Stress. The LC is activated by physiological and environmental stressors and is thought to play a role in cognitive aspects of the stress response (47). Corticotropin-releasing factor (CRF) acts through activation of the LC-norepinephrine (NE) system to increase the LC neuronal firing rate, thereby contributing to coordination of physiological and behavioral responses to stress (48, 49). Apart from the well-known descending LC-spinal pathway, which is important for pain control, an increase in LC discharge under stress conditions could potentially lead to a serious autonomic failure such as hypertensive challenge (50, 51). Our results suggest that endogenous opioids may serve to counterbalance the adverse effect of stress on the LC–NE system, while achieving analgesia.

Phospholipase C Signaling in the Opioid-Induced Analgesia. Previously, we have reported that the thalamic PLCβ4 regulates the firing properties of thalamocortical neurons through a mechanism mediated by T-type calcium channels (15). Interestingly, PAG-LC projection neurons showed no LTS responses, and their firing was not attenuated by the opioids in the absence of PLCβ4 (Fig. 3). These observations demonstrate that PLCβ4 is required for fine control of opioid responses in the descending pain pathway. Indeed, numerous studies have reported that the PLCβ isoforms are activated by the Gβγ, which is liberated by μORs activation, and induce IP_3 and diacylglycerol formation. This, in turn, results in intracellular Ca^{2+} release from IP_3-sensitive Ca^{2+} stores, PKC activation, and VDCC inhibition (52–57). These conclusions are consistent with prevailing views regarding the importance of the PLC system in opioid-signaling pathways (58, 59).

Additional studies will be required to define the antinociceptive function of other PLC systems in the PAG during the pain transmission. Importantly, our findings suggest that the

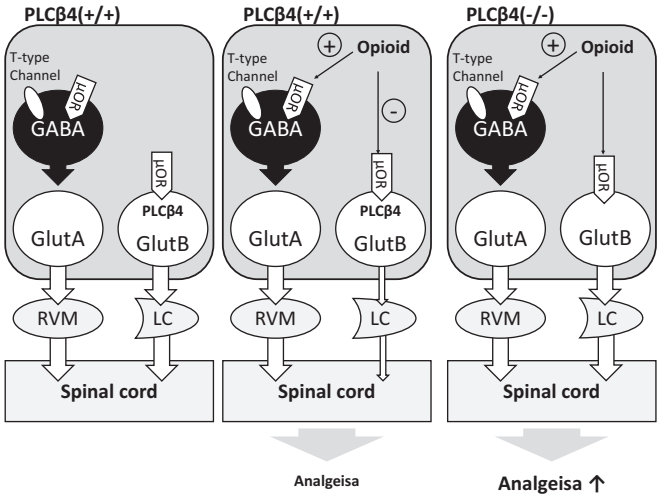

Fig. 6. Working model of the PAG descending pain-gating pathway for the opioid analgesic circuitry. (*Left*) The two distinct pathways in the PAG bifurcation model: PAG-RVM projection neurons and PAG-LC projection neurons. (*Middle*) The physiological role of the PAG-RVM pathway in positive control of analgesia (⊕) proposed in the disinhibition model, and the new proposed model of the PAG-LC pathway for negative control of antinociceptive effects (⊖) in wild-type mice. (*Right*) PAG-LC excitatory transmission showed persistent activity under opioidergic conditions in PLCβ4($^{-/-}$) mice, leading to an enhanced analgesic effect. The thickness of the arrows indicates the relative activity of individual transmissions.

current "Yin-and-Yang" model at the PAG provides a therapeutic target in pain control (Fig. 6, *Right*).

Materials and Methods

Animal care and all experiments were conducted in accordance with the Institutional Review Board of Institute for Basic Science (IBS), Korea for the ethical guidelines of Animal Care and Use. Detailed descriptions of study methods are provided in *SI Appendix, Materials and Methods*.

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