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RESEARCH ARTICLE

# Amygdala-Hippocampal Phospholipase D (PLD) Signaling As Novel Mechanism of Cocaine-Environment Maladaptive Conditioned Responses

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## Abstract

**Background:** Drug-environment associative memory mechanisms and the resulting conditioned behaviors are key contributors in relapse to cocaine dependence. Recently, we reported rat amygdala phospholipase D as a key convergent downstream signaling partner in the expression of cocaine-conditioned behaviors mediated by glutamatergic and dopaminergic pathways. In the present study, 1 of the 2 known upstream serotonergic targets of phospholipase D, the serotonin (5-hydroxytryptamine) 2C receptor, was investigated for its role in recruiting phospholipase D signaling in cocaine-conditioned behaviors altered in the rat amygdala and dorsal hippocampus.

**Methods:** Using Western-blot analysis, amygdala phospholipase D phosphorylation and total expression of phospholipase D/5-hydroxytryptamine 2C receptor were observed in early (Day-1) and late (Day-14) withdrawal (cocaine-free) states among male Sprague-Dawley rats subjected to 7-day cocaine-conditioned hyperactivity training. Functional studies were conducted using Chinese Hamster Ovary cells with stably transfected human unedited isoform of 5-hydroxytryptamine 2C receptor.

**Results:** Phosphorylation of phospholipase D isoforms was altered in the Day-1 group of cocaine-conditioned animals, while increased amygdala and decreased dorsal hippocampus phospholipase D/5-hydroxytryptamine 2C receptor protein expression were observed in the Day-14 cocaine-conditioned rats. Functional cellular studies established that increased p phospholipase D is a mechanistic response to 5-$HT_{2C}$R activation and provided the first evidence of a biased agonism by specific 5-hydroxytryptamine 2C receptor agonist, WAY163909 in phospholipase D phosphorylation 2, but not phospholipase D phosphorylation 1 activation.

**Conclusions:** Phospholipase D signaling, activated by dopaminergic, glutamatergic, and serotonergic signaling, can be a common downstream element recruited in associative memory mechanisms altered by cocaine, where increased expression in amygdala and decreased expression in dorsal hippocampus may result in altered anxiety states and increased locomotor responses, respectively.

**Keywords:** amygdala, dorsal hippocampus, phospholipase D, cocaine, conditioned hyperactivity

## Introduction

An important hallmark of cocaine addiction is the high propensity of relapse to active use despite extended periods of abstinence (Berke and Hyman, 2000). This relapse can be attributed to a pathological "hijacking" of learning and memory mechanisms in the reward pathway (Kelley, 2004; Hyman, 2005; Hyman et al., 2006). Exposure to cocaine-related cues activates brain areas important in associative memory (Breiter et al., 1997; Kruzich and See, 2001) and the expression of conditioned behaviors.

The involvement of amygdala and hippocampal brain regions has been successfully modeled in preclinical rodents. Reversible

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inactivation of the basolateral amygdala (BLA) disrupted both formation and expression of the cocaine-conditioned behavior (Kruzich and See, 2001), while electrical stimulation of the BLA was sufficient to reinstate cocaine-seeking behavior in the rat (Hayes et al., 2003). Disruptions in the hippocampus and BLA result in absence of stimulus control in drug-seeking as seen in cocaine-conditioned place preference (Isaac et al., 1989; Hiroi and White, 1991; Brown and Fibiger, 1993; Tzschentke, 2007), cocaine self-administration (Whitelaw et al., 1996), and cocaine reinstatement models (Fuchs et al., 2007).

Certain conditioned responses observed to cocaine-related stimuli in drug addicts (Ehrman et al., 1992) resemble Pavlovian attributes of conditioned behavior to cocaine-associated cues (Pert, 1994). In preclinical rodent models, cocaine-associated environmental response can be investigated as conditioned locomotor behavior in the absence of the drug (Carey and Damianopoulos, 1994; Bardo et al., 1995; Krishnan et al., 2011).

The cocaine-conditioned hyperactivity (CH) model (Barr et al., 1983) differentiates between drug-induced and drug cue-induced increases in locomotor activity in rats (Carey and Damianopoulos, 1994; R. Carey and Gui, 1997; Carey et al., 2008) and mice (Brabant et al., 2003). By repeated cocaine administration prior to placing the animals in a specific environment (eg, locomotor activity monitor), an association of the stimulus environment occurs to the behavioral effects of cocaine, namely hyperactivity, such that later reintroduction into the stimulus environment, without cocaine injection, results in the expression of the hyperactivity. The present study, therefore, employed this behavioral paradigm to investigate the signaling mechanisms recruited by cocaine environment- conditioned responses. Our group recently reported a key role for amygdala phospholipase D (PLD) as a downstream convergent target for dopaminergic and glutamatergic signaling in the expression of cocaine-conditioned response in late (Day-14) withdrawal (drug-free) state (Krishnan et al., 2011). PLD, a lipid-modifying enzyme, catalyzes the conversion of phosphatidyl choline into choline and phosphatidic acid (Cockcroft, 2001; Exton, 2002; Frohman, 2015). But PLD performs other cellular signaling roles, including regulation of exocytosis (Hughes et al., 2004; Huang et al., 2005), endocytosis (Du et al., 2004), and neurotransmitter release (Humeau et al., 2001), all of which are important mechanisms in the neurochemical basis of learning and memory (Bennett and Scheller, 1993). Using CH, the present study addresses the hypothesis that PLD signaling is key to cocaine environment-associated changes in memory mechanism. We also addressed the potential interaction between PLD and serotonergic signaling in the expression of cocaine-conditioned behaviors.

The 5-hydroxytryptamine (5-HT) 2C receptor (5-$HT_{2C}$R) (Di Giovanni and De Deurwaerdere, 2015) is 1 of 14 identified receptors for serotonin (5-HT) (Meneses, 2015) and is expressed in the amygdala and hippocampus. Interestingly, the complexity of the downstream signaling by this G-protein coupled receptor has been linked to multiple signaling pathways that are G-protein dependent and independent (reviewed in Millan et al., 2008), making it necessary to establish a downstream signaling mechanism to better define the specificity of its action. While the activation of phospholipase C via $G_{q/11}$ is most commonly studied (Hoyer et al., 2002), effects of the 5-$HT_{2C}$R mediated by downstream activation of other phospholipases, including PLD (McGrew et al., 2002, 2004), are largely overlooked.

To test the above, we studied: (1) CH in rats following 7-day repeated cocaine administration in early (Day-1) and late (Day-14) withdrawal (drug-free) states; (2) protein expression levels of 5-$HT_{2C}$R and the 2 mammalian isoforms of PLD in the amygdala and hippocampus in both drug-free states following cocaine-CH training; (3) functional association; and (4) activation of the PLD by 5-$HT_{2C}$R as proof of concept in the recruitment of this novel pathway in the expression of conditioned behaviors to cocaine.

## Methods

### Behavioral Studies

#### *Animals*

Forty-eight adult male Sprague-Dawley rats (Harlan Inc., Indianapolis, IN) weighing 225 to 325 g were used. Rats acclimated 5 to 7 days in a colony room at a constant temperature (21–23°C) and humidity (45–50%) on a 12-hour-light/-dark cycle (lights on 7:00 AM to 12:00 PM). Rats were housed 2 per cage with food and water ad libitum. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 2011) and with the approval of the Institutional Animal Care and Use Committee at University of Texas Medical Branch Galveston.

#### *Drugs*

Cocaine HCl, a gift from the National Institute of Drug Abuse (Research Triangle, NC), was dissolved in 0.9% NaCl.

#### *Apparatus*

Cocaine-conditioned locomotor activity was monitored and quantified under low light conditions (Valle, 1970).

#### *Experimental Design*

Rats were habituated to the locomotor activity monitors for 30 minutes each day for 4 consecutive days after saline (0.9% NaCl) injections. To establish CH, rats were placed in the locomotor activity chamber at the same time once each day for 7 consecutive days. Rats in the control group (n = 8) were injected with saline prior to placement in the activity monitors, and the activity was monitored for 30 minutes. At the termination of the session, rats were returned to their home cage and removed 4 hours later and injected with saline and placed back into their home cage. Rats in the unpaired group (n = 8) were injected with saline prior to placement in the activity monitors, and activity was monitored for 30 minutes. At the termination of the session, rats were returned to their home cage and received an injection of cocaine (15 mg/kg, i.p.) 4 hours later. Rats in the paired group (n = 8) were injected with cocaine (15 mg/kg, i.p.) immediately prior to placement in the activity monitors, and activity was recorded for 30 minutes. At the termination of the session, rats were returned to their home cage and received an injection of saline 4 hours later. Rats then remained in their home cage until the test; one cohort (n = 24, 8 animals per group) was tested after 1-day withdrawal (Day-1 group). After the Day-1 drug-free state, the animals were placed in the locomotor activity chamber after an injection of saline, and locomotor activity was recorded for 30 minutes.

Another cohort of 24 rats (n = 8 each for control, unpaired and paired) underwent the same habituation and training as mentioned above, but were kept for 14 days following their last dose of cocaine in their home cage with food and water ad libitum before testing (Day-14 group).

#### *Biochemical Assay*

Immediately after the termination of the CH test (for both Day-1 and Day-14 groups), the animals were anesthetized using chloral hydrate solution (400 mg/kg). Subsequent decapitation and microdissection of the brain to obtain amygdala and dorsal

hippocampus (dHC) were performed using the rat brain stereotaxic coordinates (Paxinos and Watson, 2013). The brain regions were isolated into microcentrifuge tubes, immersed in liquid nitrogen to prevent degradation, and stored at -80°C till further use. A crude synaptosomal prep was made from these brain tissues, and subsequent Western blotting was performed using standard procedures. A uniform concentration (10–20 µg) of protein was diluted in Laemmli sample buffer (6X) and heated for 20 minutes at 70°C (Chinese hamster ovary [CHO] cell extracts were denatured for 5 minutes to prevent aggregation that occurred on longer duration of heating).

Antibodies (45 µg/reaction) were covalently crosslinked onto protein A/G resin according to the manufacturer's instructions (Pierce Crosslink Immunoprecipitation Kit, Pierce Biotechnology, Rockford, IL), and immunoprecipitation studies were conducted using standard protocols.

#### *Antibodies*

Primary antibodies include: 5-$HT_{2C}$R detected by mouse monoclonal D-12 (sc-17797, Santa Cruz; 1:100); 5-$HT_{2C}$R detected by goat polyclonal N-19 (sc-15081, Santa Cruz; 1:250); PLD1 detected by rabbit polyclonal H-160 (sc-25512, Santa Cruz; 1:100); PLD2 detected by rabbit polyclonal H-133 (sc-25513, Santa Cruz; 1:100); monoclonal mouse anti-b-actin (MAB1501, Chemicon International, Temecula, CA, 1:5000); monoclonal mouse anti pan-cadherin (C-19, Abcam, Cambridge, MA, 1:5000); Phospho-PLD1 (Thr147, pPLD1T147) (#3831, Cell Signaling Technology, Danvers, MA, 1:1000); Phospho-PLD1 (Ser561, pPLD1S561) (#3834, Cell Signaling Technology, Danvers, MA, 1:1000); Phospho-PLD2 (Phospho-Tyr169, pPLD2Y169) (A8400, Assay Biotech, Sunnyvale, CA, 1:1000). Secondary antibodies included infrared-labeled goat anti-mouse (IRDye 680; 926–32220, LI-COR Biosciences, Lincoln, NE); goat anti-rabbit (IRDye 800; 827–08365, LI-COR Biosciences); donkey anti-goat (IRDye 800CW; 605-731-125, Rockland Immunochemicals, Inc., Gilbertsville, PA) and sheep anti-mouse (IRDye 680, Rockland Immunochemicals).

#### *Data Analysis*

Data from the activity monitors was organized into mean total counts (± SEM) for the dependent measure of total horizontal activity (ambulation and fine movements) recorded during the test session. A 1-way ANOVA (GraphPad InStat software for Windows V.3.01, GraphPad Software Inc., La Jolla, CA) followed by Tukey's posthoc analysis was used to analyze CH measures at both drug-free states. Time course data were broken down into 3 separate 10-minute time points. The criterion for statistical significance was set at $P < .05$.

Membranes were imaged using the Odyssey Infrared Imaging System (LI COR Biosciences) at 700 and/or 800 nm at 169 µm resolution. The integrated intensity of each band was analyzed with the Odyssey Infrared Imaging System Application version 2.1 Software. The ratio of 5-$HT_{2C}$R or PLD band intensity to pan-cadherin band intensity was determined for each sample for normalization. Further analysis to study the time-dependent changes between Day-1 and Day-14 was conducted as follows. For each antibody, normalized control values described above were averaged. Unpaired and paired values were divided by the respective average control value for each respective antibody and converted to percent increase or decrease and further assessed for changes.

### Cellular Studies

CHO K1 cell lines, stably transfected with nonedited human 5-$HT_{2C}$R (5-$HT_{2C}$R-CHO) cells in the p198–DHFR–Hygro vector containing a hygromycin resistance gene, were generous gifts from Drs. K. Berg and W. Clarke from the University of Texas Health Science Center at San Antonio (San Antonio, TX). Two lines of 5-$HT_{2C}$R-CHO cells were employed in the present experiment: the "low"-expressing CHO-1C19 cells express ~200 fmol/mg of the 5-$HT_{2C}$R protein, while the "high"-expressing CHO-1C7 cells express 5 to 10 pmol/mg of the 5-$HT_{2C}$R protein (Berg et al., 1994, 1999). Protein expression in 1C19 cells was assessed at 200 fmol/mg protein, which approximates physiological levels in the brain (Berg et al., 2001). Cells were grown at 37°C, 5% $CO_2$ and 85% relative humidity in GlutaMaxα-MEM (Invitrogen, Carlsbad CA), 5% fetal bovine serum (Atlanta Biologicals, Atlanta GA), and 100 µg/mL hygromycin (Mediatech, Manassas VA) and were passaged when they reached 80% confluence in 150-$cm^2$ tissue culture plates before extracting protein.

#### *Drugs*

Serotonin (5-HT; Acros Organics, Thermo Fisher Scientific, Rockford, IL) was dissolved in 1X Dulbecco's Phosphate Buffered Saline (DPBS; Cellgro, Thermo Fisher Scientific, Rockford, IL). WAY163909 [(7b*R*, 10a*R*)-1,2,3,4,8,9,10,10a-octahydro-7b*H*-cyclopenta-(b)(1,4)diazepino (6,7,1hi)indole], a gift from Pfizer (New York, NY), was dissolved in 0.9% NaCl. SB242084 [6-chloro-5-methyl-1-((2-(2-methylpyrid-3-yloxy) pyrid-5-yl)carbamoyl) indoline dihydrochloride, Tocris Bioscience, Ellisville MO] was dissolved in 1X DPBS containing 10 mmol/L citric acid (Sigma, St. Louis, MO) and 8% 2-hydroxypropyl-β-cyclodextrin (Trappsol Hydroxypropyl Beta Cyclodextrin, pharmaceutical grade; Cyclodextrin Technologies Development, Alachua, FL) with the final pH adjusted to 5.6. SB216641 [N-[3-[3-(Dimethylamino) ethoxy]-4-methoxyphenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1′-biphenyl]-4-carboxamide hydrochloride, Tocris Bioscience, Ellisville MO] was dissolved in 1X DPBS.

Confluent cells in the 150-$cm^2$ plates were starved with serum/antibiotic free medium overnight at 37°C. The next day, the media was aspirated and replaced with Hank's Balanced Saline Solution (HBSS; Mediatech, Manassas VA). Drugs were diluted in HBSS at the specified concentrations and applied to the cells for the requisite time as specified in Results. At the end of the treatment, cells were rinsed with HBSS and scraped in cellular protein buffer (pH 7.4) containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, plus protease inhibitor cocktail, and phosphatase inhibitor 1 and 2 cocktails (10 µL/mL Sigma-Aldrich) and 1 µM dithiothreitol (Sigma-Aldrich). Centrifugation was performed at 2000 *g* at 4°C for 10 minutes to pellet the cells. Following a second wash, a second centrifugation at 20,000 *g* for 30 minutes was performed. The pellet obtained was resuspended in the above recipe of cellular protein buffer plus 1% Nonidet P-40 (Sigma-Aldrich) detergent. Protein concentration was estimated using the BCA assay. Western-blot analysis was performed as described in the earlier sections, except that actin was the loading control used for coimmunoprecipitation studies, since cadherin was not detected in cellular preparations.

## Results

### Robust CH Is Observed in Both Day-1 and Day-14 Drug-Free States

We tested the hypothesis that CH provides a robust measure of cocaine-induced conditioned responses at Day-1 (Figure 1B-C) and Day-14 (Figure 1E-F) drug-free states. Total horizontal activity over the entire 30-minute interval (the sum of all ambulatory and fine movements – motions that interrupted the infrared

beams) was significantly increased in the paired group of animals (942.1±63.2) compared with the unpaired (642.1±53.7) or control (505.9±54.1) group in Day-1 drug-free state (Figure 1B). The apparent increased locomotor activity trend in the unpaired group of animals compared with control was accounted by the variability between animals (Figure 1C). Increase in locomotor activity as a percent of the last habituation day in the paired group of animals registered ~50% increase (156.7±10.4) compared with either unpaired (102.6±6.8) or control (97.6±9.9) groups in the Day-1 cohort.

In the Day-1 drug-free state, the paired group of animals demonstrated an increase of ~50% in their horizontal locomotor activity (156.7±10.4) compared with either unpaired (102.6±6.8) or control (97.7±10.0) groups. The increase in locomotor activity was significant for each 10-minute epoch ($P<.05$) compared with both unpaired and control groups (data not shown).

In the Day-14 drug-free state, the CH in the paired group (843.0±39.0, n=8) was significantly different compared with the unpaired (698.8±43.4, n=8) or the control (604.3±36.06, n=8) group (Figure 1E). Increase in locomotor activity as a percent of the last habituation day in the paired group of animals still registered ~50% increase (143.1±10.5) compared with either unpaired (101.3±11.6) or control (101.9±11.5) groups (Figure 1F) in the Day-14 cohort and was not different from the increased CH levels in the Day-1 drug-free state. These results suggest that cocaine-environment association elicits a robust conditioned response that is quantified at the same levels between Day-1 and Day-14 cocaine-free days.

Thus, the CH paradigm uniquely provided an opportunity to address whether PLD and 5-HT$_{2C}$R signaling occurs in response to pharmacological effects of cocaine or the conditioned response to the cocaine-associated environment or both. Studying the effect on PLD and 5-HT$_{2C}$R expressions at 2 distinct time points addressed the next step of verifying whether these key therapeutic targets were associated with cocaine-conditioned responses.

## CH Is Associated with Elevated Day-14, but Not Day-1, Expression of Amygdala PLD and 5-HT$_{2C}$R While Increased Phosphorylation of PLD Isoforms Are Observed in Both Drug-Free States in the Paired Group of Animals

Changes in phosphorylation states (pPLD) were used, in addition to protein expression profiles, as a measure of PLD signaling (Kim et al., 1999; Watanabe and Kanaho, 2000; Z. Xie et al., 2000a, 2000b; Hu and Exton, 2003, 2005). For both Day-1 (clear background) and Day-14 (dark background) drug-free states, increased levels of phosphorylation were observed in the paired group of animals (cross-hatch bars) compared with unpaired (slanted bars) at the threonine 147 (pPLD1T147, Figure 2B, middle), serine 561 (pPLD1S561, Figure 2B, last) in PLD1 isoform, and tyrosine 169 (pPLD2Y169, Figure 2C, last) in the PLD2 isoform. Interestingly, crude synaptosomal levels for both PLD1 (Fig 2B, first) and PLD2 (Fig 2C, first) were increased by 50% in Day-14 (dark background) but not Day-1 (clear background) drug-free states in the paired (hatched bars) compared with the unpaired (slanted bars) group. 5-HT$_{2C}$R expression in the paired group (Fig 2A, hatched bars) is elevated by 50% only in the Day-14 (dark background), not in the Day-1 (clear background), drug-free state compared with the unpaired group (slanted bars).

Absolute levels of crude synaptosomal 5-HT$_{2C}$R/PLD fractions in control/unpaired amygdala were indistinguishable from naïve animals (not exposed to the environment, injections, or handling; data not shown), verifying that the neurochemical changes observed are a result of conditioned responses to cocaine. 5-HT$_{2C}$R-PLD levels in the dHC (Fuchs et al., 2007; X. Xie et al., 2010) were assessed next.

## CH Is Associated with Diminished Day-14, but Not Day-1, Expression of dHC PLD and 5-HT$_{2C}$R with Differential Changes in the Phosphorylation States of the PLD Isoforms in the Paired Group of Animals

Interestingly, crude synaptosomal expression levels of 5-HT$_{2C}$R, PLD1, and PLD2 in the dHC demonstrated a contrasting profile of phosphorylation as well as expression states compared with the amygdala (Figure 3). Contrary to the increased amygdala profile, 5-HT$_{2C}$R expression in the paired group (Figure 3A, hatched bars) is diminished by 50% only in the Day-14 (dark background), not in the Day-1 (clear background), drug-free state compared with the unpaired group (slanted bars). Again, contrasting to the increased amygdala profile, decreased phosphorylation states in the dHC were observed in the paired group of animals (cross-hatch bars) compared with unpaired (slanted bars) at the threonine 147 (pPLD1T147, Figure 3B, middle), serine 561 (pPLD1S561, Figure 3B last) in PLD1 isoform and tyrosine 169 (pPLD2Y169, Figure 3C, last) in the PLD2 isoform Day-1 (clear background) drug-free state.

In contrast to the changed amygdala profile, phosphorylation states in the dHC for the PLD1 isoform (Figure 3B, middle and last) did not show any significant increase or decrease in the paired group (cross hatched bars) in the Day-14 (dark background) drug-free state. However, the overall dHC crude synaptosomal expression for PLD1 (Figure 3B, first) and PLD2 (Figure 3C, first) levels were decreased by 50% in the paired (cross-hatched bars) compared with the unpaired (slanted bars) animals in the Day-14 (dark background) drug-free state. Similar to the amygdala profile, there were no changes in the paired (cross hatched bars) compared with unpaired (slanted bars) groups observed in either PLD1 (Figure 3B, first) or PLD2 (Figure 3C, first) in the Day-1 (clear background) drug-free state. Thus, it is possible that the lack of a diminished phosphorylation profile in Day-14 drug-free state could be attributed to floor-level reduction in expression of the PLD1 isoform. Intriguingly, the diminished phosphorylation state of the PLD2 isoform Y169 (Figure 3C, last) was observed in both the Day-1 (clear background) and Day-14 (dark background) drug-free states. One possible explanation is that PLD2 isoform, being the constitutively expressed isoform with high basal activity (Slaaby et al., 2000), has sufficient basal activity despite the decrease for observing a change in the phosphorylation state. Next, we tested the hypothesis that 5-HT$_{2C}$R can functionally activate PLD using 2 approaches: coimmunoprecipitation studies and cell culture experiments.

## CH Is Characterized by Increased Amygdala and Decreased Hippocampal 5-HT$_{2C}$R-PLD1 Association

Qualitative coimmunoprecipitation studies established a functional association between 5-HT$_{2C}$R and PLD (Figure 4). Increased association of PLD1 (Figure 4A) was observed in the 5-HT$_{2C}$R pulldowns from amygdala crude synaptosomal fractions of paired compared with control and unpaired groups. A similar increased association was demonstrated in the reverse immunoprecipitation scheme using PLD1 antibody for pulldowns (Figure 4B). These results mirrored the increased expression

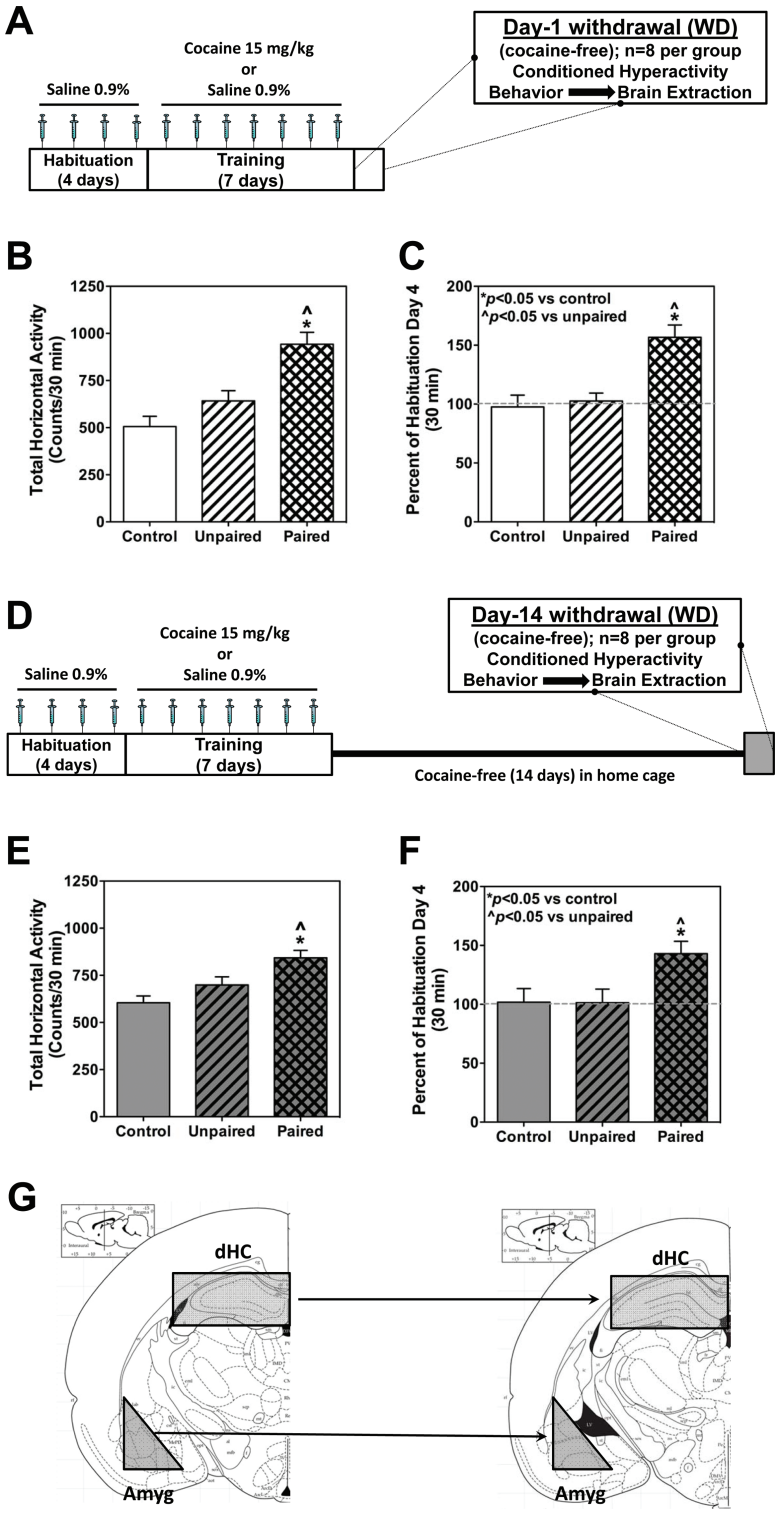


**Figure 1.** Cocaine-induced conditioned hyperactivity is observed in both (B-C) Day-1 (clear background) and (E-F) Day-14 (dark background) drug-free states. Panels A and D provide a schematic of the training and testing regimen used in the conditioned hyperactivity paradigm. Data represent the mean total horizontal activity counts (±SEM) summed over the 30-minute test session in rats conditioned to 15 mg/kg of cocaine in (B) Day-1 drug-free state (cohort of 24 animals, n = 8/group) or (E) Day-14 drug-free state (another cohort of 24 animals, n = 8/group). While increase in conditioned hyperactivity in the paired group of animals in Day-1 (C) and Day-14 (F) drug-free states, as a percent of the total horizontal activity on habituation day 4, remains the same, the analysis also demonstrates an absence of hyperactivity in the unpaired group of animals compared with control, thus validating the use of this paradigm to delineate the signaling mechanisms affected by cocaine pharmacology in contrast to cocaine-conditioned memory. Following Day-1 or Day-14 test, the animals were immediately sacrificed and the brain removed and sectioned to isolate specific brain regions. (G) A schematic of the coronal section from -2.5 mm (left) to -3.5 mm (right) bregma (modified from Paxinos and Watson, 2013). After isolating this section, the shaded rectangular and triangular cuts were performed with razor blades to isolate the dorsal hippocampus (dHC) and amygdala (Amyg), respectively, for further biochemical analysis. *P < .05 vs control; ^ P < .05 vs unpaired.

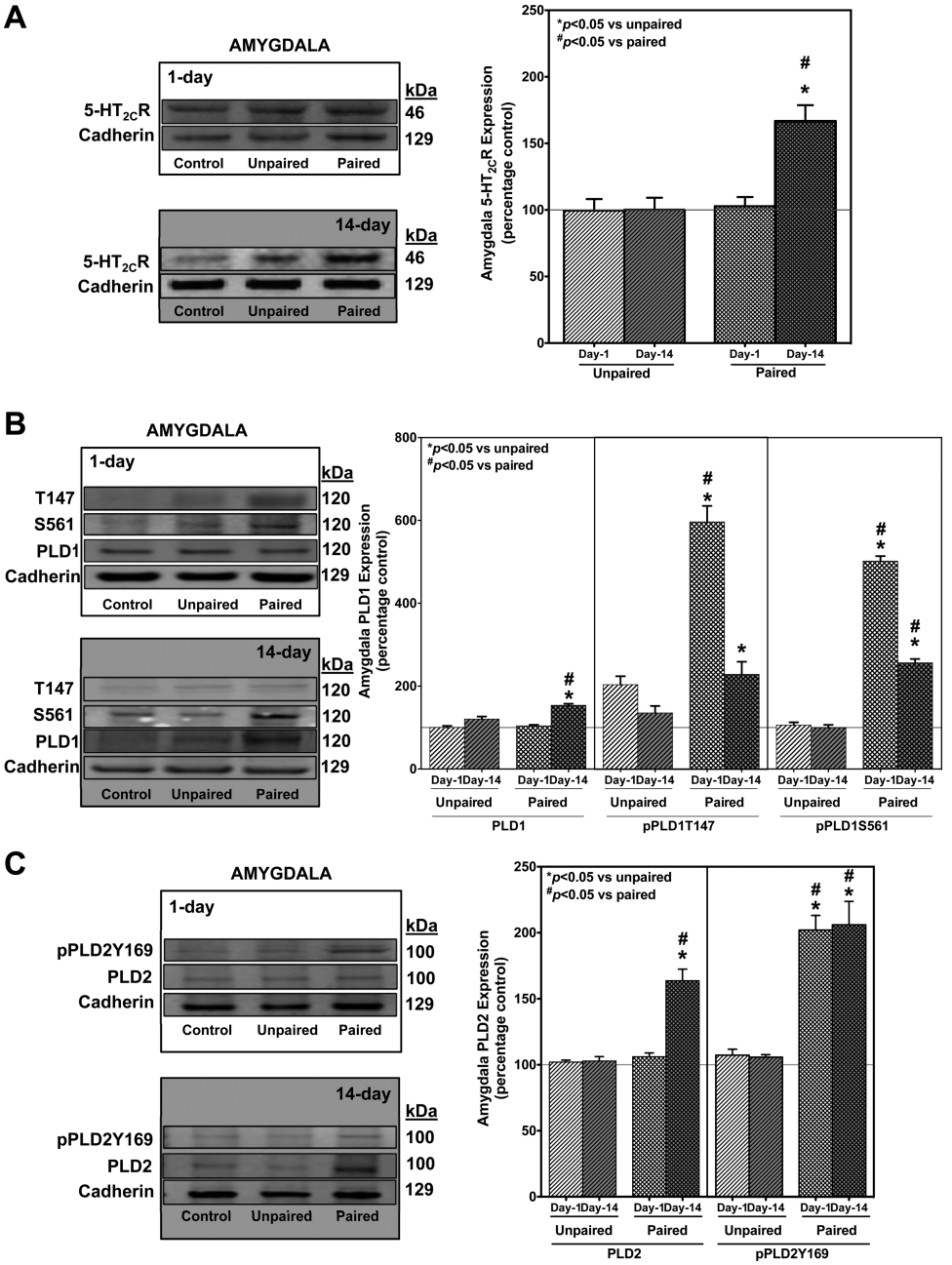


**Figure 2.** Amygdala crude synaptosomal membrane phospholipase D (PLD)1/PLD2 isoforms show elevated phosphorylation states in the Day-1, while both phosphorylation and expression levels are increased in the Day-14 drug-free states in the paired group following conditioned hyperactivity (CH). 5-$HT_{2C}$R expression remains unchanged in Day-1 and increases in the Day-14 drug-free state in the paired group following CH. Results represent the percent change in expression of the unpaired and paired compared to control group (±SEM). For both Day-1 (clear background) and Day-14 (dark background) drug-free states, increased levels of phosphorylation were observed in the paired group of animals (cross-hatch bars) compared to unpaired (slanted bars) at the [B] pPLD1T147 (middle panel), pPLD1S561 [last panel] in PLD1 isoform and [C] pPLD2Y169 (last panel) in the PLD2 isoform. Interestingly, crude synaptosomal levels for both PLD1 (B, first panel) and PLD2 (C, first panel) were increased by 50% in Day-14 (dark background), but not Day-1 (clear background) drug-free states in the paired (hatched bars) compared to the unpaired (slanted bars) group. 5-Hydroxytryptamine (5-HT) 2C receptor (5-$HT_{2C}$R) expression in the paired group (A, hatched bars) is elevated only in the Day-14 (dark background), not in the Day-1 (clear background), drug-free state compared with the unpaired group (slanted bars). $^{\#}P < .05$ vs paired; $^{*}P < .05$ vs unpaired; n = 8 animals/group.

profile observed in the Day-14 paired group. On the other hand, decreased expression in dHC corresponds with decreased 5-$HT_{2C}$R-PLD1 association in the paired group (Figure 4C-D).

While reciprocal coimmunoprecipitations were attempted with PLD2 antibody, 5-$HT_{2C}$R expression was not observed. Interestingly, PLD2 expression (data not shown) in 5-$HT_{2C}$R pulldowns mirrored the expression of PLD1 in the amygdala and dHC. Perhaps the epitope recognized by PLD2 antibody was limiting the ability of the present study to detect 5-$HT_{2C}$R expression in the PLD2 pulldowns.

## Activation of Human 5-$HT_{2C}$R Results in Increased PLD Phosphorylation States in Vitro

Functional 5-$HT_{2C}$R-PLD signaling was assessed by the effect of acute activation of human 5-$HT_{2C}$R (5-$HT_{2C}$R-CHO cells) on pPLD

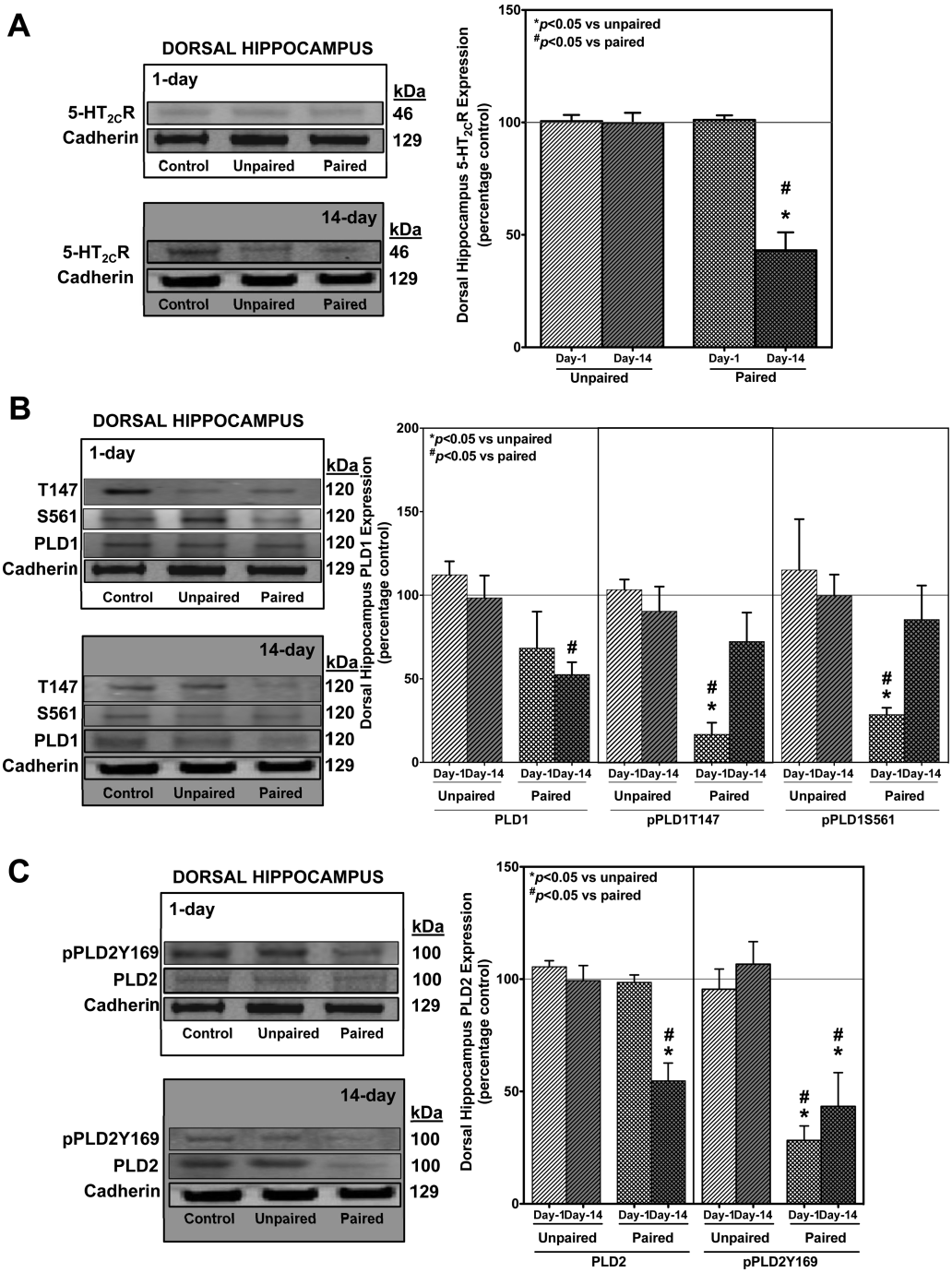


**Figure 3.** Dorsal hippocampus (dHC) crude synaptosomal membrane phospholipase D (PLD)1/PLD2 isoforms show reduced phosphorylation states in the Day-1, while either decreased (PLD2) or unchanged (PLD1) phosphorylation and decreased PLD1/PLD2 expression in the Day-14 drug-free states in the paired group following conditioned hyperactivity (CH). 5-Hydroxytryptamine (5-HT) 2C receptor (5-$HT_{2C}$R) expression remains unchanged in Day-1 and decreases in the Day-14 drug-free state in the paired group following CH. Results represent the percent change in expression of the unpaired and paired compared to control group (±SEM). 5-$HT_{2C}$R expression in the paired group (A, hatched bars) is diminished by 50%, only in the Day-14 (dark background), not in the Day-1 (clear background) drug-free state compared with the unpaired group (slanted bars). Decreased phosphorylation states were observed in the paired group of animals (cross-hatch bars) compared with unpaired (slanted bars) at (B) pPLD1T147 (middle panel), pPLD1S561 (last panel) in PLD1 isoform, and (C) pPLD2Y169 (last panel) in the PLD2 isoform Day-1 (clear background) drug-free state. Phosphorylation states of the PLD1 isoform (B, middle and last panel) did not show any significant increase or decrease in paired group (cross hatched bars) in the Day-14 (dark background) drug-free state. However, the overall dHC crude synaptosomal expression for PLD1 (B, first panel) and PLD2 (C, first panel) levels were decreased by 50% in the paired (cross-hatched bars) compared with the unpaired (slanted bars) animals in the Day-14 (dark background) drug-free state. No changes in the paired (cross hatched bars) compared with unpaired (slanted bars) groups were observed in either PLD1 (B, first panel) or PLD2 (C, first panel) in the Day-1 (clear background) drug-free state. Diminished phosphorylation state of the PLD2 isoform Y169 (C, last panel) was observed in both the Day-1 (clear background) and Day-14 (dark background) drug-free states. $^{\#}P < .05$ vs paired; $^{*}P < .05$ vs unpaired; n = 8 animals/group.

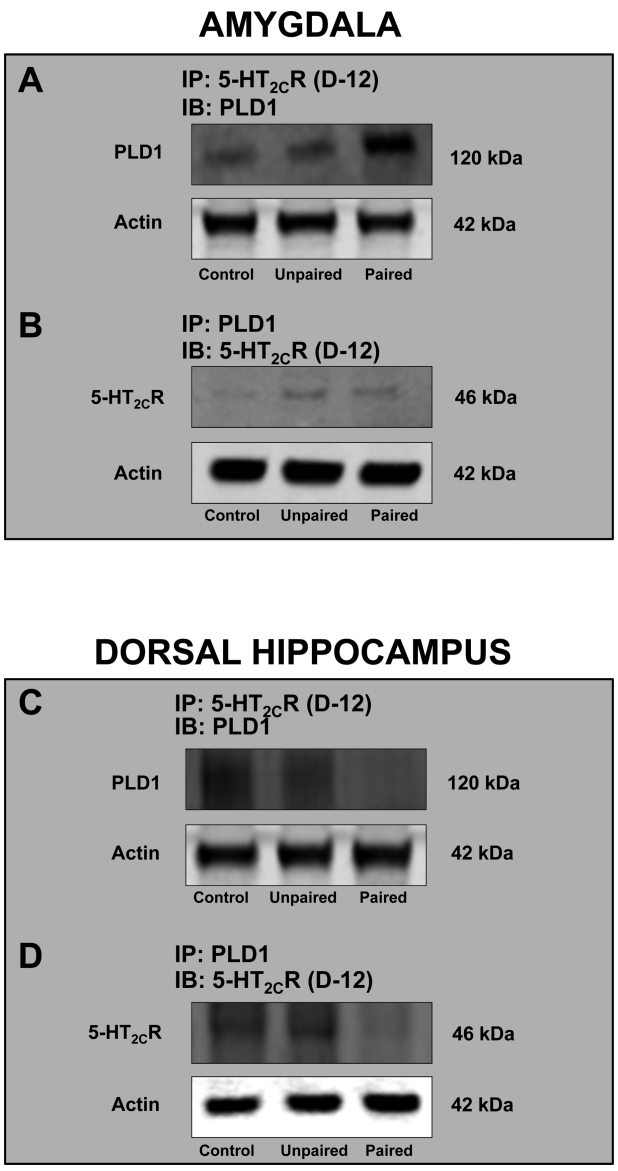


**Figure 4.** Functional (immunoprecipitation) studies demonstrate that phospholipase D (PLD)1 expression tracks 5-hydroxytryptamine (5-HT) 2C receptor (5-$HT_{2C}$R) expression in crude synaptosomal fractions of the amygdala and hippocampus in the paired group of animals in Day-14 drug-free state. (A, C) Synaptosomal fractions were immunoprecipitated (IP) with 5-$HT_{2C}$R antibody and immunoblotted (IB) with PLD1, and the converse was also performed (C-D) in both the amygdala (A-B) and the hippocampus (C-D). Uniform loading was verified by measuring β-actin. Each experiment was repeated twice. These studies establish that increased association in the amygdala and decreased association in the hippocampus of 5-$HT_{2C}$R-PLD at the crude synaptosomal levels are involved with the expression of conditioned hyperactivity.

expression. After reaching 80% confluence, cells were serum-starved overnight at 37°C to minimize the downregulation of the 5-$HT_{2C}$R (Berg et al., 2001). Cells were treated with endogenous ligand, 5-HT (2 nM or 1 μm), or the selective 5-$HT_{2C}$R agonist WAY163909 (10 nM or 10 μM) in vehicle (1X DPBS) for 5 minutes in the presence or absence of the selective 5-$HT_{2C}$R antagonist SB242084 (300 nM) or the 5-$HT_{1B}$R antagonist SB216641 (1 μM), applied 10 minutes prior to agonist application. 5-$HT_{2C}$R-CHO cells are reported to express 5-$HT_{1B}$R (Berg et al., 1994). No significant decrease in pPLD1 states (data not shown) was observed in the presence of the 5-$HT_{1B}$R antagonist. Overall expression of PLD1 or PLD2 remained unchanged (Figure 5). Increasing phosphorylation levels of PLD1 isoform were observed at T147 (Figure 5A), S561 (Figure 5C), and PLD2 at Y169 (Figure 5E) with increasing concentrations of 5-HT. Interestingly, application of WAY163909 resulted in increased phosphorylation only at the 10-μM concentration at the Y169 site on PLD2 (Figure 5F) but did not affect the phosphorylation states of T147 (Figure 5B) and S561 (Figure 5D) at any concentration. We addressed the hypothesis that increased receptor levels using the "high"-expressing CHO-1C7 may increase the signal-noise ratio and demonstrate robust levels of elevations in phosphorylation states, especially by WAY163909. However, the results mirrored the expression in the "low"-expressing CHO-1C19 cells, suggesting that perhaps WAY163909 may be demonstrating biased agonism by selectively activating PLD2, not PLD1, isoform downstream to human 5-$HT_{2C}$R.

## Discussion

The present study demonstrated: (1) there is a ~50% increase in the conditioned locomotor response in both Day-1 and Day-14 groups following 7-day CH; (2) this involves increased phosphorylation states (Day-1, Day-14) and 50% increase in the expression (Day-14) of PLD in amygdala crude synaptosomal fractions; (3) decreased PLD phosphorylation states (Day-1) and (50%) 5-$HT_{2C}$R/PLD expression (Day-14) in the dHC; (4) increased (amygdala) and decreased (dHC) crude synaptosomal 5-$HT_{2C}$R/PLD association in Day-14 paired group; and (5) biased agonism profile for the 2 PLD isoforms via activation by endogenous (5-HT) and a specific (WAY163909) activator of human 5-$HT_{2C}$R stably expressed in CHO cells.

### Robust CH Is Observed Irrespective of the Withdrawal Period

CH in the present study was chosen for its ability to quantitatively differentiate between pharmacological and conditioned responses to cocaine. Conditioned locomotor activity provides a direct measure of cue-attributed salience without the confounding variable of goal-seeking behavior and motivation state (Olmstead et al., 2001). Habituation sessions, prior to cocaine conditioning, were important for discounting rat locomotor activity that occurs when placed in novel environments (Alex and Pehek, 2007). This allowed us to utilize the 4th habituation day to eliminate intervariability among the rats and demonstrate that locomotor activity (Figure 1C, F) is increased significantly (~50%) in the Day-1 and Day-14 drug-free states exclusively in the paired group. An incubation effect (increase in cue-induced activity with increasing withdrawal time) that is reported in self-administration paradigms (Tran-Nguyen et al., 1998; Neisewander et al., 2000; Lu et al., 2004) was not observed in this noncontingent drug administration study.

### CH Increases Amygdala PLD/5-$HT_{2C}$R Signaling

The present study is the first to show significant changes in the 5-$HT_{2C}$R-PLD expression profiles after a short (Day-1) and longer (Day-14) period of drug-free state. Increased phosphorylation at pPLD1T147 can occur via PKCα (Kim et al., 1999) and is present in the pleckstrin homology domain that increases PLD enzymatic (lipolytic) activity (Lee et al., 2005) and the rate of interaction with other proteins (Sung et al., 1999). Additionally, simultaneous activation at pPLD1T147 and pPLD1S561 (located

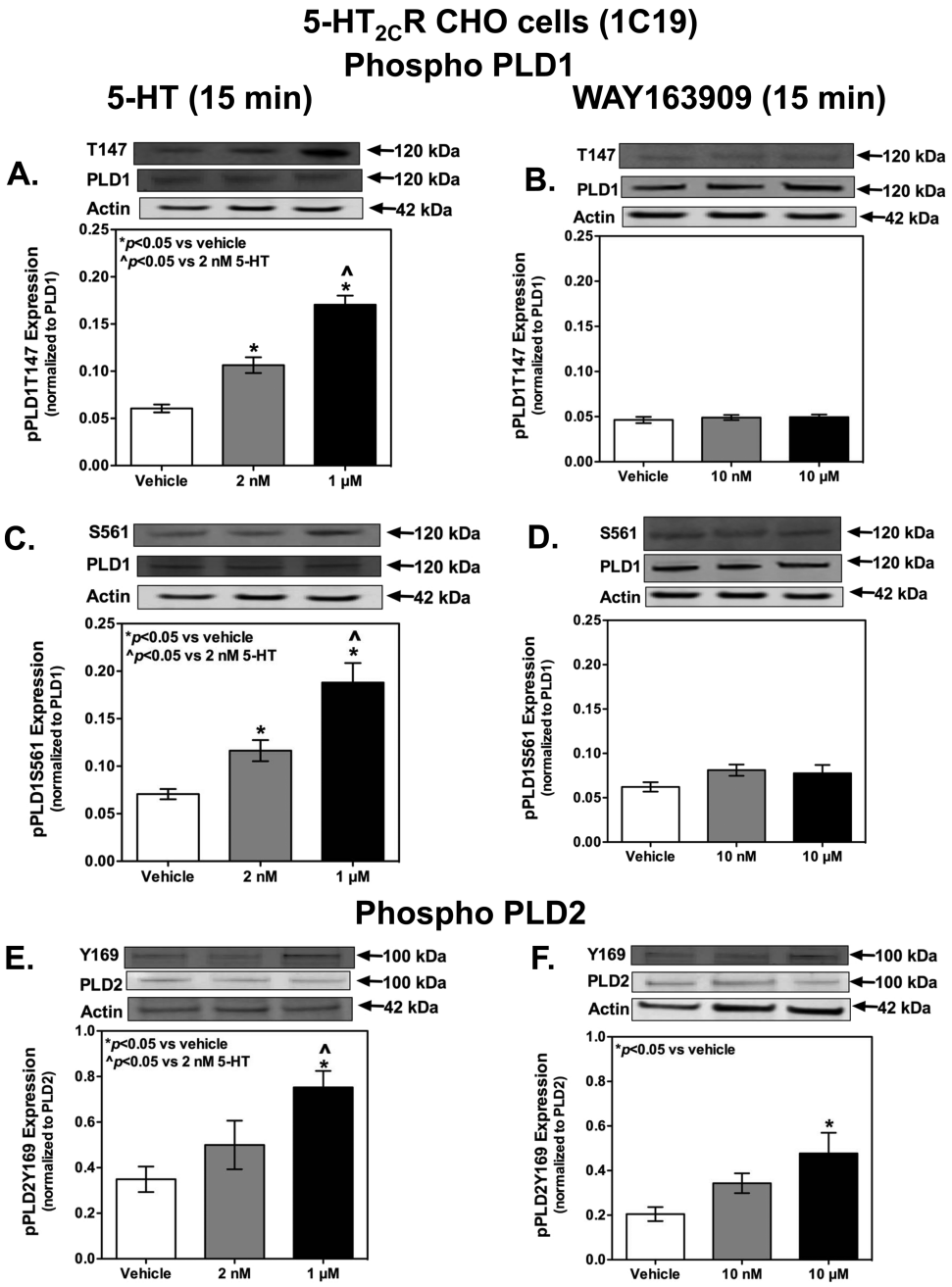


**Figure 5.** Endogenous (5-hydroxytryptamine [5-HT]-) or selective agonist (WAY163909-) mediated activation (15 minutes) of stable but "low-expressing" (~250 fmol/mg) unedited human isoform of the 5-hydroxytryptamine (5-HT) 2C receptor (5-$HT_{2C}$R) in Chinese hamster ovary (CHO)-K1 cells increases phosphorylation states of the 2 different phospholipase D (PLD) isoforms in a dose-dependent manner. Whole cell homogenates were analyzed by Western blot (n = 3–6 samples/each). Densitometric analyses were conducted on the (A-B) pPLD1T147 (120 kDa), (C-D) pPLD1S561 (120 kDa), (E-F) pPLD2Y169 (100 kDa), (A-D) PLD1 (120 kDa), and (E-F) PLD2 bands. Interestingly, 5-HT activation results in increased phosphorylation states for all 3 phosphorylation sites (A, C, E) tested, however, WAY163909 activation results in a significant increase only in the Y169 phosphorylated state at the highest concentration (F). Results represent the mean density (±SEM) of the respective IR band of each phosphorylated form normalized to the total protein levels. Actin was used as a loading control. *P < .05 vs vehicle; ^P < .05 vs 2 nM 5-HT.

in the negative regulatory loop exclusive to the PLD1 isoform) increases endogenous PLD1 activity (Kim et al., 1999).

Direct phosphorylation at the pPLD2Y169 by PKCδ (Han et al., 2002) at the pleckstrin homology domain of PLD2 can bind to SH2/SH3 containing tyrosine kinases (Ahn et al., 2003; Choi et al., 2004), resulting in increased PLD2 activity. Thus, the increased phosphorylation states of pPLD1T147 and pPLD1S561 levels as well as increased expression of PLD1/PLD2 and 5-$HT_{2C}$R (Figure 2) could trigger PKC/PLD-dependent downstream events in response to CH, some of which have been discussed below.

## CH Decreases dHC PLD/5-$HT_{2C}$R Signaling

The hippocampus is well established for its role in associative memory of context-specific information (Burgess et al., 2001; Davachi and DuBrow, 2015). It is also implicated in the associative memory mechanisms underlying addiction (White, 1996;

Crombag et al., 2008; Marchant et al., 2014). The present study investigated the dHC (also referred to as septal hippocampus; posterior hippocampus in primates) because of its preferential role in spatial learning and memory (Moser and Moser, 1998; Bannerman et al., 2004) vs the ventral hippocampus (or temporal hippocampus; anterior hippocampus in primates), which is implicated in anxiety (Bannerman et al., 2004; Engin and Treit, 2007; Koob and Volkow, 2010; Allsop et al., 2014; Strange et al., 2014) and is the subject of our future investigations of effects on anxiety-like behaviors due to cocaine conditioning.

CH reduces both phosphorylation and expression of PLD1 isoforms (Figure 3), suggesting that downstream signaling is attenuated in this brain region during conditioned responses. Since the overall PLD1 isoform expression was decreased by 50%, it is very possible that any changes in the phosphorylation states may be occluded from our analyses. A decrease noted at both withdrawal states in the phosphorylation state of PLD2 (Y169, Figure 3C) supports the above possibility, since basal PLD2 isoform expression is greater than PLD1 (Slaaby et al., 2000).

### 5-HT$_{2C}$R/PLD Brain Region-Specific Association Tracks with the Protein Expression Profiles

Reciprocal coimmunoprecipitation studies (Figure 4) established that crude synaptosomal fractions of PLD protein expression tracks that of 5-HT$_{2C}$R in the amygdala and dHC for the paired group of animals in the Day-14 drug-free state. Based on this study, we investigated some of the known downstream signaling targets of PLD, such as G-protein-independent RhoA, that could underlie 5-HT$_{2C}$R activation (McGrew et al., 2004). Since we failed to see a band for RhoA in any of the lanes (data not shown), the interaction may be G-protein coupled. Next, we tested for $G_{12/13}$ implicated in 5-HT$_{2C}$R-mediated PLD activation (McGrew et al., 2002, 2004) and observed that only $G_{13}$ expression was observed in the paired group of animals in the amygdala (data not shown), suggesting that a specific G-protein-coupled signaling is recruited. Since mTOR (mammalian target of rapamycin), a serine and threonine kinase physically associates with PLD (Sun and Chen, 2008; Foster et al., 2014) where it is central to neuroadaptation signaling mechanisms mediated by drugs of abuse (Neasta et al., 2014), we investigated the pulldowns for mTOR expression. We did not observe mTOR (data not shown), presumably because it is not membrane associated. Future studies of downstream signaling targets using cytoplasmic and nuclear fractions will be key to establish signaling downstream to PLD.

### PLD Phosphorylation Levels Increase in Response to Increasing Concentrations of Human 5-HT$_{2C}$R Agonists in CHO Cells

Using human 5-HT$_{2C}$R in CHO cells, we demonstrated a functional activation of PLD where we observe specificity in the phosphorylation states for the individual isoforms (Figure 5). While PLD2, the constitutively expressed basal isoform, showed increased phosphorylation to WAY163909, there was no activation of PLD1, thus indicating a possible biased agonism in 5-HT$_{2C}$R-PLD-mediated signaling.

### A Model for Amygdala/dHC PLD Signaling in CH

Increase in amygdala 5HT$_{2C}$R/PLD expression/association may increase anxiogenic responses (Vicente and Zangrossi, 2012, 2014). BLA infusions of the nonselective 5-HT$_{2A/2B/2C}$R antagonist, ritanserin, prevents the anxiogenic response associated with systemic MK212 (5-HT$_{2A/2C}$R agonist) administration (de Mello Cruz et al., 2005), while mCPP (5-HT$_{2B/2C}$R agonist) infusion into the BLA/CeA complex (but not dHC/vHC) enhances elevated plus maze associated anxiogenic behavior (Cornelio and Nunes-de-Souza, 2007), suggesting that amygdala 5-HT$_2$R receptors play a positive modulatory role in expression of anxiogenic behaviors. Importantly, the anxiogenic responses are completely blocked by preinfusion of SDZ SER 082 (at doses that preferentially blocked 5-HT$_{2C}$R), further localizing the effect of the anxiogenic responses to 5-HT$_{2C}$R action specifically in the amygdala (Cornelio and Nunes-de-Souza, 2007). Most importantly, a recent study provides direct evidence for our observations, where administration of a 5-HT$_{2C}$R agonist in the BLA increased anxiety-like behavior in cocaine-conditioned rats (Pockros-Burgess et al., 2014). However, increased anxiety is routinely associated with reduced locomotor activity and/or exploration in paradigms such as elevated plus maze and bright open field. Such cessation was not observed in CH, because the animals were placed back into a familiar (conditioned) rather than novel environment typically used in studies of anxiety. Such a hypothesis is supported by another study where overexpression of 5-HT$_{2C}$R in the forebrain, with highest levels in the dHC, results in decreased wheel running activity as well as open field activity (Kimura et al., 2009). Thus, the opposite scenario of a decreased 5-HT$_{2C}$R expression that we observe in the dHC could contribute towards cocaine-conditioned locomotor activity in CH.

Future studies that address a direct causal effect between CH-associated specific conditioned behaviors of anxiety and increased locomotor responses to cocaine-cue-induced memory will be important in addressing whether the 5-HT$_{2C}$R-PLD signaling pathway will be amenable to therapeutic intervention against cocaine addiction.

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## Statement of Interest

None.

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