SUPPLEMENTAL ONLINE MATERIAL

Background: Role of Phosphotidyl Inositol/Protein Kinase C (PI/PKC) Signaling in Neuropsychiatric Disorders

A substantial literature has recently implicated dysregulation of PKC signaling in the etiology of bipolar disorder. For example, a polymorphism which confers susceptibility to bipolar disorder has been identified in the gene encoding for phospholipase C, the initial enzyme in the PKC cascade \((S1)\). Increased levels of \(G_{\alpha q}\) proteins \((S2)\), altered hydrolysis of PI \((S3)\), and increased PKC activity \((S4, 5)\), particularly the alpha, gamma and zeta isoforms of PKC have been observed in tissue from bipolar patients. Bipolar disorder may also be associated with the gene encoding for RGS4, a protein that inhibits Gq signaling \((S6)\), and which is regulated by stress exposure in animals \((S7)\).

Dysregulation of PI/PKC signaling has now been found in schizophrenia as well. Mirnics et al first observed that the transcript for RGS4 is substantially lower in prefrontal cortical tissue from schizophrenic patients \((S8)\). A linkage between RGS4 and schizophrenia has now been confirmed by several other labs \((S6, 9-11)\). Highly elevated levels of calcyon, a protein that positively couples dopamine D1 receptors to PI/PKC signaling, has also been observed in the prefrontal cortex of patients with schizophrenia \((S12)\). Either reduced RGS4 and/or increased calcyon expression could disinhibit PKC activity. The relationship between altered internal calcium/PKC signaling and schizophrenia has recently been reviewed \((S13)\).

Importantly, most of the medications currently used to treat bipolar disorder and schizophrenia reduce PI/PKC signaling. Li and VAL decrease PKC activity \((S14)\), while
atypical antipsychotics such as clozapine, olanzapine and risperidone block $\alpha_1R$ and 5HT2A receptors which are coupled to PI/PKC signaling. However, the link between PI/PKC actions and neuropsychiatric symptomology is not understood.

**Materials and Methods**

**Biochemical Analysis**

*Preparation of frontal cortical slices for PKC activity assay*

Male Sprague Dawley rats (300-350g) were decapitated and their brains were rapidly placed into ice-cold cutting saline (110mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 28 mM NaHCO$_3$, 7 mM MgCl$_2$, 0.5 mM CaCl$_2$, 5 mM glucose and 0.6 mM ascorbate, saturated with 95% O$_2$/ 5% CO$_2$). 400 µm coronal slices were prepared with a Tissue Chopper and the frontal cortex was dissected out. Frontal cortical slices were transferred to a 1:1 solution of cutting saline and normal ACSF (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 25 mM glucose, saturated with 95% O$_2$/ 5% CO$_2$) and maintained at room temperature (RT) for 45 min. The slices were transferred to 100% normal ACSF (at RT) for 45 min, and finally to ACSF (at RT) for 1 hr prior to addition of drug. The alpha-1 adrenergic receptor ($\alpha_1R$) agonist, phenylephrine (PE, final concentration 10 µM), phorbol 12-myristate 13-acetate (PMA, final concentration 1µM), FG7142 (final concentration 10µM), were prepared in DMSO and incubated with the slices for 5 min. The PKC inhibitor chelerythrine (CHEL; final concentration 10µM) was applied 30 minutes prior to the addition of other drugs. Slices were removed and immediately frozen on dry ice and stored at –80°C until assayed.
Preparation of whole frontal cortex for Western blotting

FG7142 was suspended in a vehicle (VEH) containing 0.1 ml ethanol, 0.1 ml Tween-80, 0.8 ml saline, and 0.12 g hydroxybetacyclodextrin (Research Biochemicals Inc., Natick, MA). FG7142 was administered interperitonially (i.p.) to rats (10 mg/kg) 30 minutes before decapitation. FG7142 is a commonly used pharmacological stressor that reproduces all aspects of a physiological stress response in animals and humans (e.g. increased glucocorticoid and catecholamine release, freezing behavior in rodents; (S15, 16). Our research has shown comparable effects of FG7142, loud noise stress and restraint stress on both prefrontal cortical function and neurotransmitter release ((S17, 18) and unpublished data). Frontal cortex was dissected and frozen immediately on dry ice and stored at –80°C until processed.

Sample preparation

Prefrontal cortical slices from each condition were homogenized in approximately 100 µl of ice-cold lysis buffer A (20 mM Tris, pH 7.5, 1 mM EGTA, 1 mM EDTA, 2.5 mM Na₄P₂O₇, 1 mM beta-glycerophosphate, 1 mM DTT, 10 µl/ml protease inhibitor cocktail, 10µl/ml phosphatase inhibitor cocktail I & II (Sigma-Aldrich, Inc.). Whole frontal cortex samples were homogenized in 1ml of lysis buffer A. The homogenates were centrifuged at 100,000g for 30 min at 4°C. The resulting supernatant was used as the cytosolic fraction. The pellet was resuspended in an appropriate volume (100µl for slices, 0.5ml for frontal cortex) of buffer A containing 0.15% Triton X-100, 150mM NaCl and centrifuged again. The resulting supernatant was used as the membrane fraction. The protein concentration of each condition was assessed and an appropriate volume of homogenization buffer was added to normalize the protein content between samples.
**PKC Activity Assay**

PKC activity was determined with Protein Kinase C Biotrak Enzyme Assay System (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer’s instructions with minor modifications. The incubation mixture (55µl) contained: 25 µl sample, 50mM Tris-HCl (pH 7.5), 0.4mM peptide, 5mM Ca²⁺, 6mM Mg²⁺, 10µg/ml PMA, 0.1mg/ml phosphatidyl-L-serine, 0.2mM ATP and 0.5µCi [γ-³³P]ATP. The mixture was incubated at 37°C for 15 minutes. 10µl of stop reagent (0.3M H₃PO₄ containing carmosine red) was added to the mixture to terminate the reaction. A 35-µl aliquot of the incubation mixture was transferred onto a phosphocellulose binding paper disc (Whatman P-81), which was then washed in 75mM H₃PO₄ and dried. The paper disc was put into a scintillation vial with 10ml of Bio-Safe II counting cocktail. The radioactivity of [³³P]-peptide was measured in a LS6500 scintillation counter. One unit of PKC was defined as the amount of the enzyme that catalyzed the transfer of 1 pmol of phosphate from [γ-³³P]ATP into the peptide at the above conditions in 1 minute. Basal activity of PKC is defined as 100% in the control group; drug-treated groups are shown as % of control.

**Western blotting**

5x loading buffer (0.3 M Tris, pH 6.8, 50% glycerol, 10% sodium dodecyl sulfate, and 12.5% β-mercaptoethanol,) was added to the sample aliquot, and heated at 95°C for 5 min. 1µg of sample was loaded onto 10% SDS- polyacrylamide gels and resolved by standard electrophoresis. Gels were blotted electrophoretically to nitrocellulose membrane in a transfer tank using a constant current of 350 mA for 3 hrs at 4°C. The membrane blots were blocked for 1 hr at room
temperature in 5% dry milk dissolved in tris-buffered saline (TTBS; 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20). The blots were incubated with the appropriate antibody in blocking buffer overnight at 4°C (anti-PKCα, Santa Cruz Biotechnology, Inc., diluted 1:500; or anti-phospho-PKCα, Upstate Cell Signaling Solutions, 1:1000), washed four times with TTBS for 10 min each wash and then incubated with an HRP-conjugated anti-rabbit secondary antibody for 1 hr at room temperature (Santa Cruz Biotechnology, Inc., diluted 1:3000). After 4 more washes, the blots were developed with Enhanced Chemiluminescence (Amersham Biosciences, Arlington Heights, IL) and visualized with Kodak Biomax MR imaging film.

Data analysis

Densitometric analysis of immunoreactivity was conducted with a Kodak Image Station 440CF. All results are expressed as mean ± SEM. Differences were analyzed by ANOVA Fisher’s PLSD and considered significant when P<0.05.

Behavioral Studies

Subjects

All procedures were approved by the Yale Institutional Animal Care and Use Committee.

Rodents: Male Sprague Dawley rats weighing 240-280g when received (approximately 2 months old) were initially pair-housed in filter frame cages. The animals were fed a limited diet of Purina rat chow (16 grams/rat/day) immediately following behavioral testing. Water was available ad libitum. Food rewards during cognitive testing were highly palatable miniature chocolate chips.

Monkeys: Seven female and 1 male rhesus monkeys (Macaca mulatta), ranging in age
from 13 years to over 30 years, were individually housed and maintained on a diet of Purina monkey chow supplemented with fruit. Highly palatable food rewards (e.g. peanuts, raisins or chocolate chips) were utilized during testing to minimize the need for dietary regulation.

**Cognitive testing**

Special care was taken to habituate animals to all stressful procedures (handling, drug injections, etc.). Rats were trained and tested on the delayed alternation task in a T-maze, using the methods described in Birnbaum et al. (S19). On the first trial, animals were rewarded for entering either arm of the maze. Thereafter, for a total of 10 trials per session, rats were rewarded only if they entered the maze arm that was not previously chosen. Between trials the choice point was wiped with alcohol to remove any olfactory clues. Performance on the delayed alternation task is dependent upon the length of the delay between trials, thus the delay was raised as needed to maintain each individual animals performance at approximately 70% correct. This allowed us to observe improvement or impairment in performance following drug administration. For the control, spatial discrimination task, rats were trained in a similar manner as the delayed alternation task. However, in the control task half of the rats were rewarded only when they entered the left choice arm and half were rewarded for entering only the right choice arm. Once the rats performed above 60% correct for two consecutive days, they were administered the test drug. A 10 second delay between trials was used for this task to mirror the typical delay for the delayed alternation task, although performance on this task is not delay dependent.

The monkeys had been previously trained on the 2-well delayed response task as described in Avery et al. (S20) using a Wisconsin General Testing Apparatus. During delayed response
testing, the animal watches as the experimenter baits one of two foodwells with a food reward. The foodwells are then covered with identical cardboard plaques, and an opaque screen is lowered between the animal and the foodwells for a specified delay. At the end of the delay, the screen is raised and the animal is allowed to choose. Five different delay lengths were quasi-randomly distributed over 30 trials for each daily test session. The shortest of these delays was less than 1 second (the "0" sec delay). The remaining delays were adjusted for each individual monkey to yield baseline performance of about 70% across all delays (i.e. 18-22 trials correct of the possible 30 trials). The animals were tested twice a week with 3-4 days separating each test session.

_Cannulae Implantation_

Following behavioral training, rats were implanted with guide cannulae to allow drug infusions into the prefrontal cortex (stereotaxic coordinates from bregma and skull surface: anterior +3.2 mm, lateral ±0.75 mm, ventral –1.7 mm). The infusion needles projected 2.8 mm below the guide cannula such that the infusion site was –4.5 mm ventral to skull surface. For the anatomical control experiment, several rats received infusions directed at the cingulate and secondary motor cortex located dorsal to the prefrontal cortex to control for diffusion of the drug to non-prefrontal cortical sites (infusion needles projected 0.3 mm below the guide cannula such that the infusion site was –2.0 mm ventral to skull surface). Following surgery rats were individually housed and allowed to recover for at least 1 week. Topical antibiotic powder was applied to the wound margin to facilitate healing. Drug treatments were administered only after the animal recovered from surgery and achieved stable performance (60-80% correct) for two
consecutive days. All rats received all drug treatments within each experiment, therefore any rat that showed signs of infection or lost its cannulae were not included in data analysis.

**Drug administration**

Phorbol 12-myristate 13-acetate (PMA, Sigma Chemicals Co., St. Louis, MO) was initially dissolved in dimethyl sulfoxide (DMSO, 1 mg/ml) and further diluted in saline or water to the final dilution. PMA was infused intracortically in rats 10 min prior to behavioral testing. A dose of PMA was found for each individual rat that impaired delayed alternation testing (0.05, 0.5, 5 or 25 pg/0.5 µl).

CHEL (Alexis Co., San Diego, CA) was dissolved in water and infused intracortically (0.3 µg/0.5 µl) in rats 5, 10 or 15 min prior to behavioral testing for the PE/CHEL, PMA/CHEL and FG7142/CHEL experiments, respectively. For monkeys, CHEL was administered orally (0.03 µg/kg) on a cracker 60 min prior to behavioral testing. There is currently no direct evidence that CHEL enters the brain after oral administration in monkeys. Thus, efficacy in the current study provides indirect evidence of bioavailability.

NPC-15437 (1.0 µg/0.5 µl; Sigma Chemicals Co., St. Louis, MO) was dissolved in saline and infused into the rat prefrontal cortex 15 min prior to behavioral testing.

The α₁R agonist, PE (Sigma Chemicals Co., St. Louis, MO) was dissolved in saline and infused intracortically (0.1 µg/0.5 µl) in rats 5 min prior to behavioral testing.

The α₁R agonist, cirazoline (CIRAZ, Tocris-Cookson Inc., Ballwin, MO) was dissolved in saline and administered intramuscularly (i.m.) to monkeys 30 min prior to behavioral testing. A dose of CIRAZ was determined for each animal that reliably impaired their delayed response testing (0.001 µg/kg - 10 µg/kg).
FG7142 was suspended in a VEH containing 0.1 ml ethanol, 0.1 ml Tween-80, 0.8 ml saline, and 0.12 g hydroxybetacyclodextrin (Research Biochemicals Inc., Natick, MA). FG7142 was administered interperitonially (i.p.) to rats (10 to 20 mg/kg) and i.m. to monkeys (0.2 mg/kg to 2.0 mg/kg) 30 min prior to behavioral testing. A dose of FG7142 was determined for each animal that reliably impaired working memory performance but allowed completion of the test session.

Lithium carbonate (Li, Sigma Chemicals Co., St. Louis, MO) was administered orally mixed into peanut butter to monkeys three times: approximately 24 hours, 18 hours and 3 hours before behavioral testing. The monkeys received a dose of Li similar to that which has a therapeutic response in humans (5.0 – 7.5 mg/kg).

Valproate (VAL; also known as valproic acid; Sigma Chemicals Co., St. Louis, MO) was administered orally (2.5 mg/kg mixed in peanut butter or marshmallow fluff on a small piece of cracker) 60 min prior to cognitive testing. Given the very acidic nature of this compound, intramuscular injections were unsuitable, and higher doses were not tolerated with oral dosing.

All animals were acclimated to drug administration (oral, i.m., i.p. or cortical infusion) prior to receiving drug treatments. Within each experiment, the order of dose administration was determined quasi-randomly, and the experimenter testing the animal was unaware of the treatment condition. Rats were administered drugs after they had performed stably for 2 consecutive days (60-80% correct). A washout period of at least 7 days occurred between drug treatments. Monkeys were required to return to stable baseline performance for 2 consecutive testing days prior to new drug treatment. A
washout period of at least 10 days occurred between drug treatments for monkeys.

**Electrophysiology**

*Oculomotor Delayed Response Task*

Studies were performed on two adult male rhesus monkeys (*Macaca mulatta*) trained on the spatial oculomotor delayed response (ODR) task as previously described (S21). This task requires the subject to make a memory-guided saccade to a remembered visuo-spatial target (Fig. 1SA). After a 3-second inter-trial interval (ITI), a central light-emitting diode (LED) was illuminated as the fixation target. A trial began when the subject fixated at the central spot for 0.5 seconds (fixation period). After the fixation period, a cue LED was illuminated for 0.5 seconds (cue period) at one of eight peripheral targets located at an eccentricity of 13° with respect to the fixation spot. After the cue was extinguished, a 2.5-second delay period followed. The fixation cue remained lit during the cue and delay periods and the subject was required to maintain central fixation throughout both the cue presentation and the delay period. At the end of the delay, the fixation spot was extinguished which instructed the monkey to make a memory guided saccade to the location where the cue LED had been shown prior to the delay period. A trial was considered successful if the subject’s response was completed within 0.5 seconds of the offset of the fixation LED and was within 2° of the correct cue location. The subject was rewarded with fruit juice immediately after every successful response. The position of the stimulus was randomized over trials such that it had to be remembered on a trial-by-trial basis. The subject’s eye position was monitored with the
ISCAN Eye Movement Monitoring System (ISCAN, Burlington, MA), and the ODR task was generated by the TEMPO real-time system (Reflective Computing, St. Louis, MO).

**Fig. 1S.** Oculomotor delayed response task (A) and the region of recording (B).

(A) Trials began when the monkey fixated a central spot for 0.5 sec. A cue was presented in one of eight locations for 0.5 sec and a delay period lasted 2.5 sec. When the fixation spot was extinguished, the monkey was required to make a saccade to the location of the remembered cue. (B) The large circle represents the position of the cylinder of monkey H (blue) and monkey J (red); the dark grey area represents the region of recording.

**Recording Locus**

Prior to recording, the animal underwent a magnetic resonance image (MRI) scan to obtain exact anatomical co-ordinates of brain structures, which guided placement of the chronic recording chambers. MRI-compatible materials were used for the implant so that the position of the recording chambers could be confirmed by MRI after implantation.
Based on the MRI, the recording chamber for monkey H was positioned over the prefrontal cortex centered 4mm medial to the principal sulcus and 2mm posterior to its caudal end (Fig. 1SB). The recording chamber for monkey J was positioned over the prefrontal cortex centered 2mm medial to the principal sulcus and 2mm anterior to its caudal end (Fig. 1SB). Task-related cells obtained in this study were located in a small area ranging from 0-3 mm anterior to the caudal end of the principal sulcus and –1-2 mm medial to the principal sulcus.

**Pharmacology, physiology and data acquisition**

PE (Sigma Pharmaceuticals) and CHEL (Alexis Pharmaceuticals) were dissolved in 1 ml of triple distilled water (adjusted with HCl to pH3.5-4.0) at a concentration of 0.01M and stored in aliquots of 50 µl at –70°C.

Iontophoretic electrodes were constructed with a 20 µm pitch carbon fiber (ELSI, San Diego, CA) inserted in the central barrel of a seven-barrel non-filamented capillary glass (Friedrich and Dimmock, Millville, NJ). The assembly was pulled using a custom electrode puller and the tip was beveled to obtain the finished electrode. Finished electrodes had impedances of 0.3-1.0 MΩ (at 1kHz) and tip sizes of 30-40 µm. The outer barrels of the electrode were then filled with 3 drug solutions (two consecutive barrels each) and the solutions were pushed to the tip of the electrode using compressed air. A Neurophore BH2 iontophoretic system (Medical Systems Corp., Greenvale, NY) was used to control of the delivery of the drugs. The drug was ejected at currents that varied from 25-75 nA. Retaining currents of –3 to –5 nA were used in a cycled manner (1sec on, 1 sec off) when not applying drugs, and current balancing was not required because of the
low impedance of the electrode. Drug ejection did not create noise in the recording, and there was no systematic change in either spike amplitude or time course at any ejection current.

The electrode was mounted on a MO-95 micromanipulator (Narishige, East Meadow, NY) in a 25-gauge stainless steel guide tube. The dura was punctured using the guide tube to facilitate access of the electrode to cortex. Extra-cellular voltage was amplified using a custom low-noise preamplifier (SKYLAB) and band-pass filtered (180Hz-6Khz, 20dB gain, 4-pole Butterworth; Kron-Hite, Avon, MA). Signals were digitized (20.83kHz, micro 1401, Cambridge Electronics Design, Cambridge, UK) and acquired using the Spike2 software (CED, Cambridge, UK). Neural activity was analyzed using waveform sorting by a template-matching algorithm, which made it possible to isolate more than one unit at the same recording site. Post-stimulus time histograms and rastergrams were constructed online to determine the relationship of unit activity to the task. Unit activity was measured in spikes per second. If the rastergrams displayed task-related activity, the units were recorded further and pharmacological testing was performed.

Data were first collected from the cell under a control condition in which at least eight trials at each of 8 cue locations were obtained. Upon establishing the stability of the cells’ activity, this control condition was followed by PE application. Dose-dependent effects of the drug were tested in two or more consecutive conditions. Drugs were continuously applied at a relevant current throughout a given condition. Each condition had ~ 8
(typically 10 or more) trials at each location for statistical analyses of effects. CHEL was applied coincidently with PE, after the PE response was well-established.

Data analysis

For purposes of data analysis, each trial in the ODR task was divided into four epochs – Fixation, Cue, Delay and Response (Saccade). The Fixation epoch lasted for 0.5 sec. The Cue epoch lasted for 0.5 sec and corresponds to the stimulus presentation phase of the task. Delay lasted for 2.5 sec and reflects the mnemonic component of the task. The Response phase started immediately after the Delay epoch and lasted ~1.5 sec.

Two-way analysis of variance, ANOVA, was used to examine the spatial tuned task-related activity with regard to: (1) different periods of the task (cue, delay, response vs. fixation) and (2) different cue locations. In the present study, we encountered various types of delay-related activity. Some cells were activated for the preferred direction during the cue presentation and continued their firing during the delay period, but were only active in the response period for the nonpreferred direction. Others would exclusively show activity only in the mnemonic period. Delay-related activity usually terminated just before the saccade. All data were binned in 40ms windows. One-way ANOVAs were employed to assess the effect of the drug application on cells displaying delay-related activity.

Supplementary Data
Representative PMA (0.05, 0.5, 5 or 25 pg/0.5 µl) dose/response curves are shown in Fig. 2S. The 5 pg/0.5 µl dose maximally impaired most animals without side effects, and was thus the focus of control experiments.

**Fig. 2S.** Dose response curves (0.05 to 5 pg) for the phorbol ester, PMA, infused into prefrontal cortex of three representative rats. Response to PMA was compared to VEH infusion. Results represent percent correct on the delayed alternation task. Most rats were maximally impaired by the 5 pg dose. One rat (#205) was slightly less sensitive to PMA and was maximally impaired by a higher dose (25 pg) of PMA.

Infusions of PMA (5 pg/0.5 µl) dorsal to the prefrontal cortex (-2.0 mm DV) had no effect on delayed alternation performance (Fig. 3S). When the infusion cannula were subsequently lowered into the prefrontal cortex (-4.5 mm DV) of the same animals, performance was impaired (Fig. 3S). Thus, the PMA-induced impairment of working memory was anatomically specific for the prefrontal cortex. Infusions dorsal to the
prefrontal cortex are particularly important, as drug can flow up the outside of the cannula to reach more dorsal structures.

**Fig. 3S.** Effects of (A) PMA infusions (0.5 or 5 pg/0.5 µl; -2.0 mm DV) dorsal to the prefrontal cortex in the region of the anterior cingulate/secondary motor cortex compared to (B) PMA infusions (5 pg/0.5 µl; -4.5 mm DV) within the prefrontal cortex on delayed alternation performance in rats. Results represent mean ± S.E.M. percent correct. * significantly different from dorsal infusion, P= 0.007.

The effects of PMA were also dependent on the cognitive challenges of the task. PMA infusions (5 pg/0.5 µl) into the prefrontal cortex had no effect on rats performing the control spatial discrimination task (Fig. 4S), which does not require prefrontal cortical function.
Fig. 4S. Effects of PMA infusions (5 pg/0.5 µl; -4.5 mm DV) into the prefrontal cortex of rats performing a control task, spatial discrimination, that does not depend upon prefrontal cortical cognitive function. Results represent mean ± S.E.M. percent correct. PMA was not significantly different from VEH; P=0.587.

NPC-15437, like CHEL, significantly reversed the detrimental effects of FG7142 on delayed alternation performance in rats (Fig. 5S).

Fig. 5S. Effects of infusions of the PKC inhibitor, NPC-15437 (1.0 µg/0.5 µl) into the prefrontal cortex on the cognitive deficit induced by the pharmacological stressor, FG7142 in rats performing the delayed alternation task. Results represent
mean ± S.E.M. percent correct. * significantly different from VEH +VEH, P= 0.003, † significantly different from FG7142 + VEH, P= 0.048.

Pretreatment with VAL significantly protected performance from the detrimental effects of CIRAZ in monkeys (Fig. 6S).

**Fig. 6S.** Effects of VAL pretreatment (2.5 mg/kg, p.o.) on the detrimental effects of CIRAZ (CIR) administration in monkeys performing the delayed response task. Results represent mean ± S.E.M. percent correct. * significantly different from VEH +VEH, P= 0.034, † significantly different from CIR + VEH, P= 0.02.

The population response to the effects of iontophoretic application of PE is shown in Fig. 7SA; PE suppressed delay-related firing. The population response to the iontophoretic application of PE + CHEL is seen in Fig. 7SB; CHEL normalized cell firing. The population response to CHEL by itself is shown in Fig. 7SC; CHEL had little effect on its own.
Fig 7S. Population response to PE and CHEL. (A) Normalized average firing rates for 25 neurons with significant delay-related activity examined during control (blue), $\alpha_1$R agonist PE (orange) condition. Iontophoresis of PE dramatically attenuated the delay-related activity at population level. (B) Nine of the above delay-related neurons were subsequently co-applied with PE and CHEL. Co-application of CHEL with PE restored the delay activity (green). (C) Effects of iontophoresis of the PKC inhibitor, CHEL, on delay-related activity by itself. Iontophoresis of a low level of CHEL (15 nA) had little effect on the delay activity (red).
References and Notes


