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Alleviating Neuropathic Pain Hypersensitivity by Inhibiting PKMζ in the Anterior Cingulate Cortex

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Synaptic plasticity is a key mechanism for chronic pain. It occurs at different levels of the central nervous system, including spinal cord and cortex. Studies have mainly focused on signaling proteins that trigger these plastic changes, whereas few have addressed the maintenance of plastic changes related to chronic pain. We found that protein kinase M zeta (PKMζ) maintains pain-induced persistent changes in the mouse anterior cingulate cortex (ACC). Peripheral nerve injury caused activation of PKMζ in the ACC, and inhibiting PKMζ by a selective inhibitor, ζ-pseudosubstrate inhibitory peptide (ZIP), erased synaptic potentiation. Microinjection of ZIP into the ACC blocked behavioral sensitization. These results suggest that PKMζ in the ACC acts to maintain neuropathic pain. PKMζ could thus be a new therapeutic target for treating chronic pain.

One key function of the brain is to learn and memorize sensory experiences and then adapt subsequent behavioral responses. Investigations into the molecular mechanisms of such processes provide information for our basic understanding of brain functions (1). Synaptic plasticity is the major cellular model used for understanding these mechanisms. In addition to numerous studies on synaptic mechanisms for learning and memory, these same synaptic mechanisms may be responsible for pathological pain conditions (2–4). For example, nerve injury triggers long-term plastic changes along sensory pathways, from the peripheral sensory terminals to the spinal cord dorsal horn, amygdala, and cortex (3–7). These plastic changes contribute to pain perception, fear, and memory. The persistent nature...
of chronic pain results in resistance to commonly used analgesics (6). Therefore, revealing the molecular mechanisms that are involved in the maintenance of pain-related long-term synaptic plasticity changes is critical for developing effective chronic pain treatments.

Multiple protein kinases are thought to contribute to the induction of long-term potentiation (LTP) and initial consolidation of information storage. Among them, only protein kinase M zeta (PKMζ) maintains persistent synaptic changes (8–10). PKMζ, an atypical isoform of protein kinase C (PKC), has been detected in many regions of the brain, including the frontal cortex (11). In the hippocampus, LTP induction triggers the synthesis of PKMζ and activation of PKMζ is critical for late-phase LTP (L-LTP) and memory consolidation (8, 12–15).

The anterior cingulate cortex (ACC) is a key cortical area involved in chronic pain (2, 16, 17). We first examined whether, in the ACC, peripheral nerve injury causes changes in PKMζ (18). As reported previously (17, 19), behavioral allodynic response was increased 3 days after nerve injury when compared with the response in sham-treated mice (Fig. 1A). The levels of PKMζ in the ACC were significantly increased after nerve injury (sham-operated versus nerve injury group; n = 9 mice for each group, unpaired t test, t16 = −2.51, P < 0.05; Fig. 1, B and C). Because PKMζ is activated by phosphorylation, we also conducted experiments to detect possible changes in the level of phosphorylated PKMζ (p-PKMζ). Consistently, the level of p-PKMζ was also significantly increased (n = 8 for each group, unpaired t test, t14 = −2.26, P < 0.05; Fig. 1, B and D). These data suggest that peripheral nerve injury increases PKMζ activity in the ACC.

To determine whether such changes are long-lasting, we examined PKMζ and p-PKMζ levels 7 and 14 days after nerve injury. Although behavioral allodynia persisted at these time points, the protein levels of PKMζ returned to baseline (Fig. 1C), indicating that the regulation of the amount of PKMζ is short-lasting. However, the p-PKMζ level remained increased two-way analysis of variance (ANOVA), F1,28 = 6.10, P < 0.05; Fig. 1D), suggesting that PKMζ activity could contribute to the maintenance of neuropathic pain. To investigate whether the changes in PKMζ protein levels or activity were a generalized phenomenon in the central nervous system, we also examined the levels of PKMζ and p-PKMζ in the hippocampus and spinal dorsal horn 3 days after nerve injury. PKMζ and p-PKMζ levels in the hippocampus and spinal cord did not change after nerve injury (fig. S1), suggesting that changes in PKMζ are affected in a regionally specific manner in response to peripheral nerve injury.

What could be the possible second messengers that trigger the activation and synthesis of PKMζ in the ACC? In the ACC, calmodulin-stimulated adenyl cyclase 1 (AC1) is critical for ACC LTP and behavioral sensitization caused by peripheral nerve injury (16, 20). Thus, we examined whether AC1 acts upstream of PKMζ in the ACC after nerve injury by using AC1 gene knockout mice (AC1−/−). AC1−/− mice did not show any significant behavioral sensitization 3 days after nerve injury (Fig. 1A). Similar amounts

![Fig. 2. Bilateral microinjections of ZIP into the ACC decreased allodynia in mice with peripheral nerve injury. (A) The schematic view of the microinjection experiments. (B) Location of microinjection sites in the ACC. Solid circles represent ZIP injection sites, whereas open circles represent saline injection sites. The right image shows an example of the location of the cannula tip in one ACC slice with hematoxylin and eosin staining. The injection site is indicated by an asterisk. (C) Microinjection of ZIP (blue) into the ACC decreased the positive response rate in the nerve injury animal, whereas saline microinjection (black) into the ACC had no effect on the response level. Open grey circles represent the summary control data of positive response level tested from the saline and ZIP group. The red arrows indicated the time point of ZIP injection. *P < 0.05; error bars, SEMs. (D) Two hours after injection, ZIP still has an analgesic effect. However, the allodynia response recovered to preinjection level 24 hours after injection. (E) ZIP has no effect on the traveled distance in the open field test. (F) The location of microinjection sites in the ACC in the conditioned place preference experiments. Solid or open circles indicate the location of the injection that did or did not induce place preference, respectively. (G) The changed time in the ZIP paired chamber was significantly different from that of the saline paired chamber. *P < 0.05. (H) The effect of ZIP infusion into the ACC on contextual fear memory. Representative brain slices show the location of the injection site. ZIP was infused into the ACC 2 hours before memory retrieval, which was tested 3, 7, and 18 days after conditioning. (I) ZIP did not change the response latency in the tail-flick (TF) test. (J) Microinjection of ZIP into the ACC had no effect on the paw-withdrawal threshold in normal mice.](image-url)
and regionally selective analgesic effect on nerve injury–related tonic pain.

What is likely to be the synaptic mechanism responsible for the analgesic effects produced by ZIP in neuropathic pain? PKMζ can potentiate postsynaptically the amplitude of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor-mediated excitatory postsynaptic currents (EPSCs) (21). Because glutamatergic synaptic transmission in the ACC is increased after nerve injury (17), we speculated that PKMζ may contribute to the maintenance of enhanced synaptic transmission induced by nerve injury. First, we recorded AMPA receptor-mediated EPSCs in layers II and
III of the ACC 3 or 7 days after nerve injury (24). After obtaining a stable baseline, we bath-applied ZIP (5 μM) to block PKMζ activity. The evoked EPSCs (eEPSCs) were significantly decreased in six of seven experiments, and total mean responses were reduced to 83 ± 6% (mean ± SEM) of baseline after 10 min of bath application of ZIP (n = 7; Fig. 3, A and C). In contrast, ZIP did not affect the amplitude of eEPSCs recorded in neurons from sham-operated mice (n = 6; Fig. 3, B and C).

The ZIP-induced reduction of eEPSCs may be due to a decrease in either the AMPA receptor unitary conductance (γ) or number of active channels. Peak-scaled nonstationary fluctuation analysis (NSFA) has been developed to distinguish between these parameters (23, 25, 26). As a control, we performed manipulations that should affect either the number of active channels or the single-channel current amplitude but not the single-channel conductance. Similar to previous reports (26), NSFA was able to detect a reduction in the number of active channels induced by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (0.5 μM) (n = 4, paired t test, t3 = 6.60, P < 0.05; Fig. 3D) and the change of single-channel current amplitude after an alteration in membrane potential (n = 6 per holding potential; Fig. 3E). NSFA was performed on five of seven neurons where ZIP produced substantial inhibition of the eEPSCs (mean 76 ± 5%); we found that the number of active channels decreased to 65 ± 11% (n = 5, paired t test, t4 = 6.59, P < 0.05) of the baseline (Fig. 3, F and G), whereas γ was unaffected. This effect was not observed in slices obtained from sham-operated mice (Fig. 3G). These results suggest PKMζ may exert its effect by increasing the number of AMPA receptors (AMPARs) at synapses in the ACC. A biochemical assay also showed that ZIP infusion reduced postsynaptic GluA1, one component of AMPARs, selectively in the nerve injury group (n = 4 per group, unpaired t test, t6 = 7.06, P < 0.001; fig. S3).

Sensory synaptic transmission in the spinal cord dorsal horn plays important roles in chronic pain and so could be an additional site of action of ZIP, although PKMζ and p-PKMζ levels in the spinal cord did not change in response to nerve injury. To examine the effects of ZIP on spinal sensory transmission, we recorded eEPSCs from neurons in lamina II of the spinal cord after dorsal root stimulation. We found that peripheral nerve injury significantly increased the amplitudes of eEPSCs at 1.5 and 2 times of the threshold-stimulating intensity (two-way ANOVA, F1,97 = 10.00, P < 0.01; Fig. 3, H to J). In contrast to the ACC, bath application of ZIP (5 μM) to the spinal cord did not distinguish between EPSCs recorded from sham and nerve injury mice (Fig. 3K, two-way ANOVA, F1,68 = 1.02, P > 0.05). In both cases, there was a reduction in sensory transmission (Fig. 3K), which may be due to a constitutive activity of PKMζ in spinal sensory neurons. Consistent with these findings, intrathecal injection of ZIP (2 nmol/μl, 5 μl per animal) did not produce any analgesic effect (n = 4, paired t test, t3 = 0.52, P > 0.05).

The ACC is a heterogeneous cortical area, in which not all neurons would be expected to respond to neuropathic pain. We therefore used transgenic mice in which the expression of green fluorescent protein (GFP) is controlled by the promoter of the c-fos gene (27, 28), because c-fos is known to be activated in sensory neurons after injury (29). In these mice, we found that many

![Image](https://www.sciencemag.org/science-vol330-3-december-2010/art/1403/jz/full/1403)
ACC neurons were activated 3 and 7 days after nerve injury (Fig. 4, A to C). We performed whole-cell patch-clamp recordings from FosGFP-positive pyramidal cells (“green” cells) that were activated by allodynic pain and compared these with nonactivated neighboring neurons and neurons in sham-operated FosGFP mice (Fig. 4, D and E). The amplitudes of eEPSCs stimulated at the same intensity in the FosGFP-positive neurons were significantly greater than those of FosGFP-negative neurons in the ACC of nerve injury and sham-operated mice (two-way ANOVA, $F_{2,59} = 17.25, P < 0.05$, Fig. 4F). ZIP (5 μM) significantly reduced the amplitude of eEPSCs ($n = 7$, paired t test, $t_6 = 2.85, P < 0.05$; Fig. 4, G and H) in FosGFP-positive pyramidal cells but had no effect on eEPSCs in either the FosGFP-negative neurons from the same nerve injury group or in neurons from the sham group ($n = 5$, nerve injury, $t_4 = –0.63$; $n = 6$, sham, $t_4 = –1.45$; paired $t$ test, $P > 0.05$; Fig. 4, G and H). Furthermore, ZIP had no effect on the eEPSCs recorded on the FosGFP-positive neurons from the primary somatosensory cortex of mice with nerve injury. Therefore, PKMζ is required for the maintenance of L-LTP in the ACC. To test whether nerve injury–triggered plasticity shares some common mechanism with L-LTP in the ACC, we performed occlusion experiments. Indeed, synaptic potentiation was significantly reduced in slices of nerve injury mice as compared with those of control mice (unpaired $t$ test, $t_{12} = 3.52, P < 0.05$; Fig. 4, I and J).

We found that PKMζ is critical for the maintenance of long-term plasticity in the ACC and contributes to neuropathic pain by modulating excitatory synaptic transmission within this cortical structure. Its role in maintaining synaptic potentiation is a critical part in allodynia resulting from nerve injury. Therefore, PKMζ in ACC synapses provides a potential new therapeutic target for the treatment of chronic pain.

**References and Notes**

18. Materials and methods are available as supporting material on Science Online.

**Supporting Online Material**

www.sciencemag.org/cgi/content/full/330/6009/1400/DC1

**Materials and Methods**

Figs. 51 to 54

**References**

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**Micro-Optical Sectioning Tomography to Obtain a High-Resolution Atlas of the Mouse Brain**

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The neuroanatomical architecture is considered to be the basis for understanding brain function and dysfunction. However, existing imaging tools have limitations for brainwide mapping of neural circuits at a mesoscale level. We developed a micro-optical sectioning tomography (MOST) system that can provide micrometer-scale tomography of a centimeter-sized whole mouse brain. Using MOST, we obtained a three-dimensional structural data set of a Golgi-stained whole mouse brain at the neurite level. The morphology and spatial locations of neurons and traces of neurites could be clearly distinguished. We found that neighboring Purkinje cells stick to each other.

One of the most important aims in neuroscience is to obtain an interconnection diagram of the whole brain. In mammals, individual neurons are considered the basic units of the brain, but the complex functions of the brain depend more on the fine anatomical architecture of a very large number of neurons and their connections ([1]). Although modern neuroscience has made great progress in brain studies at both the system and cellular levels, our empirical knowledge of neuroanatomical connectivity remains inadequate, limiting the progress of brain studies ([2]). Thus, it is necessary to gain new insights into the morphology, localization, and interconnectivity of neural circuits throughout the whole brain at an appropriate resolution. Individual synapses are the finest functional element in circuits, but it is currently not technologically feasible to study the brainwide connectivity of complex vertebrate organisms (e.g., mice) at the synaptic level. In contrast, mesoscale techniques are currently more feasible and applicable for understanding specific neural functions ([2]).

Light microscopy has remained a key tool for neuroscientists to observe cellular properties at...