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Food Restriction Induces Synaptic Incorporation of Calcium-Permeable AMPA Receptors in Nucleus Accumbens

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Abstract

Chronic food restriction potentiates behavioral and cellular responses to drugs of abuse and D-1 dopamine receptor agonists administered systemically or locally in the nucleus accumbens (NAc). However, the alterations in NAc synaptic transmission underlying these effects are incompletely understood. AMPA receptor trafficking is a major mechanism for regulating synaptic strength, and previous studies have shown that both sucrose and d-amphetamine rapidly alter the abundance of AMPA receptor subunits in the NAc postsynaptic density (PSD) in a manner that differs between food-restricted and *ad libitum* fed rats. The present study examined whether food restriction, in the absence of reward stimulus challenge, alters AMPAR subunit abundance in the NAc PSD. Food restriction was found to increase surface expression and, specifically, PSD abundance, of GluA1 but not GluA2, suggesting synaptic incorporation of GluA2-lacking Ca²⁺-permeable AMPARs (CP-AMPARs). Naspmp, an antagonist of CP-AMPARs, decreased the amplitude of evoked EPSCs in Nac shell, and blocked the enhanced locomotor response to local microinjection of the D-1 **receptor** agonist, SKF-82958, in food-restricted, but not *ad libitum* fed, subjects. Although microinjection of the D-2 **receptor** agonist, quinpirole, also induced greater locomotor activation

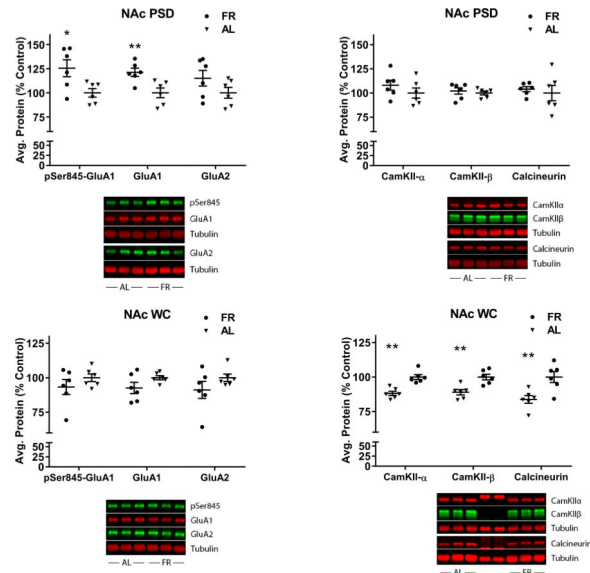
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in food-restricted than *ad libitum* fed rats, this effect was not decreased by Nasp. Taken together, the present findings are consistent with synaptic incorporation of CP-AMPA receptors in D-1 receptor expressing medium spiny neurons in NAc as a mechanistic underpinning of the enhanced responsiveness of food-restricted rats to natural rewards and drugs of abuse.

Graphical abstract



Increased surface expression and postsynaptic density abundance of GluA1, but not GluA2, in the nucleus accumbens of food-restricted (FR) relative to *ad libitum* fed (AL) rats, suggest synaptic insertion of Ca²⁺-permeable AMPARs. Electrophysiological support for this conclusion is provided, as are pharmacological results indicating involvement of these AMPARs in the enhanced behavioral response of food-restricted rats to D-1, but not D-2, dopamine receptor stimulation.

Keywords

neuroplasticity; reward; dieting; addiction

Introduction

Maintenance of energy homeostasis in an ecology of food scarcity is facilitated by neural and behavioral adaptations that increase the probability of food acquisition and consumption. Yet, food restriction driven by sociocultural pressures, cosmetic or health concerns, is a common practice (Kruger *et al.*, 2004; Bish *et al.*, 2005). Under these circumstances, the physiology of food scarcity may be induced within an environment that is abundant with food, associated cues, and proxies for food in the form of drugs that target the same neural substrate (Di Chiara 2005; Volkow *et al.*, 2008). It is therefore not surprising that dieting is a risk factor for binge pathology (Stice *et al.*, 2008), leads to rebound weight gain exceeding the weight lost (Mann *et al.*, 2007; Pietilainen *et al.*, 2012), and increases risk of initiating and relapsing to drug abuse (Krahn *et al.*, 1992; Cochrane *et al.*, 1998; Austin &

Gortmaker, 2001; Cheskin *et al.*, 2005; Seo & Jiang, 2012) Moreover, the prevalence of drug abuse among individuals with eating disorders is disproportionately high (Jonas *et al.*, 1987; Pissetsky *et al.*, 2008; Root *et al.*, 2010). The causal relation between food restriction and binge eating, drug reward sensitivity, and incentive effects of drug-associated cues and contexts have been confirmed in animal models (Carroll *et al.*, 1979; Hagan & Moss, 1997; Cabeza de Vaca & Carr, 1998; Zheng *et al.* 2012; D'Cunha *et al.*, 2013).

Mechanistic studies suggest that effects of food restriction (FR) on responsiveness to drugs of abuse and associated environments are mediated by stable neuroadaptations rather than time since the last meal or circulating levels of metabolic hormones (Zheng *et al.*, 2013). A variety of changes have been observed in the mesoaccumbens “reward” pathway, including decreased ventral tegmental tyrosine hydroxylase mRNA and protein levels (Pan *et al.*, 2006), decreased basal extracellular (Pothos *et al.*, 1995) and evoked release (Stouffer *et al.*, 2015) of dopamine (DA), and decreased mRNA levels of NAc neuropeptides known to be synthesized by D-1 receptor-expressing medium spiny neurons (MSNs) (Haberny & Carr, 2005). These findings suggest that FR is accompanied by DA conservation. Yet, within NAc, increased intracellular signaling, gene expression, and behavior in response to D-1 receptor stimulation suggest compensatory upregulation (Carr *et al.*, 2003; Haberny *et al.*, 2004; Haberny & Carr; 2005a), with potential to enhance the effects of reward stimuli that induce extracellular DA concentrations adequate to stimulate the relatively low affinity D-1 receptor (Gerfen & Surmeier, 2011; Richfield *et al.*, 1989).

One downstream effect of D-1 receptor stimulation is phosphorylation of the glutamatergic AMPA receptor GluA1 at Ser845. Relative to *ad libitum* fed (AL) subjects, FR subjects display increased pSer845-GluA1 in response to injection of a D-1 receptor agonist, d-amphetamine, sucrose consumption, and exposure to a cocaine-paired environment (Carr *et al.*, 2010; Liu *et al.*, 2011; Peng *et al.*, 2014). This is significant from standpoints of neuronal excitability and behavior. Phosphorylation at Ser845 increases peak current and channel open probability (Roche *et al.*, 1996; Banke *et al.*, 2000), facilitates trafficking of GluA1-containing AMPARs to the cell surface (Shi *et al.*, 2001; Esteban *et al.*, 2003; Man *et al.*, 2007), and stabilizes membrane Ca²⁺-permeable AMPARs (CP-AMPARs) (He *et al.*, 2009). Indeed, sucrose consumption and d-amphetamine administration were shown to increase the abundance of AMPAR GluA1 and GluA2 in the NAc postsynaptic density (PSD) with a greater effect in FR than AL rats (Peng *et al.*, 2011; Peng *et al.*, 2014). Yet, these same studies suggested that FR, in the absence of reward stimulus challenge, may increase GluA1 but not GluA2 in the PSD. Specifically, control FR subjects handled and given access to water rather than sucrose, or injected intraperitoneally with saline-vehicle rather than amphetamine, displayed elevated levels of GluA1 relative to AL subjects. The purpose of the present study was therefore to test the hypothesis that FR induces synaptic incorporation of CP-AMPARs. This particular response to FR would be of high interest, considering that these receptors are relatively rare (Reimers *et al.*, 2011), are driven into the synaptic membrane as a homeostatic response to deprivation of excitatory input *in vitro* (Thiagarajan *et al.*, 2005), and mediate synaptic strengthening and behavior formation *in vivo* (Rumpel *et al.*, 2005; Whitlock *et al.*, 2006). To test the hypothesis, diet groups receiving no unusual stimulation prior to brain harvesting were compared for AMPAR surface expression, subunit protein levels in the NAc PSD, and whole cell indicators of calcium signaling and

calcineurin, as they have been implicated in GluA1 trafficking in cultured neurons. Next, electrophysiological methods were used to substantiate biochemical findings suggestive of FR-induced insertion of GluA2-lacking AMPARs. Finally, locomotor activation elicited by D-1 and D-2 receptor stimulation in NAc was challenged with the selective antagonist of CP-AMPA receptors, Naspam. The electrophysiological and microinjection studies focused on the NAc shell, based on the critical involvement of this region in reward-related learning (Gambarana *et al.*, 2003) and reinforcing effects of abused drugs (Ikemoto, 2007).

Materials and Methods

Subjects and food restriction

All subjects were male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA) weighing 350–400 g at the time of arrival in the central animal facility where they were housed in individual plastic cages with free access to standard pelleted lab chow (Rodent Diet #5001, Lab Diet, St. Louis, MO) and water unless otherwise noted. The animal room was maintained on a 12-h light/dark cycle, with lights on at 0600 h. Half the subjects in each experiment were placed on a chronic food restriction regimen whereby daily food allotment was initially limited to 10 g of chow, delivered at 1700 h, until a 20% decrease in body weight was attained. Attainment of the target body weight typically took 15–20 days. Daily feeding was then titrated to clamp body weight at that value for 1–2 additional weeks until brain harvesting in the biochemistry and electrophysiology experiments, or 1 week until initiation, and an additional 3 weeks through completion, of the behavioral experiment.

All experimental procedures were approved by the New York University School of Medicine Institutional Animal Care and Use Committee and were performed in accordance with the “Principles of Laboratory Animal Care” (NIH publication number 85-23). All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques.

Brain harvesting and sample preparation

Subjects were sacrificed between 1000 and 1200 h by brief exposure to CO₂ and decapitation by guillotine. Brains were rapidly extracted. For protein cross-linking, NAc was rapidly dissected on ice and minced with a razor blade. The sample from one hemisphere was added to an eppendorf tube containing ice-cold artificial CSF spiked with cross-linking reagent, bis (sulfosuccinimidyl) suberate (BS³) (Pierce, Rockford, IL), while the contralateral sample from each subject was placed in a tube without the BS³ cross-linker. For subcellular fractionation, NAc and caudate-putamen (CPu) were dissected on ice and bilateral samples from three rats per treatment condition were pooled in separate tubes for fractionation, yielding a total of six tubes per brain region for the FR and AL diet conditions.

Protein cross-linking and Western blotting to measure cell surface expression

Analysis of cell surface and intracellular AMPARs was achieved by using a BS³ cross-linking method previously described by Boudreau and Wolf (2005). Minced NAc tissue of one hemisphere was incubated with or without 2 mM BS³ cross-linker for 30 min at 4°C, end-over-end rotation. Reaction was terminated by the addition of 100 mM glycine for

10min at 4°C followed by a 2 min centrifugation to pellet tissue. Supernatant was removed and pelleted tissue was sonicated in ice-cold lysis buffer (25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 0.1% NP-40, 1 μM okadaic acid, 1 mM Na₃VO₄, and 1X protease inhibitors). Total lysates were spun at 20,000 × *g* for 2 min at 4°C, aliquoted and stored at –80°C until use. Samples (30 μg) were separated by electrophoresis on precast 4-20% SDS-PAGE gels (Lonza, Rockland, ME) and transferred to a PVDF membrane (PerkinElmer, Boston, MA) for 2.5 hr at 100V. Membranes were blocked for 60 min with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and incubated overnight at 4°C with mouse monoclonal anti-GluA1 (1:1000; MAB2263, Millipore, Temecula, CA), rabbit polyclonal anti-GluA2 (1:1000; PA1-4659, Thermo Scientific, Rockford, IL), or mouse monoclonal anti-α-tubulin (1:10,000; T6199, Sigma-Aldrich, St. Louis, MO) diluted in Odyssey Blocking Buffer with 0.2% Tween-20. Blots were stripped and reprobed in reverse order with GluA1 or GluA2 antibody. Detection of band densities was optimal with chemiluminescent detection rather than the Li-COR infrared imaging system because of less background signal and signal amplification with the use of horseradish peroxidase-labelled secondary antibodies (Thermo Scientific, Rockford, IL; 1:6000). Immunoblots were developed with Supersignal West Pico and Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) using a film developer. Band densities were quantified using Image Studio Lite Ver 5.0 following software instructions (LI-COR, Lincoln, NE). Samples (±BS³ or +BS³ alone) were run at least three times and averaged across runs. Changes in AMPAR surface and intracellular band densities were normalized to tubulin levels and expressed as the percentage of AL control (–BS³ for ±BS³ gels or +BS³ for +BS³ gels) on each gel.

Subcellular fractionation and Western blotting to measure PSD abundance

Protease inhibitor cocktail and phenylmethanesulfonyl fluoride (PMSF) were added to 0.32 M sucrose solution containing 1 mM NaHCO₃, 1 mM MgCl₂, and 0.5 mM CaCl₂ (Solution A). Brain tissue was rinsed, homogenized, and subsequently diluted to 10% weight/volume in Solution A. After being well mixed, 50 μl of the whole-cell homogenates were stored at –80 °C until use.

The whole-cell homogenate was centrifuged at 2000 *g* for 10 min, after which intact cells and nuclei formed a pellet at the bottom of the tube. The supernatant was saved, and the pellet was resuspended in Solution A. The homogenate was again centrifuged at 1400 *g* for 10 min. The supernatant was collected and combined with the previously collected supernatant. They were centrifuged together at 1400 *g* for 10 min and then at 13,800 *g* for 30 min. The pellet was collected and homogenized in 0.32 M sucrose solution containing 1 mM of NaHCO₃, protease inhibitor cocktail, and PMSF (Solution B). This homogenate was placed on a sucrose gradient and centrifuged for 2-h at 82,500 *g*. The synaptosomal layer was collected from the interface of the 1 and 1.2 M sucrose layers. The sample was then resuspended in Solution B and centrifuged at 82,500 *g* for 45 min. After centrifugation, the upper liquid was discarded, and the pellet was resuspended in a solution of 25 mM Tris, at pH 7.4.

To isolate the PSD fraction, an equal volume solution containing 1% Tritron X-100, 0.32 M sucrose, and 12 mM Tris, at pH 8.1 was added to the resuspended sample. The mixture was then rocked at 4 °C for 15 min, followed by centrifugation at 13,800 *g* for 30 min. After centrifugation, the upper liquid was discarded, and the pellet was resuspended in a solution of 25 mM Tris, at pH 7.4 with 2% SDS and stored at –80 °C until use.

This method of PSD purification yields a fraction enriched in proteins that are preferentially localized or novel to the PSD (Jordan *et al.*, 2004). To validate the purity of our PSD fraction and rule out contamination of endosomal membranes where AMPARs are interchanged, we probed for the early endosomal marker, rab5.

Proteins (6–10 µg/lane) were separated by electrophoresis on precast 4–12 % sodium dodecyl sulfate polyacrylamide gels (Lonza, Rockland, ME). Precision Plus Protein standard molecular weight markers (Bio-Rad) were loaded to estimate the size of the target proteins and to ensure complete transfer of proteins from gel to membrane. Proteins were electrophoretically transferred to Protran nitrocellulose membranes (Whatman, Mobile, AL) for 1.75 h at a constant voltage of 100 V. Membranes were blocked for 60 min with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) with shaking at room temperature. Membranes were then probed overnight at 4 °C using primary antibodies for target proteins or the protein loading control, α -tubulin. Primary antibodies were diluted in Odyssey Blocking Buffer with 0.2 % Tween 20. After probing with primary antibodies and washing with TBST buffer (3×5 min), membranes were incubated for 1 h at room temperature with IRDye 680RD Goat anti-Mouse IgG (H+L) and IRDye 800CW Goat anti-Rabbit IgG (H+L) (1:16,000; LI-COR Biosciences) in Odyssey Blocking Buffer with 0.2 % Tween 20. Proteins were visualized using an Odyssey CLx infrared imaging system, and bands were quantified using the Image Studio software (LI-COR Biosciences). At least 3 gels were run to obtain averaged results for each target protein in each diet group and separate gels were run for NAc and CPu. Target proteins were normalized to total tubulin in the corresponding lane. Primary antibodies used included mouse monoclonal anti-GluA1 (1:1000; MAB2263, Millipore, Temecula, CA), rabbit polyclonal anti-phospho-Ser845-GluA1 (1:1000; AB5849, Millipore), rabbit polyclonal anti-GluA2 (1:1000; PA1-4659, Thermo Scientific, Rockford IL), mouse monoclonal anti-CaMKII- α (1:1000; 05-532, Millipore), rabbit polyclonal anti-CaMKII- β (1:1000; ab34703, Abcam, Cambridge, MA), rabbit polyclonal anti-calceineurin (1:10,000; ab3673, Abcam), rabbit polyclonal anti-rab5 (1:1,000; ab18211, Abcam), and mouse monoclonal anti- α -tubulin (1:10,000; T6199, Sigma-Aldrich, St. Louis, MO).

Electrophysiology

Rats were deeply anaesthetized with ketamine (100 mg/kg, i.p.), decapitated, and the brain quickly placed into ice-cold dissection buffer containing (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7 MgCl₂ 6 H₂O, 25 NaHCO₃, 10 dextrose, bubbled with 95% O₂ / 5% CO₂ (pH 7.4). Coronal slices of the nucleus accumbens (300 µm) were prepared with a vibratome (Leica, VT1200S), placed in room temperature artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1.5 MgSO₄ 7H₂O, 26 NaHCO₃, and 10 dextrose) for <30 min; then warmed and kept at 33–35°C for >1 hr before use. For experiments, slices were transferred to the recording

chamber and superfused ($2.0\text{--}2.5\text{ ml min}^{-1}$) with oxygenated ACSF at $33\text{--}35^\circ\text{C}$ containing $50\text{ }\mu\text{M}$ picrotoxin, to block inhibitory transmission, to isolate EPSCs.

Somatic whole-cell recordings were made from shell region medium spiny neurons in voltage-clamp with a Multiclamp 700B amplifier (Molecular Devices) using IR-DIC video microscopy. Patch pipettes ($4\text{--}6\text{ M}\Omega$) were filled with intracellular solution (in mM: 125 Cs-gluconate, 2 CsCl, 5 TEA-Cl, 4 Mg-ATP, 0.3 GTP, 10 phosphocreatine, 10 HEPES, 0.5 EGTA, and 3.5 QX-314). Data were filtered at 2 kHz, digitized at 10 kHz, and analyzed with Clampfit 10 (Molecular Devices). Extracellular stimulation ($0.01\text{--}1\text{ ms}$, $5\text{--}150\text{ }\mu\text{A}$, 0.2 Hz) was applied with a small glass bipolar electrode $0.05\text{--}0.5\text{ mm}$ from the recording electrode. After $>5\text{ min}$ of baseline recording, a solution containing the GluA2-lacking, CP-AMPA antagonist Naspm ($200\text{ }\mu\text{M}$) was superfused for 10 min. Changes in excitatory postsynaptic current (EPSC) amplitude were measured before and after drug application at a holding potential of -70 . Spontaneous EPSCs in these recordings were detected automatically with a template EPSC, and the average of all spontaneous event amplitudes and rates during the entire duration of each recording was computed. TTX was not included in the bath for these measurements.

Cannula implantation and intracerebral microinjection

Rats were anesthetized with ketamine (100 mg/kg , i.p.) and xylazine (10 mg/kg , i.p.) and stereotaxically implanted with two chronically indwelling guide cannulas (26 ga ; Plastics One, Roanoke, VA) which were placed bilaterally 2.0 mm dorsal to injection sites in the NAc medial shell (1.6 mm anterior to bregma; 2.1 mm lateral to the sagittal suture, tips angled 8° toward the midline, 5.8 mm ventral to skull surface). Cannulas were permanently secured to the skull by flowing dental acrylic around them and four jeweler's screws

Solutions were loaded into two 30 cm lengths of PE-50 tubing attached at one end to $25\text{-}\mu\text{l}$ Hamilton syringes filled with distilled water and at the other end to 31-gauge injector cannulas, which extended 2.0 mm beyond the implanted guides. The syringes were mounted on the twin holders of a Harvard 2272 microliter syringe pump which delivered the $0.5\text{ }\mu\text{l}$ injection volumes over a period of 100 s . One minute following completion of injections, injector cannulas were removed from guides, stylets were replaced, and animals were returned to test chambers where the post-injection locomotor activity test began 5-min after completion of the microinjections.

Histology

Upon completion of behavioral testing, rats were euthanized with CO_2 and decapitated. Brains were removed and fixed in 10% buffered formalin for at least 48 hr. Frozen coronal sections, $40\text{ }\mu\text{m}$ thick, were cut on a Reichert-Jung Cryostat, thaw-mounted on gelatin coated glass slides and stained with cresyl violet. Microinjection sites were determined by visual inspection of sections under an Olympus SZ40 microscope. Data obtained from subjects that did not have cannulas accurately placed bilaterally in the NAc shell, or on the shell/core or shell/olfactory tubercle border, were eliminated from the analysis. Accordingly, two subjects from each of the diet groups were removed from the study.

Drugs

SKF-82958 (Sigma-Aldrich) was dissolved in a small volume of DMSO and diluted with sterile 0.9% saline (final DMSO concentration 5%) and microinjected bilaterally in a dose of 2.0 µg or 5.0 µg. Quinpirole (Sigma-Aldrich) was dissolved in sterile 0.9% saline and microinjected bilaterally in a dose of 2.0 µg or 5.0 µg. 1-Naphthylacetyl spermine (Naspm; Sigma-Aldrich) was dissolved in sterile 0.9% saline and microinjected bilaterally in a dose of 25.0 µg alone or in combination with SKF-82958 or quinpirole. Drug doses were selected based on their previous effectiveness in differentiating AL and FR subjects in studies of electrical brain stimulation reward and/or pilot testing (Carr *et al.*, 2010, Peng *et al.*, 2014).

Locomotor activity testing

Following recovery from surgery, twelve of the twenty four subjects to be tested for effects of the D-1 **receptor** agonist, SKF-82958, were assigned to the food restriction regimen. During the 3-4 week period required for attainment and stabilization of target body weight, subjects in both diet groups were habituated to handling, transport between vivarium and laboratory, gentle restraint on a foam cushion and a mock microinjection procedure. In the week preceding the experiment, subjects underwent four habituation sessions in the locomotor activity test chamber, on separate days, which were identical to actual test sessions in format and duration, with the exception that mock intracerebral microinjections were made.

Activity was monitored in a Lucite test chamber (25.4 × 30.5 × 30.5 cm) with a textured plastic floor. Automated data collection was accomplished by 12 infrared photo beam detectors along the length of the test chamber plus 12 beams on the vertical axis (Accuscan, Columbus, OH). Each test session began with a 15 min pre-injection measurement of horizontal activity and stereotypy in the test chamber, followed by removal and intracerebral microinjection, which was followed by a 30 min post-injection measurement.

Over a 3 week period, subjects underwent 6 test sessions. In the first two test sessions, conducted 3-4 days apart, subjects received bilateral microinjection of SKF-82958 (2.0 µg) or SKF-82958 in solution with 1-naphthylacetyl spermine (Naspm; 25.0 µg; Sigma-Aldrich) with counterbalancing of treatment order. In the second set of two test sessions subjects received bilateral microinjection of SKF-82958 at the higher dose of 5.0 µg with or without Naspm (25.0 µg). Again, treatment order was counterbalanced and sessions were conducted 3-4 days apart. No effects of vehicle or Naspm alone were expected based on previous NAc shell microinjection studies (Carr *et al.*, 2010; Peng *et al.*, 2014), however, one week after completion of testing with SKF-82958, two additional sessions were run in which subjects were microinjected with Naspm (25.0 µg) or vehicle (5% DMSO in sterile 0.9% saline).

Following observation that Naspm reversed the enhanced responses of FR rats to microinjection of SKF-82958, a new group of 8 AL and 9 FR rats were prepared and tested for the effects of the D-2 **receptor** agonist, quinpirole, at doses of 2.0 and 5.0 µg, with and without Naspm (25.0 µg).

Statistical analysis

Western blot and electrophysiological results were analyzed by t-tests for independent or matched samples as appropriate. Behavioral results were analyzed by 3-way or 2-way mixed design ANOVA.

Data obtained in this study and summarized in Results are available at: <https://figshare.com/s/11e39d2364e5f57018e1>.

Results

Food restriction increases surface expression of GluA1

In the absence of BS³ cross-linker, immunoblot analysis of AMPARs in FR rats revealed a small increase in the level of the monomeric form of GluA1 ($9.5 \pm 1.6\%$, $t(16)=3.08$, $p<.01$) but not GluA2 ($t(16)=1.17$, $p=.26$), when compared to AL fed rats (Fig 1A and C). BS³ treatment of paired, bilateral NAc led to the detection of high-molecular weight species, indicative of surface AMPAR expression, with a corresponding decrease of intracellular (I) AMPARs (Fig 1B and D).

Detection of surface AMPARs with BS³ treatment revealed the presence of an approximate 600kDA and 300kDA band, the latter conflicting with previously reported findings (Boudreau & Wolf, 2005). However, Lv XF *et al.* (2015) reported the formation of multiple high-molecular weight aggregates suggesting that slight differences in methodology, including differences in primary antibodies employed, may yield detection of various quaternary structures.

Further evaluation of BS³ cross-linked samples suggested a trend of decreased intracellular GluA1 in FR rats ($9.9 \pm 3.7\%$, $p=.19$) with a significant increase in surface expression (S) of GluA1 ($22.5 \pm 7.9\%$, $t(18)=2.16$, $p<.05$) but not GluA2 ($t(18)=0.85$, $p=.41$) in FR rats relative to AL rats (Fig 2A and C). Increased surface expression (S) of GluA1 was associated with a significant increase in surface/intracellular (S/I) ratio ($22.2 \pm 8.0\%$, $t(18)=2.17$, $p<.05$) while S/I ratio of GluA2 was unchanged ($t(18)=.86$, $p=.40$). Moreover, total AMPAR expression (surface + intracellular) again showed a slight increase in GluA1 ($10.7 \pm 3.5\%$, $t(18)=2.1$, $p<.05$) with no change in GluA2 ($t(18)=.24$, $p=.81$). Together, these findings suggest that the FR-induced increase in surface GluA1 reflects a shift of GluA1 from intracellular to surface compartments, and is associated with a slight increase in total GluA1.

Food restriction increases GluA1 abundance in the PSD

The method of subcellular fractionation used in this study is a standard protocol that yields a fraction enriched in proteins preferentially localized or novel to the PSD, and in which endosomes are disrupted by detergent, solubilizing endosomal AMPA receptors (Jordan *et al.*, 2004). Purity of the current PSD fraction was confirmed by probing for the endosomal marker, rab5, and demonstrating little or no contamination (Fig 3).

In the NAc PSD, FR rats displayed elevated levels of GluA1 ($t(10)=3.49$, $p=.005$) and pSer845-GluA1 ($t(10)=2.9$, $p=.015$), but not GluA2 ($t(10)=1.54$, $p=.15$), relative to AL rats

(Fig 4, top left). None of these proteins differed between diet groups in the whole cell homogenate (GluA1: $t(10)=1.71$, $p=.11$; pSer845-GluA1: $t(10)=1.1$, $p=.29$; GluA2: $t(10)=1.31$, $p=.22$) (Fig 4, bottom left). The discrepancy from the slight increase in total GluA1 seen in BS³ cross-linked tissue (above) may result from differences in sample processing, particularly solubilization of the receptor and the centrifugation step to pellet insoluble material. Consequently, the case for FR altering synthesis or degradation of AMPARs is equivocal, while a FR-induced increase in synaptic incorporation and Ser845 phosphorylation of GluA1 is supported.

In the NAc whole cell homogenate (WC), FR rats displayed lower levels of CaMKII- α ($t(10)=5.06$, $p=.00004$), CaMKII- β ($t(10)=3.91$, $p=.003$), and calcineurin ($t(10)=3.34$, $p=.007$) relative to AL rats (Fig 4, bottom right). None of these differences between diet groups were seen in the PSD fraction (CaMKII- α ($t(10)=1.1$, $p=.30$); CaMKII- β ($t(10)=0.6$, $p=.58$); calcineurin ($t(10)=0.5$, $p=.63$) (Fig 4, top right). These results suggest that FR is associated with decreased Ca²⁺ signaling and decreased level of the phosphatase that dephosphorylates pSer845-GluA1.

The differences between diet groups seen in the NAc PSD were not seen in the CPu PSD (not shown; GluA1: $t(10)=1.8$, $p=.10$; pSer845-GluA1: $t(10)=0.7$, $p=.49$). Nor were the effects of FR on CaMKII- α or calcineurin in NAc seen in the CPu WC (CaMKII- α ($t(10)=0.87$, $p=.40$); calcineurin ($t(10)=1.56$, $p=.15$). Unlike NAc, in which FR decreased levels of CaMKII- β , levels were increased in CPu ($t(10)=2.67$, $p=.023$).

Food restriction induces CP-AMPA incorporation

Previous studies indicate that various changes in physiological state can lead to upregulation of synaptic CP-AMPA in NAc core neurons (Goffer *et al.*, 2013; Tukey *et al.*, 2013). Here we investigated whether FR increases CP-AMPA incorporation in NAc shell neurons, based on the critical involvement of this region in reward-related learning (Gambarana *et al.*, 2003) and reinforcing effects of abused drugs (Ikemoto, 2007). Whole-cell recordings were made from neurons in NAc shell brain slices from either FR or AL rats. EPSCs were isolated with picrotoxin (50 μ M) to block inhibitory transmission and evoked via extracellular stimulation. For each cell, the average reduction in EPSC amplitude over a 3-10 minute period after Nasp^m washin was compared to baseline. EPSCs in neurons from FR rats were much more sensitive to Nasp^m washin (200 μ M) than EPSCs recorded from neurons of AL animals. Specifically, EPSC amplitude was unaffected by Nasp^m in slices from AL animals ($t(8)=0.12$, $p=.9$) but reduced in slices from FR animals ($t(9)=3.2$, $p=.01$; Fig 5B). Summary data across all cells, expressed as percentage of control amplitude, indicated that EPSCs from AL animals were unaffected by Nasp^m ($t(11)=0.73$, $p=.47$), while EPSCs from FR animals were reduced compared to their own baseline ($t(6)=2.7$, $p=.02$) and compared to AL animals ($t(17)=2.1$, $p=.033$; Fig 5C). When spontaneous activity in these cells was examined, events recorded from FR animals had higher amplitudes ($t(20)=6.8$, $p<.0001$; Fig 5D, top) and were more frequent ($t(20)=4.8$, $p=.0001$; Fig 5D, bottom) than in cells from AL animals. However, Nasp^m washin did not significantly decrease spontaneous EPSC parameters in FR subjects (frequency $t(8)=1.39$, $p=.20$; amplitude ($t(8)=1.01$, $p=.38$).

CP-AMPARs mediate enhanced D-1 receptor-stimulated locomotor activity in food-restricted rats

In the 15-min monitoring/habituation periods that preceded each intracranial injection and behavioral test session, there were no differences in locomotor activity across test days ($F_{5,90}=0.5$, $p=.77$), between diet groups ($F_{1,18}=1.78$, $p=.2$), or interactions between test day and diet ($F_{5,90}=0.25$, $p=.93$). The locomotor-activating effect of SKF-82958 was dose-related ($F_{Dose;1,18}=44.3$, $p<.0001$), and greater in FR than AL rats ($F_{Diet;1,18}=10.58$, $p=.004$). Nasp_m decreased the response to SKF-82958 ($F_{Nasp\ m;1,18}=11.06$, $p=.009$), with a significant interaction between Nasp_m treatment and diet ($F_{Nasp\ m \times Diet;1,18}=5.73$, $p=.028$), reflecting the greater blocking effect of Nasp_m in FR relative to AL rats (Fig 6, top). Estimation of marginal means indicated that activity scores of FR rats in response to both doses of SKF-82958 in the absence of Nasp_m were outside the 95% confidence interval for scores obtained in the presence of Nasp_m. This was not the case for either dose of SKF-82958 in AL rats. Analysis of the follow-up test in which Nasp_m was compared to vehicle microinjection indicated that Nasp_m increased locomotor activity ($F_{1,18}=27.65$, $p<.0001$) with no effect of diet ($F_{1,18}=1.7$, $p=.21$) and no interaction between microinjection treatment and diet ($F_{1,18}=1.05$, $p=.32$).

Following completion of the experiment just described, a control experiment, with new subjects, was conducted to test whether Nasp_m also decreases behavioral responsiveness to D-2 receptor stimulation (Fig 7). The locomotor-activating effect of quinpirole (top) was dose-related ($F_{Dose;1,15}=8.97$, $p<.01$), and greater in FR than AL rats ($F_{Diet;1,15}=16.54$, $p<.001$). There was no main effect of Nasp_m ($F_{Nasp\ m;1,15}=0.07$) and no interaction between Nasp_m treatment and diet ($F_{Nasp\ m \times Diet;1,15}=2.1$, $p=.17$).

Discussion

Results of the present study confirm the prediction that FR increases surface expression and, specifically, the abundance of GluA1, but not GluA2, in the NAc PSD. The finding that FR also increased pSer845-GluA1 in the PSD is significant in several respects. Phosphorylation at Ser845 increases GluA1 peak current and channel open probability (Roche *et al.*, 1996; Banke *et al.*, 2000; Goel *et al.*, 2011), is a necessary step in the synaptic trafficking of GluA1 (Shi *et al.*, 2001; Esteban *et al.*, 2003; Man *et al.*, 2007; Goel *et al.*, 2011), maintains surface stability of CP-AMPARs (He *et al.*, 2009), and is a requirement for synaptic incorporation of GluA1 during both LTP (Esteban *et al.*, 2003; Oh *et al.*, 2006) and homeostatic synaptic scaling (Goel *et al.*, 2011; Kim & Ziff, 2014). Thus, a sustained increase in pSer845 may be instrumental in trafficking and maintaining the increased abundance of GluA1 in the PSD of FR rats.

The present electrophysiological results strengthen interpretation of the biochemical results as reflecting synaptic incorporation of GluA2-lacking, CP-AMPARs. In cultured neurons deprived of excitatory input, the amplitude and frequency of spontaneous mEPSCs are increased (Thiagarajan *et al.*, 2002, 2005), and are sensitive to Nasp_m (Thiagarajan *et al.*, 2005; Sutton *et al.*, 2006). Here, Nasp_m markedly and selectively decreased the amplitude of evoked EPSCs in MSNs of FR rats. FR was also shown to increase the amplitude and frequency of spontaneous EPSCs recorded from NAc medium spiny neurons (MSNs).

However, these changes in spontaneous activity were not significantly decreased by Nasp^m. The absence of Nasp^m effect on amplitude is somewhat surprising given that the density of CP-AMPA receptors should contribute to this amplitude. This result could indicate that additional mechanisms play a role at the level of synapses recruited during spontaneous versus evoked activity. For example, the synapses activated during spontaneous release but not those activated during evoked release could incorporate more Nasp^m-insensitive AMPA channels in FR rats. In support of this possibility, differential modulation of evoked and spontaneous activity have previously been reported (Scanziani et al., 1992; Owen et al., 2013).

Together with the biochemical data, the electrophysiological findings suggest that CP-AMPA receptors are absent from the PSD under basal conditions but are incorporated during FR. Moreover, they seem to account for the enhanced behavioral response of FR subjects to D-1 receptor stimulation. The selective effect of Nasp^m on the otherwise enhanced D-1 **receptor** agonist-induced locomotor activation in FR rats adds to previous findings that Nasp^m blocked the enhanced rewarding effects of drugs microinjected into the NAc shell of FR rats (Carr et al., 2010; Peng et al., 2014). The finding that Nasp^m, alone, moderately increased locomotor activity across diet groups is suggestive of a nonspecific effect; importantly it did not obscure the strong and selective decreasing effect of Nasp^m on locomotor activity induced by D-1 receptor stimulation in FR rats.

In addition to LTP, in which CP-AMPA receptors are incorporated into the synaptic membrane during the initial stage of targeted synaptic strengthening (Plant et al., 2006; Yang et al., 2010), more widespread synaptic incorporation of CP-AMPA receptors occurs in response to suppression of excitatory input to neurons in culture (Thiagarajan et al., 2005, 2007; Lee, 2012; Kim & Ziff, 2014) and cortical and brainstem sensory neurons *in vivo* (Goel et al., 2011; Jitsuki et al., 2011; Sarin et al., 2012). This homeostatic synaptic scaling is triggered by decreased Ca²⁺ signaling (Thiagarajan, 2002; Kim & Ziff, 2014), and decreased activity of the Ca²⁺-dependent phosphatase, calcineurin, which normally dephosphorylates pSer845-GluA1 and enables endocytosis. The resultant stabilization of pSer845-GluA1 increases synaptic expression of CP-AMPA receptors (Kim & Ziff, 2014). There is an interesting parallel in the current observations of decreased whole cell levels of CaMKII- α , CaMKII- β , and calcineurin, with increased pSer845-GluA1 in the PSD. In a prior study it was found that brief restraint combined with intracerebroventricular injection of either saline or D-1 **receptor** agonist produced greater phosphorylation of CaMKII in NAc of FR as compared to AL rats (Haberny & Carr, 2005b). The decreased levels of total CaMKII under basal conditions along with increased stimulus-induced phosphorylation suggest compensatory upregulation of the stimulated response. Thus, the present findings, in combination with key findings in the literature, suggest a mechanistic scheme to be investigated in continuing molecular studies. Specifically, the D-1 receptor, which regulates Ca²⁺ influx to MSN dendritic spines via NMDA and L-type Ca²⁺ channels (Moyer et al., 2007), may be in a predominant low affinity state relative to D-2 receptors (Richfield et al., 1989), and therefore subnormally stimulated by the low tonic extracellular (Pothos et al., 1995) and evoked DA release (Stouffer et al., 2015) in FR subjects. A compensatory synaptic incorporation of CP-AMPA receptors in D-1 receptor-expressing MSNs would be enabled, at least in part, by decreased calcineurin and increased pSer845-GluA1 in response to decreased Ca²⁺ signaling.

Behaviorally, the consequence would be increased incentive effects of food, drugs and cues that stimulate a DA surge. An important caveat to this speculative scheme is that phosphorylation and synaptic incorporation of GluA1 is regulated by calcineurin anchored to the postsynaptic scaffold protein, AKAP150 (Sanderson et al., 2012). In the present study, differences in calcineurin levels between diet groups were seen in the whole cell but not the PSD fraction where calcineurin is mediating these key regulatory activities.

Considering that food is the most essential and frequently consumed natural reward, particularly among rats that are individually housed in a predictable environment, it may be speculated that current observations reflect a homeostatic response to sustained reward deprivation. Interestingly, four other conditions associated with increased synaptic abundance of CP-AMPA receptors in NAc are cocaine abstinence (Boudreau & Wolf, 2005; Conrad et al., 2008), amphetamine abstinence (Jedynak et al., 2015), chronic pain (Goffer et al., 2013) and, most recently, withdrawal from 'junk food' (Oginsky et al., 2016). In cocaine abstinence these receptors are directly involved in escalation of cocaine-seeking and relapse (Conrad et al., 2008), while in chronic pain they ameliorate concomitant behavioral depression (Goffer et al., 2013). Chronic FR, psychostimulant withdrawal, junk food withdrawal, and chronic pain may all be viewed as aversive or reward-deficiency states and the resultant upregulation of AMPAR trafficking in NAc may be a common homeostatic response that facilitates resolution or restoration of motivational-affective balance. This hypothesis becomes especially plausible if increased CP-AMPA receptor abundance is associated with D-1 receptor-expressing MSNs. Optogenetic and chemogenetic analyses indicate that excitation of D-1 **receptor**-expressing MSNs increases behavioral activation and reward, while excitation of D-2 **receptor**-expressing MSNs exerts opposite effects (Ferguson et al., 2011; Lobo et al., 2011; Yawata et al., 2012). The present finding that Nasp^m decreased behavioral activation induced by D-1, but not D-2, **receptor** agonist microinjection in NAc shell provides support for the idea that insertion of CP-AMPA receptors might occur selectively in D-1 **receptor**-expressing MSNs. At the same time, the enhanced, but Nasp^m-insensitive, behavioral response to quinpirole in FR rats indicates that there is likely more than one neuroadaptation contributing to the net effect of FR on DA transmission and behavior. The recently demonstrated increase in D-2 receptor protein expression in NAc synaptoneuroosomes of FR rats points to a potential mechanistic basis of the enhanced response to quinpirole (Jones et al., 2016).

In summary, it is proposed that increased synaptic incorporation of CP-AMPA receptors during FR serves to enhance the behavioral response to acute reward stimuli. In addition, due to increased D-1 receptor signaling and Ca²⁺-dependent signaling via CP-AMPA receptors, reward stimulation may also have increased capacity to induce neuroplastic changes that ingrain the associated reward-directed behavior (Mangiavacchi & Wolf, 2004; Asrar et al., 2009; Witgen et al., 2010). In the wild, the adaptive value of upregulated reward learning and strengthening of food acquisition/consummatory behavior is clear. In an environment where severe dieting is voluntary or a component of an eating disorder, and there is occasional breakthrough gorging on palatable food, or use of pharmacological proxies, those behaviors may be subject to strengthening that endures, contributing to an increased vulnerability to overeating, bingeing, and/or abusing drugs.

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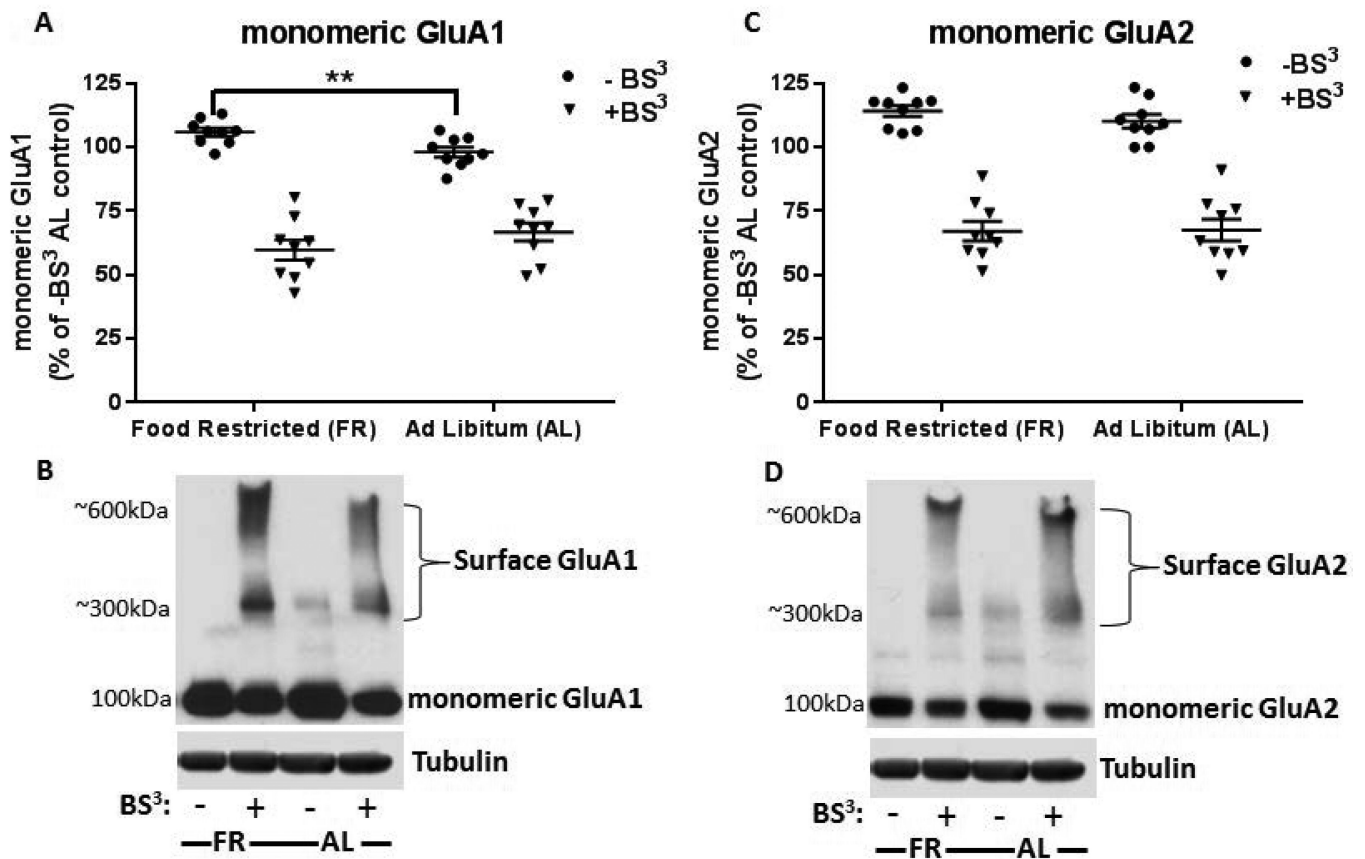


Figure 1.

Effect of chronic food restriction (FR) on AMPAR subunit expression and distribution in nucleus accumbens (NAc) as assessed by BS3 cross-linking studies. A and C: Quantification of monomeric AMPAR proteins, normalized to α -tubulin, revealed an increase in uncrosslinked (-BS3) monomeric GluA1 (A) but not GluA2 (C) with no change following BS3 cross-linking (+BS3) when compared to ad libitum (AL) fed control. B and D: Shown are representative immunoblots depicting the levels of uncrosslinked, monomeric AMPARs (total) while cross-linked AMPARs were observed as both high-molecular weight bands of 300- 600kDA (surface) and unmodified, monomeric bands of 100kDA (intracellular). Data obtained from individual subjects are expressed in comparison to the normalized control and displayed with mean \pm s.e.m, n=9 per group, **p<.01.

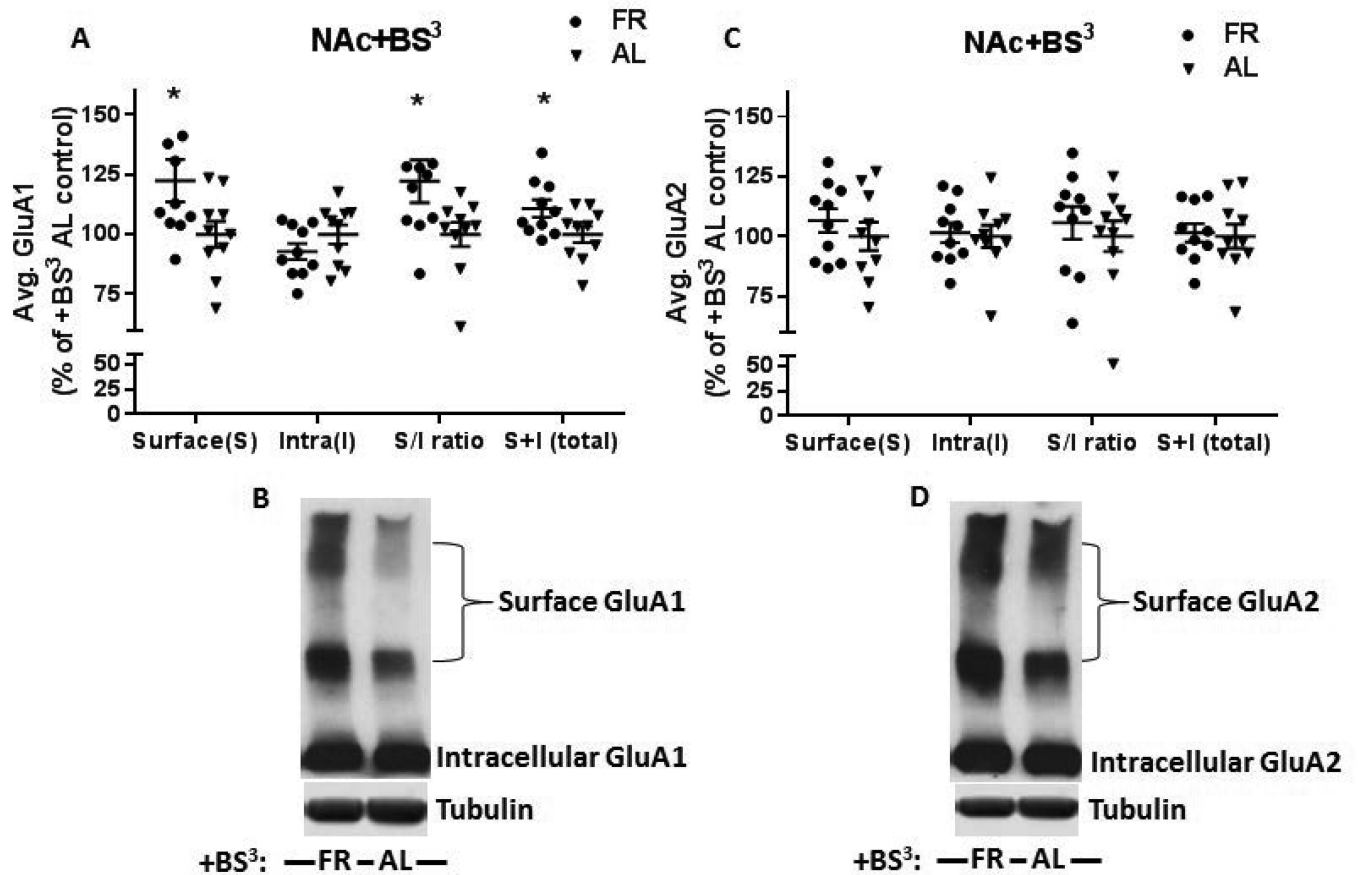


Figure 2.

BS3 cross-linking of NAc from FR and AL rats revealed differential distribution of AMPARs in surface (S) and intracellular (I) compartments. A and C: Quantification of GluA1 (A) showed increased surface (S) and total (S+I) expression with a concomitant increase in surface/intracellular (S/I) ratios. No significant change in intracellular (I) pools was observed in FR rats when compared to AL control. Band densities are normalized to α -tubulin and expressed as percentage of AL control. Evaluation of GluA2 (C) revealed no change in surface (S), intracellular (I), surface/intracellular (S/I) ratio, or total (S+I). B and D: Shown are representative immunoblots of BS3 cross-linked samples. Data obtained from individual subjects are expressed in comparison to the normalized control and displayed with mean \pm s.e.m., n=10 per group, *p<.05.

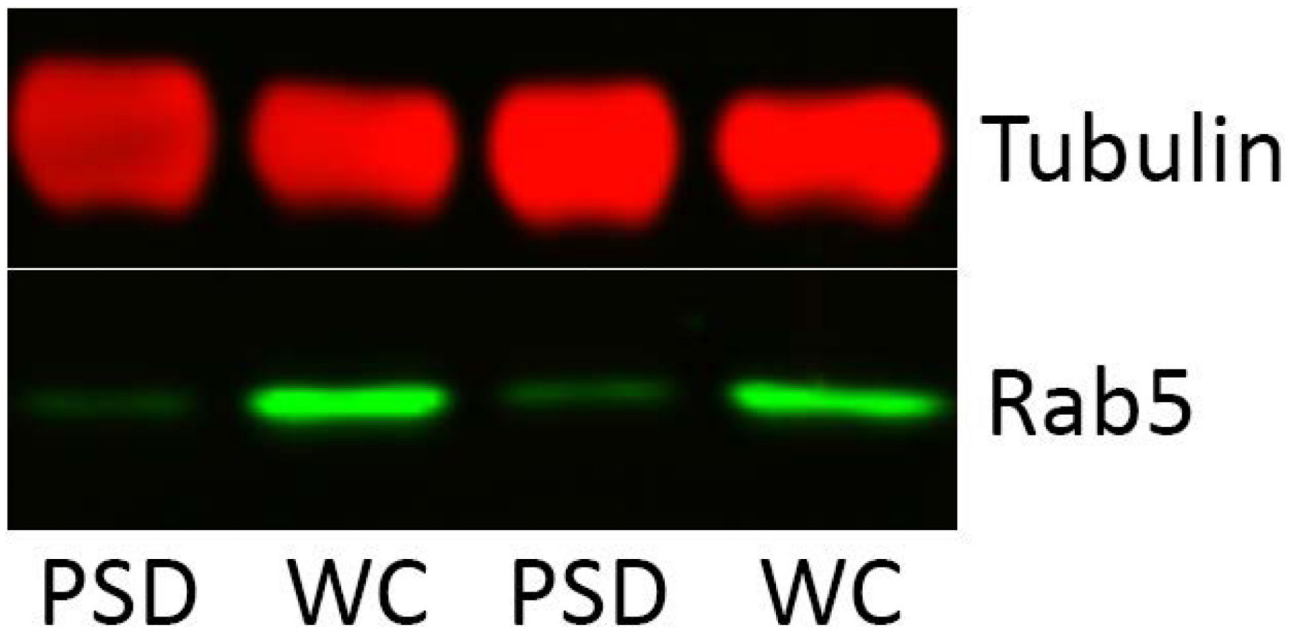


Figure 3. Whole cell homogenate (WC) and postsynaptic density fractions (PSD) of nucleus accumbens were probed for the endosomal marker rab5 to confirm purity of the PSD fraction.

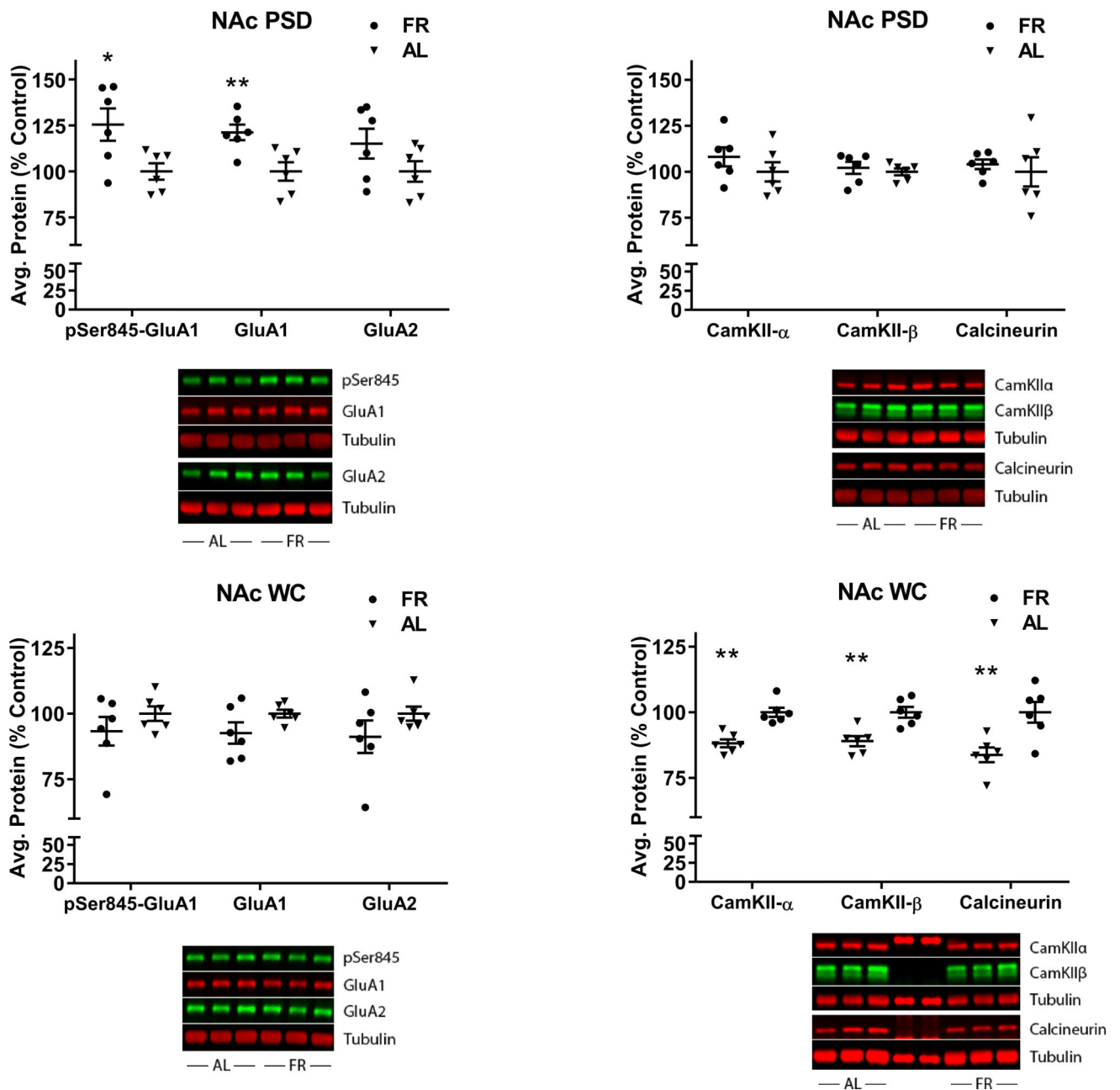


Figure 4.

Effects of chronic food restriction (FR) on abundance of pSer845-GluA1, GluA1, GluA2, CaMKII- α and - β , and calcineurin in the postsynaptic density (PSD) and whole cell homogenate (WC) fractions of nucleus accumbens (NAc). Following densitometry, intensities of bands corresponding to the target protein for each sample were divided by the intensities of the corresponding α -tubulin bands. Data obtained from individual samples are expressed in comparison to the normalized control and displayed with mean \pm s.e.m. Representative immunoblots are included beneath each graph. $n=18$ per group, with three NAc pooled per tube for fractionation. FR increased pSer845-GluA1 and GluA1 in the PSD

(top left) but not the WC (bottom left). FR decreased CaMKII- α and $-\beta$, and calcineurin in the WC (bottom right) but not the PSD (top right). * $p < .05$; ** $p < .01$.

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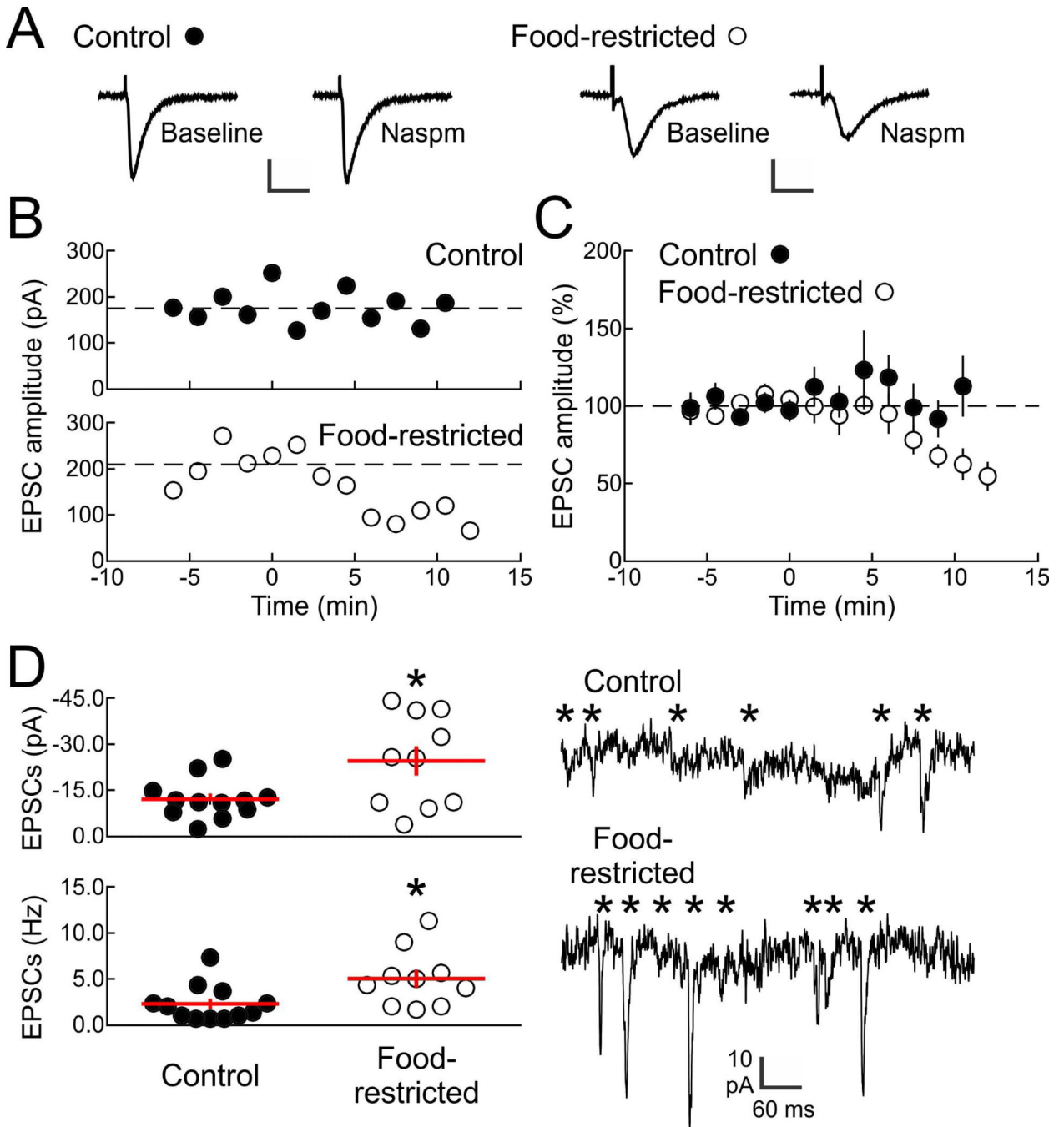


Figure 5. Incorporation of CP-AMPA receptors into NAc shell excitatory synapses after food restriction. **A:** Example evoked EPSCs from NAc shell neurons of AL control (left, scale: 30 ms, 100 pA) and FR (right, scale: 20 ms, 250 pA) rats. EPSCs were isolated in picrotoxin (50 μ M) and measured before and after Naspam washin (200 μ M). **B:** Time course of Naspam washin for example cell from AL control animal (top, filled circles) and FR animal (bottom, open circles). EPSCs from AL animal were unaffected by Naspam 3+ min after washin (baseline amplitude: 174.1 ± 9.9 pA, amplitude 3+ minutes after Naspam washin: 176.3 ± 13.2 pA,

t(8)=0.12, p=.9), while EPSCs from FR animal were reduced (baseline amplitude: 208.4±24.6 pA, amplitude 3+ minutes after NaspM washin: 117.1±16.4 pA, t(9)=3.2, p=.01). NaspM washin occurred at time 0. C: Summary data across all cells showing that EPSCs from AL animals were unaffected by NaspM 3+ min after washin (109.1±12.4% of control amplitude, n=12 neurons, t(11)=0.73, p=.47), while EPSCs from FR animals were reduced (70.6±11.0% of control amplitude, n=7 neurons, t(6)=2.7, p=.02 compared to baseline; t(17)=2.1, p=.033 compared to neurons from AL animals). NaspM washin occurred at time 0. D: Analysis of spontaneous EPSCs recorded in AL and FR animals. Top left: summary of mean spontaneous EPSC amplitudes (AL: -12.1±1.8 pA, FR: -24.6±4.8 pA, t(20)= 6.8, *p<.0001). Bottom left: summary of mean spontaneous EPSC frequencies (AL: 2.3±0.6 Hz, FR: 5.0±1.0 Hz, t(20)=4.8, *p=.0001). Right: example recordings of spontaneous EPSCs. * individual detected spontaneous events.

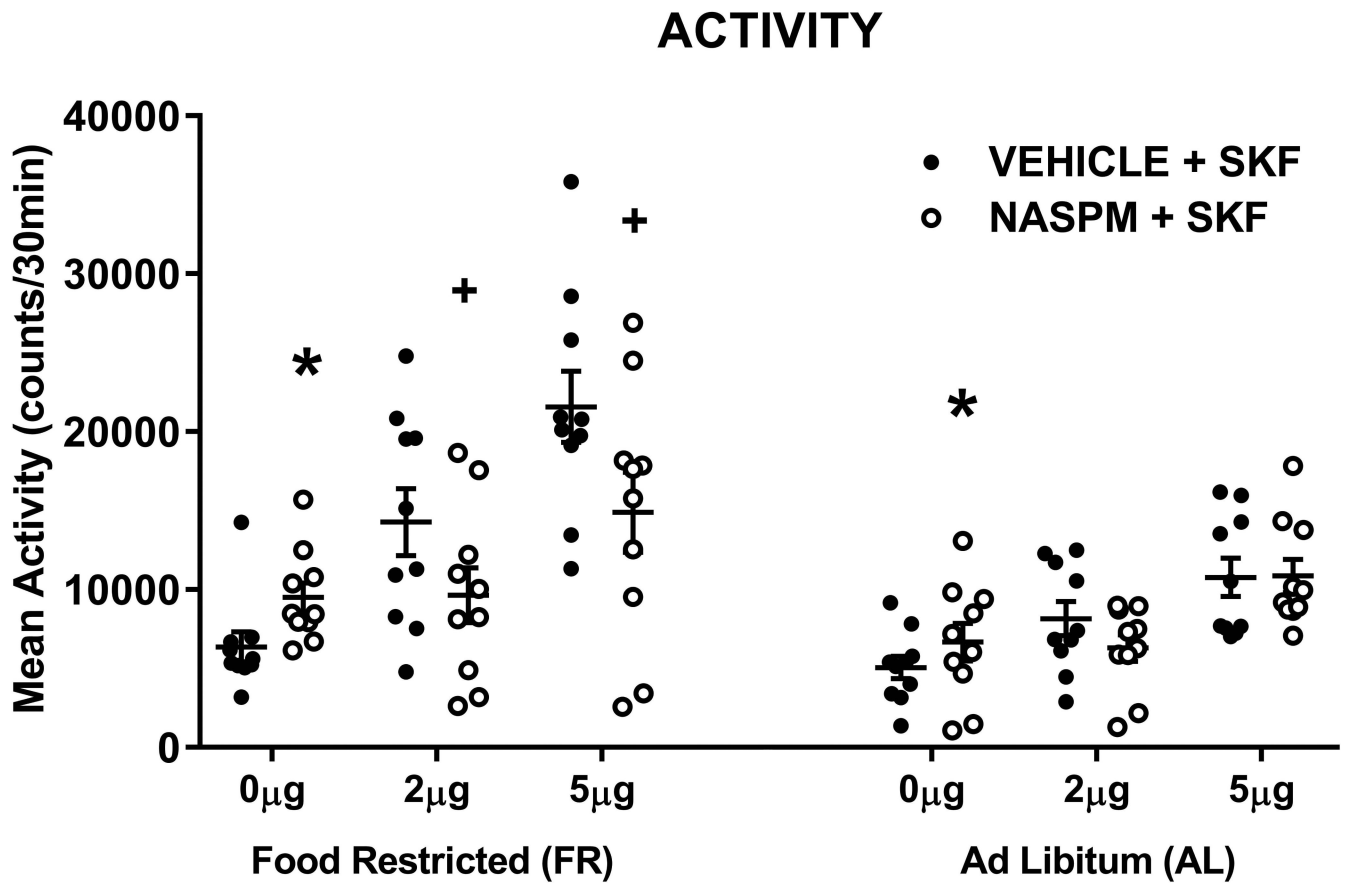


Figure 6. Effects of SKF-82958, microinjected in nucleus accumbens shell, in the absence and presence of Naspm (25.0 µg), on locomotor activity in food-restricted (FR) and ad libitum fed (AL) rats. Data from individual subjects are displayed with mean (\pm s.e.m.) counts shown for the 30-min post-injection tests. * Naspm increased locomotor activity relative to saline vehicle, $p < .0001$; + Naspm decreased SKF-induced locomotor activity, $p < .01$. $n = 10$ per diet group.

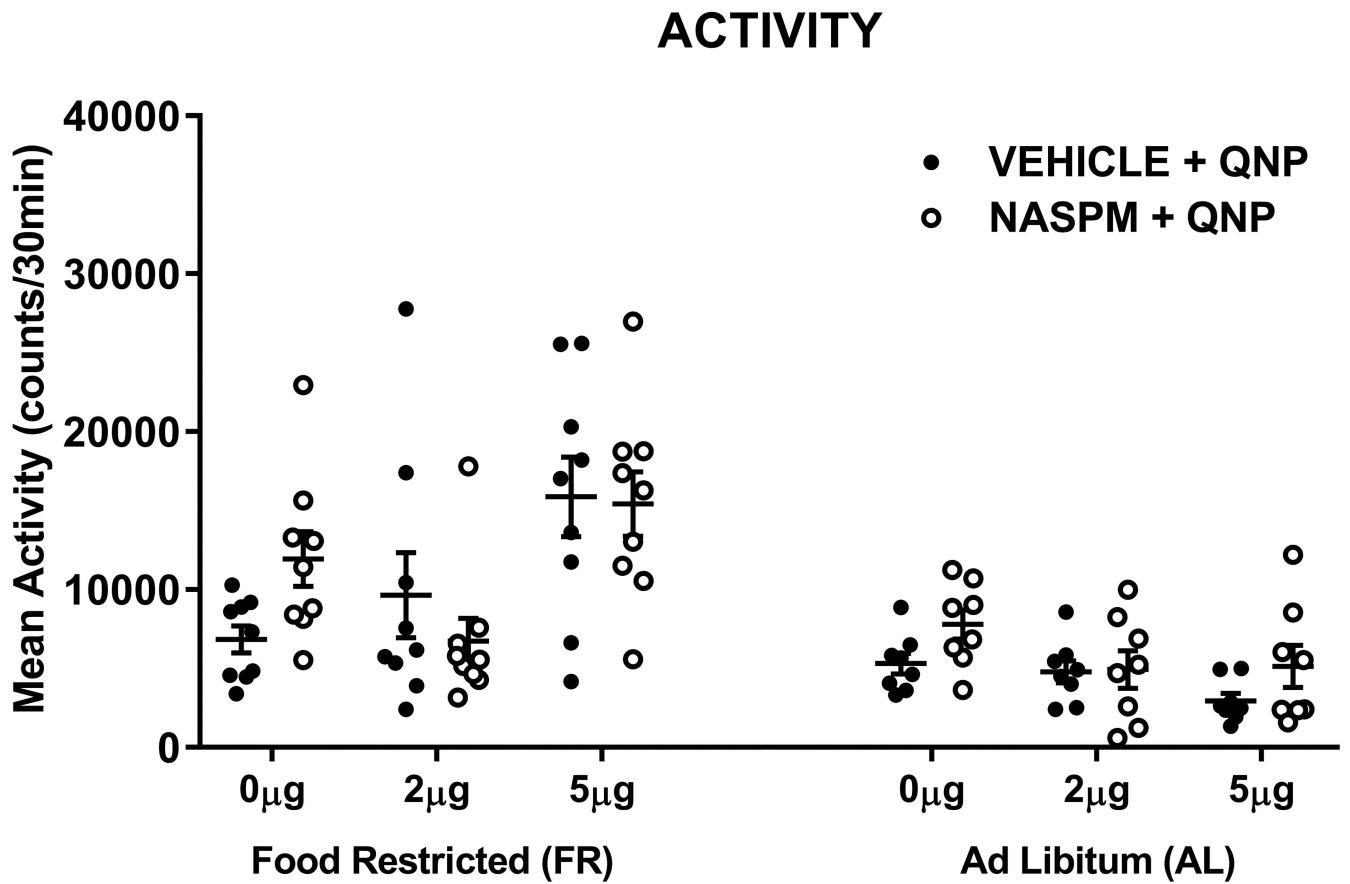


Figure 7. Effects of quinpirole, microinjected in nucleus accumbens shell, in the absence and presence of Naspm (25.0 µg), on locomotor activity in food-restricted (FR) and ad libitum fed (AL) rats. Data obtained from individual subjects are displayed with mean (\pm s.e.m.) counts shown for the 30-min post-injection tests. Naspm did not affect quinpirole-induced locomotor activity. $n=8$ (AL) and 9 (FR) per diet group.