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**A CORRELATED BEHAVIORAL AND BIOCHEMICAL ANALYSIS INVESTIGATING  
GONADAL INFLUENCE ON PREFRONTAL CORTICAL FUNCTION THROUGH  
MODULATION OF NMDA RECEPTOR TRAFFICKING**

A Thesis Presented

by

**Surya Bhamidipaty**

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

**Master of Science**

in

**Biochemistry and Cell Biology**

Stony Brook University

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Neuronal activity in the prefrontal cortex (PFC) underlies working memory processes in the brain. Reports have suggested critical roles for gonadal steroid modulation of this PFC function in adult male rats (Kritzer et al 2007), yet the exact mechanisms have yet to be defined. Given the clinical implications of male PFC vulnerability with schizophrenia, this is an important question to be addressed. This thesis seeks to gain insight as to whether hormone control over NMDA-receptor mediated glutamate signaling may be part of the way hormones influence the PFC's complex operations. This will be accomplished in interrelated studies using gonadally intact control and gonadectomized male rats. First, Barnes Maze behavioral testing for spatial working memory recently shown to be impaired in gonadectomized (GDX) rats, will be used to determine whether a behavioral rescue in subjects can be ascertained by locally blocking NMDA receptors using (2*R*)-amino-5-phosphonopentanoate (APV, an

NMDA antagonist) within the PFC. Complementary studies will evaluate the subcellular distribution profiles for the NR1 subunit of the NMDA receptor in PFC of control (SHAM) and GDJ rats. In both studies, overexpression/overactivity of NMDA receptors at PFC synapses is expected in GDJ relative to control rats. To date, the data collected indeed support the hypotheses that reduction of NMDA over-activity via APV in GDJ restores PFC-dependent behaviors depicted by an enrichment of NR1 proteins at the membrane and at synapses in GDJ PFC relative to controls.

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## ABBREVIATIONS

ABC	Avidin-Biotin-Peroxidase Complex
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	D-2-amino-5-phosphonovaleric acid
BM	Barnes Maze
CNS	Central Nervous System
DA	Dopamine
ECL	Enzyme Linked Chemiluminescence
ER	Endoplasmic Reticulum
GDX	Gonadectomized
GluR	Glutamate Receptor
GluR1	Type 1 receptor AMPA subunit
ITI	Inter-trial-interval
NMDA	N-methyl-D-aspartate
NR1	NMDA Receptor Subunit 1
PFC	Prefrontal Cortex
PSD	Postsynaptic Density
TBST	Tris-buffer saline with 0.1% Tween (TBST)

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## **1. GENERAL INTRODUCTION**

It is well known that gonadal steroids exert potent structural and functional effects on the mammalian CNS, including those that influence cognitive information processing and other functions mediated by the cerebral cortex. To better understand the substrates that mediate gonadal steroid effects on cortical information processing, the studies of this thesis will determine whether the effects of hormone manipulations (gonadectomy, GDX) in adult male rats on spatial working memory functions of the PFC stem from dysregulation of PFC NMDA signaling. To do this, the Barnes Maze behavioral testing paradigm will be used to compare the performance of GDX and sham-operated control (SHAM) rats that receive bilateral intra-PFC infusion of saline or the NMDA antagonist D-2-amino-5-phosphonovaleric acid (APV) prior to testing. These studies will test the hypothesis that androgen regulation of NMDA receptors in the PFC is a source of what normally supports PFC behavior in male rats - and drives abnormal PFC behavior seen after GDX. Companion studies will use biochemical fractionation and qualitative immunoblotting to explore the related hypothesis that GDX alters NMDA function by causing increased trafficking of the receptor to cell membrane/synaptic surface within the PFC. As discussed further below, these findings may be relevant for understanding specific patterns of increased male vulnerability in certain mental illnesses and neurological disorders which are known to involve the PFC.

## **1.1. The Prefrontal Cortex and Working Memory**

The behavioral process that will be investigated in Barnes Maze testing is spatial working memory, an executive function that is dependent on the PFC. Executive operations, such as this, are broadly defined as one's ability to guide thought and action concurrently with internal goals; however, scientists have yet to clearly define how the capacity for coordinated and purposeful behavior arises from neural states. For years, researchers have placed emphasis on the mechanisms by which executive control guides behavior by studying the PFC. Disruptions within PFC circuitry have demonstrated pathologies of schizophrenia, ADHD, Parkinson's Disease, and various forms of psychosis and addictions (Nasser et al. 2002, Arnsten 2000).

Working memory is more specifically defined as the ability to hold and manipulate information while carrying out a wide range of cognitive tasks (Baddeley & DeSalla 1996; Baddeley & Hitch 1974). Furthermore, of the numerous interconnected systems that comprise working memory, one system temporarily holds visuo-spatial information, and it is the transient nature of the information that separates working memory from other types of memory such as semantic or procedural memory, which are respectively longer-lasting forms of memory (Baddeley & Hitch 1974). It has been established that PFC neuronal activity holds transient information that is essentially used as a template to guide action (Goldman-Rakic 1991; Fuster 1991). The distinction between working memory and other forms of memory in the brain has been emphasized by a number of investigators. Goldman-Rakic (1996) stated that although damage to

the PFC does not impair knowledge about the world or long-term memory, it does impair the ability to use such knowledge to guide behavior. Spatial working memory is a type of working memory for locations in space that is experimentally accessible in animal models including rats. As discussed in the next section, gonadal hormones, specifically androgens in male rats, strongly influence this behavioral construct.

## **1.2. Androgens and the Prefrontal Cortex**

Within the past couple of decades, the PFC and its neural circuitry have been repeatedly identified as sensitive to the influence of gonadal hormones (Keenan et al., 2001). Thus, when examining PFC-dependent spatial learning and working memory tasks, reports depict an advantage for adult male Sprague Dawley rats over females (Dawson et al., 1975; Van Hest et al., 1988). In order to study isolated androgen contributions on cognitive function in adult male rats, gonadal hormones are removed via surgical methods, GDX, and then systematically replaced. Accordingly, GDX in adult male rats has been shown to significantly impair performance in tests of spatial (Daniel et al., 2003; Kritzer et al., 2007) and non-spatial (Ceccarelli et al., 2001) working memory. Additionally, the same reports indicate that androgen, but not estrogen, replacement in GDX rats prevents the exhibited behavioral impairments. In order to begin to understand how the structural and/or neurochemical actions of gonadal steroids impact PFC information processing, the studies of this thesis ask the novel question of whether the adverse effects of GDX for PFC spatial working memory can be prevented not by hormone replacement, but by restoring neurochemical

order to the cortex. The focus is on NMDA-receptor mediated glutamate systems which are introduced in the next section.

### **1.3. Glutamate Receptors**

Glutamate is the major excitatory neurotransmitter that is ubiquitous throughout the mammalian central nervous system (CNS) activating both ion-channel-forming (ionotropic) and G-protein-coupled (metabotropic) glutamate receptors (GluRs). The ionotropic GluRs are further grouped into three categories defined by their agonist selectivity and sequence homology:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type receptors, N-methyl-D-aspartate (NMDA)-type receptors, and kainate receptors (Dingledine et al., 1999). Contributing to the vast structural and functional heterogeneity necessary for carrying out functional roles in glutamatergic neurotransmission, a variety of subunits of have been identified within each category of ionotropic GluR (Nusser, 2000).

To date, many studies have depicted strong correlations between various GluRs and sites of neurotransmitter release and their subsequent impact on postsynaptic responses. Within the past few years, reports have suggested that the number, density, and location of the GluRs with respect to the site of neurotransmitter release also have grave influences on postsynaptic responses (Nusser, 2000). It is now generally accepted that the regulation of GluR number, density, and/or synaptic location could be more significant to the overall diverse properties of glutamatergic synapses. My experiments ask whether hormones regulate behaviors of the PFC by changing the membrane availability of its

NMDA receptors These receptors are heteromeric ligand-gated ion channels (see Figure 1) assembled from three subunit families within the endoplasmic reticulum (ER) requiring co-synthesis of NR1 and NR2 proteins (McIlhinney et al. 1998). Each heteromeric receptor is composed of one essential NR1 subunit and one or more of the modulatory NR2 subunits (Rao et al., 1997). One question addressed within these studies is whether hormones influence the synaptic availability of these receptors, presumably via androgen influences on NMDA receptor trafficking in the PFC. This concept is now introduced in the next section.

#### **1.4. Receptor Trafficking**

Receptor trafficking is important for regulating neural signaling. Defined by the movement of proteins from one subcellular compartment to another, studies have revealed evidence for glutamate receptors quickly moving around neurons changing receptor density, compositions, and availability in response to released neurotransmitters (Collingridge et al, 2004). Using endocytic and exocytic mechanisms, most receptors are inserted into and removed from the plasma membrane respectively while moving intracellularly from their site of synthesis, to their site of function (plasma membrane), and finally to their respective sites of degradation (Collingridge et al, 2004). Numerous reports exemplify the importance of intracellular trafficking events (Malenka et al., 2003; Isaac et al., 2004; Bernard et al., 2006) depicting strong correlations between irregular receptor trafficking with neurological disorders such as Parkinson's disease, Alzheimer's disease, and addiction (Wolf et al., 2003; Snyder et al., 2005).



Once synthesized, NMDA receptor complexes are typically targeted to the post-synaptic membrane where they can interact with components of the postsynaptic density. The postsynaptic density (PSD) serves primarily as a large macromolecular complex comprised of signaling, anchoring, and scaffolding elements (Wyszynski et al. 1997; Lin et al. 1998). Studies have shown that NMDA receptors can also be targeted to extrasynaptic and intracellular sites. Ultimately, it is suggested that the differences in NMDA receptor subunit composition is what drives receptors to different sites i.e. localizing to excitatory synapses - where they associate with postsynaptic membranes, and/or also extrasynaptic and intracellular sites (Petralia et al., 1994). As summarized below, my hypothesis for this project is that GDX elicits an abnormally high number of these receptors being localized to the cell membrane and synaptic sites of the PFC.

### **1.5. Summary of Working Model**

The studies presented within this thesis build upon evidence showing that the function of the PFC in males is modulated through gonadal steroid actions (Janowsky et al. 2000; Kritzer et al. 2007). More recent evidence suggests that this may be due to hormones regulating the localization and activity of NMDA receptors within the PFC. My hypothesis is that GDX induces an oversensitivity to NMDA stimulation which contributes to observed behavioral impairments. In turn, I believe these behavioral impairments result from an increased amount of NMDA receptors being expressed at the cell surface/synaptic sites in the PFC. I will address these in related studies demonstrating reversal of GDX induced

behavioral abnormalities through an NMDA receptor blockade. Furthermore, I will depict enhanced localization of NMDA receptor proteins at membrane- and synapse-linked compartments in GDX PFC.

## **2. MATERIALS/METHODS**

### **2.1. BEHAVIORAL ASSAYS**

#### **2.1.1. Animal Subjects**

To complete this study, a total of 16 adult male Sprague-Dawley rats (Taconic Farms) were used. Animals were housed in pairs of like treatment under a 12:12 h light:dark cycle with food and water available ad libitum. 8 animals were gonadectomized (GDX) while the other 8 animals underwent sham surgery (SHAM). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were designed to minimize animal use and discomfort.

#### **2.1.2. Surgeries**

All surgeries were carried out under proper aseptic conditions using intraperitoneal injections of ketamine (0.9 mg/kg) and xylazine (0.5 mg/kg) as anesthesia. Rats were monitored during recovery from surgery and given 0.03mg/kg buprenorphine to manage post-operative discomfort.

(1) Sham surgery or gonadectomy (GDX) was performed 28 days before behavioral testing. For both procedures, the sac of the scrotum and the underlying layers of tunica were incised. For GDX, the vas deferens was also bilaterally ligated and the testes removed. Incisions were closed using sterile wound clips.

(2) Craniotomy surgeries placed cannulae 3.2 mm anterior to Bregma in mPFC using coordinates Paxinos ad Watson (1998) (See Figure 2). This

surgery entailed anesthetizing rats and securing them within a stereotaxic frame (Kopf Instruments). Once fixed, an incision was made to expose the skull, and burr holes were drilled in order to insert the cannula tip (Plastics One, Roanoke, VA) -2.2mm to the skull with the infusion probe extending 2mm beyond. The cannula was then secured to the skull using shallow screws and dental cement. In order to maximize efficiency, all craniotomy surgeries were performed by the same person (MNL). Subjects were given 5 days for post-operative recovery before behavioral testing.

### **2.1.3. Euthanasia/PFC Dissections**

Animals were euthanized via rapid decapitation and their brains were quickly removed over wet ice. For PFC collection, the frontal parts of the hemispheres were dissected at the level bregma 3.2, according to the coordinates of Paxinos and Watson (1998) (see Figure 2). The basal parts at the level of rhinal fissures were also removed. The PFC brain structures were weighed and quickly frozen in -80C until ready for biochemical assay. To maximize efficiency, all microdissections were performed by the same person (MFK) within 5 minutes from decapitation.

### **2.1.4. Barnes Maze Behavior & Drug Infusion Studies**

The Barnes maze (BM), a dry-land maze test, is designed for assessing spatial learning in rodents relying on their innate preference for dark confined spaces over brightly lit/open areas (Barnes, 1979; Pompl et al., 1999). The maze is an open circular platform, made of white acrylic with a diameter spanning 4

feet across (See Figure 3). Additionally, there are 12 evenly spaced holes around the periphery, one of which leads to a recessed goal chamber. A Logitech Carl Zeiss HD1080P video camera was hung from the ceiling directly above the platform recording all behavioral trials. Underneath one of the holes is the entrance to a darkened escape box that is not visible from the surface of the board thus allowing the rodents to learn the location of a designated escape box. The escape box dimensions spanned 4 inches deep, 9 inches long, and 8 inches wide.

On the BM, animals use extra-maze visio-spatial cues to escape from bright open surfaces to a small dark escape box located under a platform. Each rat was tested four times per day for three consecutive days to find the escape box (with the exception being the teaching trial/habituation of the first BM session, having only three trials). Each trial allowed 3 minutes to locate the escape hole, and 2 minutes to sit in the escape box. If the rat was unable to find the escape box within the allotted 3 minutes, it was put/directed into the escape box by the experimenter. After freely entering or being placed into the escape box, rats were given two minutes in the escape box to ultimately reinforce the escape box being a 'reward' from the open bright surface of the maze. The rats were then taken out of the escape box and placed back in their home cage for fifteen-minute inter-trial-intervals (ITI). In each trial, latency to locate and enter the escape box was measured. Learning/memory performance is measured by the number of pokes (errors) or head hovers over a hole with a decrease in number of errors over the period of training considered to be a better

performance. The BM performance scores have been demonstrated to be impaired with age and specific pathologies (Prut et al., 2007).

#### **2.1.4.a. BM Parameters for Habituation Training**

During the teaching trial/habituation of the first BM session, animals gain familiarity to the testing apparatus while being trained to locate the escape box in three trials, interspaced by 15-minute ITIs during which they are placed back in their respective home-cages. During the first trial, the animal was placed in the escape box for 2 minutes. The second trial consisted of placing the animal directly in front of the hole's entrance to the escape box, facilitating the rat to enter the escape box when necessary. The rat would then sit in the escape box for 2 minutes. Finally, the third trial consisted of creating a path using physical barriers (empty cages) to direct the rat directly to the escape box hole from the center of the maze. No latency times were recorded for the teaching trials. In between each trial (habituation and testing trials), the escape box and the maze were cleaned with 70% ethanol solution.

#### **2.1.4.b. BM Testing Acquisition Parameters for Consecutive Testing Days 1 & 2, and One Week Follow Up for Long Term Memory**

All subjects were tested for two consecutive days consisting of four trials each day. During each trial, the rat is placed under a start box in the center of the maze for 30 seconds. The start box was then removed and the rat was allowed to explore freely to find the escape box. Testing sessions ended after the animal had entered the escape box or when a pre-determined time (3 minutes) had

elapsed. If the animal did not find the escape box during the given time period, it was guided and placed in the escape box for 2 minutes at the end of the trial. All subjects were re-tested 7 days later using the same behavioral testing parameters to assess long-term memory.

#### **2.1.4.c. BM Drug Infusion Studies**

**Drug/Saline injections:** Post-craniotomy recovery and immediately prior to BM Testing, vehicle infusions were made directly into cortex using the Infusion probe within the cannula (See Surgeries for Cannula Placement). Infusions utilized a 28-gauge polypropylene syringe (Millepore, Billerica, MA) connected to a syringe pump controller (BASi Bee Hive controller, West Lafayette, IN) with 1 meter (0.12mm diameter) of Teflon tubing. APV (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile MQH<sub>2</sub>O and prepared as a stock solution. Infusions of drug (APV, 10ug/uL) or Saline were delivered at a rate of 0.5uL/min for 1 min. All animals received 1 min APV or saline infusion into the probe within the cannula regulated by monitoring the syringe pump controller. After vehicle infusion, animals were immediately subjected to 1 additional min with syringe pump controller (nothing being infused into probe) to ensure proper delivery of appropriate vehicle: drug or saline.

**BM Testing:** Barnes Maze behavioral testing was conducted as per aforementioned testing parameters (2.1.4.a. and 2.1.4.b.) 15 minutes post-infusion.

## **2.2. BIOCHEMICAL & MOLECULAR ASSAYS**

### **2.2.1. Subcellular Fractionation**

Biochemical fractionation was performed as described previously (Lin et al., 1998; Wyszynski et al., 1998) with the following modifications.

Tissue was dounce homogenized in ice-cold TEVP buffer [containing 10mM Tris-HCl, pH 7.4, 5mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, and 1mM EDTA containing 320 mM sucrose]. Homogenates were then centrifuged at 800 x g (Denville 260D Brushless Microcentrifuge at 4C) for 30 minutes to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 1,000 x g for 30 minutes to obtain a synaptosomal fraction (P2) which was subsequently hypo-osmotically lysed, and then centrifuged at 25,000 x g for 1 hour (Ultracentrifuge) to pellet a synaptosomal membrane fraction (LP1). The resulting supernatant (LS1) was centrifuged at 165,000 x g for 3 hours to obtain a synaptic vesicle-enriched fraction (LP2). Concurrently, the supernatant (S2) was also centrifuged at 165,000 x g for 3 hours to obtain a cytosolic fraction (S3), and a light membrane/microsome-enriched fraction (P3). After each centrifugation the resulting pellet was rinsed briefly with ice-cold TEVP buffer before subsequent fractionations in order to prevent crossover contamination (See Figure 4).

### **2.2.2. Protein Quantification**

A cell lysis buffer was prepared containing the following: 1 % (v/v) triton-X-100, 0.5 mM EDTA, 15 mM NaCl, 2 mM Tris base, and before use, one tablet of Roche Applied Sciences "cComplete Mini, EDTA-free Protease Inhibitor Cocktail



Tablet” was added to 10ml lysis buffer solution to inhibit proteolytic and phosphatase activity. The total protein concentration was determined by the Bradford method (Bradford, 1976), individual measurements were recorded at 562nm using a Milton Spectronic 1001 Plus. After protein calculations, all fractions were aliquoted and stored at –80C.

### **2.2.3. Antibodies**

Commercially available antibodies and their appropriate dilutions used in these studies are listed in Table 1. These antibodies have previously been used for Western blot experiments and were reported not to demonstrate cross-reactivity with other mGluRs (Gill et al., 1999). For each antibody, gels were run and the blots were probed under identical conditions.

### **2.2.4. Sample/Lysate Preparation for Immunoblot**

Samples lysates were prepared by combining solubilized homogenate with 4X sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulfate, 5% beta-mercaptoethanol, pH 6.8) and heating at 95C for 4 minutes. For the quantification of proteins in subcellular fractionation experiments, equal amounts (10ug) of protein from each fraction were loaded into each lane of the gel.

### **2.2.5. Preparation of 10% Resolving/3% Stacking Gels**

The gel cast was assembled with 1.5 mm spacers and tested with MQH<sub>2</sub>O for leaks. 10 % gels were prepared for separation using 25 % (v/v) separating

buffer (0.55 M Tris base, 0.4 % (w/v) SDS pH adjusted to 8.8 with HCl), 31.25 % (v/v) acrylamide/bis-acrylamide solution, 42.6 % (v/v) MQH<sub>2</sub>O, 0.1 % (v/v) TEMED, and 1 % (v/v) Ammonium persulphate (APS). This solution was immediately transferred into the gel cast and overlaid with ethanol to prevent gel dehydration. Once the resolving gel set, the ethanol was removed and washed two times with MQH<sub>2</sub>O. The stacking gel (3 %) was prepared using 25 % (v/v) stacking buffer (1.64 M Tris base, 0.4 % (w/v) SDS pH adjusted to 6.8 with HCl), 12.5 % (v/v) acrylamide/*bis*-acrylamide, 61.5 % (v/v) MQH<sub>2</sub>O, 0.1 % (v/v) TEMED and 1 % APS (v/v). Upon addition of the stacking gel overlaying the resolving gel, a gel comb (for 12 wells) was carefully inserted. After the gel set, the gel comb was carefully removed and the lanes were briefly washed using MQH<sub>2</sub>O.

#### **2.2.6. Western Blot**

Methods followed as described in Dunah et al., 1996 with the following modifications. The gel assembly was taken out of the casting apparatus and put into the gel tank submerged with running buffer (0.025M Tris base, 0.192M Glycine, 0.1 % SDS).

Lysates (See Sample Prep) were loaded into wells and run against protein standards (Amersham ECL DuellVue). Gels received a constant 180 V for 40 min for clean protein separation. Upon separation, nitrocellulose membranes were cut appropriately to cover the size of the gels, and pre-soaked in transfer buffer (0.025 M Tris base, 0.192 M Glycine, 20 % methanol (v/v)) before being overlaid atop of the gels. The sponges and blotting paper were also soaked in cold transfer buffer. Gels were carefully removed from the electrophoresis tank and

loaded into another gel tank alongside an ice pack before starting the transfer electrophoresis held at a constant 100V for 1 hour under light protected conditions. After the transfer was complete, the membranes were blocked overnight on a rocker at 4C in 5% milk in Tris-buffer saline containing 0.1% Tween-20 (TBST).

After sufficient blocking for non-specific binding, the blot membranes were incubated with primary antibodies for one hour at room temperature (see Table 1) in 3% milk in TBST. Preceding incubation in appropriate secondary antibody (see Table 1), membranes were washed 3X (10min each wash) with TBST. After 1 hour secondary antibody incubation at room temperature, the blots were then washed 3X again using TBST, and incubated for 30 minutes at room temperature in Vectastain ABC system. The incubation in the ABC system allowed for avidin to act as a bridge to biotinylated enzymes which would later be illuminated via chemiluminescent detection. Membrane/blots were subsequently washed with MQH<sub>2</sub>O prior to incubation in ECL substrate. Detection was made in a dark room using enzyme linked chemiluminescence (ECL) detection reagents from Amersham (UK). The HRP antibody conjugate catalyzes luminol in alkaline conditions which excites the luminol to ultimately decay in a chemiluminescent reaction. ECL reagents were mixed in a 1:1 ratio and overlaid atop of the blots at 0.125 ml/cm for 1 minute before development. Films were scanned using an Alphatek AX 300SE X-Ray Film Processor.

### **3. RESULTS**

#### **3.1. Experiment 1 – Barnes Maze Behavioral Results**

Behavioral assessment using the Barnes Maze test depicted gonadal influence/GDX-induced deficit on spatial learning and working memory. Thus, this pilot investigation revealed that on average (4 trials  $\pm$ SEM), SHAM made  $6.33\pm 4.17$  primary errors,  $3.67\pm 2.02$  secondary errors, and took  $39.67\pm 29.08$  seconds (latency) to find the goal hole. Additionally, from the first to the last trial, the amount of time taken to find the target hole decreased, and thus improved by  $84.85\%\pm 12.5$ . In contrast, GDX exhibited  $38.23\% \pm 5.2$  more total errors,  $50.47\% \pm 24.76$  more time, and only showed a  $15.56\%\pm 9.8$  improvement in latency to find the goal from trial 1 to trial 4 (See Figure 5a,b,&e).

Notably these group-specific patterns of behavior were virtually reversed when APV was infused (See Figure 5c,d,&f). Thus, upon APV infusion, performance in the SHAM group became markedly worse; these animals made  $12.25\pm 4.15$  total errors with a  $21.36\% \pm 10.9$  improvement in behavior from trial 1 to trial 4. In contrast, performance in the GDX rats was very clearly improved; thus GDX given APV made only  $6.75\pm 4.52$  total errors and showed a remarkable  $81.26\% \pm 6.99$  improvement the time it took them to find the goal from trial 1 to trial 4.

#### **3.2. Experiment 2 – Subcellular Distribution of NR1**

The reliability of these data depend on adequate separation of subcellular

compartments via differential centrifugation. To standardize for these conditions, antibodies against three proteins were used as fractionation markers: synaptophysin, syntaxin, and calnexin (See Figure 6a-c). Synaptophysin is a synaptic vesicle glycoprotein involved with the fusion of synaptic vesicles to the presynaptic membrane (Devoto and Barnstable, 1987). As expected, synaptophysin was concentrated in the synaptosomal membrane (LP1), synaptic vesicle enriched fraction (LP2), and light membrane (P3) fractions. Syntaxin (Fig. 6b), a protein involved with the docking of synaptic vesicles (Bennett et al., 1992; Barinaga, 1993), was also dense in both the light membrane (P3) and synaptic vesicle-enriched (LP2) fractions, but demonstrated much lower levels in the synaptosomal membrane compartment (LP1). Calnexin, a calcium-binding protein glycoprotein known to interact with newly synthesized glycoproteins in the endoplasmic reticulum (David et al., 1993), was concentrated in the light membrane compartment (P3) but was less prominent in the synaptosomal membrane (LP1) and/or synaptic vesicle-enriched (LP2) fractions.

With evidence of adequate separation, these biochemical fractionation and immunoblotting techniques were then applied to pooled PFC tissues from SHAM and GDX rats and probed with an antibody against the NR1 subunit of NMDA receptors. These analyses revealed markedly different subcellular distribution profiles for the NR1 between the two groups SHAM and GDX (Figure 6d&e). Specifically, the relative concentration of NR1 subunits in membrane and synaptic fractions was visibly greater in GDX tissue compared to SHAM (Control). All samples were loaded at 10ug per lane as demonstrated using

beta-actin as a positive loading control (Figure 6d). These data are provisionally interpreted as confirming hypotheses for functionally relevant GDX-induced increases in synaptic availability of NMDA in the PFC.

## **4. DISCUSSION**

### **4.1. General Discussion**

The general hypothesis for these studies was that GDX leads to a marked increase of NMDA receptor expression/localization on the cell membrane/synapse space in the PFC that functionally disrupts its ability to carry out spatial working memory functions. The studies presented here validate this hypothesis in a two-fold study. First, it was shown that GDX-induced working memory deficits can be attenuated by blocking/reducing activity at PFC NMDA receptors. Subsequently, NMDA NR1 subunits were shown to be abnormally concentrated in membrane/synaptic fractions of PFC tissue from GDX rats in comparison to SHAM. Although the changes in protein expression do not directly support a change in receptor function, both sets of findings are at least consistent with GDX inducing an increase in glutamate receptor availability which could well be the basis for the NMDA over-sensitivity observed in previously microdialysis studies (Aubele and Kritzer, 2011), and indicated here in behavioral studies. This study thus marks an initial and potentially important step for elucidating mechanisms involved with receptor trafficking in response to androgen sensitivity within the PFC.

### **4.2. Behavioral findings**

Learning in the Barnes maze was assessed in this study using three different parameters: (1) the number of errors taken place before entering the target escape box, (2) the latency/time necessary to reach the escape box, and (3) the path length used to enter the escape box. Studies have shown that

rodents sometimes directly navigate to the escape hole immediately upon beginning a trial (by chance), and at other times investigate the goal hole but then leave without entering it (Reiserer et al. 2007). Both can add significant variance in the data. In order to control for this, this study quantified the number of errors made before the first encounter of the escape hole as “primary errors.” Reports have indicated that such “primary errors” are a better indication for whether or not the rat has learned the location of the escape hole during testing. Similarly, path length and latency were recorded as “primary path length” and “primary latency,” along with also recording total values (total errors/ latency/ path length) before entering escape hole.

Comparisons for infusion groups (Sham (+) Saline, Sham (+) APV, GDX (+) Saline, GDX (+) APV) were made (See Figure 5) illustrating APV’s ability to ameliorate cognitive deficits generally exhibited by GDX via behavioral impairment. Although the number of animals was low with high variance, the behavioral data nonetheless support APV’s ability to rescue behavioral impairment due to GDX. Further testing with a larger number of animals is necessary in order to statistically validate these intriguing, but preliminary findings

### **4.3. Subcellular distribution of NR1**

Analyses of GDX effects on the localization of NMDA receptors in the PFC used differential centrifugation to separate proteins present at synaptic sites from those found in intracellular cytoplasmic and vesicular pools. Despite using a well-

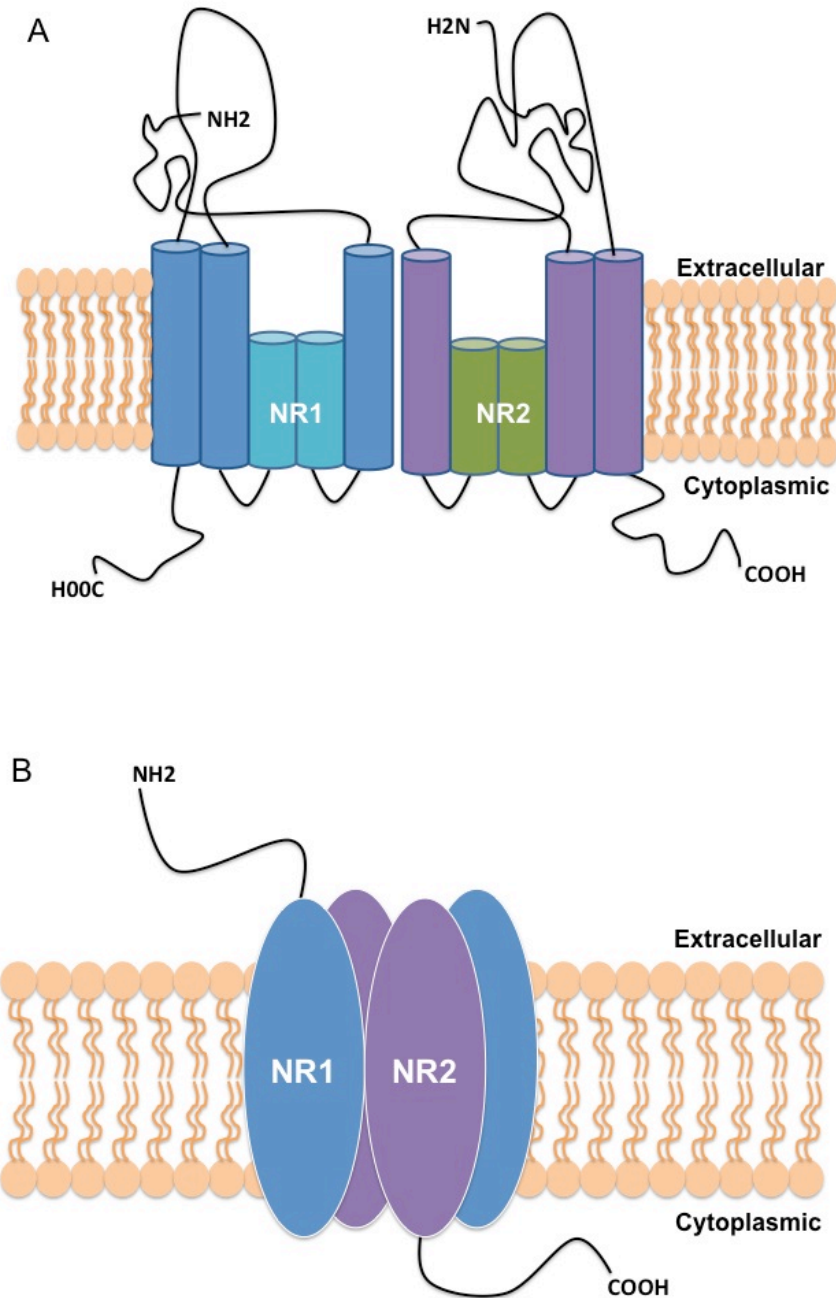


cited biochemical fractionation protocol described by Dunah and Standaert, 2001, considerable experimental effort was nonetheless required to overcome problems encountered in attaining clean, distinct subcellular separations, and in reliably retrieving an LP2 fraction (containing synaptic vesicles and trafficking organelles for membrane proteins and neurotransmitter receptors (Sudhof, 1995; Takamori et al., 2000)) from the relatively small amounts of PFC starting material available (see Figure 4).

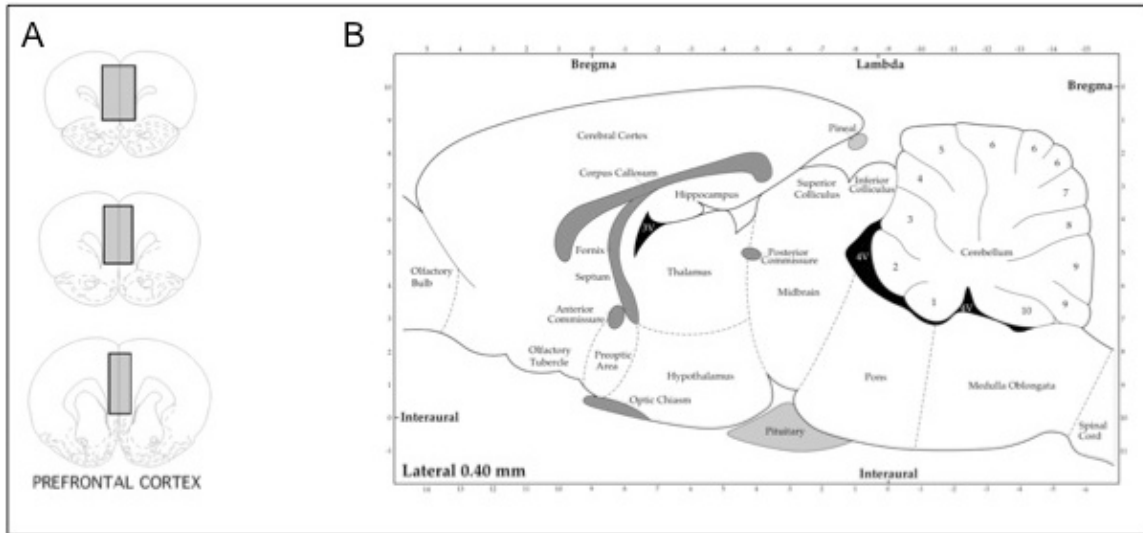
Once efficient subcellular fractionation was achieved, antibodies to the NR1 subunit were used to assess the compartmental distributions of NMDA glutamate receptors. Since the glutamate receptor NR1 subunit is an integral membrane protein, numerous reports have confirmed its localization to the three distinct membrane-linked compartments (LP1, LP2, and P3). These results correlate with and validate/support other studies showing glutamate receptor localization to be at post-synaptic sites and intracellular vesicular compartments (Petralia et al., 1994). Studies in SHAM cohorts corroborated these results also highlighting the particular abundance of NR1 in the P3 fraction which is known to contain organelles such as ER, Golgi, and mitochondria (Schapira, 1998). Furthermore, it has been suggested that the P3 fraction represents newly synthesized glutamate receptors destined for post-translational modifications. In the GDX rats, NR1 was also in these same three fractions LP1, LP2, and P3. However, in contrast to SHAM where NR1 was most pervasive in the P3 fraction, GDX subjects demonstrated a marked increase of NR1 within the LP1 and LP2 fractions. Ultimately, this suggests that within GDX subjects, there are

proportionately more NMDA receptors in transit and/or localized to the postsynaptic density validating the NMDA over-activity that also characterizes these animals.

## FIGURES



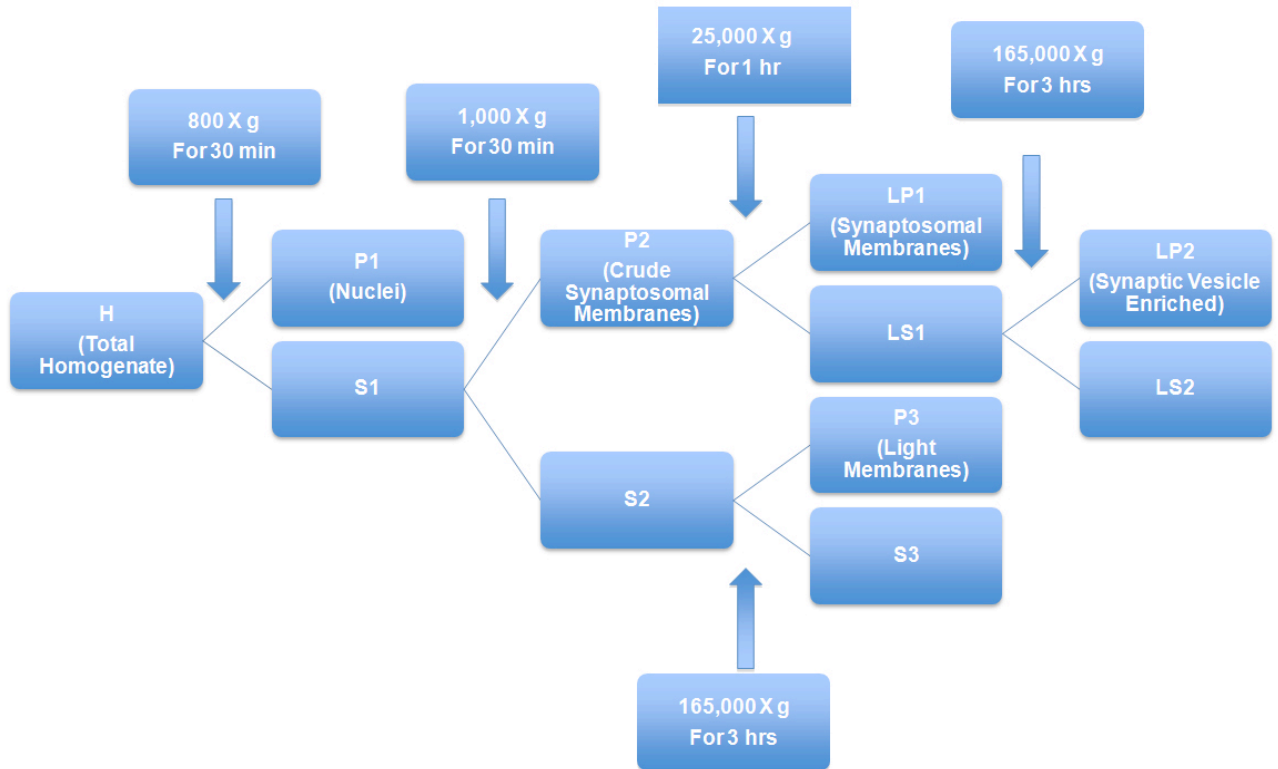
**Figure 1:** Glutamate NMDA receptors. (A) Heteromeric ionotropic receptors formed by two NR1 and two NR2 subunits that bind glycine and glutamate respectively. (Only two subunits of tetrameric complex depicted), (B) Heteromeric NMDA receptor complex within membrane.



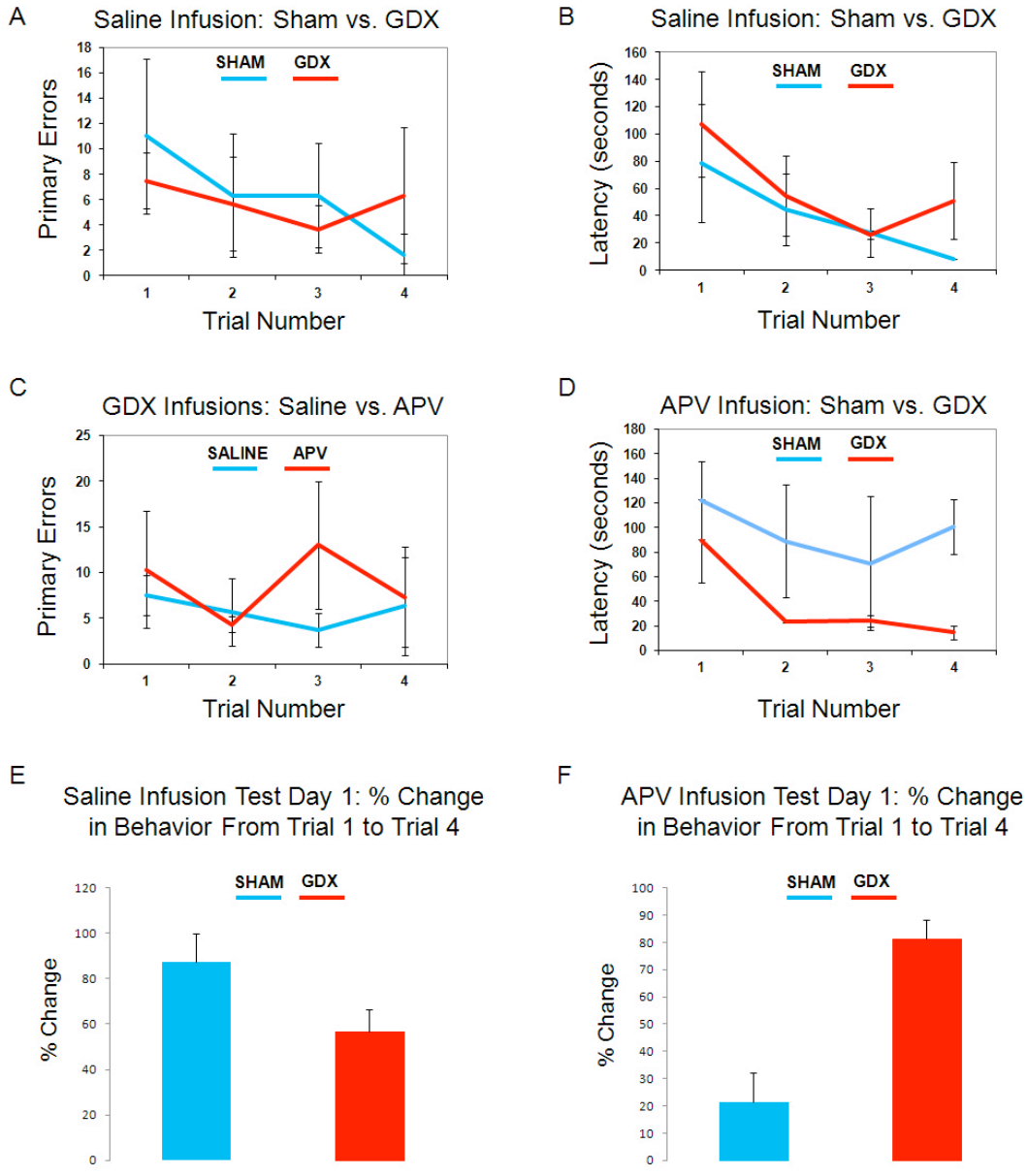
**Figure 2:** Guidelines used for rat Prefrontal Cortex Microdissections. (a) General Prefrontal Cortex Region, (b) Coordinates used for defining microdissected region. (Image reproduced from Paxinos and Watson (1998) with copyright permissions.)



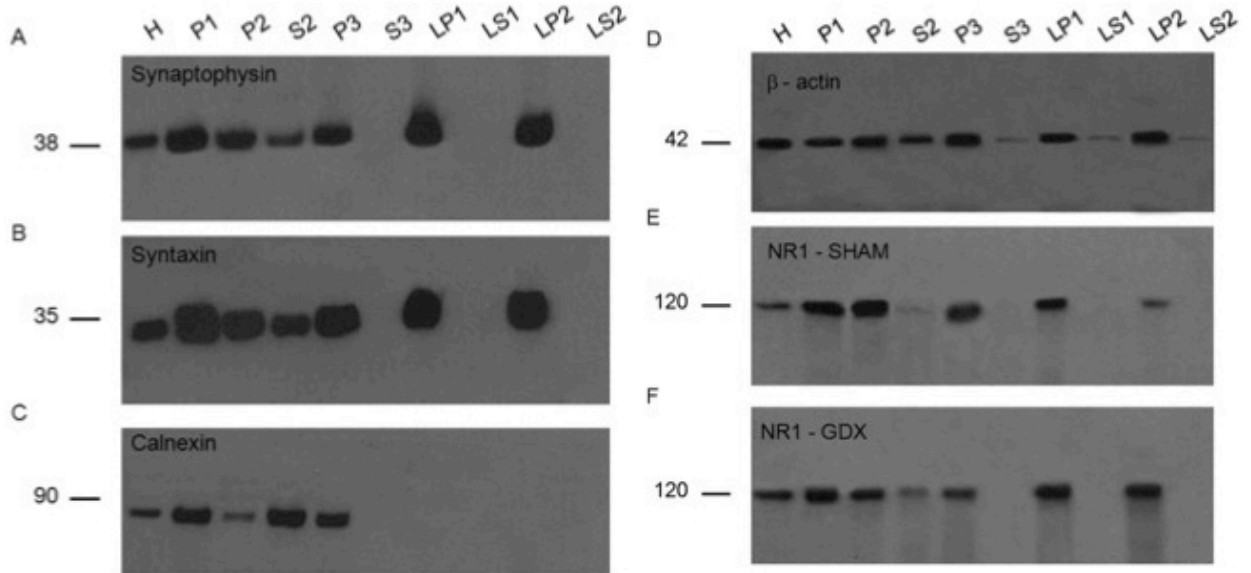
**Figure 3:** Barnes Maze used for Behavioral Testing. Maze dimension include a four feet diameter with twelve evenly spaced holes surrounding the periphery – one of which leads to a recessed goal chamber. Logitech Carl Zeiss HD1080P video camera hung directly above the platform to record all behavioral trials.



**Figure 4:** Schematic for Subcellular Fractionation characterizing the fractionated subcellular compartments of pooled rat Prefrontal Cortex. The procedure for the subcellular separation of proteins as depicted is described in the Materials and Methods Section.



**FIGURE 5:** Barnes maze behavioral data acquisition in Experiment 1 (mean  $\pm$  SEM; 4 trials per day/per animal). Upon intra-saline infusion, performance improvement of SHAM in contrast to GDX represented by a decrease in number of primary errors (A) and decrease in latency (B) over the course of four trials. Percent Change in behavior demonstrates a greater improvement for saline-infused SHAM animals as demonstrated by decreases in latency and errors over the course of four trials (E). SHAM and GDX rats given intra-APV infusion prior to behavioral testing demonstrated reverse behavioral performance (F) with GDX showing fewer primary errors (C) and markedly improved latency (D).



**Figure 6:** Characterization and subcellular distribution of NR1 in Sham and GDX pooled rat PFC. The isolated biochemical fractions (described in Materials and Methods) from PFC tissues were separated by SDS-PAGE, and the blots were probed with antibodies against synaptophysin (A), syntaxin (B), and calnexin (C), and  $\beta$ -actin (D). NMDA receptor subunit NR1 is differentially distributed between subcellular compartments within the PFC of Sham (E) and hormone manipulated (GDX) rat PFC (F). Total protein (10  $\mu$ g) from each fraction was loaded in each lane as represented by uniform distribution of  $\beta$ -actin (D). The positions and sizes of molecular weight markers are indicated in kilodaltons.



## TABLES

**TABLE 1: Antibodies Used in Study**

<b>Antibody</b>	<b>Type</b>	<b>Company</b>	<b>Dilution</b>	<b>Incubation Time</b>
Synaptophysin	Primary	Sigma	1:1000	1 hr/Room Temp
Syntaxin	Primary	Sigma	1:200,000	1 hr/Room Temp
Calnexin	Primary	Santa Cruz	1:5000	1 hr/Room Temp
NR1	Primary	Millipore	1:200	1 hr/Room Temp
Beta-actin	Primary	Sigma	1:20,000	1 hr/Room Temp
Biotinylated horse anti-mouse	Secondary	Vector	1:20,000	1 hr/Room Temp
Biotinylated horse anti-rabbit	Secondary	Vector	1:10,000	1 hr/Room Temp

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