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PROTEIN KINASE C α MEDIATES A NOVEL FORM OF PLASTICITY IN THE ACCESSORY OLFACTORY BULB

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Abstract

Modification of synapses in the accessory olfactory bulb (AOB) is believed to underlie pheromonal memory that enables mate recognition in mice. The memory, which is acquired with single-trial learning forms only with coincident noradrenergic and glutamatergic inputs to the AOB. The mechanisms by which glutamate and norepinephrine (NE) alter the AOB synapses are not well understood. Here we present results that not only reconcile the earlier, seemingly contradictory, observations on the role of glutamate and NE in changing the AOB synapses, but also reveal novel mechanisms of plasticity. Our studies suggest that initially, glutamate acting at Group II metabotropic receptors and NE acting at α_2 -adrenergic receptors inhibit N-type and R-type Ca²⁺ channels in mitral cells via a G-Protein. The N-type and R-type Ca²⁺ channel inhibition is reversed by activation of α_1 -adrenergic receptors and protein kinase C α (PKC α). Based on these results, we propose a hypothetical model for a new kind of synaptic plasticity in the AOB that accounts for the previous behavioral data on pheromonal memory. According to this model, initial inhibition of the Ca²⁺ channels suppresses the GABAergic inhibitory feedback to mitral cells, causing disinhibition and Ca²⁺ influx. NE also activates phospholipase C (PLC) through α_1 -adrenergic receptors generating inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Calcium and DAG together activate protein kinase C α (PKC α) which switches the disinhibition to increased inhibition of mitral cells. Thus, PKC α is likely to be a coincidence detector integrating glutamate and NE input in the AOB and bridging the short-term signaling to long-term structural changes resulting in enhanced inhibition of mitral cells that is thought to underlie memory formation.

The ability to change the strength of synapses allows organisms to learn and form memories. Much of our knowledge of such plasticity comes from studies on long-term potentiation and depression (Malenka and Bear, 2004), which are thought to underlie memory formation during multiple-trial learning. Synaptic plasticity underlying single-trial learning, on the other hand, is less well studied. We have investigated the mechanism of synaptic plasticity in the accessory olfactory bulb (AOB) that is associated with the formation of the pheromonal memory underlying mate recognition in mice.

Female mice form a robust, long-lasting memory for the pheromones of their mate during a sensitive period around the time of mating. Exposure to pheromones from a male that is genetically dissimilar to her mate causes a recently mated female to return to estrus, blocking her pregnancy (Brennan and Keverne, 1997). The recognition of her mate's pheromones prevents the mate from causing abortion of his own offspring, and is therefore vital for reproductive success. The male pheromones are detected by the sensory neurons in the

vomeroneasal (VN) organ, which project to mitral/tufted cells (henceforth referred to as 'mitral cells') in the AOB (Mori, 1987; Shepherd, 2004; Ma, 2007), via the VN nerve. Mitral cells project from the AOB to the corticomedial amygdala, which in turn projects via the bed nucleus of the stria terminalis to the medial hypothalamus. Activation of this pathway by pregnancy blocking pheromones from an unfamiliar male leads to a decrease in prolactin secretion from the pituitary and a consequent reduction in circulating progesterone and failure of embryo implantation. Mitral cell activity in the AOB is regulated by inhibitory feedback from granule cell interneurons at reciprocal dendrodendritic synapses (Shepherd, 2004). Memory formation is hypothesized to result from long-lasting increases in the inhibitory feedback on those mitral cells that respond to the mating male's pheromones (Brennan and Keverne, 1997), preventing them from activating the pregnancy blocking pathway. In support of this hypothesis, GABA release in the AOB has been shown to increase during and after pheromonal memory formation (Brennan et al., 1995).

Memory formation requires the association of pheromonal input mediated by glutamate release from the VN nerve onto mitral cells and the release of NE in the AOB from locus ceruleus projections, which signal that mating has occurred (Brennan and Keverne, 1997). Infusion of Group II (mGluR₂/mGluR₃; henceforth referred to as mGluR_{2/3}) metabotropic glutamate receptor agonist DCG-IV into the AOB obviates the requirement for NE and induces memory formation during exposure to male pheromones, in the absence of mating (Kaba et al., 1994). DCG-IV has been found to presynaptically inhibit GABA release from granule cells in AOB slices *in vitro* (Hayashi et al., 1993). The decrease in GABA release caused by mGluR_{2/3} activation is inconsistent with the long term increase in mitral cell inhibition that is hypothesized to underlie mate recognition. Thus far no satisfactory explanation has been obtained for the mechanism by which activation of mGluR_{2/3} or adrenergic receptors in the AOB causes increased inhibition of mitral cells which is thought to underlie memory formation. We therefore investigated the cellular effects of glutamatergic and adrenergic signaling using acute AOB slices with a view to identify the mechanisms of synaptic plasticity in the AOB that might explain pheromonal learning.

EXPERIMENTAL PROCEDURES

Recordings from AOB slices

Balb/c mice were obtained from Charles River (Wilmington, MA) and used for experiments using a protocol approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. Female mice between 2–4 weeks of ages were used for optimal patch-clamp experiments in AOB slices. Our previous studies have shown that in these mice all the major components of the glutamatergic and adrenergic signaling pathway are expressed at levels comparable to the AOB in adult female mice (Skinner et al., 2008). Sagittal sections (200 μ M) were prepared using a vibratome in an oxygenated and chilled solution (incubation solution) containing 124 mM NaCl, 5 mM KCl, 10 mM Glucose, 2 mM CaCl₂, 2.9 mM MgSO₄, 3 mM NaH₂PO₄ and 23 mM NaHCO₃ (pH 7.4). The slices were recovered for at least 1 h in the incubation solution. The extracellular solution contained 105 mM NaCl, 2.5 mM KCl, 20 mM Glucose, 2 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄ and 26 mM NaHCO₃ (pH 7.4). Whole cell recordings were carried out using a patch electrode pulled from borosilicate glass with a resistance of 4–6 M Ω , filled with a solution containing CsCl 130 mM, 2 mM MgCl₂, 20 mM N-[2-hydroxy-ethyl] piperazine-N'-2-ethanesulfonic acid (HEPES), 4 mM ATP, 0.5 mM GTP, 11 mM EGTA, 1 mM CaCl₂; pH 7.2 (Edwards et al., 1989; Hayashi et al., 1993; Jia et al., 1999). Mg-ATP was used in the internal solution in order to minimize the run-down of Ca²⁺ channels (Kepplinger KJF and Romanin C, 2005). Voltage-clamp recordings were done with a Multiclamp 700A amplifier. The microscope was equipped with infrared differential interference optics and a charge-coupled device camera which allowed the

experimenter to visually identify the AOB neurons and to guide the patch electrodes. Mitral cell and granule cell layers were readily distinguishable in the AOB slices. Mitral cells, which are considerably larger than granule cells, were identified based on their size and morphology. After a gigaohm seal and whole cell access was achieved, the series resistance was between 10 and 20 m Ω and was compensated (60–80%) by the amplifier. Cells with more than 20% variation in input resistance were discarded. High threshold Ca²⁺ currents were recorded under voltage-clamp in the presence of tetrodotoxin (TTX) (1 μ M) and triethylammonium (20 mM). For the study of current-voltage relationships, mitral cells were held at –80 mV and currents were recorded in response to +10mV steps.

Spontaneous and evoked IPSC measurements from mitral cells were made with a pipette solution containing (in mM): 125 KCl, 2MgCl₂, 2 CaCl₂, 10 EGTA, 2 NaATP, 0.5 NaGTP, and 10 HEPES, adjusted to pH 7.3 with KOH. All data were filtered at 1 kHz and digitized at a sampling rate of 10 kHz. Data were required at a holding potential of 0mV in the presence of 1 μ M TTX (Schoppa et al., 1998). For stimulation of the glomerular layer for measurement of evoked IPSCs, a bipolar tungsten stimulating electrode (Frederick Haer & Co, Brunswick, ME), was used. Stimulus pulses were generated by Digidata1322A (Axon Instruments, Sunnyvale, CA) which triggered a stimulus isolation unit (Maximum 2 mA stimulation with duration of 100 μ sec).

While space clamp concerns cannot be eliminated from studies of neurons in slice preparations, we took the following steps to assess and minimize it. Poor space clamp is indicated by an increase in the delay to onset of inward current with increasing magnitude of the command pulse, or by rapidly inactivating inward currents that were elicited by steps from depolarizing test potentials back to the holding potential. Cells that included these response features were eliminated from our analysis. In addition, all reported quantitative measurements were taken at steady state (250 msec after the test pulse) to minimize the effect of inadequate space clamp on the transient phase of the current. Voltage output of the amplifier was recorded in parallel with current traces to monitor escape voltage transients (Halliwell et al., 1994; Graef et al., 2009).

Drug application

The concentrations of the pharmacological and other reagents used were as follows:

BAPTA (1,2-Bis [2-aminophenoxy] ethane-N,N,N',N'-tetraacetic acid): 10 mM; Bisindolylmaleimide I (Bis I): 2 μ M; Bisindolylmaleimide V (Bis V): 2 μ M; Chelerythrine: 10 μ M; Cirazoline: 10 μ M; Clonidine: 5 μ M; DCG-IV: 0.5 μ M; GDBPS: 100 μ M; GTP γ S: 100 μ M; Glutamate: 500 μ M; HBDDE (2, 2', 3, 3' 4, 4'-Hexahydroxy-1, 1'biphenyl6-6'-dimethanol dimethyl ether: 50 μ M; L-CCG-I: 10 μ M; LY333531 (low): 10 nM; LY333531 (high): 1 μ M; LY379268: 10 μ M; NE: 30 μ M; Nifedipine: 10 μ M; Picrotoxin: 200 μ M; PMA (phorbol myristate acetate): 500 nM; PTX (pertussis toxin): 1 μ g/ml; Recombinant PKC α : 2 μ M; Recombinant PKC γ : 2 μ M; PKC α inhibitor (Compound 6): 20 μ M; SNX-482: 100 nM; UK14304: 5 μ M; U73122: 10 μ M; U73343: 10 μ M; ω -conotoxin-GVIA: 1 μ M; ω -conotoxin-MVIIIC: 1 μ M. Stock solutions of Bis I, Bis V, LY333531, nifedipine, picrotoxin, PMA, U73122, U73343, UK14304 were prepared in dimethyl sulfoxide (DMSO). For these reagents the same volume of DMSO alone was used for controls. All other reagents were dissolved in distilled water.

For testing the effect of PTX, we used the A-protomer (the subunit containing enzymatic activity) (Biomol, Plymouth Meeting, PA) in the patch pipette. The A-protomer, because of its small size (28 K) can be efficiently dialysed into cells and has been used successfully in several studies (Elmslie, 1992; Kurachi et al., 1986; Xie and Lewis, 1997). The A-protomer was pre-incubated with 10 mM dithiothreitol at 37°C for 10 min to convert it to its

enzymatically active form and was diluted in the internal solution (1 $\mu\text{g/ml}$ final concentration) along with 1 mM nicotinamide adenine dinucleotide (NAD). For testing the effect of protein kinase C isoforms, purified recombinant PKC α and PKC γ (Upstate USA Inc., Charlottesville, VA) were diluted in the internal solution (2 μM final). Heat-inactivated PTX and PKC isoforms were used as controls. PKC α Compound 6 (kindly provided by David Larsen, Albert Einstein School of Medicine, New York) and the peptides (Quality Controlled Biochemicals, Hopkinton, MA) were included in the patch pipette.

For testing the effect of ω -conotoxin-GVIA, ω -conotoxin-MVIIC, SNX-482 and picrotoxin, the AOB slices were incubated in ACSF containing the toxin for 15 min.

Data were collected from the same cells while using drugs that could be washed out. For experiments with drugs that could not be washed out or those with irreversible effects (PTX, ω -conotoxin-GVIA, ω -conotoxin-MVIIC, SNX-482, picrotoxin, GDBPS and GTP γ S, recombinant PKC α and PKC γ , and PKC α , PKC γ), different cells were used for data collection with each reagent and the experiments were carried out under comparable conditions.

Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test (pairwise multiple comparison procedures) while comparing multiple groups for treatment effect. In these instances, the F and the P values for the overall comparison are given first and then the P values from the post-hoc Tukey test are given next to the groups being compared. For comparison between two groups, Student's t-test was used. Cumulative probability distributions of IPSC amplitude and frequency were analyzed using Kolmogorov-Smirnov test. The values are expressed as mean $\pm$ standard error. The P-values between 0.05 and 0.01 are indicated by one asterisk (*) or a number sign (#) in the figures; P-values less than 0.01 are indicated by two asterisks (**) or number signs (##). The P-values indicate significant difference with respect to controls unless otherwise stated in the text or otherwise indicated in the figures. Comparisons between other groups are stated in the text where necessary and in the figures such comparisons are indicated by lines between bars that are being compared.

RESULTS

mGluR_{2/3} and α_2 -adrenergic receptors (α_2 -ARs) inhibit Ca²⁺ currents in the mitral cells of the AOB

In the AOB, mGluR_{2/3} receptors disinhibit mitral cells by presynaptic inhibition of GABA release from the granule cell side of the reciprocal synapses. The mechanism underlying disinhibition is unclear. One possibility is that mGluR_{2/3} close voltage-gated Ca²⁺ channels (Swartz, 1993; Catterall, 2000), thus controlling glutamate release from mitral cells or GABA release from granule cells, either of which leads to disinhibition of the mitral cells. The presence of mGluR_{2/3} in mitral cells has been demonstrated through previous immunocytochemical experiments (Ohishi et al., 1998; Sahara et al., 2001). To test the idea that mGluR_{2/3} reduces the Ca²⁺ current, we isolated the high-threshold Ca²⁺ current in mitral cells in the whole cell mode under voltage-clamp conditions and added mGluR_{2/3} agonist L-CCG-I (10 μM) (Hayashi et al., 1992). Typical Ca²⁺ currents in untreated (control) and L-CCG-I-treated mitral cells are shown in Fig. 1 A & B. We also added two other mGluR_{2/3} agonists LY379268 (Monn et al., 1999) or DCG-IV (Hayashi et al., 1993)(10 μM each) to the bath. All the three agonists L-CCG-I ($56.7 \pm 7.6\%$; $P < 0.01$; $n = 11$), LY379268 ($56.7 \pm 9.9\%$; $P < 0.01$; $n = 6$), and DCG-IV ($58.3 \pm 14.5\%$; $P < 0.05$; $n = 5$) significantly reduced the Ca²⁺ current relative to controls (100%) (Fig. 1C).

The current exhibited characteristic properties of Ca^{2+} currents: we were able to substitute Ba^{2+} as charge carrier, and the current was blocked by Cd^{2+} ($1.6 \pm 1.4\%$, $P < 0.001$ relative to control [100%]; $n = 9$) (Supplementary Fig. S1). The run-down (decrease over time) of Ca^{2+} channels is a commonly observed phenomenon as has been reported previously (Kepplinger KJF and Romanin C, 2005). When we recorded the Ca^{2+} current in several mitral cells ($n=17$) without any treatment, we observed an average of 7.5% run down at the end of a 15-min period.

To test whether α_2 -ARs control Ca^{2+} channels (Li et al., 1998) in the AOB, we isolated the Ca^{2+} current and applied α_2 -AR agonists clonidine or UK14304 ($5 \mu\text{M}$ each) (Andorn et al., 1988) to the bath. Both clonidine ($52.5 \pm 9.4\%$; $P < 0.01$; $n = 10$) and UK14304 ($48.3 \pm 13.3\%$; $P < 0.01$; $n = 6$) significantly inhibit the Ca^{2+} current relative to controls (100%) (Fig. 1D)

Inhibition of Ca^{2+} channels by $\text{mGluR}_{2/3}$ is mediated by a G-protein

The effect of $\text{mGluR}_{2/3}$ on Ca^{2+} channels could be mediated by direct coupling via G-proteins as has been shown in cultured cortical neurons (Swartz, 1993). If the reduction in Ca^{2+} current were mediated by a G-protein, reagents that inhibit G-proteins should prevent the reduction in the Ca^{2+} current caused by L-CCG-I. Inclusion of inhibitors of G-proteins $\text{GDP}\beta\text{S}$ or pertussis toxin (PTX) (Swartz, 1993; Ikeda et al., 1995) in the patch pipette not only reduced the inhibition but significantly increased the Ca^{2+} current relative to controls but inclusion of a G-protein activator $\text{GTP}\gamma\text{S}$ significantly reduced the Ca^{2+} current. In the presence of $\text{GDP}\beta\text{S}$ or PTX, $\text{mGluR}_{2/3}$ agonist L-CCG-I failed to decrease the Ca^{2+} current (Fig. 1E) (see Supplementary Table S1 for details). These results indicate that $\text{mGluR}_{2/3}$ acts on Ca^{2+} channels via an inhibitory G-protein. Time course experiments with $\text{GDP}\beta\text{S}$ showed an increase in the Ca^{2+} current relative to controls whereas time course with $\text{GTP}\gamma\text{S}$ showed a decrease in the Ca^{2+} current further supporting the role of a G-protein in Ca^{2+} channel inhibition (Control at 4 min: 100%; At 10 min, Control: $96 \pm 2.1\%$, $\text{GDP}\beta\text{S}$: $135.6 \pm 8.1\%$, $P < 0.01$; $\text{GTP}\gamma\text{S}$: $62.0 \pm 8.7\%$, $P < 0.01$; At 20 min: Control: $96 \pm 1.4\%$, $\text{GDP}\beta\text{S}$: $145.4 \pm 7.9\%$, $P < 0.01$; $\text{GTP}\gamma\text{S}$: $36.0 \pm 6.8\%$; $P < 0.01$) (Fig. 1F). In the presence of $\text{GDP}\beta\text{S}$ and PTX, the Ca^{2+} currents were higher than untreated controls indicating that these reagents removed tonic inhibition (Swartz, 1993; Yang and Tsien, 1993; Zhu and Ikeda, 1994) of Ca^{2+} channels by G-proteins. Further increase in the Ca^{2+} current with the addition of L-CCG-I suggests that perhaps $\text{mGluR}_{2/3}$ activation causes a cycle of G-protein activation and formation of additional hetero-trimers because of $G\alpha$ association with $G\beta\gamma$ which in turn should increase the efficacy of $\text{GDP}\beta\text{S}$ and PTX. Overall, the results indicate that $\text{mGluR}_{2/3}$ receptors inhibit Ca^{2+} channels by direct coupling through G-proteins.

Mutual occlusion of $\text{mGluR}_{2/3}$ and α_2 -AR effects on the Ca^{2+} current

Since agonists of both $\text{mGluR}_{2/3}$ and α_2 -AR cause reduction in the Ca^{2+} current, we tested whether these receptors exert their inhibitory effect on the Ca^{2+} current through similar or dissimilar mechanisms. We first administered L-CCG-I, the $\text{mGluR}_{2/3}$ agonist followed by clonidine, the α_2 -AR agonist. As before, L-CCG-I reduced the Ca^{2+} current ($62.8 \pm 8.5\%$; $P < 0.05$) relative to controls (100%) but clonidine did not have an additive effect on Ca^{2+} current ($58.8 \pm 7.5\%$; $P = 0.1873$; $n = 8$) (Fig. 2A). The converse experiment (administration of clonidine followed by L-CCG-I) yielded similar results (Control: 100%; Clonidine: $53.9 \pm 5.9\%$, $P < 0.01$; Clonidine + L-CCG-I: $51.1 \pm 6.0\%$; $P = 0.38$ Clonidine + L-CCG-I compared to Clonidine alone; $n = 7$; paired t-test) (Fig. 2B). Thus $\text{mGluR}_{2/3}$ and α_2 -AR occlude each other's effect. It is possible, however, that occlusion could occur if either L-CCG-I or clonidine had caused maximal inhibition of Ca^{2+} current possible under the recording conditions.

The Ca^{2+} current in mitral cells is mediated by N- and R- type Ca^{2+} channels

Next, we sought to characterize the type of Ca^{2+} channels that mediate the Ca^{2+} currents in mitral cells. The Ca^{2+} current was reduced to $43.2 \pm 3.70\%$ compared to controls (100%) in

the presence of ω -conotoxin-GVIA (1 μ M), an N-type Ca^{2+} channel blocker (Tsien et al., 1988) (Fig. 3). Although the inhibition of the Ca^{2+} current by ω -conotoxin-GVIA was significant ($P < 0.01$; $n = 6$) a portion of the current remained resistant to the toxin. ω -conotoxin-MVIIIC (P/Q-type Ca^{2+} channel blocker, 1 μ M) and nifedipine (L-type Ca^{2+} channel blocker, 10 μ M) are without effect (Supplementary Fig. S1) suggesting that the Ca^{2+} current is not likely to be mediated by P/Q or L-type channels. Therefore, we tested the effect of SNX-482, an R-type Ca^{2+} channel blocker (Newcomb et al., 1998). SNX-482 reduced the Ca^{2+} current to $59.9 \pm 5.3\%$ relative to controls (100%) (Fig. 3). To test whether N-type and R-type channels carry bulk of the Ca^{2+} current, we applied both ω -conotoxin-GVIA and SNX-482. Together these two reagents reduced the Ca^{2+} current to $3.1 \pm 1.2\%$ relative to controls (100%) (Fig. 3).

PKC activation reverses mGluR_{2/3} and α_2 -AR-mediated decrease in the Ca^{2+} current

Activation of mGluR_{2/3} or α_2 -AR reduces the Ca^{2+} influx via a G-protein and thus could cause a reduction in glutamate release from mitral cells or GABA release from granule cells. Either or both of these effects would lead to disinhibition of mitral cells in the AOB. In order for pheromonal memory to form, however, disinhibition has to be converted to increased inhibition. A molecule that can potentially reverse the action of mGluR_{2/3} or α_2 -AR is PKC, which is known to uncouple G-proteins from N-type (Swartz, 1993; Zamponi et al., 1997) and R-type (Bannister et al., 2004) Ca^{2+} channels. To test whether PKC has an effect on Ca^{2+} currents, we applied an activator of PKC, phorbol 12-myristate-13-acetate (PMA). We observed that PMA (500 nM) reverses the decrease in the Ca^{2+} current by L-CCG-I (ANOVA: $F_{(2,30)} = 14.72$, $P < 0.001$; Control: 100%; L-CCG-I: $56.5 \pm 7.3\%$, $P < 0.05$; L-CCG-I + PMA: $145.0 \pm 18.8\%$; $P < 0.01$ compared to L-CCG-I and $P < 0.01$ compared to control; $n = 11$) (Fig. 4A). To test whether PMA acts through a Ca^{2+} -sensitive PKC, we carried out the experiments with a Ca^{2+} chelator BAPTA (10 mM). When BAPTA was present in the internal solution, PMA application did not reverse the L-CCG-I-mediated inhibition of the Ca^{2+} current (ANOVA: $F_{(2,18)} = 8.134$, $P = 0.003$; Control: 100%; L-CCG-I: $55.5 \pm 7.7\%$, $P < 0.01$; L-CCG-I + PMA: $57.0 \pm 13.3\%$, $P = 0.992$ L-CCG-I + PMA compared to L-CCG-I alone) (Supplementary Fig. S2). We studied the current-voltage relationships of L-CCG-I and PMA. We noticed that PMA leads to rapid activation of the Ca^{2+} current at more negative test pulses and the increase in current amplitude occurs over the entire range of test pulses (Fig 4B top panel). Activation of Ca^{2+} current at negative test pulses has been shown to be enhanced because of removal of tonic inhibition of Ca^{2+} channels by G-proteins (Swartz, 1993). We also tested the effect of L-CCG-I and PMA in time course experiments. In these experiments L-CCG-I was maintained in the bath for 10 min after which PMA was added to bath for 4 min. L-CCG-I decreased the Ca^{2+} current and PMA reversed the effect of L-CCG-I and increased Ca^{2+} current. The increase in the Ca^{2+} current was maintained as long as PMA was present in the bath and gradually decreased after washout of PMA (At 15 min, L-CCG-I: $42.9 \pm 7.9\%$, $P < 0.01$; at 19 min, L-CCG-I + PMA: $131.1 \pm 10.2\%$; $P < 0.05$, L-CCG-I + PMA at 19 min compared to L-CCG-I at 15 min; $n = 8$) (Fig. 4B bottom panel).

We carried out similar experiments on the effect of α_2 -AR agonist clonidine on the Ca^{2+} current. The current-voltage relationships of clonidine and PMA and the time course studies with these reagents were similar to that observed with L-CCG-I and PMA. (ANOVA: $F_{(2,21)} = 13.22$, $P < 0.001$; Control: 100%; Clonidine: $52.8 \pm 12.8\%$, $P < 0.05$; Clonidine + PMA: $132.0 \pm 13.5\%$, $P < 0.01$ Clonidine + PMA compared to Clonidine alone; $P < 0.05$ Clonidine + PMA compared to control; $n = 8$) (Time course: at 15 min, Clonidine: $35.5 \pm 6.8\%$; $P < 0.01$; at 19 min, Clonidine + PMA: $125.5 \pm 8.4\%$; $P < 0.05$, Clonidine + PMA at 19 min compared to Clonidine alone at 15 min; $n = 8$) (Fig. 4C & D).

The time course of PMA effect on the Ca^{2+} current is similar to that observed in frog sympathetic neurons (Yang and Tsien, 1993). The extent of the Ca^{2+} current with L-CCG-I +

PMA and Clonidine + PMA is comparable to the effect of PMA alone (see next section) suggesting reversal of Ca^{2+} channel inhibition which is in agreement with recent studies on rat sympathetic neurons (Diaz-Cardenas et al., 2008).

The α isoform of PKC mediates the effect on the Ca^{2+} currents in mitral cells

There are twelve mammalian PKC isoforms (Mellor and Parker, 1998) and different PKC isoforms appear to have specific roles in other systems (Sajikumar et al., 2005; Sossin, 2007; Sacktor, 2008). To understand the precise role of PKC in AOB plasticity, it is important to identify the PKC isoform critical for uncoupling $\text{mGluR}_{2/3}$ and α_2 -AR from Ca^{2+} channels and for increases in Ca^{2+} current. To identify the PKC isoform, first we standardized our experiment using bath application of chelerythrine (10 μM) which inhibits all isoforms of PKC. We found that chelerythrine blocked the PMA-mediated increase in the Ca^{2+} currents. We narrowed down the list of PKC isoforms for our study by reasoning that one of four Ca^{2+} - and diacylglycerol (DAG)-dependent (classical) PKC isoforms (α , βI , βII , γ) might act on Ca^{2+} channels because glutamate receptors can cause a rise in intracellular Ca^{2+} and adrenergic receptors can stimulate production of DAG. Blockade of the PMA effect in reversing the L-CCG-I-mediated inhibition of the Ca^{2+} current in the presence of BAPTA (see Supplementary Fig. S2) ruled out participation of novel PKCs, which are DAG-dependent but Ca^{2+} -insensitive (Mellor and Parker, 1998), further supporting a role for classical PKC isoforms. An inhibitor specific to PKC α and PKC γ HBDDE (Kashiwada et al., 1994; Mathur and Vallano, 2000) significantly inhibited the increase in Ca^{2+} current caused by PMA whereas an inhibitor of βI and βII isoforms of PKC, LY 333531 (10 nM), had no effect (Fig. 5A). At higher concentrations LY333531 can inhibit PKC α and PKC γ (Shen et al., 2003). Application of 1 μM LY333531 inhibited the Ca^{2+} current (Fig. 5A). Furthermore, a highly potent and specific inhibitor of PKC α , Compound 6 (20 μM) (Lee et al., 2004) ($P < 0.05$; $n = 11$), blocked the PMA effect on Ca^{2+} current (Fig. 5B) (see Supplementary Table S2 for details). Moreover, inclusion of purified recombinant PKC α but not PKC γ in the patch pipette resulted in an increase in Ca^{2+} current (At 10 min, Control: $98.3 \pm 1.7\%$; PKC α : $190.7 \pm 11.4\%$, $n = 7$, $P < 0.01$; PKC γ : $102.5 \pm 2.4\%$, $P = 0.279$; At 15 min, Control: $97.5 \pm 1.7\%$; PKC α : $200.4 \pm 11.0\%$, $P < 0.01$; PKC γ : $98.3 \pm 1.7\%$, $n = 6$, $P = 0.752$) (Fig. 5C, top panel). Application of a PKC inhibitor chelerythrine inhibited the increase in Ca^{2+} current caused by purified PKC α and inhibitory effect is reversed after the washout of the drug (At 10 min [just before chelerythrine application], $195.0 \pm 9.0\%$; $P < 0.01$ relative to baseline; At 15 min (just before washout of chelerythrine): $109.8 \pm 2.3\%$, $P < 0.01$, 15 min compared to 10 min; at 20 min [i.e. after washout of chelerythrine for 5 min], $152.5 \pm 8.0\%$; $P < 0.01$, 20 min compared to 15 min) (Fig. 5C, bottom panel).

Our finding that PKC α but not the closely related isoform PKC γ causes an increase in Ca^{2+} current suggested that a unique region of PKC α might play a role in mediating its effect on Ca^{2+} channels. The C-terminus of PKC α is known to interact with a PDZ domain containing protein PICK1 (protein interacting with C-kinase 1) (Staudinger et al., 1997) which is believed to localize PKC α close to the target of phosphorylation. If this idea is correct, then a PKC α peptide that would compete with the endogenous protein should block the PMA-induced increase in Ca^{2+} current. We tested a peptide with the unique C-terminal 11 amino acids of PKC α . As control of specificity, we also tested 11-amino acid C-terminal peptide from PKC γ . We found that PKC α peptide significantly inhibited the PMA-mediated increase in the Ca^{2+} current relative to controls ($P < 0.01$; $n = 7$) while PKC γ peptide had no effect ($P = 0.903$; $n = 7$) (Fig. 5D) (see Supplementary Table S2 for details).

α_1 -adrenergic receptors increase the Ca^{2+} current via phospholipase C (PLC) and PKC

Our results on reversal of Ca^{2+} current inhibition by PKC α suggest that the kinase must be activated by stimuli that induce pheromonal memory. Classical isoforms of PKC such as PKC α require Ca^{2+} and DAG for activation (Mellor and Parker, 1998). We hypothesized that

in addition to its action on α_2 -AR, NE also acts on α_1 -adrenergic receptor (α_1 -AR) activating PLC. Activated PLC produces DAG that can activate PKC α . We tested this hypothesis using cirazoline, an agonist of α_1 -AR (Gellai and Ruffolo, Jr., 1987). We found that cirazoline caused an increase in the Ca^{2+} current. This increase was abolished by PLC inhibitor U73122 (Bleasdale et al., 1990) (ANOVA: $F_{(2,21)} = 56.48$, $P < 0.001$; Control: 100%, Cirazoline: $168.8 \pm 9.7\%$, $P < 0.001$; Cirazoline + U73122: $65.0 \pm 7.3\%$, $P < 0.001$ Cirazoline + U73122 compared to Cirazoline alone, $n = 8$) whereas an inactive analogue U73343 had no effect on the increase in the Ca^{2+} current caused by cirazoline application (Control: 100%, Cirazoline: $157.3 \pm 9.4\%$; $P < 0.05$; Cirazoline + U73343: 156.7 ± 8.8 ; $P > 0.05$ Cirazoline + U73343 compared to Cirazoline alone) (Fig. 6A). The PLC inhibitor U73122 alone did not have an effect on the Ca^{2+} current suggesting that inhibition caused by U73122 was through a specific inhibitory effect on PLC (Control: 100%, U73122: $98.8 \pm 5.8\%$, $P = 0.97$, $n = 7$) (Supplementary Fig. S3). Therefore, PKC activation is likely to be mediated by PLC activation through α_1 -AR. Activation of PKC would in turn increase the Ca^{2+} current. If α_1 -AR-mediated increase on the Ca^{2+} current is mediated by PKC, inhibition of PKC should decrease the α_1 -AR-mediated increase in the Ca^{2+} current. Indeed, the PKC inhibitor chelerythrine significantly reduced the effect of cirazoline on the Ca^{2+} current (ANOVA: $F_{(2,14)} = 8.73$, $P = 0.002$; Control: 100%, Cirazoline: $151.9 \pm 8.4\%$, $P < 0.01$; Cirazoline + Chelerythrine: $96.0 \pm 9.1\%$, $P < 0.01$ Cirazoline + Chelerythrine compared to Cirazoline alone) (Fig. 6B). To obtain additional evidence for a role of PKC, we used the PKC inhibitor Bis I and an inactive analogue Bis V. We observed that Bis I significantly reduced cirazoline-mediated increase in the Ca^{2+} current (ANOVA: $F_{(2,21)} = 12.18$, $P < 0.001$; Control: 100%, Cirazoline: $164.8 \pm 11.4\%$, $P < 0.05$; Cirazoline + Bis I: $90.9 \pm 14.1\%$, $P < 0.05$ Cirazoline + Bis I compared to Cirazoline alone) whereas Bis V did not have a significant effect (Control: 100%, Cirazoline: $148.8 \pm 11.9\%$, $P < 0.05$; Cirazoline + Bis V: $145.2 \pm 12.0\%$; $P > 0.05$ Cirazoline + Bis V compared to Cirazoline alone, $n = 7$) (Fig. 6C). These results suggest that α_1 -AR-mediated increase in the Ca^{2+} current is mediated by PLC and PKC.

NE and glutamate modulate inhibitory postsynaptic currents in mitral cells

Inhibitory transmission from granule to mitral cells controls the mitral cell output. We therefore investigated the effect of mGluR $_{2/3}$ agonist L-CCG-I and PKC activator PMA on spontaneous inhibitory postsynaptic potentials (IPSCs) observed in the mitral cells. L-CCG-I reduced both the frequency and amplitude of IPSCs, and PMA reversed the L-CCG-I effect (Amplitude, ANOVA: $F_{(2,30)} = 13.24$, $P < 0.001$; Control: 100%, L-CCG-I: $69.3 \pm 8.2\%$, $P < 0.01$; L-CCG-I + PMA: $396.2 \pm 82.8\%$, $P < 0.01$ L-CCG-I + PMA compared to L-CCG-I alone; Frequency, ANOVA: $F_{(2,30)} = 22.88$, $P < 0.001$; Control: 100%; L-CCG-I: $50.8 \pm 10.4\%$, $P < 0.01$, L-CCG-I + PMA: $144.5 \pm 13.4\%$, $P < 0.05$ L-CCG-I + PMA compared to L-CCG-I alone) (Fig. 7). The IPSCs are significantly reduced with picrotoxin, a GABA $_A$ receptor antagonist (Supplementary Fig. S4). Although PMA-mediated reversal of Ca^{2+} current inhibition caused by L-CCG-I suggests a role for PKC based on our other experiments, it is possible that PMA could have an effect on IPSCs independent of PKC. To test this possibility, we measured the effect of PMA alone on IPSCs. PMA increased the amplitude as well as frequency of IPSCs. The PMA effect was significantly inhibited by the PKC inhibitor Bis I (Amplitude, ANOVA: $F_{(2,15)} = 28.16$, $P < 0.001$; Control: 100%, PMA: $203.7 \pm 13.0\%$, $P < 0.001$; PMA + Bis I: $135.2 \pm 11.3\%$, $P < 0.001$ PMA + Bis I compared to PMA alone; Frequency, ANOVA: $F_{(2,15)} = 12.80$, $P < 0.001$; Control: 100%, PMA: $360.7 \pm 59.8\%$, $P < 0.001$; PMA + Bis I: $190 \pm 56.6\%$, $P < 0.05$ PMA + Bis I compared to PMA alone) (Supplementary Fig. S5). These results suggest that the PMA effect on IPSCs is mediated by PKC. The PMA and PKC effect on IPSCs most likely occurs through the PKC effect on Ca^{2+} channels although additional direct PKC effect on neurotransmitter release cannot be ruled out.

Next, we tested the effect of L-CCG-I and PMA on IPSC evoked in mitral cells by stimulating the glomerular layer, which evoked IPSCs because of the reciprocal dendrodendritic synapses between mitral and granule cells. When glomerular stimulation excites a mitral cell it releases glutamate onto the granule cell. Excitation of the granule cell causes it to release GABA back onto the mitral cell which causes the IPSC (Schoppa et al., 1998). We observed that L-CCG-I decreased the IPSC evoked in mitral cells by stimulating the glomerular layer, and PMA increased the evoked IPSC (ANOVA: $F_{(2,15)} = 17.79$, $P < 0.001$; Control: 100%; L-CCG-I: $53.3 \pm 6.7\%$, $P < 0.01$; L-CCG-I + PMA: $208.0 \pm 32.5\%$, $P < 0.05$ L-CCG-I + PMA compared to L-CCG-I alone) (Fig. 8A). Furthermore, application of NE reduced the evoked IPSCs Since PKC is likely to be activated by Ca^{2+} influx through glutamate receptors, we tested application of glutamate together with NE. Glutamate and NE together cause an increase in evoked IPSC (ANOVA: $F_{(2,15)} = 31.96$, $P < 0.001$; Control: 100%, NE: $31.3 \pm 7.1\%$, $P < 0.05$, NE + Glutamate: $212.0 \pm 27.1\%$; $P < 0.01$ NE + Glutamate compared to NE alone) (Fig. 8B).

DISCUSSION

The acquisition of mate recognition memory in female mice, through single-trial learning, depends on the association of glutamate release from AOB mitral cells, resulting from pheromonal input, with the mating-induced NE release in the AOB. This is postulated to result in long-lasting increase in granule cell inhibitory feedback on the AOB mitral cells that respond to the mating male (see Supplementary Fig. S6). The cellular and molecular mechanisms underlying the convergence of the glutamate and NE signals have yet to be established, however. Our findings suggest that activation of α_2 -AR and mGluR_{2/3} pathways leads to a blockade of N- and R- type Ca^{2+} channels. This most likely leads to a decreased release of glutamate onto granule cells causing decreased GABA release from granule cells which in turn results in disinhibition of mitral cells. Based on our data we infer that this disinhibition of mitral cells is switched to increased inhibition by α_1 -AR-mediated activation of PKC α , which blocks the inhibitory effect of mGluR_{2/3} and α_2 -ARs on Ca^{2+} channels in mitral cells. This study has identified the pathway for α_1 -AR activation of PKC via PLC, and is the first study to unequivocally establish the isoform of PKC (PKC α) that mediates effects on Ca^{2+} channels in a physiological context. These results also demonstrate a novel role for cross-talk between PKC and G-proteins in regulating Ca^{2+} channels critical for synaptic plasticity.

mGluR_{2/3} and α_2 -AR cause disinhibition of mitral cells in the AOB

Our findings that activation of mGluR_{2/3} receptors results in a reduction in GABA-mediated spontaneous and stimulus-evoked IPSCs in mitral cells is consistent with the previous finding that activation of mGluR_{2/3} receptors on granule cells results in disinhibition of mitral cell activity, by a presynaptic inhibition of GABA release (Hayashi et al., 1993). The observations of the present study, however, suggest that the mGluR_{2/3} pathway might also cause disinhibition by a presynaptic inhibition of glutamate release from mitral cells onto granule cells at the reciprocal synapses. Our functional studies are supported by previous immunohistochemical studies that have found high levels of mGluR_{2/3} expression in mitral cells of the AOB (Ohishi et al., 1998; Sahara et al., 2001). The effectiveness of the G-protein activator GTP- γ -S in reducing the Ca^{2+} current and of the G-protein inhibitors GDP- β -S and PTX in increasing it suggest that the mGluR_{2/3} effect on the N- and R- type Ca^{2+} channels in mitral cells is mediated through a G-protein. G-protein-mediated modulation of N-type and R-type Ca^{2+} channels is well established (Ikeda and Dunlap, 1999; Meza et al., 2007), and similar inhibitory effects of mGluR_{2/3} receptors on Ca^{2+} channels have been reported in cultured hippocampal, cortical (Swartz, 1993), and sympathetic (Ikeda et al., 1995) neurons.

Our experiments revealed that α_2 -ARs also act via a G-protein to decrease the N-type and R-type Ca^{2+} currents recorded from mitral cells, in a similar manner to mGluR_{2/3} receptors. High

levels of α_2 -AR expression in the AOB of bullfrog (Bachman et al., 1998) and mice (Skinner et al., 2008) have been shown. This is consistent with the report that NE inhibited Ca^{2+} channels in mitral cells and therefore reduced glutamate release onto granule cells (Kaba and Huang, 2005). It is also in agreement with the previous work showing that the α_2 -AR agonist clonidine inhibited Ca^{2+} currents in mitral cells from accessory and main olfactory bulbs of *Xenopus laevis* (Czesnik et al., 2001). Others have observed α_2 -AR-mediated inhibition of Ca^{2+} currents in cultured neurons from the rat main olfactory bulb (Trombley and Shepherd, 1992; Trombley, 1992) and neocortex (Timmons et al., 2004). Several studies have reported a similar disinhibitory effect of NE in the olfactory bulb (Jahr and Nicoll, 1982; Ciombor et al., 1999; Hayar et al., 2001) as well as other parts of the brain such as hippocampus (Doze et al., 1991).

We found that the effects of mGluR_{2/3} and α_2 -AR activation on mitral cell Ca^{2+} currents showed mutual occlusion, suggesting that mGluR_{2/3}- and α_2 -AR-mediated pathways converge at some point. This convergence resulting in mitral cell disinhibition may provide an explanation for how pheromonal learning can be induced by local infusions of the mGluR_{2/3} agonist DCG-IV into the AOB in the absence of mating and the associated NE release (Kaba et al., 1994).

PKC α converts the disinhibition to increased inhibition of mitral cells in the AOB

The current hypothesis explaining mate recognition postulates a long-lasting increase in inhibitory feedback on the sub-population of mitral cells receiving input from the mating male's pheromones, thus preventing the signal from being transmitted centrally to block pregnancy (see Supplementary Fig. S6 for details). Based on our results, it can be deduced that activation of PKC converts the disinhibition of mitral cells to increased inhibition. Mechanistically this could come about by PKC-mediated reversal in mitral cells of the inhibitory effect of both mGluR_{2/3} and α_2 -AR on N-type Ca^{2+} channels or stimulation of R-type channels by PKC superimposed on N-type channel inhibition. In other systems, enhancement of R-type Ca^{2+} current by PKC, independent of G-protein-mediated inhibition, has been reported (Meza et al., 2007). PKC has been shown to decouple the signaling pathway from neurotransmitter receptors to Ca^{2+} channels in cultured sympathetic neurons (Zhu and Ikeda, 1994) and non-neuronal cells (Zamponi et al., 1997). The efficacy of the specific inhibitor of PKC α in blocking the effect of PKC activation by PMA on Ca^{2+} channels, and the effectiveness of recombinant PKC α but not a closely related isoform PKC γ in stimulating the Ca^{2+} current, suggest a role for the α -isoform of PKC. Moreover, a peptide corresponding to a unique C-terminal region of PKC α (but not an equivalent PKC γ peptide) blocked the PMA effect suggesting a role for the PDZ domain-containing PICK1 (Staudinger et al., 1997), which is thought to localize PKC α in close proximity to Ca^{2+} channels, and requires further investigation.

Classical isoforms of PKC, such as PKC α require both DAG and Ca^{2+} for activation. Our finding that α_1 -AR agonist cirazoline increased the mitral cell Ca^{2+} current via PLC suggests that the α_1 -AR pathway is capable of activating PKC α in the AOB mitral cell. Expression of α_1 -AR is considerable in the AOB (Skinner et al., 2008). The α_1 -AR activation of PKC α may therefore play a critical role in reversing the inhibitory effect of mGluR_{2/3} and α_2 -ARs on glutamate release from mitral cells, allowing the inhibitory GABAergic feedback onto mitral cells to increase. We found that NE decreased the magnitude of evoked IPSCs in AOB mitral cells, but when combined with glutamate the IPSC amplitude was increased. This could be explained by the excitatory action of glutamate on mitral cells leading to elevated intracellular Ca^{2+} , which when combined with α_1 -AR-mediated production of DAG (as well as release of Ca^{2+} from intercellular stores through IP₃), would activate PKC α and increase mitral cell Ca^{2+} currents and glutamate release. Increases in glutamate release could in turn cause increased GABA release and increases in IPSC amplitude. Our findings on α_1 -AR-mediated

effects on mitral cells are in agreement with the recent report that NE increased inhibition of mitral cells in AOB slices via an α_1 -AR pathway, although action of NE was attributed only to increased GABA release from granule cells and the mechanism of α_1 -AR effect was not investigated (Araneda and Firestein, 2006). The reason these authors did not find a disinhibitory effect of NE on mitral cell activity could be due to the use of mice of C57/B16 strain, which have been found to show differences in electrophysiological properties compared to mice of other inbred strains (Nguyen et al., 2000a; Nguyen et al., 2000b). Although it is also possible that disinhibition was insubstantial in their experimental conditions, as the overall effect of adrenergic activity on the membrane potential of mitral cells was reported to be rather small (Araneda and Firestein, 2006).

A hypothetical model of cellular and molecular mechanisms of AOB plasticity underlying pheromonal memory

The results described here represent our initial attempts to identify the signaling events that occur in the AOB in response to glutamatergic and noradrenergic input. Based on our data, we propose that, in the short-term (on a milliseconds to seconds time scale), glutamate acting through mGluR_{2/3} and NE acting through α_2 -ARs, inhibit N-type/R-type Ca²⁺ channels via inhibitory G-proteins. This results in decreased glutamate release from mitral cells and consequently decreased GABA release from granule cells, which would be greatest in those mitral cells receiving pheromonal input, due to the mGluR_{2/3} influence. These short-term events depend mainly on regulation of presynaptic N-type/R-type channels (Catterall and Few, 2008; Xu et al., 2007) and are akin to those underlying presynaptic short-term plasticity in other parts of the brain (Zucker and Regehr, 2002; Lee et al., 2008).

In the intermediate-term, the disinhibition of mitral cells would lead to increased Ca²⁺ influx through possibly through extrasynaptic L-type Ca²⁺ channels (Tippens et al., 2008) as has been shown by others (Miura et al., 1997; Hsu et al., 1999), which combined with α_1 -AR-mediated activation of PLC and DAG (Zhong and Minneman, 1999) would lead to the activation of PKC α . Because α_1 -AR effect occurs through the generation of second messengers, NE action of α_1 -AR would be expected to occur over a slower time course (hundreds of seconds) (Bhalla, 2002), compared to the action of NE on α_2 -ARs. Activation of PKC α through α_1 -ARs would decouple the inhibitory effect of mGluR_{2/3} and α_2 -ARs on N-type/R-type Ca²⁺ channels, thus increasing glutamate release from mitral cells, and GABA release from their associated granule cells. The consequence would be an increase in inhibitory gain of the reciprocal synapses of those mitral cells receiving pheromonal input (see Supplementary Fig. S7).

Thus our results provide a novel alternative mechanism for memory induction, in which the association between glutamatergic pheromonal input and mating-induced NE is explained by the role of PKC α in coincidence detection in mitral cells. Moreover, activation of PKC α provides a mechanism for the maintenance of enhanced feedback inhibition onto mitral cells, during the hours following mating, until the protein synthesis-dependent changes that underlie the long-lasting, and NE-independent, maintenance of memory for the mating male have become established. Thus, activation of PKC α through PLC (Rosenzweig et al., 1993; Steidl et al., 2003; Stough et al., 2006) might extend the short-term molecular events of memory induction and bridge them to the long-term changes brought about by gene induction. In support of this idea, we have found that PLC- and PKC- mediated pathway induces gene expression in the AOB (Skinner et al., 2008). The validity of the proposed mechanisms and the role of these signaling molecules ultimately need to be tested using behavioral experiments.

The proposed mechanism has the advantage that the locus of memory induction is in the presynaptic mitral cells that actually receive pheromonal input, rather than their associated postsynaptic granule cells, and therefore potentially allows greater specificity. Persistent presynaptically mediated transmitter release has been observed in other systems such as

hippocampal neurons (Arancio et al., 1995). It does not exclude, however, a role for postsynaptic induction at the granule cell side of the reciprocal synapse by conventional mechanisms of synaptic plasticity involving ionotropic glutamatergic transmission (Wilson et al., 1996). Indeed this is supported by the finding of NMDA-dependent long-term potentiation of the glutamatergic synapse from mitral to granule cells (Kaba and Huang, 2005) and increased length of the postsynaptic density on granule cells following mating (Matsuoka et al., 2004). Moreover, it is possible that NE also has an effect on granule cells through α_2 and α_1 ARs leading to increased GABA release and thus enhanced inhibition of the mitral cells. A recent study showed an excitatory effect of α_1 ARs on granule cells (Smith et al., 2009). Additional detailed investigations will be necessary to elucidate the mechanism of plasticity in granule cells.

The coincidence detecting role of PKC α in AOB synaptic plasticity is likely to be broadly relevant to how NE influences synaptic plasticity in many other areas of the brain. The neural module representing feedback inhibition in the AOB exists in the MOB (Hildebrand and Shepherd, 1997), where it is critical for several forms of social learning (Kendrick et al., 1992; Sanchez-Andrade and Kendrick, 2008). This neural module, in slightly different forms, is also seen in other brain regions (Doze et al., 1991). Adrenergic projections are widespread in the brain (Moore and Bloom, 1979). Although the role of β -adrenergic receptors in synaptic plasticity and memory has been extensively investigated (Watabe et al., 2000; Frey et al., 2001; McGaugh and Roozendaal, 2002), much less is known regarding the mechanisms by which α -adrenergic receptors contribute to memory. The role of both α_1 -ARs and α_2 -ARs in memory formation is well established (Gibbs and Summers, 2002; McGaugh, 2004) but the signaling pathways mediating the role of these receptors is not well understood. NE-mediated disinhibition acting through α_2 -ARs is likely to enhance neuronal sensitivity to diverse sensory stimuli, increasing intracellular Ca²⁺ levels and priming them for activation of PKC α by NE acting at α_1 -ARs. PKC is known to activate gene expression through transcription factors such as Egr-1. Thus by activating gene expression cascades, PKC α can convert the transient action of NE into long-term synaptic plasticity. The model we have proposed with PKC α as a coincidence detector provides a potential new framework in which to understand strong memory formation mediated by α -adrenergic receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AOB, Accessory Olfactory Bulb
 α_1 -AR, α_1 -adrenergic receptor
 α_2 -AR, α_2 -adrenergic receptor
 BAPTA, 1,2-Bis [2-aminophenoxy] ethane-N,N,N',N'-tetraacetic acid

Bis I, Bisindolylmaleimide I
 Bis V, Bisindolylmaleimide V
 DAG, Diacylglycerol
 HBDDE, 2, 2', 3, 3' 4, 4'-Hexahydroxy-1, 1'-biphenyl-6'-dimethanol dimethyl ether
 mGluR_{2/3}, mGluR₂/mGluR₃
 NE, Norepinephrine
 NMDA, N-methyl-D-aspartic acid
 PKC, Protein Kinase C
 PKC α , protein kinase C α
 PLC, Phospholipase C
 TTX, tetrodotoxin
 PTX, pertussis toxin
 VN, vomeronasal

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Fig. 1. mGluR_{2/3} and α_2 -AR agonists reduce Ca^{2+} currents through a G-protein

(A) Ca^{2+} currents were evoked by 800-ms pulses from a holding potential of -80 mV to various test potentials. Current traces recorded without any treatment (Control) after depolarizations to potentials between -50 mV and -20 mV. (B) Superimposed traces recorded 5 min after application of $10 \mu\text{M}$ L-CCG-I with the same protocol as in A. The voltage steps and the calibration bars apply to both A and B. (C) Application of mGluR_{2/3} agonists L-CCGI ($10 \mu\text{M}$), DCG-IV ($0.5 \mu\text{M}$), and LY379268 ($10 \mu\text{M}$) causes reduction in Ca^{2+} current amplitude relative to controls (black bars). Separate control recordings were made for each agonist. (D) Application of α_2 -AR agonists clonidine ($5 \mu\text{M}$) and UK 14304 ($5 \mu\text{M}$) reduces Ca^{2+} current amplitude relative to controls (black bars). Representative intracellular traces and cumulative data are shown for each agonist. (E) GDP β S ($100 \mu\text{M}$) and PTX ($1 \mu\text{g/ml}$) increase the Ca^{2+} current while GTP γ S ($100 \mu\text{M}$) decreases the Ca^{2+} current. In the presence of GDP β S or PTX, L-CCG-I fails to reduce the Ca^{2+} current. (F) Time course of GDP β S and GTP γ S effect on the Ca^{2+} current. GDP β S and GTP γ S were included in the patch pipette. The current was allowed to stabilize for 4 min after which the time course was determined (* $P < 0.05$; ** $P < 0.01$).

Fig. 2. Mutual occlusion of L-CCG-I and clonidine effect on the Ca^{2+} currents

(A) L-CCG-I occludes the inhibitory effect of clonidine of Ca^{2+} currents. Application of L-CCG-I (10 μ M) causes significant (* P <0.05) reduction in the Ca^{2+} current compared to controls (100%). Clonidine (5 μ M) application following L-CCG-I application does not reduce Ca^{2+} current further. (B) Clonidine occludes the inhibitory effect of L-CCG-I on Ca^{2+} currents. Clonidine reduces the Ca^{2+} current significantly (** P <0.01) relative to controls (100%) but application of L-CCG-I application following clonidine does not additionally decrease the Ca^{2+} current.

Fig. 3. The Ca^{2+} current is mediated by N-type and R-type Ca^{2+} channels

The Ca^{2+} current is significantly (** $P < 0.01$) but not completely inhibited by an N-type Ca^{2+} channel inhibitor ω -conotoxin-GVIA (1 μM) and an R-type Ca^{2+} channel inhibitor SNX-482 (100 nM) (* $P < 0.05$) relative to control (100%). Together, the two drugs almost completely inhibit the Ca^{2+} current (* $P < 0.05$) relative to control.

Fig. 4. PKC activator PMA reverses the inhibition of Ca^{2+} current by mGluR_{2/3} and α_2 -AR
 (A) L-CCG-I (10 μM) reduces the Ca^{2+} current significantly (** $P < 0.01$) compared to control and PMA (500 nM) reverses L-CCG-I-mediated inhibition (## $P < 0.01$). (B) Current-voltage relationship (top) and time course (bottom) of PMA-mediated reversal of inhibition of Ca^{2+} current by L-CCG-I. (C) Clonidine reduces the Ca^{2+} current significantly (* $P < 0.05$) compared to controls and PMA reverses clonidine-mediated inhibition (## $P < 0.01$). (D) Current-voltage relationship (top) and time course (bottom) of PMA-mediated reversal of inhibition of Ca^{2+} current by clonidine.

Fig. 5. PKC α is the PKC isoform that uncouples mGluR $_{2/3}$ and α_2 -AR from Ca^{2+} channels
 (A) Inhibition of PMA-mediated increase in Ca^{2+} current (* P < 0.05 or ** P < 0.01 versus control) by chelerythrine (10 μM), HBDDE (50 μM) and high concentration (1 μM) of LY333531 (** P < 0.01 versus PMA) but not low concentration (10 nM) of LY333531. In the cumulative data shown in the bar graph, the third bar in each cluster represents the effect of the inhibitor. Representative intracellular traces are shown for each condition (1: Control; 2: PMA; 3: inhibitor). (B) Inhibition of PMA-mediated increase in Ca^{2+} current by a specific PKC α inhibitor. (C) Top Panel: Increase in Ca^{2+} current by purified PKC α but not PKC γ included in the patch pipette. Values for control time course overlap with that for PKC γ and hence are not shown in this graph. The traces at left show increase in the Ca^{2+} current with PKC α while the traces for PKC γ (right) show no change. Bottom Panel: Time course PKC α -mediated increase in Ca^{2+} current and its inhibition by chelerythrine. (D) PKC α C-terminal peptide but not PKC γ C-terminal peptide inhibits PMA-mediated increase (** P < 0.01 versus control) in the Ca^{2+} current.

Fig. 6. α_1 -AR increases Ca^{2+} current through PLC and PKC

(A) Cirazoline (10 μM), an agonist of α_1 -AR significantly (** $P < 0.01$ versus control) increases the Ca^{2+} current amplitude relative to control and a PLC inhibitor U73122 (10 μM) inhibits (### $P < 0.01$ versus cirazoline), whereas an inactive analog U73343 (10 μM) does not inhibit the cirazoline effect. In the cumulative data shown in the bar graph, the third bar in each cluster represents the effect of the reagent as indicated (in panels A & C). Representative intracellular traces are shown for each reagent. (B) Cirazoline increases the Ca^{2+} current amplitude and PKC inhibitor chelerythrine inhibits (# $P < 0.05$ versus cirazoline) the cirazoline effect. (C) A PKC inhibitor Bis I (2 μM) inhibits (### $P < 0.01$ versus cirazoline), but the inactive analog Bis

V (2 μ M) does not inhibit ($P>0.05$) the cirazoline-mediated increase ($*P < 0.05$ relative to control) in the Ca^{2+} current.

Fig. 7. mGluR_{2/3} agonist L-CCG-I decreases inhibitory transmission in the AOB and PKC activator PMA reverses the L-CCG-I effect

(A) Cumulative probability distribution showing a decrease in spontaneous IPSC amplitude in L-CCG-I treated mitral cells (significant shift to left relative to control; $P < 0.01$, Kolmogorov – Smirnov [KS] test) and an increase in amplitude in cells treated L-CCG-I + PMA (significant shift to right relative to control, $P < 0.01$, KS test). (B) Summary graph showing the decrease of IPSC amplitude with L-CCG-I (** $P < 0.01$ versus control) and reversal of L-CCG-I effect by PMA (## $P < 0.01$: L-CCG + PMA versus L-CCG-I). (C) Cumulative probability distribution of spontaneous IPSC frequency showing decrease in frequency in L-CCG-I-treated cells (significant shift to right relative to control; $P < 0.01$, KS test) and increase in frequency in cells treated with L-CCG-I + PMA (significant shift to left relative to control; $P < 0.01$, KS test). (D) Summary graph showing the decrease of IPSC frequency with L-CCG-I (** $P < 0.01$ versus control) and reversal of L-CCG-I effect by PMA (# $P < 0.05$: L-CCG-I + PMA versus L-CCG-I).

Fig. 8. Regulation of inhibitory transmission in the AOB as measured by evoked IPSCs
 (A) L-CCG-I decreases evoked IPSCs ($*P < 0.05$ versus control) and PMA reverses the L-CCG-I effect ($\#P < 0.05$: L-CCG-I + PMA versus L-CCG-I). (B) NE (30 μ M) decreases evoked IPSCs ($*P < 0.05$ versus control) and application of NE (30 μ M) + glutamate (500 μ M) increases the evoked IPSCs ($\#\#P < 0.01$: NE + Glutamate versus NE).