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ERK 在杏仁體中央被核神經元細胞興奮性角色探討

Role of extracellular signal-regulated kinase in neuronal excitability of capsular central amygdaloid neurons

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摘要

杏仁體中央被核(CeAC)可接收經由杏仁體-旁臂核(PBA)路徑傳遞，來自腦幹及脊髓的痛覺訊息，同時也接收直接由脊髓投射上來的痛覺訊息，此核區被稱為「痛覺杏仁核」已將近十年，先前研究指出佛波醇12,13-乙酸酯(PDA)可顯著促進旁臂核到CeAC的神經傳導作用，且與細胞內部的胞外訊息傳遞激脢(ERK)有關，本研究使用全細胞紀錄電生理紀錄以及藥理方式探討ERK在調節CeAC神經細胞興奮性上所扮演的角色，給予短期PDA(蛋白激脢C之活化劑)可提高動作電位發生的頻率，然而給予長期PDA卻會降低其頻率；另外短期PDA也會增加I_h-current，但長期PDA則減緩其強度，此外，給予長期PDA也會延遲動作電位發生的時間，在給予蛋白激脢C(PKC)抑制劑－Chelerythrine與GF109203X之後，短期與長期的PDA反應都不復出現，給予U0126不會影響短期PDA帶來的反應，卻逆轉了長期PDA先前的反應情形，實驗結果顯示：(1)給予PDA會經由PKC—ERK路徑作用，在不同的時間條件下，調節CeAC的神經興奮性；(2)活化ERK加劇動作電位延遲發生的情形(A型鉀離子通道)。

Keyword: 中樞神經敏感化、神經興奮性、杏仁體、杏仁體中央被核、胞外訊息調節激脢、鉀離子通道、佛波酯、蛋白激脢C。

Abstract

The capsular central amygdaloid (CeAC) nucleus acquires nociceptive specific information from the brainstem and spinal cord via the parabrachio-amygdaloid (PBA) pain pathway as well as via direct projections from the spinal cord. It has been termed as “nociceptive amygdala” for almost a decade. Previous study indicated that application of phorbol 12,13-diacetate (PDA) caused marked enhancement of synaptic transmission of parabrachial input onto CeAC nucleus and the elevation of intracellular ERK was involved. In the present study, using patch-clamp technique and pharmacological methods, the role of ERK in regulating neuronal excitability of CeAC was examined. Short-term application of the PKC activator, PDA increased the number of action potentials whereas the long-term application decreased the spike number. Beside, short-term PDA enhanced the size of I_h current but long-term PDA downsized it. In the meanwhile, long-term application of PDA also increased the first spike latency. Protein Kinase C (PKC) inhibitors, Chelerythrine and GF109203X abolished the effect of PDA in both time-scales. Application of U0126 had no effect on short-term PDA however it reversed the effect caused by long term PDA application. The result suggested that (1) PKC—ERK pathway induced by PDA regulated input-output function of neuronal excitability of CeAC in different time-scales; (2) ERK activation enhanced the delay onset of action potential (stongly

related to A-type potassium channel).

Keyword: central sensitization, neuronal excitability, amygdale, ERK, potassium channel, PDA, PKC.

Contents

Introduction

Capsular central amygdaloid nucleus and Nociception

The amygdala is consisted with several anatomically and functionally distinct nuclei. Capsular central amygdaloid nucleus (CeAC) located in central nucleus of amygdala. Central nucleus not only receives nociceptive information from the brainstem and spinal cord via the parabrachio-amygdaloid (PBA) pain pathway as well as via direct projections from the spinal cord. It also receives projection from basal lateral nucleus (BLA) and lateral nucleus (LA), which processes affective, cognitive and polymodal information from thalamus and cortical areas (Shi and Davis 1999; LeDoux 2000; Stefanacci and Amaral 2000; Price 2003). Recent studies suggested that amygdala serve as a role in modulating pain perception. There was reduction in opioid- and cannabinoid-induced anti-nociception in rhesus monkeys after bilateral lesions of the amygdaloid complex (Manning et al., 2001). Another *in vivo* experiment using electrophysiological technique combined with mechanical and thermal noxious stimulation to record the response of amygdaloid neurons then discovered that the lateral –capsular part of amygdaloid central nucleus (CeAC) had the most responses to noxious stimulation. Since CeAC play an crucial role in regulating pain perception, it was termed as nociceptive amygdala (Gauriau and Bernard, 2002; Neugubauer et al., 2004). Moreover, Neugebauer et al. (2003, 2006)

described the plastic changes in the amygdala in the model of arthritic pain (Neugebauer et al., 2003; Neugebauer et al. 2006). In the meanwhile Ikeda et al. (2007) also provided intriguing novel information about pain mechanisms in the amygdala in the mouse model of chronic neuropathic pain (spinal nerve ligation model). The study described a pain-related synaptic plasticity in the lateral capsular division (Ikeda et al., 2007). Interestingly, Ikeda et al. (2007) used an identical method to demonstrate synaptic plasticity changes and increased neuronal excitability in the amygdala in neuropathic pain that described in the arthritis pain model (Neugebauer et al. 2003, Bird et. al. 2005). These results indicated that mechanisms of chronic neuropathic pain involved an N-methyl-D-aspartate (NMDA) receptor independent form of plasticity in the CeAC (Ikeda et al., 2007), whereas increased NMDA receptor function was critical for plasticity associated with acute arthritic pain (Bird et al., 2005; Li and Neugebauer 2004). Moreover, Carrasquillo and Gereau (2007) used the formalin test as a mouse model of persistent inflammatory pain; it was found that activation of ERK in the amygdala was necessarily sufficient to induce long-lasting peripheral hypersensitivity to tactile stimulation. Pharmacological activation of ERK by PDA in the amygdala induced peripheral hypersensitivity in the absence of inflammation. Blockade of inflammation-induced ERK activation by U0126 significantly reduced long-lasting peripheral hypersensitivity associated with

persistent inflammation. In the meanwhile, Chang et al. (2011) used acid-induced muscle pain model (AIMP) of mouse to further prove that phosphorylated ERK in the CeAC prominently was increased and PBA–CeAC synaptic transmission was postsynaptically enhanced. Addition to thermal and mechanical pain, neuropathic pain, arthritis pain and inflammation induced pain and acid induced muscle pain were considered to be strongly related to CeAC. Therefore, CeAC indeed played a crucial role in regulation of pain-modulatory circuitry.

Central sensitization

The term “Central sensitization” was first brought up by Clifford Woolf in 1983 (Clifford, 1983). He demonstrated that a thermal injury in the periphery tissue was able to cause an amplification of noxious and innoxious stimuli evoked activities. More importantly, the amplification was coupled to an augmented flexion reflex response (recorded by EMG). Since reflexes are mediated by a dorsal horn – ventral horn reflex arc, it proves that somehow the central nervous system (CNS) must be involved in these amplified signals of stimulation. Moreover, he also noted that the injury on one side of paw was able to boost the reaction of reflex arc on the contra-lateral side of the body. Because the injury was occurred on the opposite of the body, peripheral mechanisms were not likely the candidate to mediate the outcome. It

indicated that the circuitry must have involved amplification of signaling in the CNS. Such mechanisms could result in pain amplification similar to what is seen in humans following an injury or in chronic pain conditions.

In addition to central sensitization occurred in dorsal horn of the spinal cord, it is also important to note that central sensitization can occur in other CNS regions as well. For instance, recent studies have shown that the amygdala plays a crucial role in the sensitization of nociceptive responses following pain induction (Neugebauer et al., 2003; Kobert et. al 2010). Neugebauer et al. (2003) described that in the arthritis pain model, the activities of couple groups of neurons in CeAC were enhanced (by extracellular single-unit recordings) and there were hypersensitivity to thermal and mechanical stimulations in peripherals. In addition, it was found that application of (*R*,*S*)-3,5-dihydroxyphenylglycine (DHPG), the mGluR agonist in the central nucleus of mice was sufficient to induce peripheral hypersensitivity in the absence of injury and it is related to ERK activation (Kobert et al., 2010). Other than amygdala, another recent study has shown that rostral ventromedial medulla (RVM) in brainstem, shows plasticity in its responses to painful stimuli after peripheral injury. This region not only sends descending fibers into the spinal cord that are able to amplify nociceptive signals, it is also considered to be involved in descending pain modulation (Carlson and Heinricher, 2007). Pharmacologically, the RVM is a major

target for cannabinoid and opioid analgesics (Walker, 2002) and it is possible that these compounds suppress pain by attenuating central sensitization-like responses in these neurons.

Central sensitization is influenced by comprehensive causes. For instance, the increased activity in glutamate receptors, including increased NMDA and AMPA receptor activity and group I mGluRs (mGluR1/5) are crucial to the formation of central sensitization (Willis, 2001; Latremoliere and Woolf, 2009). In addition, many of other molecular substrates which could be activated during central sensitization, such as PKA, PKC, CaMKII alpha and ERK play a key role in the scheme. Activation of NMDA receptor and other Ca2+ channels increases the intracellular Ca2+, so PKC and CaMKII are phosphorylated, furthering the activation of ERK and MAPKs (Ji and Strichartz, 2004). Intracellular Ca2+ plays a crucial role in generation of central sensitization. It is now considered that the cascade reactions take large part in promoting the formation of central sensitization, the ground reasons for chronic pain and neuropathic pain (Yashpal et al., 1995; Malmberg et al., 1997; Yashpal et al., 2001).

In addition to these molecular targets, neurons that signal central sensitization have also been identified. These neurons are found in lamina I of the spinal cord and they express the substance P receptor NK-1 (Mantyh and Hunt, 2004). Mantyh et al.

demonstrated that spinal administration of substance P conjugateded with saporin (toxin) was able to ablate experimental hyperalgesia in pre-clinical models showing that these neurons are crucial for the full expression of central sensitization.

Objectives

ERK was demonstrated in participation of synaptic plasticity in the model of neuropathic, arthritis and acid induced muscle pain (AIMP) in CeAC by pharmacological and electrophysiological techniques. The synaptic transmission of PBA input onto neurons of the CeAC nucleus was significantly increased in the previous studies (Ikeda et al., 2003; Neugebauer et al., 2003; Neugebauer et al., 2004; Ikeda et al., 2007; Chang et al., 2011). By using immunostaining technique , it was found that the phosphorylated ERK (pERK) in CeAC was significantly increased in the model of chronic pain induced by paw injection of formalin as well as by direct injection of PDA to CeAC (Carrasquillo et al.,2007). Moreover, PDA was proved to facilitate the synaptic transmission of PBA input onto neurons of the CeAC nucleus in both AIMP models and normal mice, and pERK in CeAC was also significantly increased in the AIMP models (Chang et al., 2011). These studies indicate that (1) the elevation of ERK plays a universal role in modulating different types of pain perception (2) ERK is perhaps activated by PKC pathway since direct injection of

PDA induced the elevation of pERK.

In the present study, several issues related to the role ERK in modulating neuronal excitability were discussed:

Issue 1: The aspects about how activation of PKC pathway by PDA affects the input-output function of neuronal excitability of CeAC.

Issue 2: The existence of PKC—ERK pathway in regulating the input-out function of neuronal excitability.

Issue3: The role of ERK in regulating the delay onset of action potential (A-type potassium channel).

Materials and Methods

Preparation of amygdala slices

The use of animals in this study was approved by the Ethical Committee for Animal Research of the National Taiwan University. Male C57BL/6 mice at the age of 4–9 weeks were used. Coronal brain slices (300μm thick) containing central amygdala (CeAL), and capsular division (CeAC) amygdaloid neurons of the right hemisphere were obtained from Male C57BL/6 mice since the hemispheric lateralization described by Fu et al. (2008) as well as Ji et al. (2010) previously.(Fu and Neugebauer, 2008; Ji et al., 2010). The mice were decapitated and their brains rapidly removed and placed in ice-cold artificial-CSF (ACSF), containing the following (in mM): 119 NaCl, 2.5 KCl,1.3 MgSO4, 26.2 NaHCO3, 1 NaH2PO4, 2.5 CaCl2, and 11 glucose, with the pH adjusted to 7.4 by gassing with 95% O2/5% CO2. All slices were cut using a vibroslicer (ZERO 1, DSK) and were kept in oxygenated ACSF (95% O2/5% CO2) at room temperature (24 –25°C) to allow recovery for at least 90 min before recording commenced. Slices were transferred to an immersion-type recording chamber mounted on an upright microscope (BX50WI, Olympus Optical) equipped with an infrared-differential interference-contrast microscopic video. The lateral nucleus of the central amygdala (CeAL), and capsular division (CeAC) were clearly identified under low magnification. The capsular

division appeared to be slightly brighter than other regions. The boundaries among different amygdaloid nuclei were easily discerned under light microscopy.

Whole-cell Patch clamp recording

Neurons in the CeAL and CeAC were recorded under visual guidance with patch pipettes pulled from borosilicate glass (1.5 mm outer diameter, 0.32 mm wall thickness; G150F-4, Warner Instruments). The patch pipettes had a resistance of 3–8MΩ when filled with a solution consisting of the following (in mM): 131 K-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA,8 NaCl, 2 ATP, 0.3 GTP, and 6.7 biocytin, with the pH adjusted to 7.2 by KOH and the osmolarity to 300–305 mOsm. Recordings were made at room temperature with an Axopatch 1D amplifier (Molecular Devices). Neurons were recorded at -60 mV. While performing current-clamp recordings, the input resistance (*R*n) was continuously monitored by applying a current pulse of 30 pA and the bridge was balanced by adjusting the *R*s compensation of the amplifier. Data were discarded when the *R*n varied by > 20% from its original value during the recording. The membrane potential (*V*m) of the recorded neurons was clamped (or held) at -60 mV. All signals were low-pass filtered at a corner frequency of 2 kHz and digitized at 10 kHz using a Micro 1401 interface (Cambridge Electronic Design)**.** Data were collected using Signal software (Cambridge Electronic Design). Neuronal excitability was measured by recording

action potentials generated by intracellular current injections (100 ms) of increasing magnitude (20 pA steps , -160pA to 200 pA) while the cell was held at a starting membrane potential of -60 mV.

Drug application.

Drugs were applied by gravity-driven superfusion of the brain slice in the ACSF (>2 ml/min). Solution flow into the recording chamber was controlled with a three-way stopcock. The chemicals used for the ACSF and internal solution were purchased from Merck. Picrotoxin, kynurenic acid, glycine, PDA, U0126, chelerythrine, and GF109203X were purchased from Sigma. PDA, U0126, Chelerythrine and GF109203X were dissolved in DMSO to stock solution. For U0126 administration, the slices were kept in U0126-containing oxygenated ACSF (95% O2/ 5% CO2) at room temperature (24 –25°C) allowing the treatment for at least 120 minutes before recording commenced. Except for U0126, drugs were all added to the ACSF and bath applied.

Data and statistical analyses.

Baseline of spike number was recorded at least 10-min bath in the base solution. For PKC inhibitors, superfusion was administrated for 10-min before recording. Data were compiled and analyzed using OriginPro 8.0 software (OriginLab, Northampton, MA, USA). Number of action potential are expressed as *original traces* or as

means ±SEM. A pair two sample *t* test was used to compare responses before (baseline) and after PDA application as well as to PKC inhibitors and U0126. The length of current injection in each frame were 100ms. First spike latency and input resistance were presented as the mean±SEM. The criterion for significance was $p < 0.05$.

Results

Identification of Capsular Central amygdaloid neuron

Before whole-cell patch clamp was commenced, the brain slices were placed under light microscope without attachment filter lens. Capsular central amygdaloid nucleus (CeAC) appeared to be brighter than other region with naked eyes (Fig1-A2). According to the previous study, CeAC can be categorized as Type 1 and Type 2 neurons (Chang et al., 2011). CeAC neurons studied in the present study were mostly Type1 neurons (Type1:Type2 = 26:2).Theses neurons showed prominent delay onset in firing the first action potential with injection of depolarizing current pulses and a voltage sag with injection of hyperpolarizing current pulses (Fig1 B). In the present study, a prominent delay onset was defined as the first peak of action potential appeared over 0.1 second after injecting 20pA depolarizing current. Since the prominent delay in onset of action potential could be ascribed to the expression of a wild variety in amount of ion channels mediating the fast activated and inactivated A-type potassium current (I_A), I_A could be the best candidate for delaying onset of action potentials in neurons (Schoppa and Westbrook,1999; Shibata et al.,2000; Burdakov et al.,2004).

PDA modulates input-output function of neuronal excitability in different time scales.

Whole-cell patch recordings were made in coronal brain slices from the right hemisphere, because accumulating evidence suggested that pain-related amygdala functions were lateralized to the right hemisphere (Carrasquillo and Gereau, 2007, 2008; Ji and Neugebauer, 2009). After successfully switched into whole-cell configuration, action potential was generated by direct intracellular current injection. Each frame of injection lasted for 100ms. The intracellular injection of hyperpolarizing and depolarizing current pulses were from 20pA to 200pA in 20pA steps (n=8 neurons).

Baseline of action potential was recorded after application of 10-min base solution containing picrotoxin, strychnine, and kynurenic acid (PSK) to block primary synaptic transmission. PDA is a type of phorbol ester, a well-known and effective PKC activator. It was applied to examine the role of PKC and the possible effect of downstream pathway, such as the cascade reaction induced by ERK (Robertson et al., 1999; Yuan et al., 2002; Carrasquillo and Gereau, 2007). The hypothesis in the present study was that 5-min PDA would induce rapid effect of PKC and 20-min PDA would be able to activate its downstream cascade reaction such as ERK. Since the consideration of number of action potential was the most direct method to inspect input-output function of neuronal excitability, the spike number was conducted in

three perspectives in the present study: first, spike number with depolarizing current injection pulse from 20pA to 200pA in 20pA steps; second, spike number induced by 80pA current injection; third, spike number induced by 120pA current injection. These parameters were chosen simply because there were individual varieties among neurons. Some neuron tend to fire action potential with smaller intensity of current injection such as 20pA, others tend to fire action potential with larger intensity of current injection such as 80pA. Not to mention the administration of certain drugs was able to influence the firing condition. Generally speaking, at the level of 80pA current injection, CeAC neurons were able to fire action potential and in most cases, and adaptation of action potential appeared at the level of 120pA current injection. All the scale bars were adjusted to the same module to facilitate the comparison of different data.

From the perspective of spike number on 20pA-200pA current injection, 5-min PDA (1.5μM) prominently increased input-output function (Fig. 2-A, 2-B1, red diamond). Spike number significantly increased with 5-min PDA (Fig. 2-A, 2-B2, *p=0.00196, Pair two sample t-test*); Besides, on 80pA and 120pA current injection with5-min PDA application, clear tendencies in elevation of spike number (Fig.2-C1, C2) were also existed. On the other hand, input-output function was significantly decreased (Fig.2-B2, *p=0.01068*) with 20-min application of PDA. Likewise, on

80pA and 120pA current injection with 20-min PDA application, spike number was decreased, especially on 80pA current injection (Fig.2-C1, *Pair two sample t-test, p=0.00139*). These data suggested that the effect of PDA could be divided at least in 2 categories, short-term and long term. The short-term effect (5-min PDA) generally increased input-out function of neuronal excitability whereas the long term effect (20-min PDA) decreased it. Since the aim of present study was to understand the role of ERK, long term effect the effect of 5-min PDA on first spike latency and input resistance were not discussed. With 20-min PDA application, the first spike latency was significant increased (*Pair two sample t-test, p<0.0001*). It was possible that 20-min PDA activated the ERK/MAPK pathway which enhanced the function of A-type potassium channel (Schrader, 2005), causing the rise of first spike latency and the decline of spike number. There was no significant difference in input resistance between baseline and 20-min PDA.

Moreover, hyperpolarized activated cation current (I_h) were also measured as sag size to conduct the role of I_h in CeAC. The I_h is caused by the activation of hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels which belong to the superfamily of voltage-gated potassium channels (Kv). HCN channels are sometimes referred to as “pacemaker channels” because they help to generate rhythmic activity within groups of heart and brain cells (Luthi et al., 1998). I_h

mediates repetitive firing in neurons and cardiac myocytes (Accili et al., 2002; Robinson, 2003). In addition, roles of I_h in the regulation of resting membrane potential (Lupica et al., 2001), membrane input resistance (Magee et al.,1998), synaptic plasticity (Beaumont et al., 2000), and dendritic integration (Berger et al., 2001) have been reported (Poolos et al., 2002). Increasing evidence implicates I_h in activity-dependent changes of neuronal excitability (Beaumont et al., 2002; Welie et al., 2004), and in certain pathological conditions such as epilepsy or neuropathic pain (Shah et al., 1998; Chaplan t al., 2003). It has been reported that I_h was also found in the BLA in rats (Womble and Moises, 1993). In the present study, 5-min PDA had no significant effect on sag size but 20-min PDA significantly reduced the sag *(*Fig. 2-E, *Pair two sample t-test, p=0.0448)*, suggesting the role of PKC downstream pathway in I_h.

Effect of Chelerythrine –PDA on capsular central amygdaloid neuron

Chelerythrine is a potent, selective and cell permeable protein kinase C inhibitor that binds to the catalytic domain of PKC. It is at least 100-fold more selective for PKCs than for other kinases (Herbert et al., 1990; Chao et al., 1998) and is broadly applied on pharmacological experiments. Here, Chelerythrine (3μM, applied by superfusion) was administrated 10-min before PDA application to examine the role of PKC and its possible effect to ERK. It was worth notice that PKC inhibition did not

necessarily indicate to complete ERK suppression. The issue about ERK involvement was awaited to be discussed.

In the view of spike number on 20pA-200pA current injection, effect of 5-min and 20-min PDA were both abolished by Chelerythrine (Fig. 3-A,3-B).The spike number was neither prominently increased nor decreased. At the level of 80pA and 120pA current injection, there were not any crucial effects on spike number after PDA application, indicating that the input-output function was inhibited by Chelerythrine in both time scales. It suggested that the elevation of spike number was caused by rapid PKC effect and the long-term decline was caused by PKC mediated reaction, pointing out the possible existence of PKC-ERK pathway in the matter of modulating input-out function. However, first spike latency was significantly increased with 20-min PDA (Fig.3-D1, *Pair two sample t-test, p=0.00161),* indicating the enhancement of A-current. It was possible that Chelerythrine inhibited about 75% of PKC activity in the concentration level of micro-molar (Herbert et al., 1990). In addition, effect of 20-min PDA alone on first spike latency was potent (Fig. 2-D1, *Pair two sample t-test, $p<0.0001$*). After long term application of PDA, small amount of PKC activated the downstream pathway. Once the downstream pathway was activated, PKC inhibition no longer applied, causing the further delay onset of action potential. There was no significant difference on input resistance with Chlerythrine and addition of 20-min

PDA. Moreover, there is no significant change after PDA administration in sag sizes caused by I_h current (Fig. 3-E)

Effect of GF109203X –PDA on capsular central amygdaloid neuron

GF109203X is a potent and selective PKC inhibitor in the concentration level of nano-molar and also a PKA inhibitor in the concentration level of micro-molar (Chernova et al., 2007). Here, GF109203X (3μM, applied by superfusion) was administrated to confirm the short-term and long-term effects of PKC as well as PKA to a greater degree. GF109203X inhibited the effect of long-term PDA (Fig. 4-A1,A2). It also inhibited short-term effect of PDA (Fig.4-B1, n=6) except for the level of 80pA and 100pA current injection. On the average number of total action potential, GF109203X was able to abolish the effect of both 5-min and 20-min PDA (Fig 4-B2). The average number of action potential was neither increased nor decreased with 5-min and 20-min PDA administration. Moreover, in the level of 120pA current injection, the effect of PDA was also abolished. However, 20-min PDA significantly reduced the spike number in the level of 80pA current injection (*Pair two sample t-test, p=0.1211).* It was possible that except for inhibiting PKC, there was selective effect of GF109203X which changed the tonic environment of neurons and affected the baseline of spike number, causing an error in data analysis. There was no

significant effect on the first spike latency and input resistance with 20-min PDA. Moreover, there is no significant change after PDA administration in sag sizes caused by I_h current (Fig. 4-E).

Effect of U0126–PDA on capsular central amygdaloid neuron

U0126 is a very selective and highly potent inhibitor of Mitogen-Activated Protein Kinase (MAPK) cascade by inhibiting its immediate upstream activators, MEK1and MEK2. It inhibits both active and inactive for of MEK1and 2, preventing the activation of ERK. U0126 (10μM) was added in oxygenated ACSF allowing the treatment going on brain slices for at least 120 minutes before recording commenced. Theoretically, U0126 should not change the result of 5-min PDA because it did not affect the function of PKC. The spike number should still somehow increase. Besides, without PKC inhibition, 20-min PDA should be able to decrease the spike number and to increase the first spike latency (Fig. 2). The hypothesis here in the U0126—PDA protocol was to examine the existence of PKC—ERK pathway activated by PDA.

From the view of spike number on 20pA-200pA current injection, both 5-min and 20-min PDA significantly increased the input-output function of neuronal excitability (Fig. 5-A,B, *Pair two sample t-test, $p<0.0001$, $n=7$*). Moreover, in the level of 80pA and 120pA current injection, there were also tendencies in elevation of

the spike number with both 5-min and 20-min PDA application. Especially in the level of 120pA injection, 5-min PDA significantly increased the number of action potential (*Pair two sample t-test, p=0.4368*). Furthermore, 20-min PDA significantly decreased the first spike latency *(Pair two sample t-test, p<0.0001).* For 20-min PDA application, U0126 not only reversed the effect on input-output function of neuronal excitability but also the first spike latency, suggesting the existence of PKC—ERK pathway activated by PDA in modulating neuronal excitability. Moreover, the reverse condition of first spike latency also indicated the role of ERK to enhance A-current. Furthermore, 20-min PDA also significantly increased the input resistance (*Pair two sample t-test, p=0.0233*). In addition, 5-min PDA had no significant effect on sag sizes but 20-min PDA significantly increases the sags *(*Fig. 5-E, *Pair two sample t-test, p=0.04747).* The result is in contrast to the control set (Fig.2, PSK and PDA) where the sag sizes significant decreases. It is interesting that application of U0126 not only reverses the outcome in both spike number and spike latency (I_A), but also reverses the outcome in I_h, suggesting that there is a strong relation between these channels, possibly modulated by the same mechanism.

Discussion

In the present study, we show that PKC activation increases neuronal excitability in short-term scale whereas decreases neuronal excitability in long-term scale. In addition, the decline of input-output function of neuronal excitability are caused by the activation of PKC—ERK pathway.

PKC activation by PDA regulates the neuronal excitability in different time scale

PDA has been shown to enhance glutamate release in many synapses (Malenka et al., 1986; Shapira et al., 1987; Parfitt and Madison, 1993; Capogna et al., 1997; Hori et al., 1999; Francis et al., 2002; Lou et al., 2008). Central nucleus of amygdala which including CeAC are predominantly composed of GABAergic neuron (Ciocchi, 2010). Therefore, application of kyneurenic acid , picrotoxin and glycine in the present study allows us to study the effect of PKC activation on voltage-gated ion channel of CeAC. The result indicates that activation of PKC at initial stages enhances the function of voltage-gated ion channels of CeAC. The enhancement is abolished by 2 different PKC inhibitors, Chelerythrine and GF109203X. However Chelerythrine and GF109203X suppress the effect of PDA application in different patterns. Chelerythrine reduces the spike number more effectively and postpones the adaptation of action potential. Furthermore, it suppresses the delay onset of action potential less effectively with long-term PDA. On the other hand, GF109203X

reduces the spike number less effectively and it has no effect to adaption of action potential. In the mean while, it suppresses the delay onset of action potential more effectively. The differences between two PKC inhibitors are probably due to (1) the selective effects of different inhibitors since there are different targets of PKC inhibitors. (2) The concentration level of PKC inhibitor: Chelerythrine inhibits about 75% of PKC activities in the concentration level of micro-molar (Herbert et al., 1990). It is possible that after long-term application of PDA, it activates certain downstream pathway and partially enhances the delay onset of action potential. On the other hand, in the concentration level of micro-molar, GF109203X acts as PKA inhibitor as well (Chernova et al., 2007). It is sensible that the spike number can be slightly increased by the co-inhibition of PKC and PKA. Hence, PKC activation plays a comprehensive role in regulating neuronal excitability. Previous study reported that superfusion of another type of phorbel ester (PMA, by whole-cell patch clamp) alone increases action potential firing in 7 neurons whereas inhibited action potential firing in 5 neurons in CeAC (Li et al, 2011). Although according to Li et al. (2011), the time scale was not the one of the parameters being considered, it is possible that the differential results were due the time occasion while recording commenced. Furthermore, another previous study (Hu et al. 2003) indicates that PKC activation by immediate PMA suppresses the transient outward current of A-type potassium

channels and enhances the neuronal excitability in dorsal horn neurons. It gives rise to the picture for the involvement of A-type potassium channel in regulating neuronal excitability of CeAC.

ERK activation by PDA decreases neuronal excitability

In the present study, we show that U0126 effectively reverses the effect of ERK activation by PDA. The spike number was significantly increased and the first spike latency was significantly decreased with U0126 and long-term PDA activation. Our result suggests that ERK activation decreases the input-output function of neuronal excitability in the absence of primary synaptic transmission. ERKs play important roles both in nociception and modulation of A-type potassium current (Hu et al., 2003a,b). It is able to modulate A-type potassium current in dorsal horn neuron and in strong connection with Kv.4.2 (Schrafer et al., 2005; Hu et al., 2006). Inhibition of ERK signaling by PD98059 decreases neuronal excitability and cannot be reversed by PMA in dorsal horn neurons (Hu et al., 2003a,b). In addition, inhibition of ERK signaling by PD98059 also reduces sustained potassium outward currents in dorsal horn neurons (Hu et al., 2003a,b). It gives rise to the picture that in addition to A-type current, sustained potassium current may be another candidate for ERK to modulate neuronal excitability. Theoretically, the neuronal excitability will be increased if the

sustained potassium current is decreased. The differential outcome between CeAC and dorsal horn neurons is possiblely caused by (1) the selective effect of drugs. The target of PD98059 is MEK1 whereas the targets for U0126 are both MEK1/2. Besides, PDA is chosen over PMA in the present study. (2) Lack of primary synaptic transmission: kyneurenic acid blocks most of the glutamatergic current. Both ionotropic and metabotropic glutamate receptors are blocked, left out the remaining voltage-gated Ca^{2+} channels. Intracellular Ca^{2+} was not sufficient to evoke the additional effect with or without ERK.

Futhermore, I_h is conducted in sag sizes, 20-min PDA application alone suppresses the I_h whereas U0126 with 20-min PDA facilitates the I_h. It is surprising that the tendency in I_h is in consistent with those in spike number and first spike latency. It is possible that in addition to Kv.4.2 (I_A), the functional target of PKC-ERK pathway includes HCN channel. On the other hand, it is known that there are two substrates act in regulating the function of HCN channel: proton and PIP_2 (Biel et al., 2009). Proton is excluded in the discussion since there is no particular manipulation of protons in the present study. PIP_2 (cleavage to IP3 and DAG) acts as an allosteric activator from the intracellular site that facilitates channel activation by shifting rest membrane potential toward more positive potentials. As a result, PIP2 adjusts HCN channel opening to a voltage range relevant for the physiological role of I_h channels.

PIP2-mediated regulation of HCN channels may be of physiological significance for the function in neuronal circuits, as enzymatic degradation of phospholipids reduces channel activation and slows down firing frequency of neurons (Cerbai et al., 1999; Qu et al., 2001; Robinson et al., 1997). The picture also leads to another possible candidate responsible for the reduction of spike number in the present study. Especially that a similar mechanism for PIP2 modulation of gating was identified in voltage-gated and inwardly rectifying K^+ channels (Baukrowitz et al., 1998; Huang et al., 1998; Oliver et al., 2004; Shyng and Nichols, 1998).

In conclusion, there are three types of voltage-dependent K^+ currents which might be the possible in regulating the neuronal excitability in CeAC in the present study: (1) transient A-type current(I_A), responsible for the delay onset of action potential, (2) sustained delayed rectifier current(I_K), responsible for the change in rest membrane potential (Akins et al., 1993; Gold et al., 1996; McFarlane et al., 1991; Everill et al., 1998, Xu, 2006) and (3) hyperpolarized activated current (I_h), mainly responsible for pace-making activity and comprehensively affect the neuronal properties. Long-term application of PDA decreased the neuronal excitability (spike number ↓ ,latency ↑ ,sag size ↓). PKC inhibition abolishes both short and long term effect. Furthermore, application of U0126 reverses the long-term outcome (spike number ↑ ,latency ↓ ,sag size ↑) and has no effect on short-term PDA, indicating the

PKC-ERK pathway is existed (Cheng et al, 2011). For the change in neuronal excitability, it is possible that PKC—ERK activation by PDA modifies the property of voltage-dependent K+ channels, A-type and sustained delay rectifier type (Fig 6), enhancing the function of voltage-gated potassium channels, so that neuronal excitability is generally decreased.

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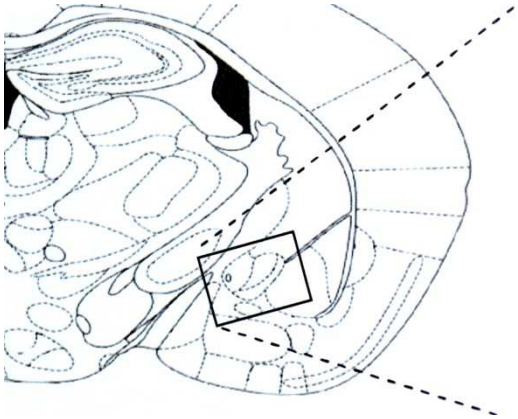
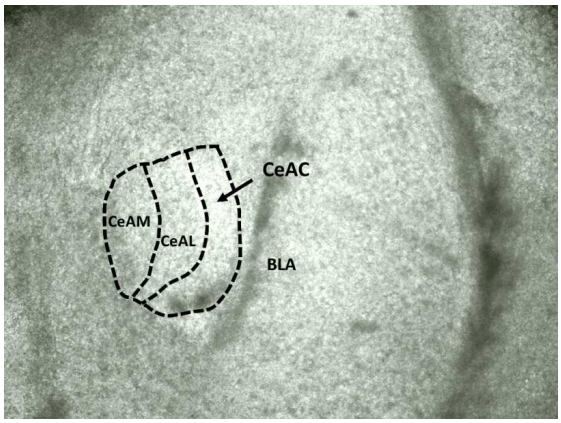
Fig. 1

A1



A2



Bregma-1.34

B

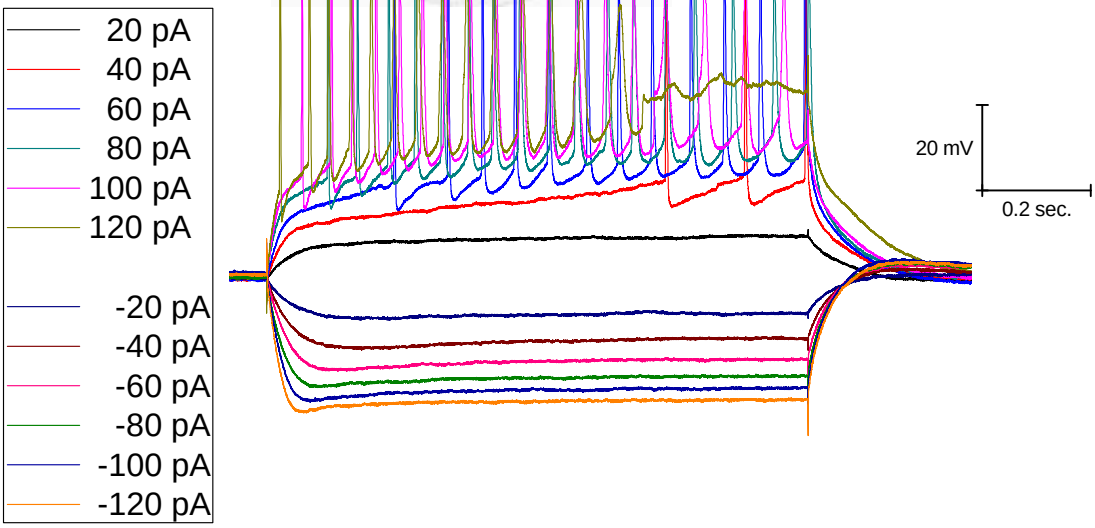
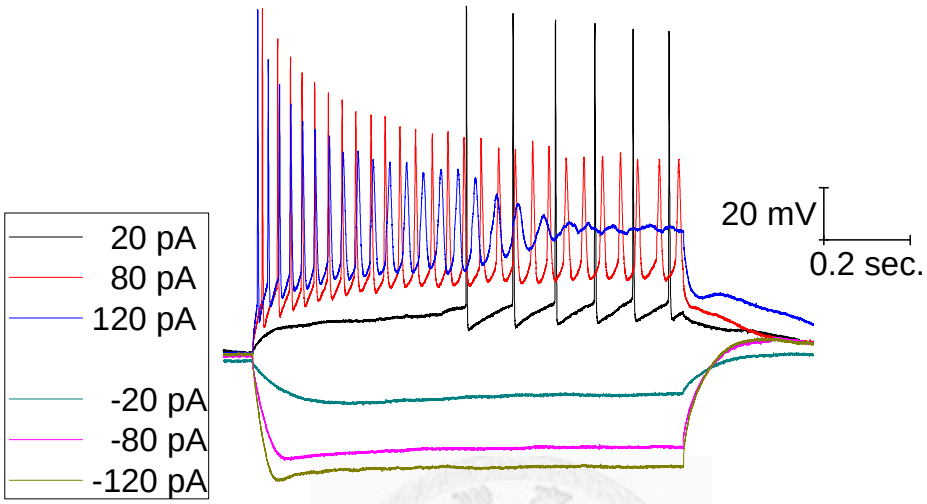


Fig1. Identification of capsular central amygdaloid neuron (CeAC). A1: Presumed CeAC area is right next to basal-lateral nucleus (BLA) of amygdale. A2: Coronal acute brain slices containing central nucleus and BLA of amygdale. CeAC appeared to be lighter than other region under light microscope (without filter lens). B. Representative examples of action potentials, recorded after at least 10-minute bath application of base solution (picrotoxin, strychinine and kynurenic acid). Prominent delay onset of action potential was generated by injecting proper intensity of depolarizing current pulse and the voltage sags were generated by injecting hyperpolarizing current pulses. Current injection beyond 120pA would generally cause adaptation of action potential.

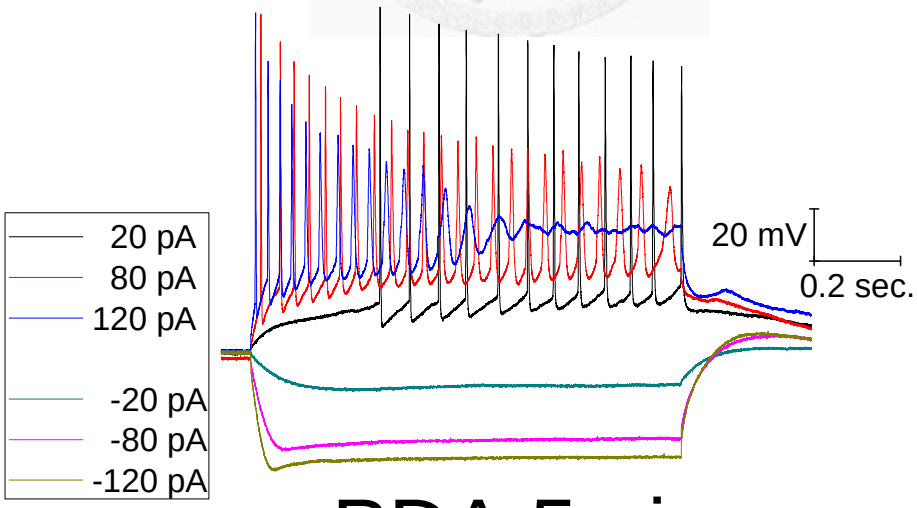
Fig. 2

A1



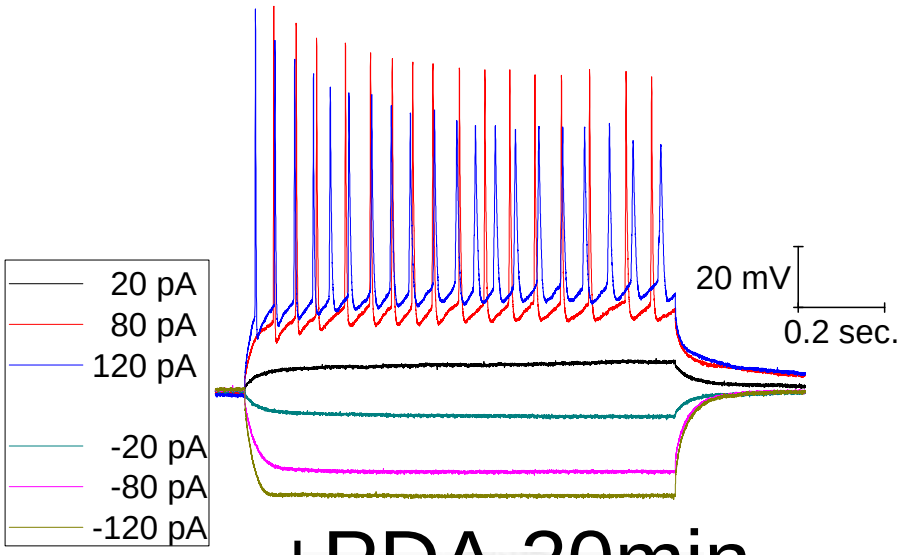
PSK

A2

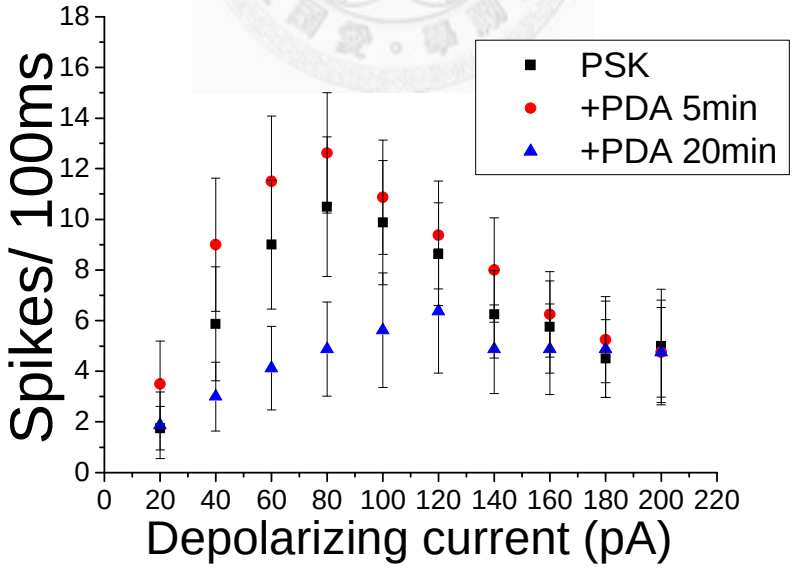


+PDA 5min

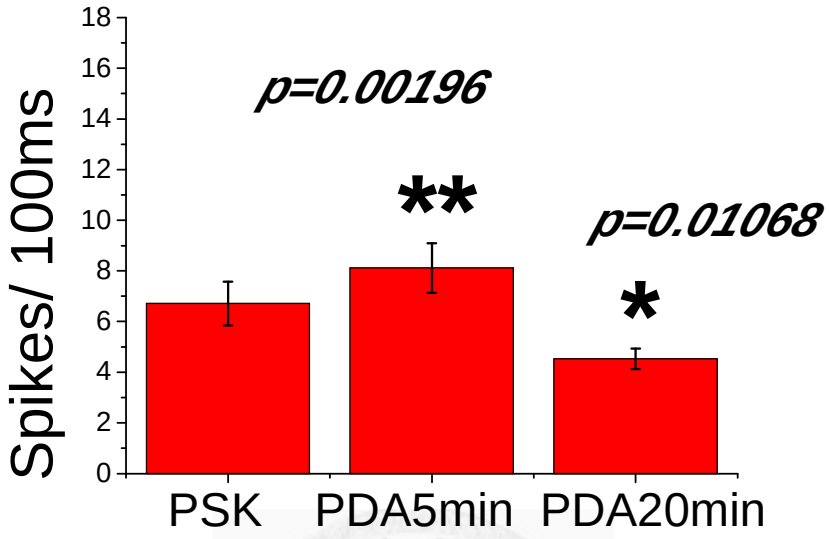
A3


20 pA
80 pA
120 pA
-20 pA
-80 pA
-120 pA
20 mV
0.2 sec.
+PDA 20min

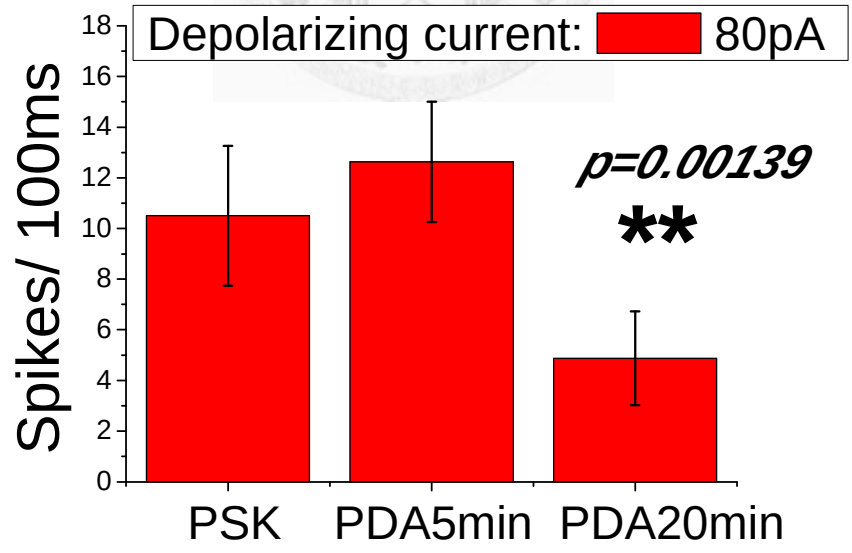
B1


PSK
+PDA 5min
+PDA 20min
Spikes/ 100ms
Depolarizing current (pA)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180
200
220

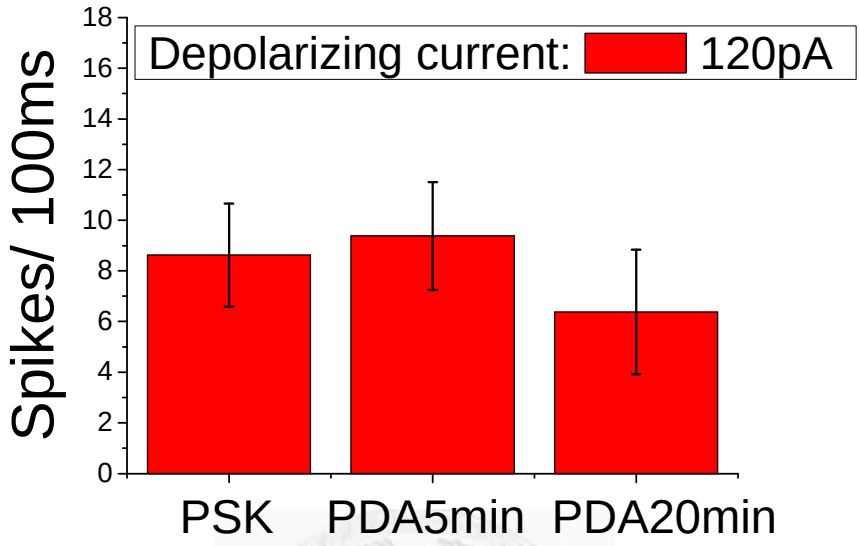
B2


Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
p=0.00196
**
p=0.01068
*
PSK
PDA5min
PDA20min

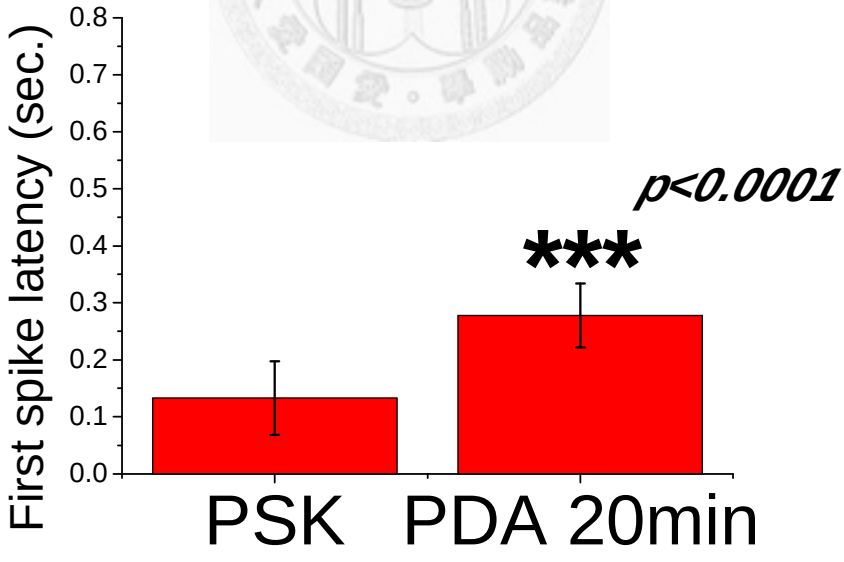
C1


Depolarizing current:
80pA
Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
p=0.00139
**
PSK
PDA5min
PDA20min

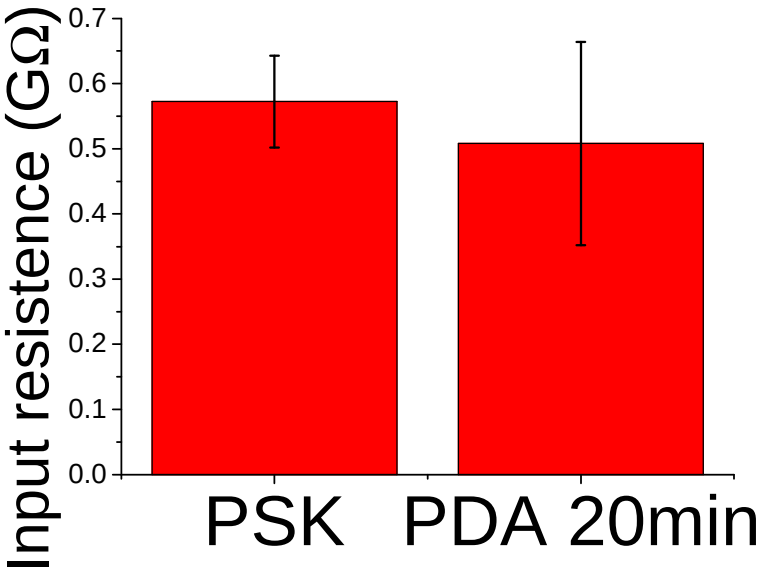
C2


Depolarizing current: 120pA
Spikes/ 100ms
0
2
4
6
8
10
12
14
16
18
PSK
PDA5min
PDA20min

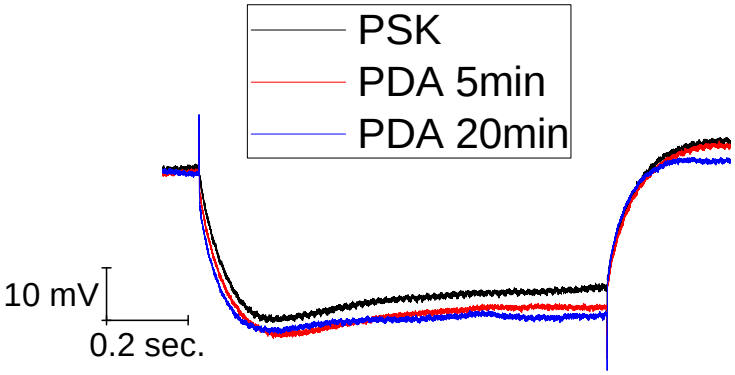
D1


First spike latency (sec.)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
p<0.0001
***
PSK
PDA 20min

D2



E1



Hyperpolaring current: -120pA

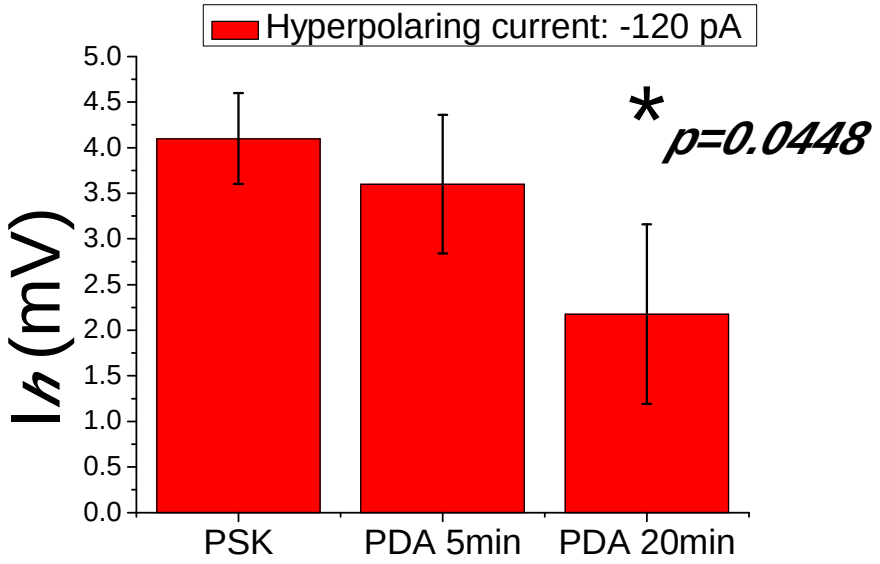
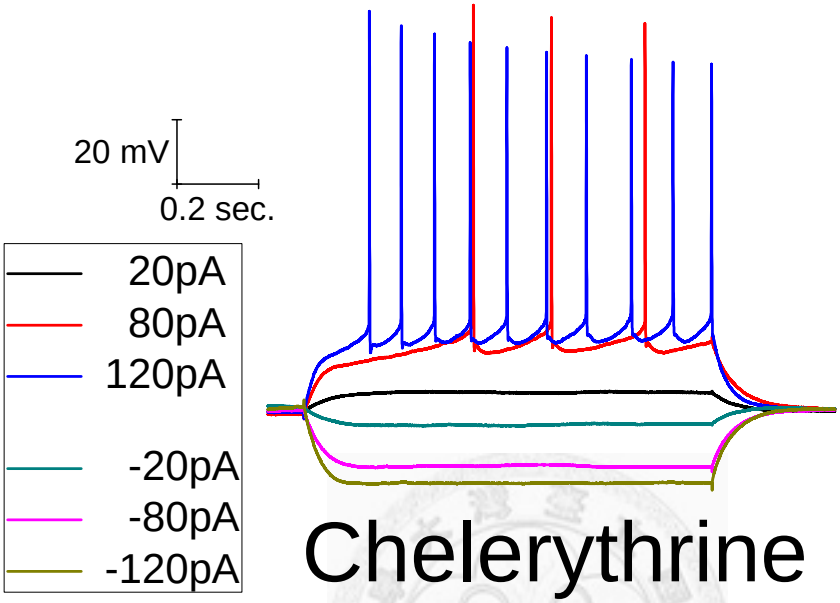


Fig2. Effect of PDA on capsular central amygdaloid neuron. A1-A3 Representatives of action potentials recorded with base solution (picrotoxin, strychnine, and kynurenic acid, PSK, 5-min and 20-min PDA application, generated by depolarizing and hyperpolarizing current pulse injection (±20, ±80, ±120pA).B1: 5-min PDA increased the spike number whereas 20-min PDA decreased the input-output function of neuronal excitability (generated by different intensity of current injection from 20pA- 200pA with 20pA interval, n=8). Symbols showed (mean ±SE) number of spikes per 100 ms calculated from recording traces. Action potential adaptation appeared around 80-120pA current injection with PDA application (red diamond and blue triangle). B2: Summary of changes in action potential firing with 5-min and 20-min PDA. 5-min PDA significantly increased *(Pair two sample t-test, p=0.00196)* the spike number in contrast to 20-min PDA application *(Pair two sample t-test,*

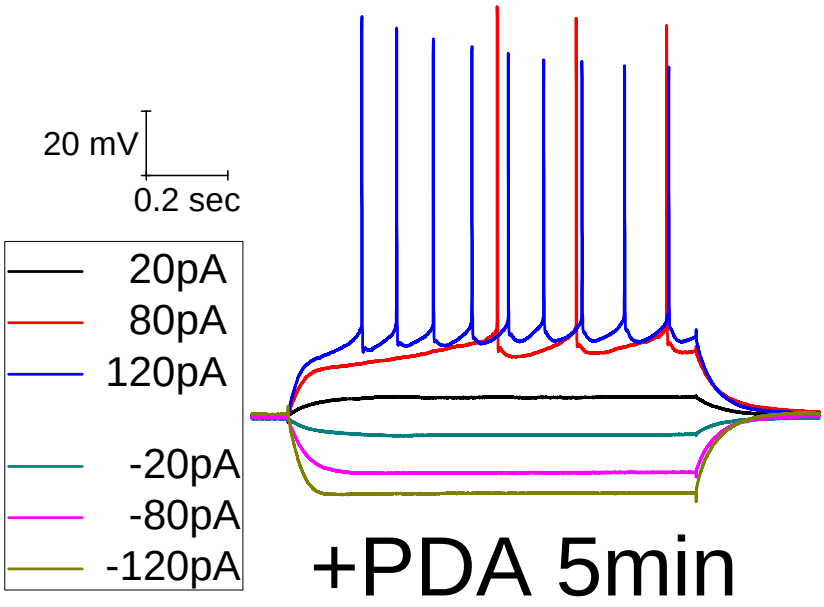
p=0.01068). C1, C2: Summary of spike number on 80pA and 120pA current injection with PSK and 20-min PDA respectively. 20-min PDA significant decreased the spike number on 80pA current injection. D1: 20-min PDA significantly increased the first spike latency *(Pair two sample t-test, p<0.0001)*. D2: Input resistance with application of base solution and 20-min PDA application. There was the tendency on decline of input resistance. E1: Representatives of sag traces produced by hyperpolarizing current with PSK, 5-min PDA and 20-min PDA application respectively (generated by hyperpolarizing current injection, -120pA).The size of sag were smaller after application of PDA. E2: Summary of sag size changes. 20-min PDA significantly reduced the size of sag *(Pair two sample t-test, p=0.0448).*

Fig. 3

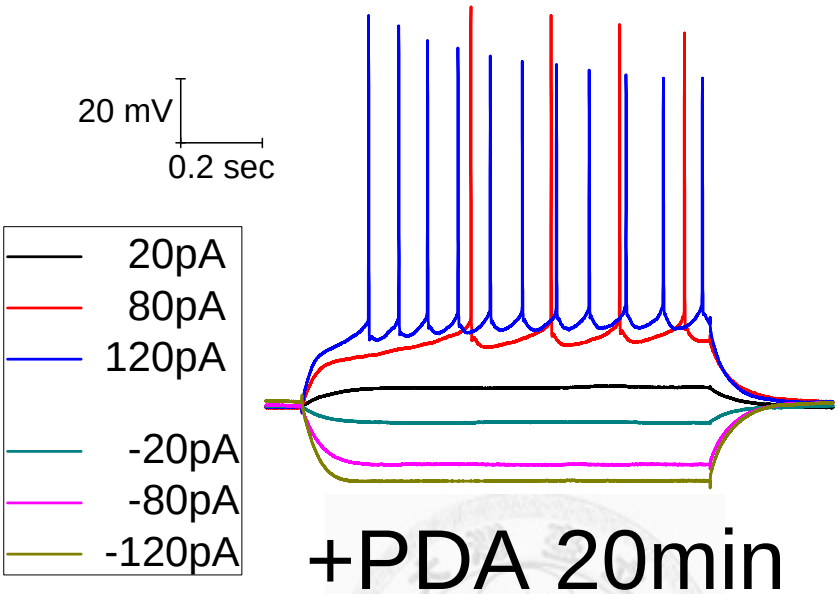
A1


20 mV
0.2 sec.
20pA
80pA
120pA
-20pA
-80pA
-120pA
Chelerythrine

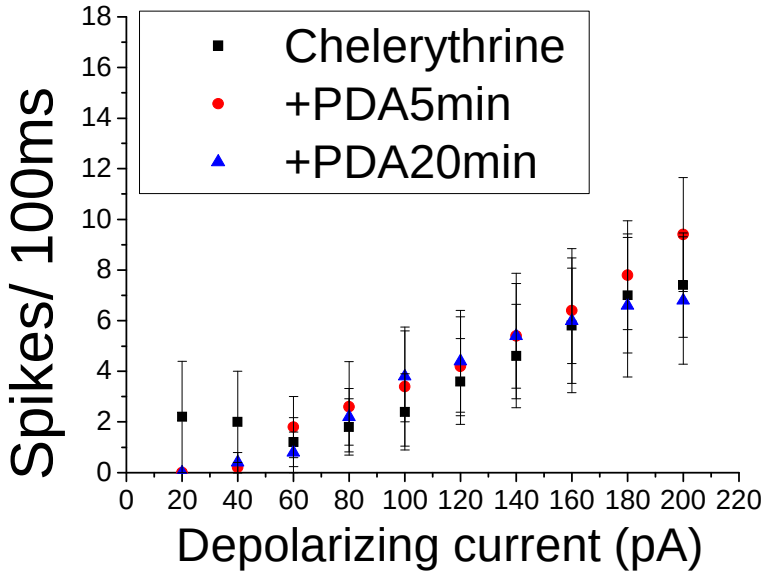
A2


20 mV
0.2 sec
20pA
80pA
120pA
-20pA
-80pA
-120pA
+PDA 5min

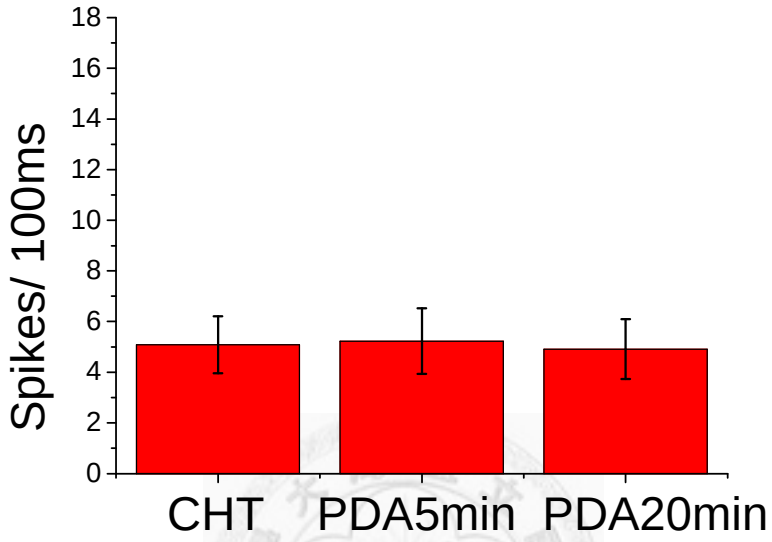
A3


20 mV
0.2 sec
20pA
80pA
120pA
-20pA
-80pA
-120pA
+PDA 20min

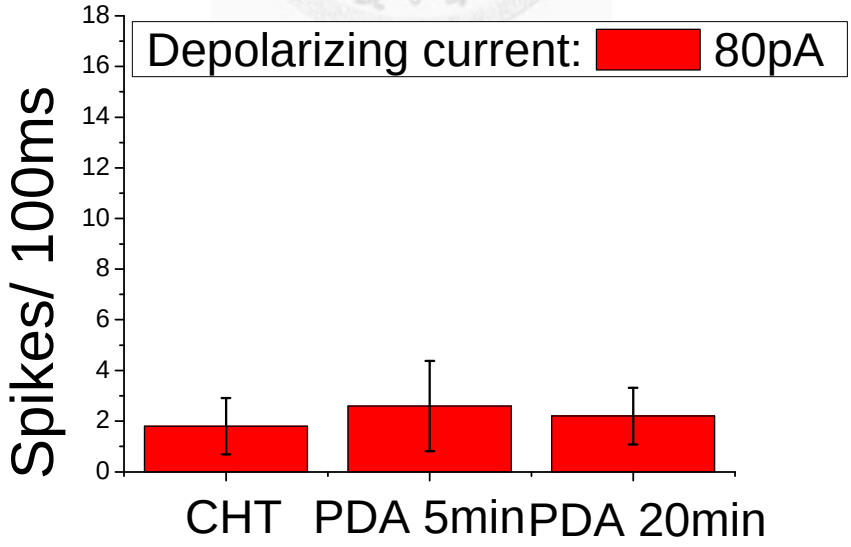
B1


Chelerythrine
+PDA5min
+PDA20min
Spikes/ 100ms
Depolarizing current (pA)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180
200
220

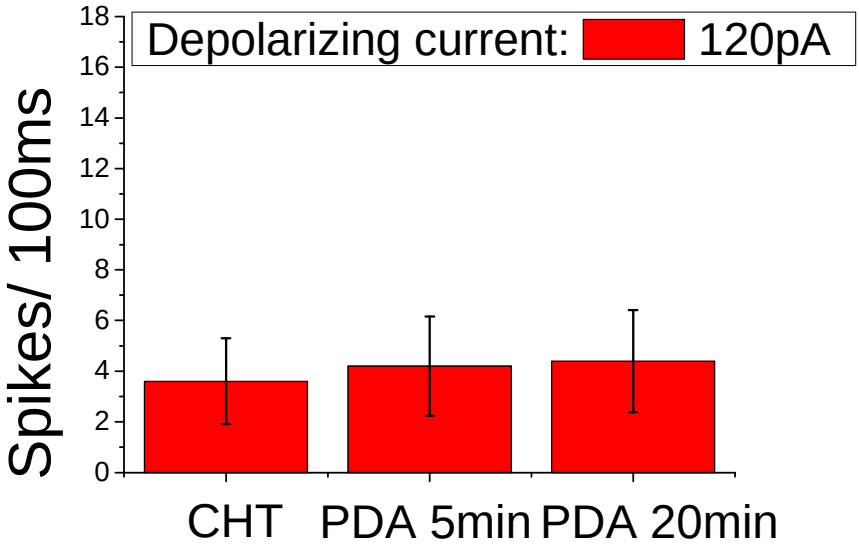
B2


Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
CHT
PDA5min
PDA20min

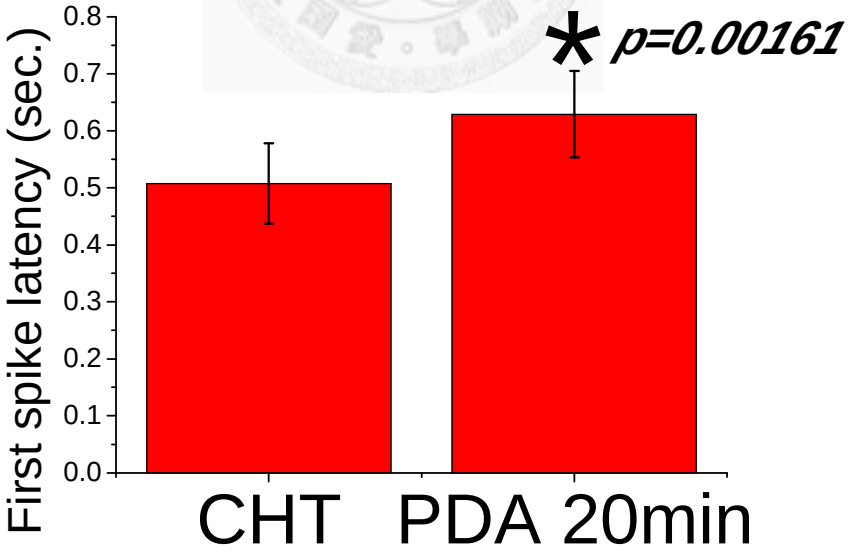
C1


Depolarizing current:
80pA
Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
CHT
PDA 5min
PDA 20min

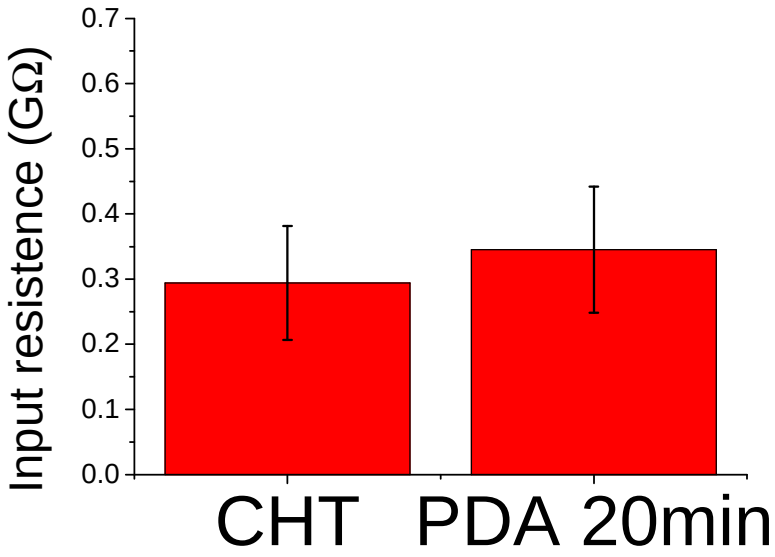
C2


Depolarizing current: 120pA
Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
CHT
PDA 5min
PDA 20min

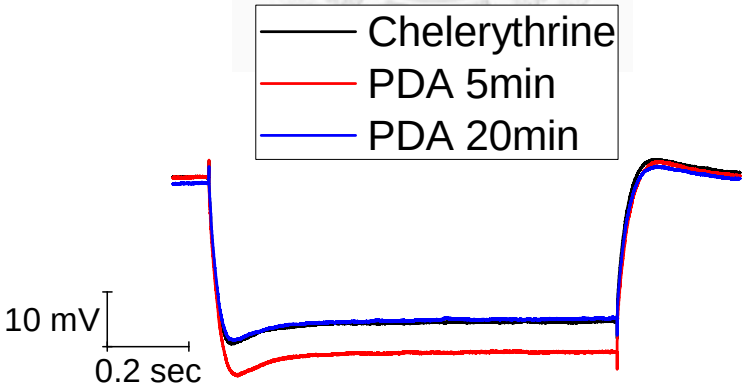
D1


* p=0.00161
First spike latency (sec.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CHT
PDA 20min

D2



E1



Hyperpolarizng current: -120pA

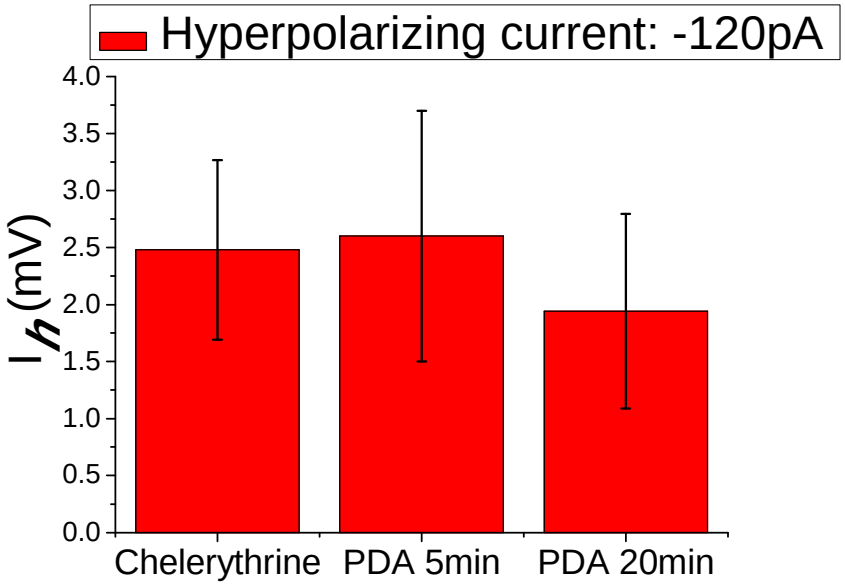
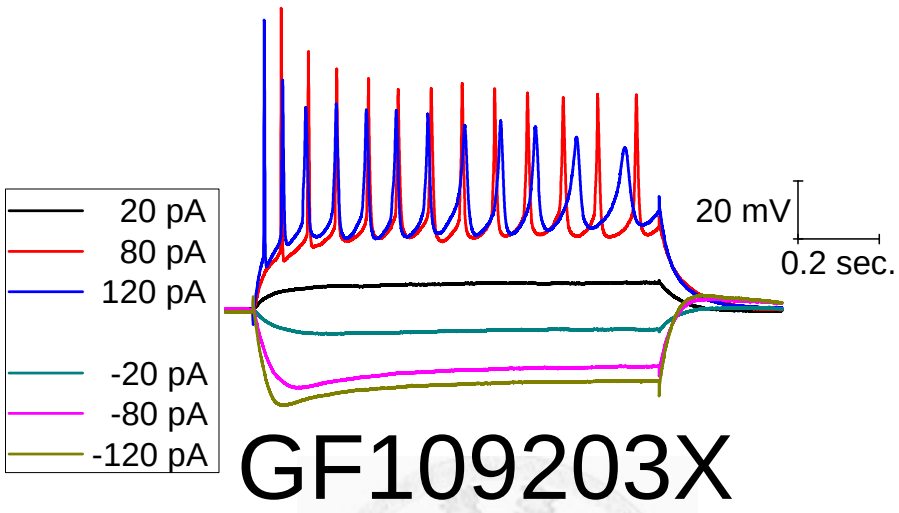


Fig3. Effect of Chelerythrine –PDA on capsular central amygdaloid neuron. A1-A3 Representatives of action potentials recorded with 10-min Chelerythrine, 5-min PDA and 20-min PDA application, generated by depolarizing and hyperpolarizing current pulse injection (±20, ±80, ±120pA).B1: Chelerythrine inhibited the effect of PDA. Spike number was neither increased nor decreased (generated by different intensity of current injection from 20pA- 200pA with 20pA interval, n=5). It also postponed the occurrence action potential adaptation. Symbols showed (mean ±SE) number of spikes per 100 ms calculated from recording traces. B2: Summary of changes in action potential firing with 10-min Chelerythrine, 5-min and 20min PDA application. Chelerythrine efficaciously inhibited the effect of PDA in both time scales. C1, C2: Summary of spike number on 80pA and 120pA current injection with Chelerythrine and 20-min PDA respectively. Chelerythrine inhibited the effect of 20-min PDA in

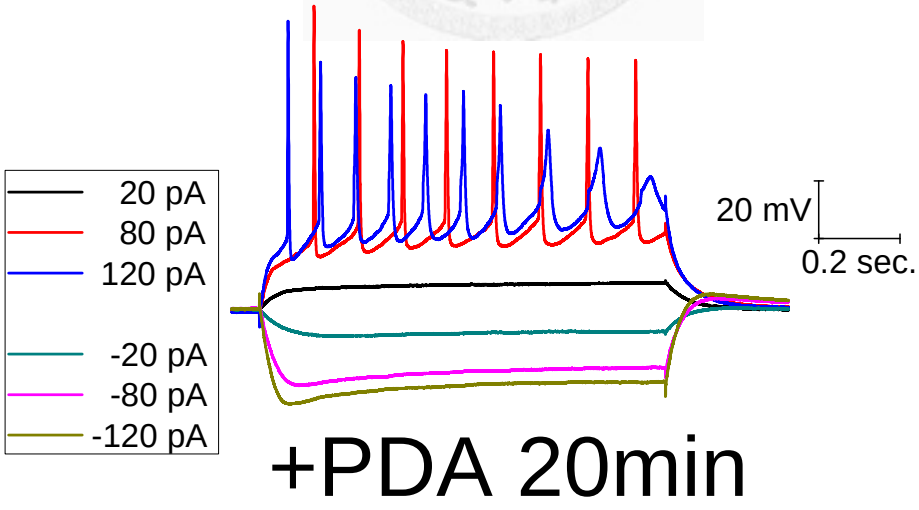
both circumstances. D1: 20-min PDA significantly increased the first spike latency *(Pair two sample t-test, p=0.00161).* D2: Input resistance with Chelerythrine and 20-min PDA application. There was no crucial change. E1: Representatives of sag traces produced by hyperpolarizing current with 10-min Chelerythrine, 5-min PDA and 20-min PDA application in turn respectively (generated by hyperpolarizing current injection, -120pA). E2: Summary of sag size changes. There is no significant changes in sag size. However, the current with 5-min PDA is more hyperpolarized.

Fig. 4

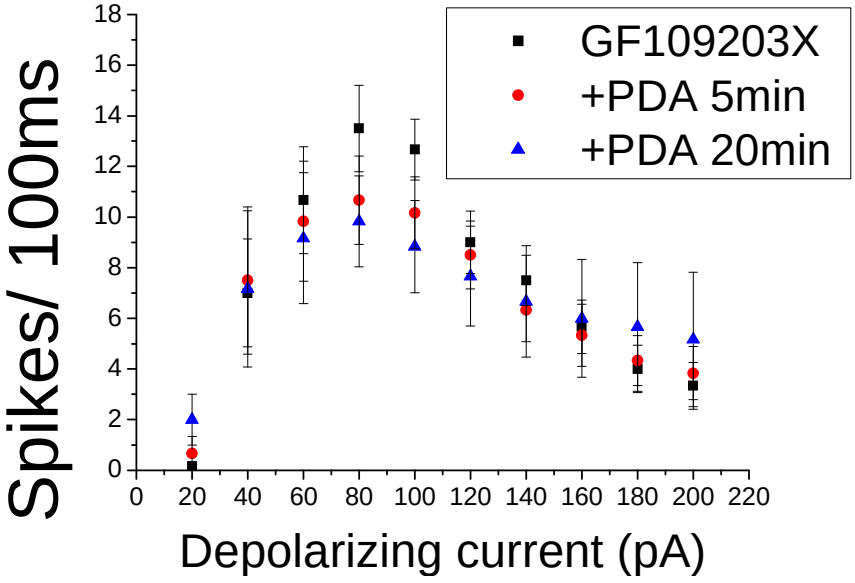
A1


20 pA
80 pA
120 pA
-20 pA
-80 pA
-120 pA
20 mV
0.2 sec.
GF109203X

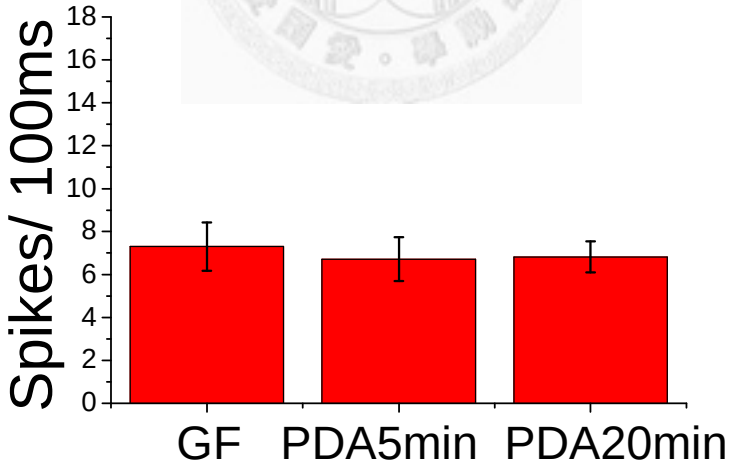
A2


20 pA
80 pA
120 pA
-20 pA
-80 pA
-120 pA
20 mV
0.2 sec.
+PDA 20min

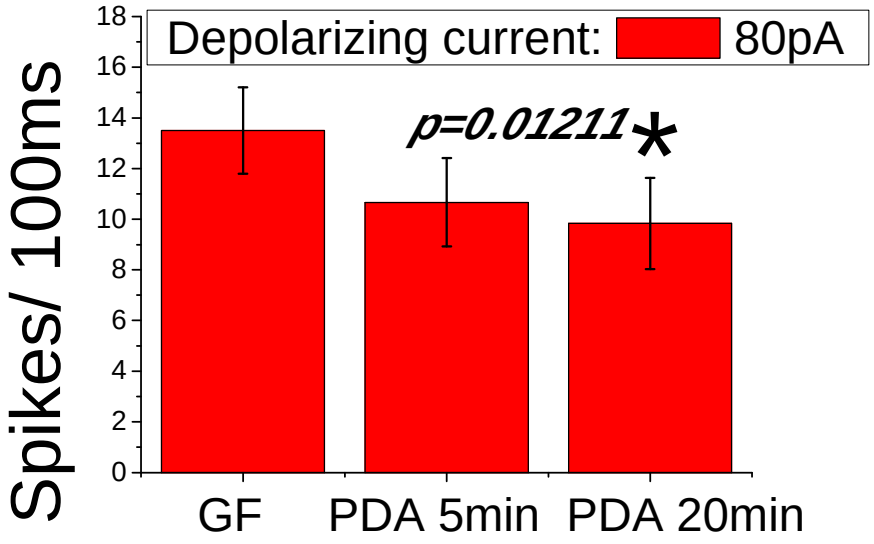
B1


Spikes/ 100ms
Depolarizing current (pA)
GF109203X
+PDA 5min
+PDA 20min
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180
200
220

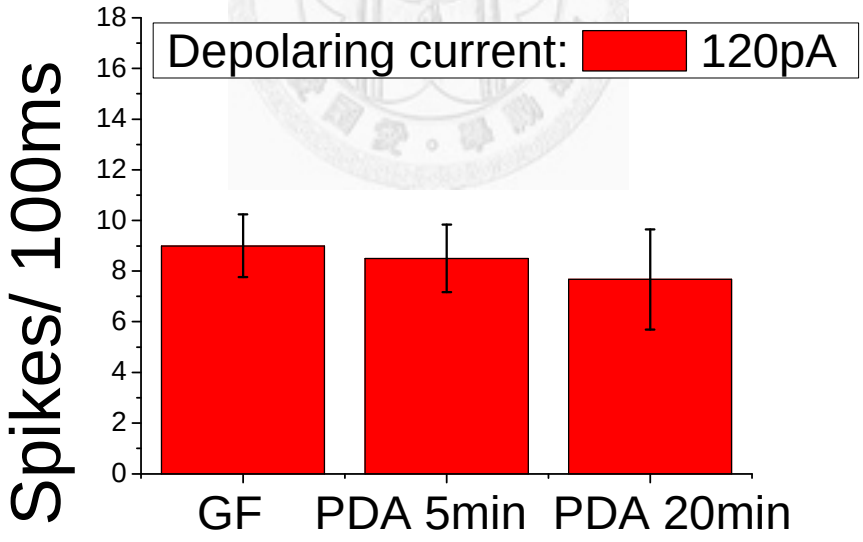
B2


Spikes/ 100ms
GF
PDA5min
PDA20min
0
2
4
6
8
10
12
14
16
18

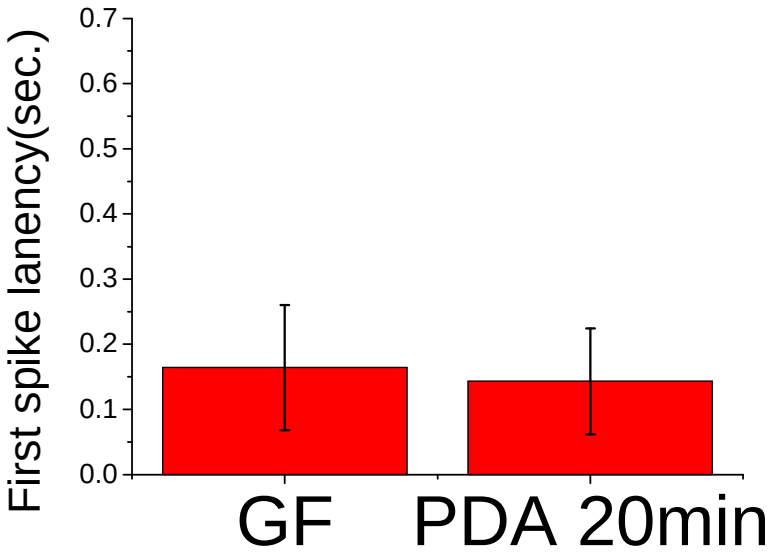
C1


Depolarizing current: 80pA
p=0.01211*
Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
GF
PDA 5min
PDA 20min

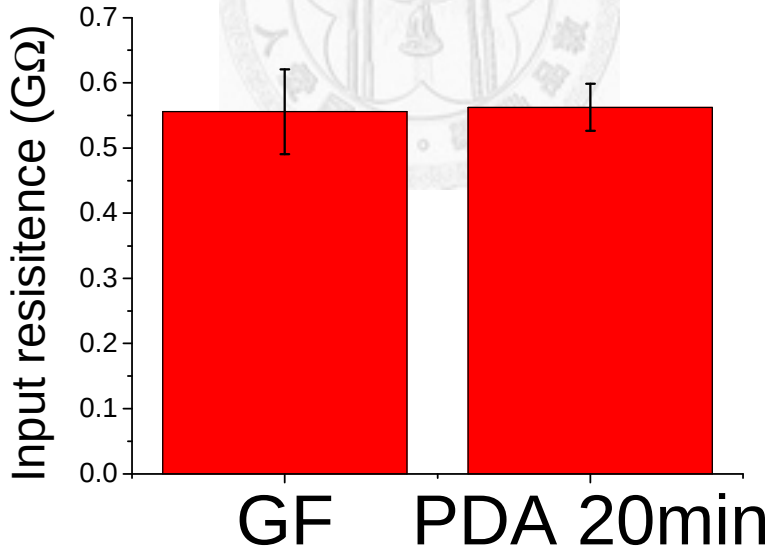
C2


Depolaring current: 120pA
Spikes/ 100ms
18
16
14
12
10
8
6
4
2
0
GF
PDA 5min
PDA 20min

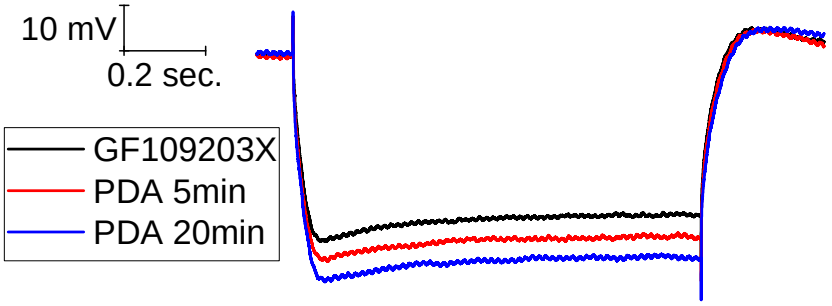
D1


First spike lanency(sec.)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
GF
PDA 20min

D2


Input resisitence (GΩ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
GF
PDA 20min

E1



E2

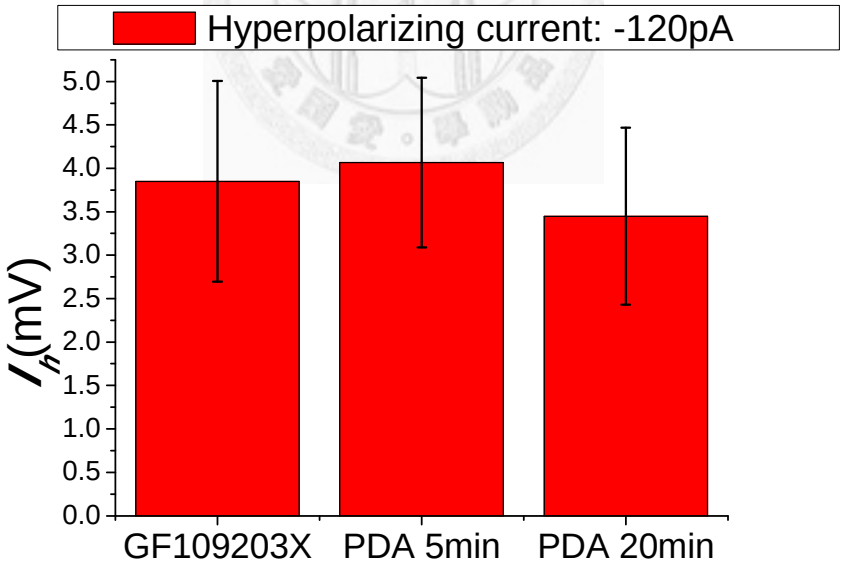
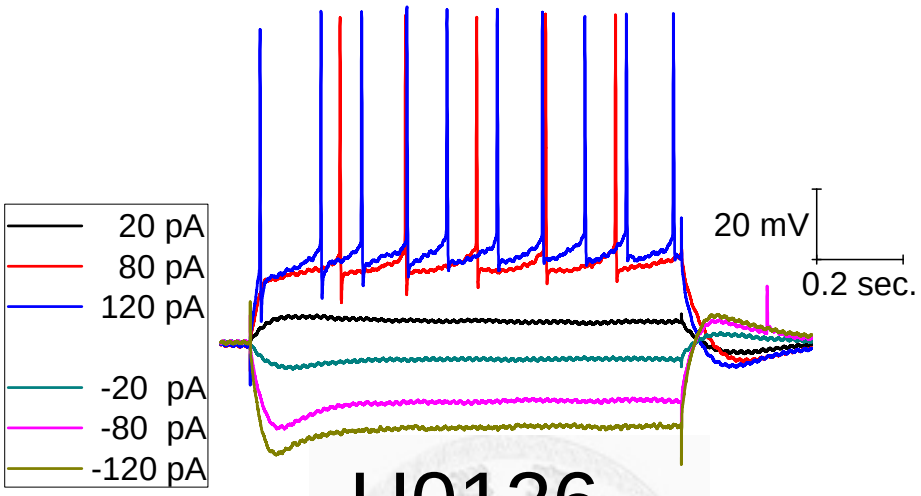


Fig4. Effect of GF109203X –PDA on capsular central amygdaloid neuron . A1, A2 Representatives of action potentials recorded with 10-min GF109203X and additional 20-min PDA application, generated by depolarizing and hyperpolarizing current pulse

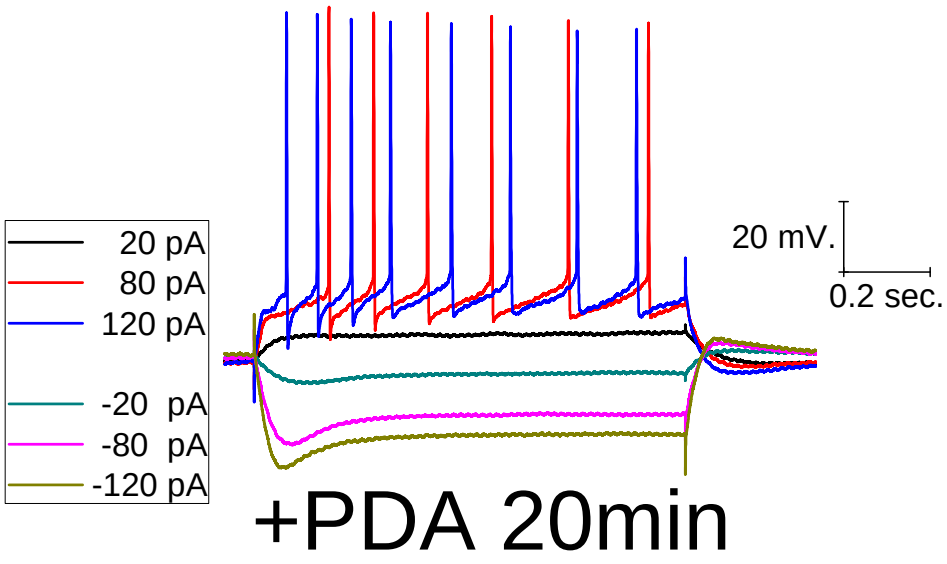
injection (±20, ±80, ±120pA). 20-min PDA did not significantly changes the action potential firing pattern. B1: GF109203X generally inhibited the effect of PDA (generated by different intensity of current injection from 20pA- 200pA with 20pA interval, n=6). Symbols showed (mean ±SE) number of spikes per 100 ms calculated from recording traces. B2: Summary of changes in action potential firing with 10-min GF109203X, 5-min and 20min PDA application. GF109203X efficaciously inhibited the effect of PDA in both time scales. C1, C2: Summary of spike number on 80pA and 120pA current injection with GF109203X and 20-min PDA respectively. GF109203X inhibited the effect of 20-min PDA in both circumstances except for 20-min PDA application on 80pA current injection *(Pair two sample t-test, p=0.01211).* D1: GF109203X successfully inhibited the effect of 20-min PDA. The first spike latency was not decreased. D2: Input resistance with GF109203X and 20-min PDA application. There was no crucial change. E1: Representatives of sag traces produced by hyperpolarizing current with 10-min GF109203X, 5-min PDA and 20-min PDA application in turn respectively (generated by hyperpolarizing current injection, -120pA). E2: Summary of sag size changes. There is no significant change in sag size. However, the current traces with 5-min and 20-min PDA application are more hyperpolarized.

Fig 5

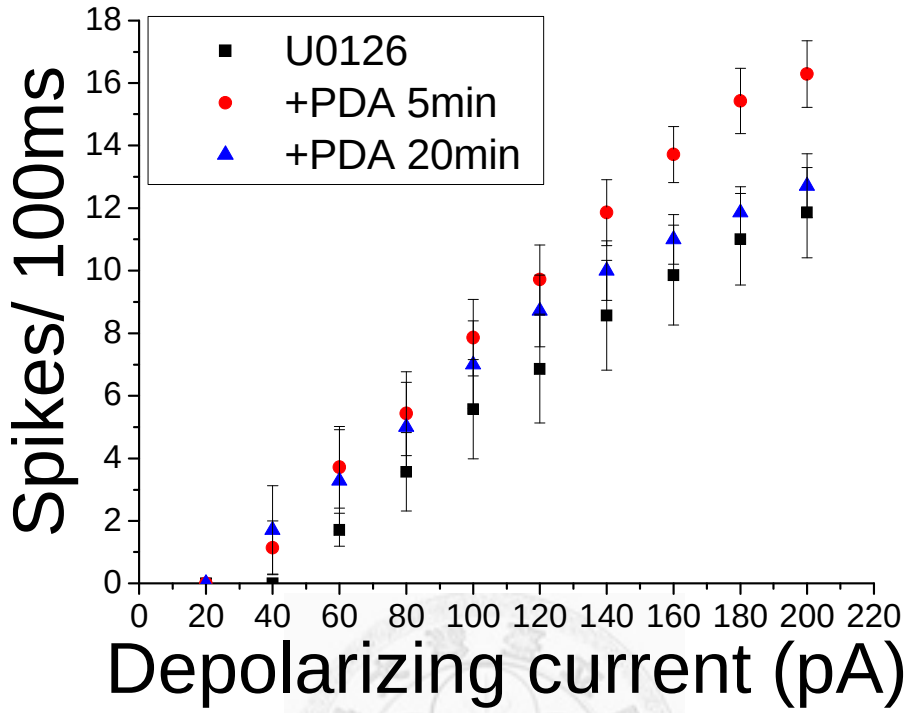
A1


20 pA
80 pA
120 pA
-20 pA
-80 pA
-120 pA
20 mV
0.2 sec.
U0126

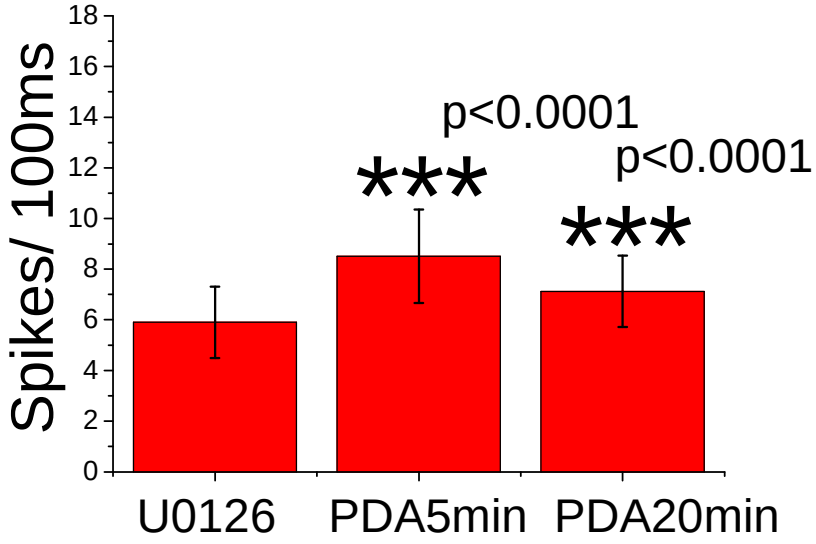
A2


20 pA
80 pA
120 pA
-20 pA
-80 pA
-120 pA
20 mV.
0.2 sec.
+PDA 20min

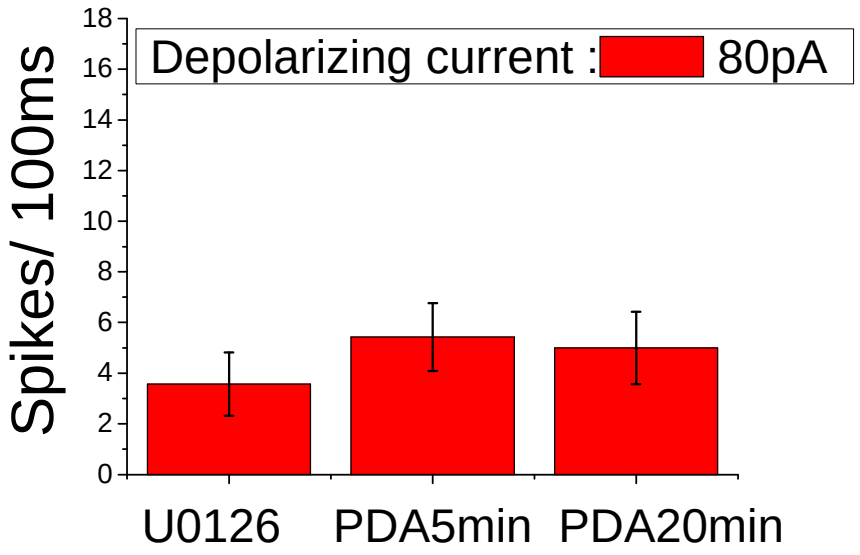
B1


U0126
+PDA 5min
+PDA 20min
Spikes/ 100ms
Depolarizing current (pA)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180
200
220

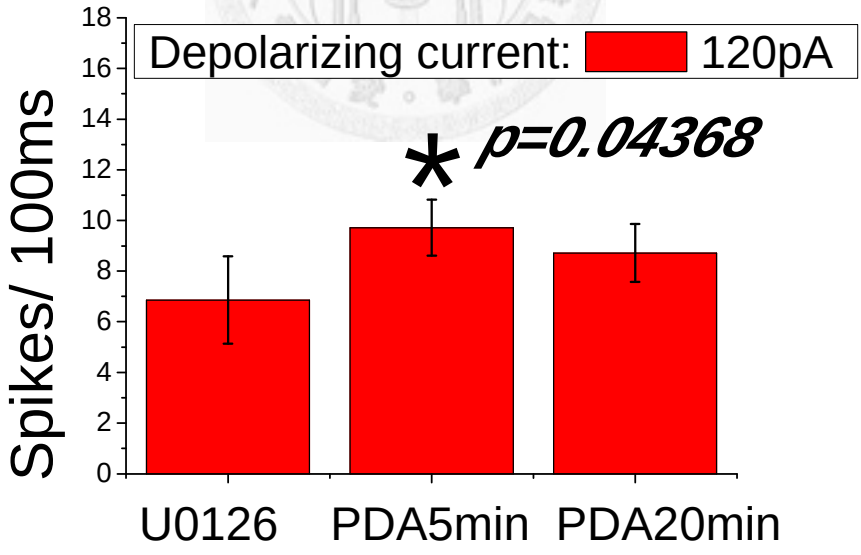
B2


Spikes/ 100ms
p<0.0001
***
p<0.0001
***
U0126
PDA5min
PDA20min

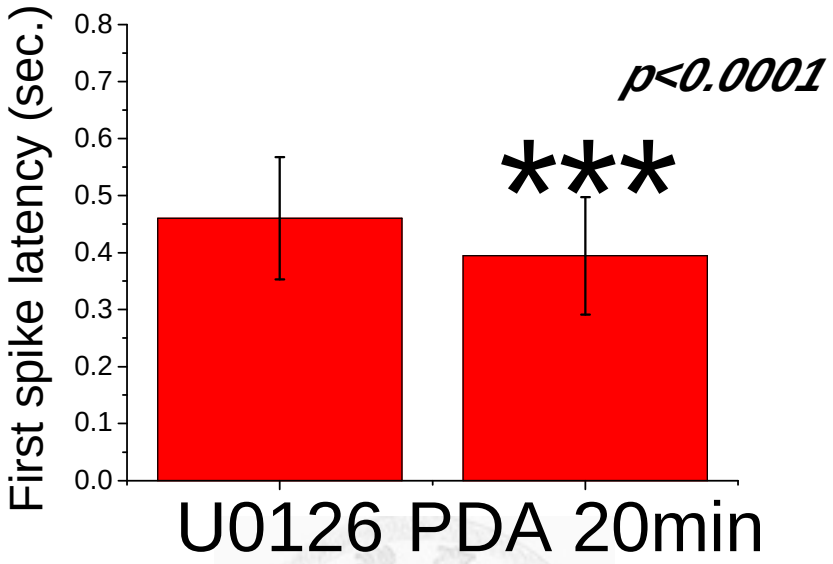
C1


Depolarizing current : 80pA
Spikes/ 100ms
0
2
4
6
8
10
12
14
16
18
U0126
PDA5min
PDA20min

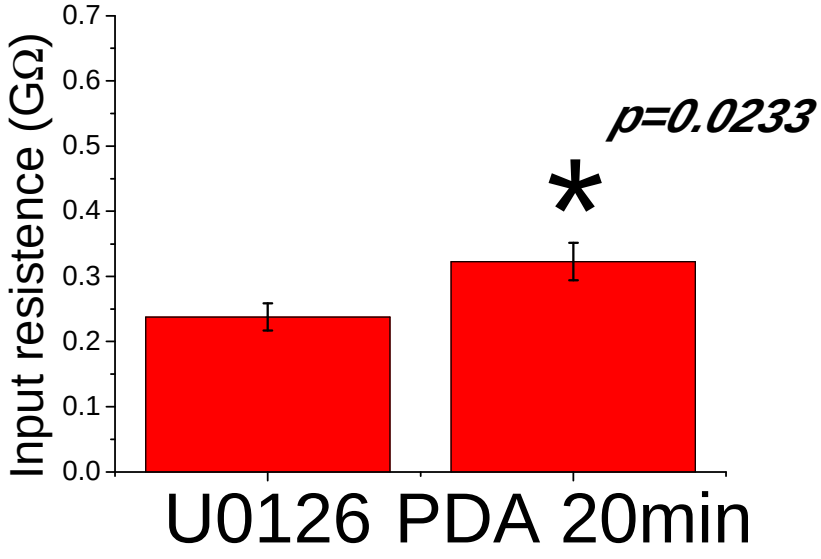
C2


Depolarizing current: 120pA
Spikes/ 100ms
*
p=0.04368
0
2
4
6
8
10
12
14
16
18
U0126
PDA5min
PDA20min

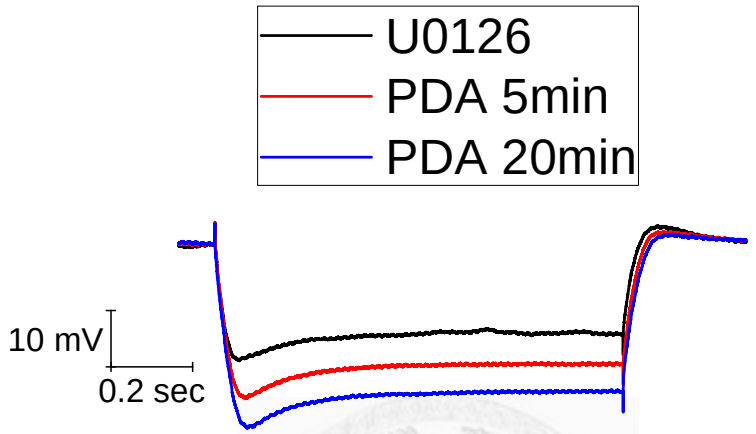
D1


First spike latency (sec.)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
p<0.0001
***
U0126 PDA 20min

D2


Input resistence (GΩ)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
p=0.0233
*
U0126 PDA 20min

E1



Hyperpolarizing current: -120pA

E2

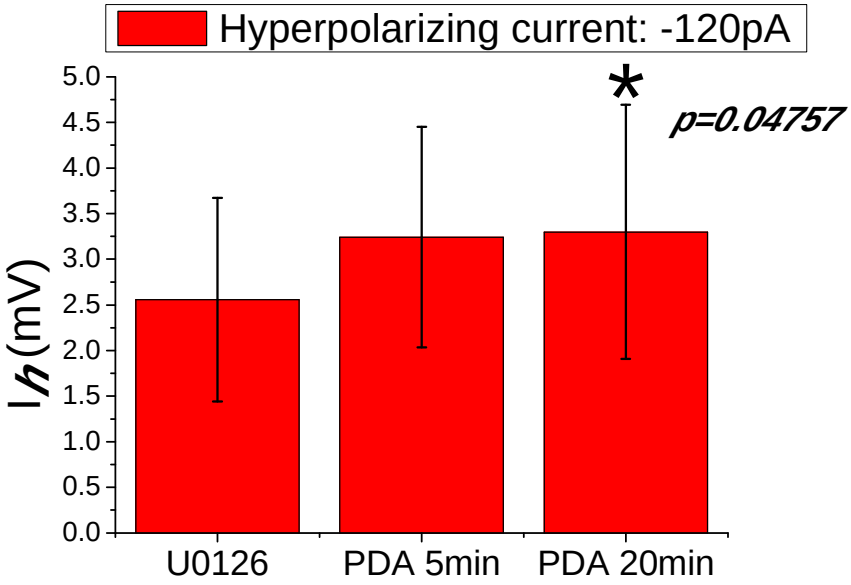


Fig. 5 Effect of U0126–PDA on capsular central amygdaloid neuron. A1, A2 Representatives of action potentials recorded with U0126 and additional 20-min PDA application, generated by depolarizing and hyperpolarizing current pulse injection (± 20, ±80, ±120pA).B1: U0126 reversed the effect of 20-min PDA (generated by different intensity of current injection from 20pA- 200pA with 20pA interval, n=7). The spike number was increased with 20-min PDA application *(Pair two sample t-test, p<0.0001)*. The effect of 5-min PDA was not changed by U0126. Spike number significantly increased with 5-min PDA *(Pair two sample t-test, p<0.0001).* Symbols showed (mean ±SE) number of spikes per 100 ms calculated from recording traces. B2: Summary of changes in action potential firing with U0126 and 20min PDA application. 20-min PDA increased the spike number. C1, C2: Summary of spike number on 80pA and 120pA current injection with U0126 and 20-min PDA respectively. 5-min PDA significantly increased the spike number on 120pA current injection. *(Pair two sample t-test, p=0.04368).* D1: U0126 reversed the effect of 20-min PDA in first spike latency. The latency was significantly decreased GF109203X successfully inhibited the effect of 20-min PDA. The first spike latency was not decreased *(Pair two sample t-test, p<0.0001).* D2: Input resistance was significant increased with and 20-min PDA application *(Pair two sample t-test, p=0.0233)*. It was in contrast to the tendency downward with application of 20-min

PDA alone. E1: Representatives of sag traces produced by hyperpolarizing current with U0126, additional 5-min PDA and 20-min PDA application respectively (generated by hyperpolarizing current injection, -120pA).The size of sag were smaller after application of PDA. E2: Summary of sag size changes. 20-min PDA significantly increased the size of sag *(Pair two sample t-test, p=0.04747).*

Fig.6

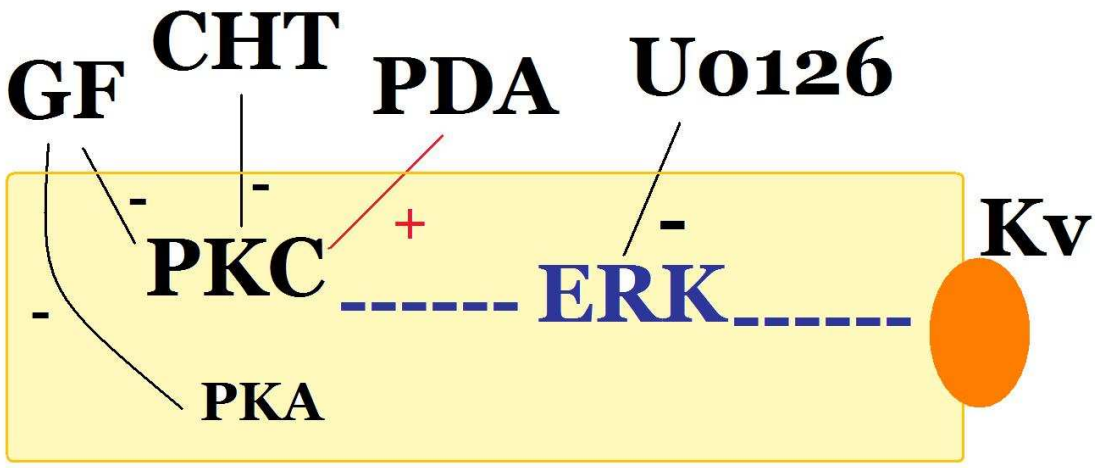


Fig.6 Diagram of rationale indicating PKC—ERK pathway activated by PDA. ERK activation was able to enhance the function of A-type type potassium channel or sustained delayed rectifier current. ERK activation by long-term PDA significantly induced the decline of spike number as well as the rise of first spike latency. PKC blockade eliminated the effect of PDA in both short-term and long-term scale. On the other hand, ERK inhibition by U0126 significantly induced the rise of spike number as well as the decline of first spike latency.