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Targeting neuronal adenylyl cyclase for the treatment of chronic pain

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Pain research is currently undergoing dramatic changes. In the area of basic pain research, new discoveries have been made towards the understanding of pain transmission, modulation and plasticity. However, many of these basic discoveries have not yet led to the development of new drugs for the treatment of chronic pain. One major reason for this disconnection is the lack of translational research and drug discovery based directly on the novel pain mechanism. In this review, I focus on activity-dependent potentiation in pain-related cortical areas and recent translational research on adenylyl cyclase subtype 1 (AC1) as a novel target for treating chronic pain. In particular, I discuss the AC1 inhibitor, NB001, which produces powerful analgesic effects in animal models of chronic pain by inhibiting chronic pain-related cortical potentiation.

Chronic pain is a major health problem, causing both economic and emotional hardship in modern society. Patients with chronic pain can be forced to quit their jobs and undergo various treatment procedures. If their chronic pain does not respond to the limited treatment available, patients often resort to the long-term use of analgesics, but still have an inability to control their pain sufficiently. This can result in emotional suffering for both the patients and their families. Thus, there is a crucial need to identify novel drugs that can help to reduce chronic pain.

Tissue or nerve damage often causes chronic pain, and several animal models have been developed to investigate the basic mechanisms of such pain. Hyperalgesia and allodynia are typical behavioral responses associated with chronic pain. In hyperalgesia, the behavioral response to noxious stimuli and the intensity of pain are increased. Allodynia is a pathological state in which the nociceptive threshold is decreased and a normally non-noxious stimulus can induce pain. The use of animal models has aided understanding of the cellular and molecular mechanisms of hyperalgesia and allodynia in chronic pain. Both peripheral and central sensitization contribute to chronic pain. Peripheral sensitization reflects the increased sensitivity of primary afferent nociceptors, and includes lowered thresholds and an increased responsiveness of the skin or internal organs. Furthermore, both during and after injury, synaptic transmission in the central nervous system (CNS) undergoes long-lasting changes. Some of these central changes are permanent, altering the perception of future sensory stimuli by the brain. Unlike hyperalgesia and allodynia, spontaneous pain is difficult to study in animal models, although some recent studies have tried to tackle this problem [1,2]. One key finding related to chronic pain is that such pain probably results from the over-excitation of pain-related sensory transmission (spinal cord), perception (cortex) and modulation (descending facilitation) [3–5]. Important questions remain regarding how synaptic plastic changes affect network activity, and how network overexcitation interacts with consciousness and the feeling of pain. From a translational point of view, the understanding of molecular and cellular mechanisms of pain transmission, perception and modulation should aid in the development of more effective drugs for alleviating chronic pain.

In this review, I summarize recent progress made by neurobiological studies of cortical plasticity in chronic pain. I also present a recent new discovery of a neuronal specific, activity-dependent adenylyl cyclase (AC) for the treatment chronic pain, including neuropathic and inflammatory pain.

The basic neuronal mechanism of chronic pain

Cumulative evidence using integrative neurobiological approaches suggests that physiological pain (or acute pain) is

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distinguished from pathological pain (chronic pain) at the cellular and molecular levels. For example, in excitatory synapses, physiological pain is probably mediated by normal sensory synaptic transmission without the activation of activity-dependent signaling pathways. By contrast, pathological pain is probably triggered by transcriptional and translational events, and might also result in structural changes and reorganization in the central synapses [6–12]. Most conventional analgesics often unselectively affect synaptic transmission (excitatory and inhibitory transmission); however, there is not a single clinical drug for the treatment of chronic pain that selectively targets pathological pain.

Recent molecular studies of different levels of the CNS have underscored the fact that the mechanisms leading to chronic neuropathic pain are much more complicated than was previously assumed. At peripheral sites, injuries trigger sensitization and induce prolonged abnormal neural activity along primary afferent fibers [13–15]. In the spinal cord, long-term potentiation (LTP) of sensory synaptic transmission in the spinal dorsal horn projecting neurons often occurs [3,6]. Recent work demonstrates that long-term synaptic plasticity also takes place in cortical areas that participate in pain perception, such as the anterior cingulate cortex (ACC), prefrontal cortex (PFC) and insular cortex [4,7,8,16–19]. Similar changes can be also found in the neurons located in subcortical areas, such as the amygdala [20,21]. Furthermore, behavioral evidence indicates that changes in cortical excitation might regulate subsequent pain transmission in the spinal cord by top-down descending facilitation modulation [22–25].

Targeting synaptic potentiation for the treatment of chronic pain

Given that that spinal and cortical potentiation contribute significantly to chronic pain, it is conceivable that proteins and ion channels that are involved in the induction and expression of plastic changes in somatosensory pathways are potential drug targets for treating chronic pain. Among the many candidates that have roles in central plastic changes, the *N*-methyl-D-aspartate (NMDA) receptor, a major glutamate receptor that is important for synaptic LTP [26,27], has been intensively investigated [28]. The NMDA receptor NR2B subunit has been selected as a potential drug target, because doing so results in fewer adverse effects than targeting other NMDA receptor subunits [28–31] (Box 1).

One possible solution to avoid NMDA receptor-related adverse effects is to target NMDA receptor downstream proteins or messengers. Many intracellular proteins have been reported to act downstream from NMDA receptors, such as AC subtype 1 (AC1), protein kinase C (PKC), calmodulin-dependent protein kinase II (CaMKII) and tyrosine kinases. Among them, calcium-stimulated AC1 has a crucial role in pain-related LTP in both the spinal cord dorsal horn and the ACC. Behaviorally, AC1- and AC8-knockout (KO) mice show reduced inflammatory, deep muscle pain and neuropathic pain, whereas other physiological functions remain intact in AC1-KO mice, including acute pain [32], hippocampal LTP and related Morris water maze performance, as well as anxiety-like behaviors and motor functions [33]. Given that that AC1 is expressed mainly in the CNS, I suggest that AC1 is a suitable neuron-specific drug target for treating neuropathic pain.

Cortical proteins as drug targets for treating chronic pain

Most pain research focuses on the spinal cord dorsal horn as a major target for treating chronic pain. One obvious consideration is the lack of adverse effects on the CNS if drugs are only acting locally. However, in reality, most analgesic drugs act on the CNS, including gabapentin, opioids and calcium channels blockers. Cumulative evidence from animal and human studies suggests that the cortex and related areas are not only important for processing pain perception, but also for undergoing plastic changes, and might have an active role in chronic pain [4,34,35]. Active cortical involvement in chronic pain is similar to the fear memory process [36,37]. Fearful information is mainly processed by the amygdala and hippocampus initially, and permanent painful or fearful information is stored in cortical areas. For tissue or nerve injury, recent evidence indicates that cortical synapses that receive noxious or painful information undergo plastic changes and, in the long term, trigger structural changes. Although there is no doubt that certain forms of chronic pain are driven mainly by abnormal peripheral inputs, many forms of chronic pain are not. Preventing further plastic changes or erasing existing plastic changes should, therefore, be the focus of new drug development.

Pain-related cortical synapses undergo LTP

Synaptic and cellular mechanisms of excitatory transmission and plasticity are best studied using *in vitro* brain slice preparation. Electrophysiological, pharmacological and genetic studies reveal that glutamate is the fast excitatory transmitter in the ACC. Most of excitatory currents in ACC pyramidal cells are mediated by glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors [38]. The glutamate receptor subunits GluK5 and GluK6 (or GluR5 and GluR6) containing Kainate receptors contribute to 10–15% of excitatory transmission. Both LTP and long-term depression (LTD) can be induced in ACC slices of adult mice or rats. Different stimulation protocols have been found for inducing long-lasting potentiation of synaptic responses in ACC cells. Both field recording and whole-cell patch-clamp recording techniques can be used to study LTP and LTD in ACC slices [39,40]. For field recording from adult rat or mouse ACC (*in vitro* slices or *in vivo* recording), glutamatergic synapses in the ACC can undergo long-lasting potentiation in response to theta burst stimulation (TBS), a paradigm more closely related to the activity of ACC neurons. LTP is also observed after peripheral injury (Fig. 1a,b), with potentiation lasting for at least 40–120 min [41]. Whole-cell patch-clamp recordings allow for better investigation of synaptic mechanisms for LTP in the ACC [40]. LTP can be induced using three different protocols, including the pairing training, the spike–excitatory postsynaptic potential (EPSP) pairing, and TBS protocol [40] (Fig. 1c,d). Unlike field recordings, LTP induced by the pairing protocol is triggered mainly by the activation of NMDA receptors but not through L-type voltage-dependent calcium channels (VDCCs) [40]. These studies provide clear evidence that LTP induced by different stimulation protocols shares some common signaling pathways. Similar LTP have been reported in other pain-related excitatory synapses, such as PFC, somatosensory cortices and the insular cortex [4,41].

BOX 1

Genetic search for novel drug target for chronic pain

Typically, the search for targets of chronic pain starts with existing signaling proteins. It is common for industry to focus on protein targets or ion channels that are important for pain transmission, such as transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors. It is believed that the input of pain from primary nociceptive fibers can be reduced, it will be possible reduce chronic pain. Even under conditions of chronic pain, some spontaneous pain might be maintained by ongoing activity from peripheral sensory fibers. Thus, reducing or inhibiting activity from these nociceptive fibers is potentially important for reducing pain. However, recent studies suggest that certain types of chronic pain are not simply the result of peripheral sensitization. In some cases, physicians cannot even identify the source of chronic pain (Fig. I).

The use of transgenic and gene KO mice provides new approaches for searching for new pain targets. Mutant mice are powerful tools for studying the neurobiological functions of a simple gene or protein before the development of an initial inhibitor or activator. Furthermore, it is possible to study the contribution of each protein isoform subtype. Often, chemical inhibitors are not highly selective. Modern neurobiological approaches provide a better assessment for the basic mechanisms of chronic pain, and possible roles of new proteins. Furthermore, the potential adverse effects of inhibiting new proteins can also be investigated under *in vivo* conditions. The use of the NMDA receptor NR2B subunit and AC1 as drug targets for chronic pain are good examples of such genetic approaches [24,50,67].

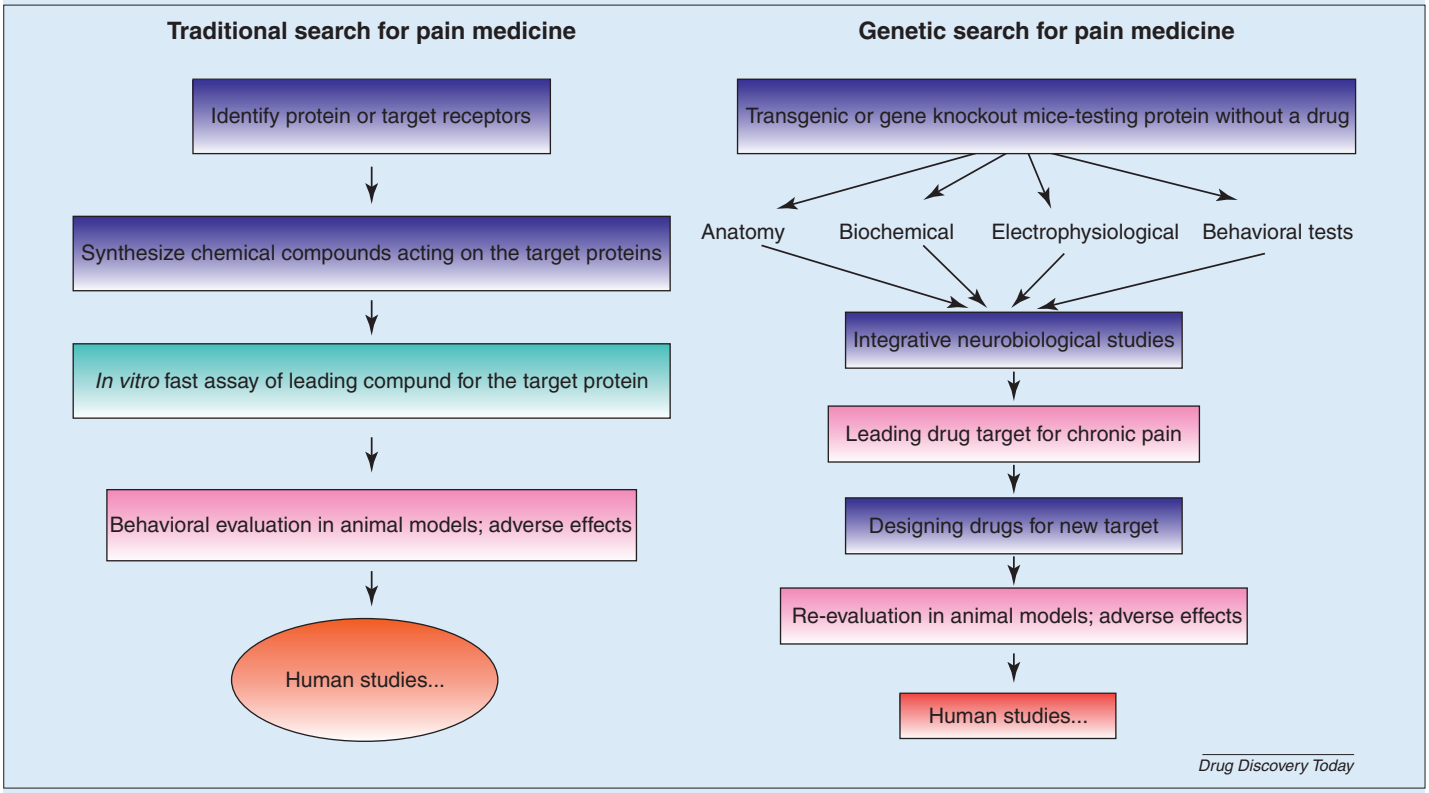

FIGURE I

LTP can be divided into at least two major phases. Most electrophysiological studies of LTP have focused on early LTP (E-LTP). Late-phase LTP (L-LTP) that lasts for at least 3 h after induction has been reported in the hippocampus [42–45], but is less investigated in pain-related cortex areas, such as the ACC. Using multiple channel field recording techniques, strong TBS is able to induce L-LTP that lasts for 3–6 h in ACC slices [46] (Chen *et al.*, unpublished data; Fig. 1e). This key mechanism shows that chronic pain and ACC LTP share similar mechanisms, as seen in occlusion experiments. If ACC LTP is already induced in mice with chronic pain, one would expect that TBS will fail to induce any further potentiation. In fact, nerve injury has been reported to occlude LTP, especially L-LTP, in ACC slices, whereas early potentiation can still be induced in slices of animal tissue with nerve injury. These experiments provide direct evidence showing that L-LTP and chronic pain share similar mechanisms in the ACC.

AC1 as a key messenger in neurons

cAMP is a key intracellular second messenger that has crucial roles in many physiological functions, such as learning and memory, chronic pain, emotional fear and drug abuse [4,45,47]. AC is the enzyme that catalyzes ATP to cAMP. Two major families of AC have been reported: nine membrane-bound ACs (AC1–9) and one soluble AC (sAC). Unique organ and cellular distributions and activation mechanisms support distinct physiological functions of each AC isoform in biological systems.

Of these ten subunits, AC1 and AC8 are two of the AC subtypes that respond positively to CaM [48]. Compared with AC8, AC1 is

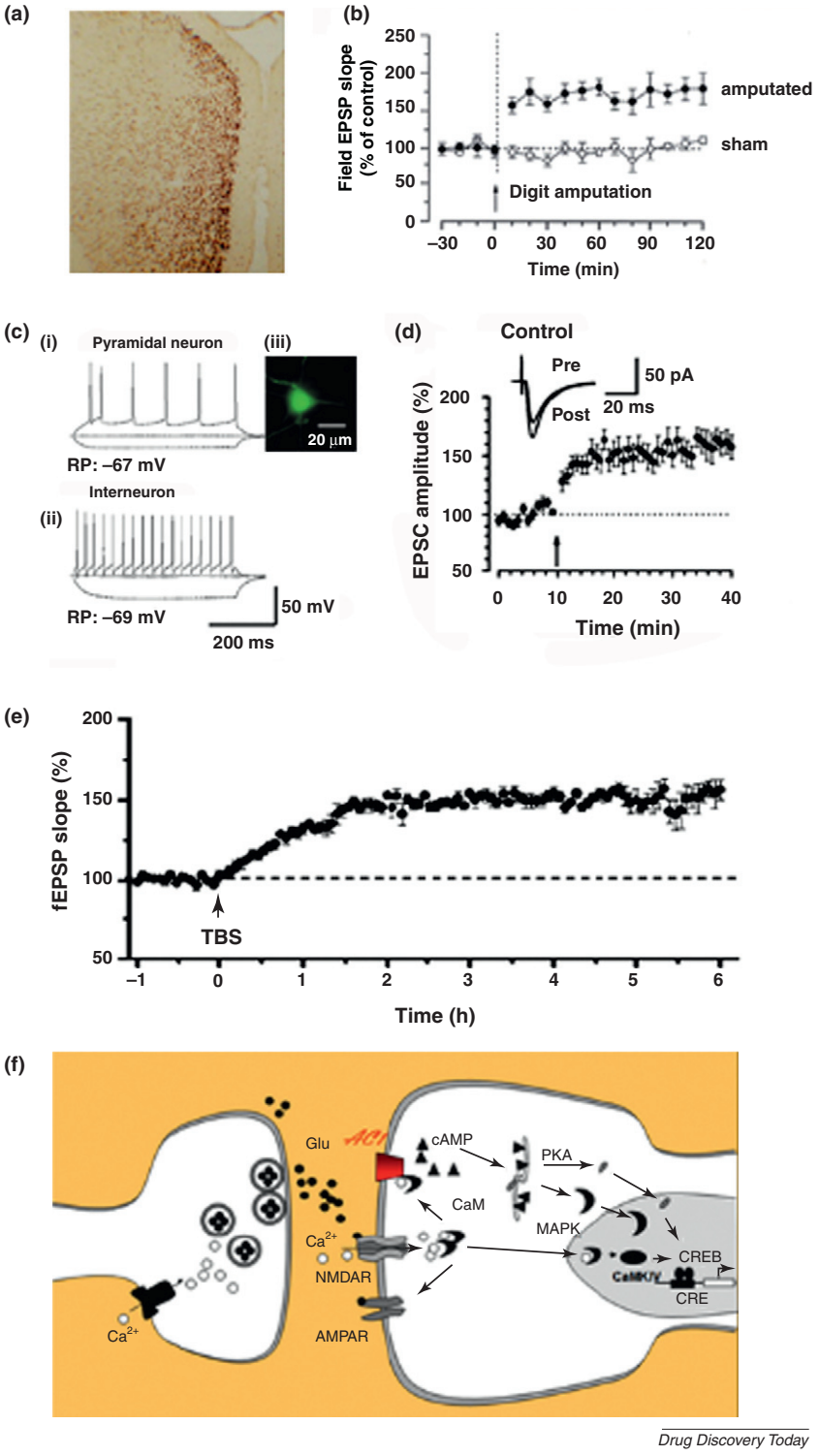

FIGURE 1

Long-term potentiation (LTP) as a cellular model for chronic pain in the anterior cingulate cortex (ACC). **(a)** Activation of immediate early genes in ACC neurons of an adult rat after peripheral injury. **(b)** *In vivo* recording of ACC LTP in adult rats after amputation of a single digit in the hind paw under anesthesia. **(c)** Current-clamp recordings to identify pyramidal neurons (i) and interneurons (ii) of adult mice by current injections of −100, 0 and 100 pA. A labeled pyramid-like neuron is shown in (iii). RP: resting membrane potential. **(d)** LTP was induced in pyramidal neurons in adult mouse ACC by the pairing training protocol (indicated by an arrow). The insets show averages of six excitatory postsynaptic currents (EPSCs) 5 min before and 25 min after the pairing training (arrow). The broken line indicates the mean basal synaptic responses. **(e)** Field recording of late-phase LTP (L-LTP) in adult mouse ACC slices. **(f)** Model of ACC LTP. Neuronal activity triggers the release of glutamate (Glu: pink circles). Subsequent activation of glutamate *N*-methyl-D-aspartate (NMDA) receptors results in increases of postsynaptic Ca^{2+} in dendritic spines. Ca^{2+} is an important intracellular signal that triggers a series of biochemical events that contribute to the expression of ACC LTP. Intracellular Ca^{2+} binds to calmodulin (CaM) and leads to the activation of calcium-stimulated adenylyl cyclases (ACs), including AC1 and calcium-CaM-dependent protein kinases (CaMKs) [e.g. protein kinase C (PKC), calmodulin-dependent protein kinase II and IV (CaMKII and CaMKIV)]. In turn, calcium-CaMKs phosphorylate glutamate

more sensitive to an increase in calcium. In the ACC, AC1 is highly expressed in cingulate neurons located in most of the ACC layers [32]. AC1 is selective for plastic changes and deletion of the gene encoding AC1 does not affect basal glutamate transmission in the ACC. By contrast, LTP induced by TBS or pairing stimulation is abolished in cingulate pyramidal cells [49]. Whole-cell patch-clamp recordings have also revealed that AC1 activity is required for the induction of LTP in ACC pyramidal cells. By using chemical design and biochemical screening, several selective inhibitors of AC1 have been identified. It has been consistently demonstrated that the pharmacological inhibition of AC1 in ACC neurons abolishes LTP induced by pairing training [50].

Recent data also show that AC1 is essential for the induction of L-LTP in the ACC synapses. In wild-type mice, TBS induced L-LTP that lasted for at least 3–6 h; by contrast, TBS failed to induce any significant potentiation in ACC slices of AC1-KO mice (Chen *et al.*, unpublished data). In addition to the contribution to the ACC, AC1 activity is likely to contribute to other pain-related cortical areas, such as PFC, insular cortex and somatosensory cortex. It has been reported that AC1 activity is required for injury-activated immediate early gene activity in these areas. Similar LTP induction protocols also induce LTP in PFC, somatosensory cortex and insular cortex areas.

AC1 contributes to behavioral sensitization and spinal facilitation and/or potentiation

The behavioral contribution of AC1 to chronic pain has been investigated using AC1-KO mice [32]. Whereas wild-type and AC1- as well as AC8-KO mice were indistinguishable in tests of acute pain, behavioral responses to peripheral injection of two inflammatory stimuli (formalin and complete Freund's adjuvant) were reduced or abolished in AC1-KO mice. AC1 also contributes to inflammation-induced activation of cAMP response element-binding (CREB). Using an acute persistent inflammatory muscle pain model, it has been shown that the behavioral nociceptive responses of both the late phase of acute muscle pain and chronic muscle inflammatory pain are significantly reduced in AC1-KO mice [51].

The role of AC1 in chronic pain-related plasticity changes is not limited to the cortex. AC1 activity has also been shown to be crucial for spinal cord facilitation and plasticity. In the spinal cord dorsal horn, application of low doses of serotonin (5-HT) to slices of tissue from young animals or the co-application of forskolin and 5-HT to slices of adult animal tissue produced long-lasting facilitation of excitatory synapses transmission between primary afferent fibers and dorsal horn neurons. This enhancement requires the recruitment or trafficking of functional AMPA receptors. Studies using AC1-KO mice found that calcium-sensitive CaM-regulated AC1 is required for the enhancement [52].

In addition, AC1 also contributes to the activation of the extracellular signal-regulated kinase (Erk) either after peripheral tissue inflammation *in vivo*, or by glutamate or substance P (SP) *in vitro* spinal cord slices. It also contributes to spinal LTP induced by pairing protocols in spinal dorsal horn neurons [53].

Cortical amplification of synaptic transmission after injury

Unlike spinal cord dorsal horn neurons or neurons in the somatosensory cortex, neurons in the ACC show a widespread and diffuse receptive field that can often cover the whole body of an organism [54]. The unique properties of ACC neurons enable one to detect long-term plastic changes in them without using selective makers. By recording AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) in pyramidal neurons in layer II/III of the ACC in mice with peripheral nerve ligation [55], it was shown that the input (stimulation intensity)–output (EPSC amplitude) curve of the AMPA receptor-mediated current was significantly shifted to the left after peripheral nerve injury, compared with that in a control group. These results suggest that excitatory synaptic transmission was increased in the ACC after peripheral nerve injury [18] (Fig. 2).

Two major synaptic mechanisms might contribute to immediate synaptic potentiation (reviewed in [56]). For presynaptic amplification, data collected using three different experimental measurements consistently suggest that presynaptic release of glutamate is significantly enhanced. Electrophysiological measurements include paired-pulse facilitation (PPF), miniature EPSC analyses and the use of MK801. PPF is a transient form of plasticity commonly used as a measure of presynaptic function, in which the response to the second stimulus is enhanced as a result of residual calcium in the presynaptic terminal after the first stimulus. After nerve ligation, there was a significant reduction in PPF in ACC neurons compared with those from control mice. In the case of AMPA receptor-mediated miniature EPSCs (mEPSCs), following peripheral nerve injury, there was an obvious increase in mEPSC frequency in ACC neurons compared with that of the control group. Finally, the blocking rate of the irreversible NMDA receptor blocker, MK-801, was measured in both control mice and mice with neuropathic pain. The blocking rate of the NMDA receptor-mediated synaptic current by MK801 is used to estimate the transmitter-release probability. A significantly faster decay time by MK801 was observed in mice with nerve ligation than in control mice. Taken together, these results indicate that the enhanced excitatory synaptic transmission in the ACC after nerve injury is to the result of an increase in the probability of presynaptic neurotransmitter release.

For postsynaptic changes, changes have been detected in the amplitude of mEPSCs, suggesting that there is an increase of postsynaptic responsiveness after nerve injury. By using western blot, no difference was found in the expression levels of both GluA1 (or GluR1) and Glu A2&3 (GluR2&3) receptors in ACC between control mice and mice with nerve ligation. However, induction of neuropathic pain by nerve ligation was found to be associated with an increase in the abundance of GluA1 subunits in

α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic (AMPA) receptors, increasing the sensitivity to extracellular glutamate. Trafficking of additional AMPA GluA1 receptors might also contribute to synaptic potentiation. *Abbreviations*: CRE, cAMP response elements; CREB, cAMP response element-binding; MAPK, mitogen-activated protein kinase.
Modified, with permission, from [40,67].

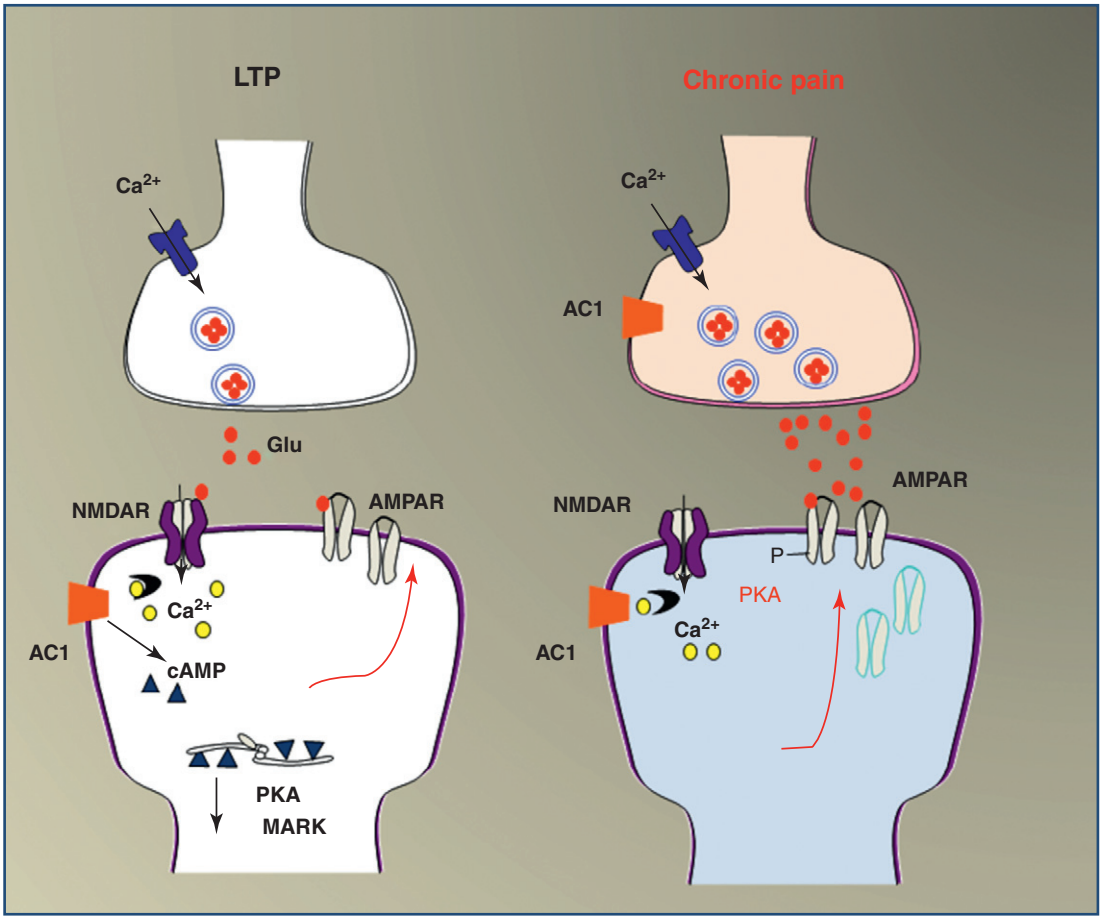

FIGURE 2

Contribution of adenylyl cyclase 1 (AC1) to anterior cingulate cortex (ACC) plasticity in chronic pain compared with long-term potentiation (LTP). Peripheral injuries lead to pre- and postsynaptic changes within ACC synapses. Presynaptic enhancements of glutamate release, as well as postsynaptic alterations in α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic (AMPA) and *N*-methyl-D-aspartate (NMDA) receptor (R)-mediated responses contribute to enhanced noxious sensory transmission within the brain. The activity of AC1 is probably required for both pre- and postsynaptic plastic changes in glutamatergic synapses. Although postsynaptic mechanisms have been investigated recently, presynaptic mechanisms remain to be investigated. *Abbreviations*: MAPK, mitogen-activated protein kinase; PKA, protein kinase A.

the membrane fraction and a corresponding decrease in the levels in the cytosolic fraction. By contrast, nerve ligation had no effect on the intracellular distribution of GluA2&3 subunits in ACC neurons. These data show that the AMPAR GluA1 subunit is redistributed in ACC neurons as a result of nerve injury.

AC1 contributes to injury-induced cortical changes

Is AC1 activity required for injury-triggered presynaptic and postsynaptic changes? In AC1-KO mice, behavioral sensitization caused by tissue inflammation or nerve injury was significantly reduced or inhibited [32]. It is thus expected that ACC pre- and postsynaptic changes triggered by injury could be prevented if these cortical changes are indeed important for behavioral sensitization in animal models of chronic pain (Fig. 2). Following nerve injury, it was shown that AC1-KO mice did not exhibit a reduction in the PPF of AMPA receptor-mediated EPSCs as shown by wild-type mice following the same injury [18]. No increase in mEPSC frequency or amplitude was observed in AC1-KO mice. Moreover, the evoked EPSCs in wild-type and AC1 KO mice were compared and there was no significant difference between the two groups. These results indicate that both pre- and postsynaptic enhancement of excitatory synaptic transmission in the ACC depend on AC1 in neuropathic pain. Fig. 2 summarizes possible synaptic mechanisms for ACC LTP and periphery injury-triggered plastic changes within the ACC.

Biochemical studies have also confirmed the importance of AC1. The expression of GluA1 and GluA2&3 was not altered in the ACC of AC1-KO mice compared to wild-type mice. Furthermore, the phosphorylation levels of the GluA1 subunit in the ACC of AC1-KO mice with neuropathic pain was not affected.

The phosphorylation of the GluA1 subunit of AMPA receptor is crucial for the synaptic expression of the receptors, their channel properties and synaptic plasticity [57]. Interestingly, the phosphorylation levels of GluA1 (at Ser 845) were found to be significantly increased in the ACC after nerve injury. These data indicate that nerve injury can increase the phosphorylation levels of GluA1 through the PKA signaling pathway [18]. cAMP might also contribute to injury-triggered enhancement of presynaptic glutamate release. Using a novel transgenic mouse model, heterologously expressing an *Aplysia* octopamine receptor (Ap oa1), it was shown

that activation of Ap oa1 by octopamine enhanced glutamatergic synaptic transmission in the ACC by increasing presynaptic glutamate release *in vitro* [58]. Bilateral microinjection of octopamine into the ACC significantly facilitated behavioral responses to inflammatory but not acute pain.

c-fos transgenic mice: recording from pain-activated cells

Activity-dependent immediate early genes, particularly c-*Fos*, have been useful for mapping injury-related neuronal activities in the CNS, including the spinal cord dorsal horn neurons and cortical neurons [32,59]. In the spinal cord dorsal horn, activation of c-Fos mimics the pattern of neurons that receives noxious inputs from periphery [59]. In ACC, activation of *c-fos* is induced by peripheral tissue inflammation or nerve injury [21]; and genetic overexpression of the NMDA receptor NR2B leads to increases in Fos activation and behavioral nociceptive responses [21]. Thus, with appropriate control, Fos can be used to identify neurons in the CNS that are activated by peripheral sensory activity, such as noxious stimuli. Thus, by using transgenic mice in which the expression of GFP was controlled by the promoter of the *c-fos* gene [47,60], it was shown that many ACC neurons were activated 3 and 7 days after nerve injury (i.e. had become GFP positive). Whole-cell patch-clamp recordings from FosGFP-positive pyramidal cells ('green' cells) that were activated by allodynic pain were compared with recordings from non-activated neighboring neurons and neurons in sham-operated FosGFP mice. The amplitudes of eEPSCs stimulated at the same intensity in the FosGFP-positive neurons were significantly greater than from FosGFP-negative neurons in the ACC of nerve injury and sham-operated mice. This finding provides direct evidence that peripheral injury triggers selective plastic changes in ACC neurons. The use of FosGFP transgenic mice will therefore provide more pain-specific plastic changes in cortical neurons in future studies.

PKMζ as a key mechanism for maintaining pain-related cortical L-LTP

Multiple protein kinases are thought to contribute to the induction of LTP and initial consolidation of information storage [47,61]. PKMζ, an atypical isoform of PKC, has been detected in many regions of the brain, including the frontal cortex [62]. In animal models of neuropathic pain, the activity of PKMζ has been found to be upregulated (Fig. 2), increasing either protein synthesis or phosphorylation. The activity of AC1 is crucial for the upregulation of PKMζ. AC1-KO mice did not show any significant changes in the amounts of PKMζ or the phosphorylation of PKMζ (p-PKMζ).

The use of a selective PKMζ inhibitor, ζ inhibitory peptide (ZIP) shows that PKMζ is crucial for the maintenance of L-LTP in ACC excitatory synapses [47]. Blocking the activities of PKMζ with ZIP 3 h after pain induction erased L-LTP [47]. Given that ACC LTP requires the AMPA GluA1 subunit, it is likely that PKMζ interacts with GluA1 to maintain LTP in the ACC. Nerve injury resulted in an increase in GluA1 and in its phosphorylation on Ser845, but not GluA2/3, in the ACC neurons [18]. Bath application of ZIP was found to decrease the amplitude of evoked EPSCs, and applying non-stationary fluctuation analysis showed that the effect of ZIP on the eEPSCs was to the result of a decrease in the number of active channels, rather than a decrease in the unitary conductance of AMPARs. Inhibiting the activities of PKMζ in the ACC decreased the protein level of GluA1 in the synapses [47]. Therefore, these results suggest that, under neuropathic pain conditions, PKMζ interacts with the GluA1 subunit of AMPARs in the ACC.

Neuron-specific AC1 as a potential drug target for treating chronic pain

For CNS drugs, it is important to avoid adverse effects in non-neuronal tissues. Many clinically proven effective drugs have been removed from the market as a result of their adverse effects in non-neuronal organs, such as the cardiovascular system and liver. There are at least three major strategies to avoid adverse effects of CNS analgesic drugs: (i) drug targets are mainly expressed in neurons; (ii) target proteins are recruited or activated in an activity-dependent manner with an almost 'silent' status under normal physiological conditions; and (iii) choosing target proteins that are crucial for chronic pain-related neuronal plasticity, but not for other cognitive functions.

However, few target proteins meet all three criteria. Based on the data reviewed so far, AC1 is a top target for treating chronic pain (see also [63,64] for the consideration of ACs as drug targets). First, AC1 is primarily expressed in neurons, and no AC1 gene expression has been found in heart, liver, or kidney cells. Second, AC1 is activated in a calcium-CaM–dependent manner. Third, it acts downstream from the glutamate NMDA receptors and contributes to chronic pain-related neuronal plasticity in the cortex and spinal cord. Finally, AC1-KO mice show reduced or blocked behavioral sensitization to non-noxious mechanical stimuli in animal models of inflammatory and neuropathic pain.

NB001 as the first generation of selective inhibitors for AC1

Using a heterologous expression system, chemical screening experiments have been performed to identify AC1 inhibitors. NB001 has been identified as a selective inhibitor for AC1 [50] (Fig. 2a,b). In both human embryonic kidney (HEK) 293 cells in which AC1 was stably expressed and adult mouse neurons, NB001 produced a dose-dependent inhibition of AC1 activity with an estimated IC_{50} of 5–8 μM. Furthermore, NB001 produces a dose-dependent inhibition of cAMP production triggered by the excitatory transmitter glutamate in ACC slices, indicating its effectiveness in inhibiting activity-dependent cAMP under physiological or pathological conditions in adult animals. Furthermore, it produced significant inhibition of glutamate-induced cAMP production in human neuroblastoma SH-SY5Y cells with an estimated IC_{50} of 8.3 μM. This is consistent with the fact that the genes encoding AC1 in mouse and humans share up to 90% homology [50]. The inhibitory effect of NB001 is subtype selective. NB001 did not significantly affect AC5–8 activity at effective inhibiting doses for AC1, and the efficacy difference between AC1 and AC5–8 is more than tenfold.

Similar to results from AC1-KO mice, NB001 inhibited sensory-related LTP in two key areas. In mouse ACC slices, postsynaptic application of NB001 completely blocked the induction of LTP in ACC pyramidal neurons. In spinal cord, NB001 prevented the induction of LTP induced by a pairing protocol. These results provide strong evidence for the crucial roles of AC1 in both spinal and ACC LTP, which is one possible cellular mechanism

contributing to neuropathic pain. LTP has at least two different major forms: E-LTP and L-LTP. Recent studies using a multiple channel recording system found that bath application of NB001 produced inhibition of L-LTP that lasted for at least 3 h after pain induction (Chen *et al.*, unpublished).

Analgesic effects of NB001 in animal models of neuropathic and inflammatory pain

Previous studies with AC1-KO mice showed that behavioral allodynia (pain experienced to a usually innocuous stimulus) in animal models of neuropathic pain and inflammatory pain was significantly reduced [32]. However, it is difficult to rule out the possible contribution of developmental defects in AC1-KO mice that might contribute to the behavioral results. With the identification of NB001 as an inhibitor for AC1, the effects of NB001 on behavioral allodynia in animal models of neuropathic pain induced by nerve ligation were examined [55]. Consistent with genetic studies using AC1-KO mice, administration of NB001 (0.1 mg/kg, given intraperitoneally) given 30 min before behavioral allodynia testing produced a significant analgesic effect. NB001 at a higher dose of 1 mg/kg produced a greater inhibition of behavioral allodynia [50,53] (Fig. 3c).

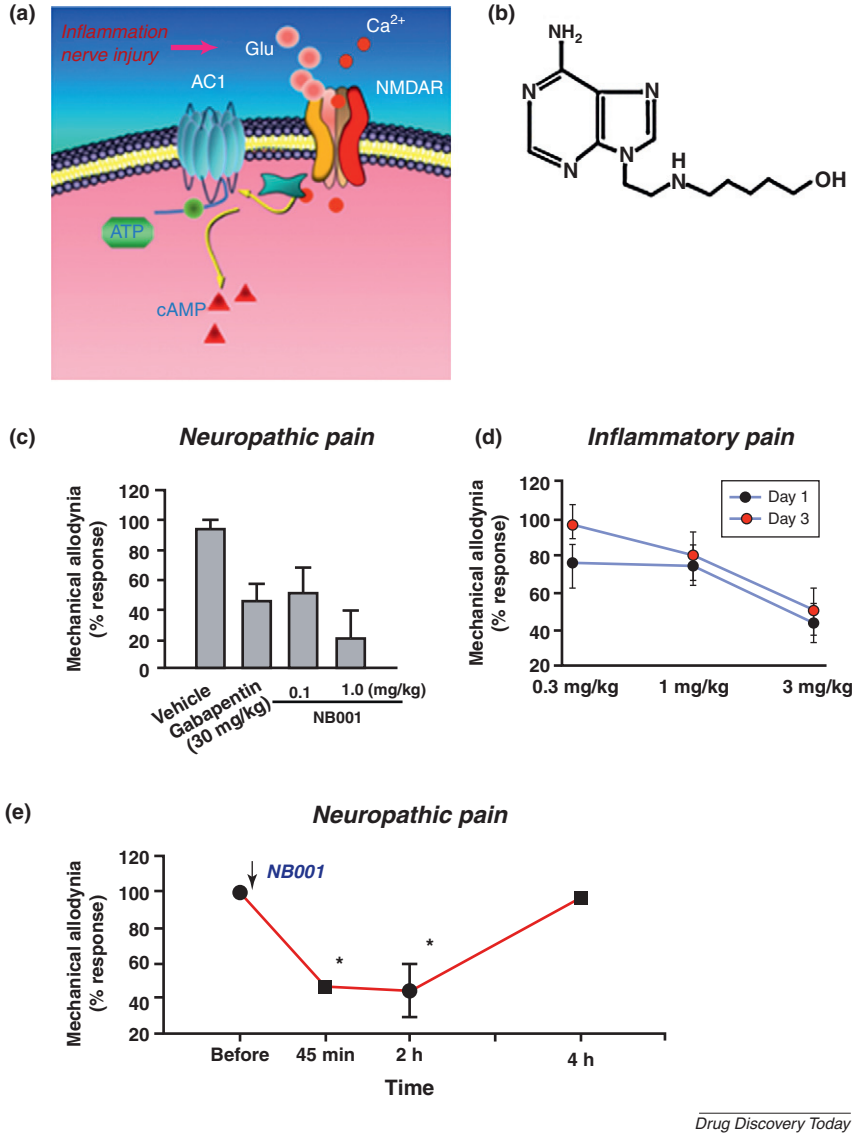

FIGURE 3

Preclinical proof-of-concept for the adenylyl cyclase 1 (AC1) inhibitor NB001 as a novel drug for treating chronic pain. **(a)** A model showing that AC1 acts downstream from the glutamate *N*-methyl-D-aspartate (NMDA) receptors and is activated in a calcium-dependent manner. **(b)** Chemical structure of NB001. **(c)** The effect of NB001 on allodynia after nerve ligation. Intraperitoneal (i.p.) administration of NB001 (0.1 mg/kg body weight) given 30 min before behavioral allodynia testing produced a significant analgesic effect. NB001 at a high dose of 1 mg/kg produced a greater inhibition of behavioral allodynia. i.p. injection of gabapentin produced significant analgesic effects in the animals with neuropathic pain. The inhibitory effects were comparable to those produced by NB001 at 0.1 mg/kg. **(d)** The effect of NB001 on inflammatory pain. At 1 day after Freund's Complete Adjuvant (CFA) injection, NB001 (1 and 3 mg/kg, i.p.) produced significant analgesic effects. The inhibitory effect is dose related, with greater inhibition being found with a higher dose of NB001. **(e)** Oral application of NB001 (1 mg/kg, 3 ml per injection) produced significant analgesic effects in adult rats with neuropathic pain ($n = 5$). Allodynia was measured at 45 min, 2 h and 4 h after oral application.
Modified, with permission, from [50].

In general, application of NB001 at different dosages did not cause any abnormal behaviors in animals. Animals injected with NB001 were calm and less responsive to behavioral allodynic measurement than were control animals. To compare these results with NB001 with the analgesic effect of currently available drugs for neuropathic pain, experiments were also performed with gabapentin. Intraperitoneal injection of gabapentin (30 mg/kg) produced significant analgesic effects in animals with neuropathic pain. The inhibitory effects were comparable to those produced by NB001 at 0.1 mg/kg. Behavioral allodynia induced by the hind-paw injection of complete Freund's adjuvant (CFA) (50%) has been commonly used for evaluating the analgesic effects of a drug on chronic inflammatory pain. One day after the CFA injection, NB001 (1 and 3 mg/kg, i.p.) produced significant analgesic effects (Fig. 3d). The inhibitory effect was dose related: greater inhibition was found with a higher dose of NB001. Similar analgesic effects were also obtained 3 days after CFA injection.

Conclusion and future directions

In conclusion, basic neurobiological evidence demonstrates that AC1 acts as a key signaling protein for triggering chronic pain-related central plasticity. Thus, inhibiting AC1 activity is likely to be one of the leading novel neural mechanisms for treating chronic pain. From a basic science point of view, AC1 is one of the most suitable candidates for attenuating or erasing (by inhibiting the activity of PKMζ) chronic pain-related cortical potentiation. Current drugs for treating neuropathic pain, such as GABApention, failed to prevent cortical potentiation and non-selectively reduced excitatory transmission (Chen *et al.*, unpublished data). More importantly, there is a significant need for improved drugs to treat patients worldwide. Many patients have to seek alternative medicine for controlling pain and suffer emotional disorders from ongoing chronic pain. Owing to the lack of novel drug treatments targeting the cortex, some patients have opted for surgical ablation of the ACC to reduce or inhibit chronic pain [65,66]. I believe that investigating the basic mechanisms underlying chronic pain will help to develop effective medication to treat patients with chronic pain.

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