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Trafficking of calcium-permeable and calcium-impermeable AMPA receptors in nucleus accumbens medium spiny neurons co-cultured with prefrontal cortex neurons

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Abstract

AMPA receptor (AMPA) transmission onto medium spiny neurons (MSNs) of the adult rat nucleus accumbens (NAc) is normally dominated by GluA2-containing, Ca²⁺-impermeable AMPAR (CI-AMPA)s. However, GluA2-lacking, Ca²⁺-permeable AMPA receptors (CP-AMPA)s accumulate after prolonged withdrawal from extended-access cocaine self-administration and thereafter their activation is required for the intensified (incubated) cue-induced cocaine craving that characterizes prolonged withdrawal from such regimens. These findings suggest the existence of mechanisms in NAc MSNs that differentially regulate CI-AMPA)s and CP-AMPA)s. Here, we compared trafficking of GluA1A2 CI-AMPA)s and homomeric GluA1 CP-AMPA)s using immunocytochemical assays in cultured NAc MSNs plated with prefrontal cortical neurons to restore excitatory inputs. We began by evaluating constitutive internalization of surface receptors and found that this occurs more rapidly for CP-AMPA)s. Next, we studied receptor insertion into the membrane; combined with past results, the present findings suggest that activation of protein kinase A accelerates insertion of both CP-AMPA)s and CI-AMPA)s. We also studied constitutive cycling (net loss of receptors from the membrane under conditions where internalization and recycling are both occurring). Interestingly, although CP-AMPA)s exhibit faster constitutive internalization, they cycle at similar rates as CI-AMPA)s, suggesting faster reinsertion of CP-AMPA)s. In studies of synaptic scaling, long-term (24 h) activity blockade increased surface expression and cycling rates of CI-AMPA)s but not CP-AMPA)s, whereas long-term increases in activity produced more pronounced scaling down of CI-AMPA)s than CP-AMPA)s but did not alter receptor cycling. These findings can be used to evaluate and generate hypotheses regarding AMPAR plasticity in the rat NAc following cocaine exposure.

Keywords

Nucleus accumbens; Medium spiny neurons; AMPA receptors; Trafficking

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1. Introduction

The nucleus accumbens (NAc), a key player in the reward circuitry of the brain, consists mainly of GABAergic medium spiny neurons (MSNs) (Meredith and Totterdell, 1999). MSNs are critical for motivated behavior, as they serve as an interface between limbic and motor systems (Sesack and Grace, 2010). AMPA-type glutamate receptors (AMPA receptors) provide the major source of excitatory drive to MSNs, which explains why cocaine-induced plasticity of AMPAR transmission has been linked to robust alterations in cocaine-related behaviors (Wolf and Ferrario, 2010; Wolf and Tseng, 2012).

AMPA receptors, which are tetramers of GluA1-4 subunits, can be classified based on whether they contain the GluA2 subunit. GluA2-containing receptors are Ca²⁺-impermeable AMPARs (CI-AMPA receptors), while those lacking GluA2 are higher-conductance, Ca²⁺-permeable AMPARs (CP-AMPA receptors; Isaac et al., 2007; Lee, 2012). CI-AMPA receptors predominate in NAc synapses of adult rodents under normal circumstances (Conrad et al., 2008; Kourrich et al., 2007; Reimers et al., 2011) and are upregulated following behavioral sensitization to cocaine (Boudreau et al., 2007; Boudreau and Wolf, 2005; Kourrich et al., 2007). Different AMPAR plasticity occurs in the ‘incubation of drug craving’ model, in which rats exhibit progressive intensification (incubation) of cue-induced drug seeking following withdrawal from extended-access drug self-administration (Lu et al., 2004). After prolonged withdrawal from extended-access cocaine self-administration, CP-AMPA receptors (predominantly homomeric GluA1 receptors) accumulate in excitatory synapses onto NAc MSNs (Conrad et al., 2008). Consistent with their higher conductance, CP-AMPA receptor accumulation increases the baseline responsiveness of NAc MSNs to excitatory drive (Purgianto et al., 2013), presumably enhancing the response of MSNs to cocaine-related cues. Accordingly, CP-AMPA receptor activation is required for expression of incubated cue-induced cocaine craving (Conrad et al., 2008; Lee et al., 2013; Loweth et al., 2014; Ma et al., 2014; Mameli et al., 2009; Wolf, 2016). Similar plasticity has recently been demonstrated in the NAc during the incubation of methamphetamine craving (Scheyer et al., 2016). These results suggest that CI-AMPA receptors and CP-AMPA receptors can be independently regulated in NAc MSNs in response to psychostimulant treatment. Indeed, subunit composition has been found to influence AMPAR trafficking in other cell types (Esteban et al., 2003; Hanley, 2014; Malinow, 2003; Passafaro et al., 2001; Shi et al., 2001; Xia et al., 2007).

The goal of the present study was to explore potential differences in CI-AMPA receptor and CP-AMPA receptor trafficking using an *in vitro* model system comprised of postnatal rat NAc neurons co-cultured with postnatal prefrontal cortex (PFC) neurons from enhanced cyan fluorescent protein (ECFP)-expressing mice. PFC neurons, which innervate NAc MSNs in the intact brain (Sesack et al., 1989), restore excitatory inputs to MSNs in the co-culture but can be distinguished from MSNs based on fluorescence (Sun et al., 2008). MSNs maintained in these co-cultures recapitulate a key aspect of the NAc after incubation of cocaine craving, namely high CP-AMPA receptor expression (Sun and Wolf, 2009). We have conducted extensive studies of AMPAR trafficking in this co-culture model (Reimers et al., 2014; Sun et al., 2008; Sun and Wolf, 2009; Wolf, 2010b), but have not systematically compared GluA1-containing receptors that include GluA2 (CI-AMPA receptors) to GluA1-containing receptors that

lack GluA2 (CP-AMPA). Here we used immunocytochemical assays to compare CI-AMPA and CP-AMPA trafficking in cultured NAc MSNs.

2. Methods

2.1. Animals

Pregnant Sprague Dawley rats (Harlan, Indianapolis, IN), obtained at 18–21 d of gestation, were housed individually in breeding cages. Postnatal day 1 (P1) pups were decapitated and used to obtain NAc neurons. PFC neurons were obtained from P1 homozygous ECFP-expressing mice [strain B6.129(ICR)-Tg(ACTB-ECFP)1Nagy/J; The Jackson Laboratory, Bar Harbor, ME]. The homozygous ECFP transgenic mouse strain was maintained by mating ECFP male and female mice in-house.

2.2. NAc/PFC co-cultures

As described previously (Sun et al., 2008), the medial PFC of ECFP-expressing P1 mice was dissociated with papain (20–25 U/mL; Worthington Biochemical, Lakewood, NJ) at 37 °C for 30 min. PFC cells were plated at a density of 30,000 cells/well onto coverslips coated with poly-D-lysine (100 µg/mL; Sigma-Aldrich, St. Louis, MO). One to three days later, the NAc from P1 rats was dissociated with papain as above, and plated at a density of 30,000 cells/well with the PFC cells. Co-cultures were grown in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM GlutaMAX, 0.5% Gentamicin and 2% B27 (Invitrogen), hereafter referred to as “media”. One-half of the media was replaced every 3–4 d. Co-cultures were used within 2–3 weeks after plating.

2.3. Drug treatments

Co-cultures were treated with 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX) (24 h; 20 µM; Tocris, Bristol, United Kingdom), (–)bicuculline methiodide (24 h; 20 µM; Tocris) or the membrane permeable protein kinase A (PKA) activator Spadenosine 3,5-cyclic monophosphorothioate triethylammonium salt (Sp-cAMPS; 15 min; 10 µM; Sigma-Aldrich) (all drugs dissolved in media). We showed previously that these CNQX and bicuculline treatments elicit synaptic scaling (Reimers et al., 2014; Sun and Wolf, 2009) and that this Sp-cAMPS treatment increases synaptic insertion of GluA1-containing AMPARs (Mangiavacchi and Wolf, 2004a; Sun et al., 2008) in cultured NAc MSNs.

2.4. Surface staining

Surface GluA1 and GluA2 were labeled by incubating live cultures with antibodies recognizing the extracellular N-terminal domains of GluA1 (N-GluA1; 1:10; PC246, aa 271–285; Calbiochem, San Diego, CA) and GluA2 (N-GluA2; 1:20; MAB397, aa 175–430; Millipore, Billerica, MA) in media (37 °C; 15 min). Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (10 min), blocked with 5% donkey serum in PBS (1 h) and incubated with Cy3-conjugated donkey anti-rabbit secondary (1:500; Jackson ImmunoResearch, West Grove, PA) and Alexa 488-conjugated donkey anti-mouse secondary (1:1000; Invitrogen) under non-permeabilized conditions (1 h).

2.5. Receptor cycling

We used a slight modification of a previously described method (Casimiro et al., 2011; Xia et al., 2006, 2007). First, cell surface GluA1 and GluA2 were labeled by incubating live cells with N-GluA1 (1:10) and N-GluA2 (1:20) antibodies in media (37 °C; 15 min). Antibodies were then removed and cells returned to the incubator to allow cycling to occur for 60 min before fixation under non-permeabilizing conditions (4% paraformaldehyde; 10 min). Controls were fixed at 0 min to quantify receptor surface expression prior to cycling. Cells were then blocked and incubated with Cy3-and Alexa 488-conjugated secondary antibodies, as described in Section 2.4, to detect remaining surface receptors.

2.6. Receptor internalization

Surface AMPARs on live neurons were labeled by incubating with N-GluA1 (1:10) and N-GluA2 (1:20) in media for 30 min. To minimize receptor trafficking, this was performed at 15 °C, using a 3% CO₂ refrigerated incubator (Tritech Research, San Diego, CA). Cells were then washed briefly with media to remove primary antibodies and brought to RT (5 or 30 min) to allow internalization of labeled AMPARs (controls were not brought to RT). Next, cells were fixed with 4% paraformaldehyde and incubated with nonconjugated goat anti-rabbit (1:50; Sigma) and goat anti-mouse (1:50; Abcam) secondary antibodies to mask the remaining surface AMPARs (i.e., AMPARs that did not internalize during the RT incubation). Cells were permeabilized (0.1% Triton X-100), blocked and incubated with Cy3-and Alexa 488-conjugated secondary antibodies (1 h) to detect newly internalized GluA1 and GluA2, respectively.

2.7. Receptor insertion

To selectively detect insertion of new GluA2-containing receptors onto the cell surface, we used a previously described modification (Sun et al., 2005) of a preblocking method (Lu et al., 2001). To block existing cell surface GluA2, live cells were incubated with N-GluA2 antibody (1:20; 30 min) followed by non-conjugated goat anti-mouse (1:50; 30 min) at 15 °C (to minimize receptor trafficking) in a 3% CO₂ refrigerated incubator. Cells were then brought to RT, in either control media or media containing SpcAMPS, to allow insertion of new AMPARs into the cell membrane. Next, cultures were washed and fixed with 4% paraformaldehyde in PBS (RT; 10 min). Newly inserted GluA2 was then detected by a second round of immunostaining with N-GluA2 antibody (RT; 1 h) and Alexa 488-conjugated secondary antibody (see Section 2.4).

2.8. Image analysis and statistics

Images were collected and analyzed as described previously (Sun et al., 2008). All experimental groups compared were from the same culture preparation and were processed simultaneously. Three to six cells per well from 4 to 6 wells were analyzed for each group. Analysis was restricted to processes of NAc MSNs, which can be distinguished from other NAc neuronal subtypes based on morphology and from ECFP-expressing PFC neurons based on fluorescence (Sun et al., 2008). MSNs were selected for analysis under phase contrast imaging to avoid experimenter bias based on the intensity of fluorescence staining. Images were acquired with a Nikon inverted microscope using a 40× oil objective, an

ORCA-ER digital camera and MetaMorph software (Universal Imaging). For each image, total area in a fixed length (15 μm) of process, located at least one soma diameter away from the soma, was measured using a threshold set based on average background fluorescence in unstained areas. The soma was excluded for all measurements. Data are presented as mean \pm SEM. Groups were compared with t-tests or two-way ANOVAs (significance set at $p < 0.05$).

3. Results

3.1. Experimental system

For all studies, we assessed AMPARs in NAc MSNs co-cultured with mPFC neurons (Fig. 1). We quantified homomeric GluA1 receptors by measuring the area of GluA1 staining that was not colocalized with GluA2 [GluA1(+)/GluA2(-)], hereafter referred to as CP-AMPAR staining. We quantified GluA1A2 receptors by measuring the area of GluA1/A2 colocalization [GluA1(+)/GluA2(+)], hereafter referred to as CI-AMPAR staining. The number of channels available on our fluorescence microscopy system limited us so that we were unable to stain for a synaptic marker as well as both GluA1 and GluA2 (see section 4.3 for more discussion). Furthermore, we acknowledge that our assessment of subunit colocalization is based on analysis in a single focal plane, rather than a 3-D reconstruction, but in the case of our surface expression studies, the assay is designed to exclusively detect cell surface receptors.

3.2. Constitutive internalization

Previously, we characterized constitutive and glutamate agonist-induced internalization of GluA1 in MSNs in cultures that contained only NAc neurons (Mangiavacchi and Wolf, 2004b). However, whether GluA1 was associated with GluA2 was not assessed. Here, we determined whether CP-AMPARs and CI-AMPARs differ in their rates of constitutive internalization using an assay that selectively detects newly internalized AMPARs (Section 2.6). Briefly, after labeling surface GluA1 and GluA2 with primary antibody, cells were brought to RT for 5 or 30 min to permit receptor internalization. After masking remaining surface receptors, newly internalized receptors were detected with fluorescent secondary antibody; controls (0-min group) were never brought to RT. We analyzed internalized area of GluA1, GluA2, CI-AMPARs and CP-AMPARs (Fig. 2).

Two-way ANOVA revealed a significant interaction of internalized area and time ($F_{(4,150)} = 2.47$, $p < 0.05$). Post-hoc comparisons (Tukey tests; see Fig. 2) indicated significant internalization of GluA1, GluA2 and homomeric GluA1 receptors over 30 min, whereas GluA1A2 CI-AMPARs showed only modest internalization that did not reach statistical significance. GluA2 internalization appeared most robust with significant increases occurring between 5- and 30-min time-points, whereas internalization of GluA1 and homomeric GluA1 receptors appeared to be plateauing more quickly. Interestingly, the amount of total internalized GluA1 at each time-point (first set of bars in Fig. 2b) roughly matches the sum of GluA1 internalized as a component of GluA1(+)/GluA2(+) receptors and a component of GluA1(+)/GluA2(-) receptors (third and fourth sets of bars, respectively). However, total internalized GluA2 (second set of bars) is substantially greater

than GluA2 internalized as a component of GluA1(+)/GluA2(+) receptors. The difference likely reflects internalization of GluA2/GluA3 receptors (see Discussion). Overall, these results suggest that homomeric GluA1 CP-AMPARs constitutively internalize at a faster rate than GluA1A2 CI-AMPARs.

3.3. Membrane insertion

We previously used a pre-blocking assay to show that GluA1 is constitutively inserted into NAc MSN membranes and that this insertion is accelerated by activating PKA to elicit GluA1 phosphorylation at serine 845 (Chao et al., 2002a, 2002b; Mangiavacchi and Wolf, 2004a; Sun et al., 2008; see Discussion). The assay involves masking surface GluA1 by incubating with primary GluA1 antibody followed by non-conjugated secondary antibody at 15 °C; then cells are warmed to RT to allow insertion of new AMPARs into the plasma membrane. Newly inserted GluA1 is detected with a second round of immunostaining, this time using a fluorescently conjugated secondary antibody.

Our original intention was to repeat these experiments but monitor both GluA1 and GluA2. Unfortunately, when we began these studies, we found that the anti-rabbit non-conjugated secondary antibody used to mask GluA1 in our previous insertion studies and in the internalization studies shown in Fig. 2 no longer effectively masked surface GluA1 (the internalization studies were the first experiments performed for this project and they were completed several years prior to attempting the insertion studies). Similar reagents from other suppliers also failed (data not shown). The inability to find an anti-rabbit non-conjugated secondary antibody that could mask surface GluA1 staining precluded analysis of GluA1 membrane insertion. However, we identified an effective anti-mouse non-conjugated secondary antibody and used this reagent, along with the mouse anti-GluA2 primary antibody, to monitor GluA2 insertion.

We compared cells that were never brought to RT (0 min group; controls), cells that were brought to RT for 15 min in normal media (15-min group) and cells that were brought to RT for 15 min in media containing the membrane-permeable PKA activator SpcAMPS (10 μ M; 15 min + SpcAMPS group) (Fig. 3). One-way ANOVA revealed a significant difference in area of GluA2 membrane insertion between the groups ($F(2,60) = 15.56$, **** $p < 0.0001$). Compared to modest constitutive insertion in the 0 min group, there was a trend towards increased GluA2 insertion in the 15 min group and robust insertion in the 15 min + SpcAMPS group; the latter group differed significantly from both the 15 min group (Tukey's, *** $p < 0.001$) and the 0 min group (Tukey's, **** $p < 0.0001$). Together with our earlier findings on GluA1 insertion, these results suggest that PKA accelerates membrane insertion of both GluA1A2 receptors and homomeric GluA1 receptors (see Section 4.4).

3.4. Changes in AMPAR surface expression during synaptic scaling

Synaptic scaling is a form of homeostatic plasticity in which a prolonged increase in activity weakens excitatory transmission, whereas prolonged activity blockade causes opposite effects (Fernandes and Carvalho, 2016; Lee, 2012; Lee et al., 2014; Turrigiano, 2012; Turrigiano and Nelson, 2004; Vitureira et al., 2012). Synaptic scaling stabilizes the activity of neuronal circuits against ongoing perturbations, including Hebbian plasticity. We

previously showed that CI-AMPA receptors but not CP-AMPA receptors undergo robust synaptic scaling in MSNs in NAc/PFC co-cultures (Reimers et al., 2014; Sun and Wolf, 2009). Here we repeated these studies to set the stage for new experiments asking whether the rate of CP-AMPA or CI-AMPA cycling is altered after synapses have scaled up or down (Section 3.5).

To elicit synaptic scaling, cells were incubated for 24 h in normal media (controls), the AMPA antagonist CNQX (to block synaptic activity) or the GABA_A receptor antagonist bicuculline (to increase synaptic activity through disinhibition). Then we measured GluA1 and GluA2 surface expression (Fig. 4). In agreement with prior findings (Sun and Wolf, 2009), CNQX scaled up surface GluA1 and GluA2 (Fig. 4b; GluA1, $t_{(40)} = 4.84$, **** $p < 0.0001$; GluA2, $t_{(40)} = 4.35$, **** $p < 0.0001$) and also increased surface CI-AMPA receptors (GluA1A2 colocalized area) but not CP-AMPA receptors (Fig. 4b; CI-AMPA receptors, $t_{(40)} = 5.65$, **** $p < 0.0001$; CP-AMPA receptors, $t_{(40)} = 0.74$, n.s.). Bicuculline scaled down surface GluA1 and GluA2, as well as surface CI-AMPA receptors (Fig. 4d; GluA1, $t_{(35)} = 4.02$, *** $p < 0.001$; GluA2, $t_{(35)} = 3.94$, *** $p < 0.001$; CI-AMPA receptors, $t_{(35)} = 4.15$, *** $p < 0.001$), as found previously (Reimers et al., 2014; Sun and Wolf, 2009). Bicuculline also produced a small decrease in surface CP-AMPA receptors ($t_{(35)} = 2.34$, * $p < 0.05$). In our prior study that used a different measure of surface CP-AMPA receptors (based on counting the number of immunoreactive puncta rather than measuring the area of immunoreactivity), we had observed a small bicuculline-induced reduction that did not achieve statistical significance (Sun and Wolf, 2009). Overall, these findings suggest that synaptic scaling in NAc MSNs is primarily mediated by changes in levels of CI-AMPA receptors.

3.5. Cycling under basal conditions and after long-term activity manipulation

Constitutive cycling refers to the net loss of receptors from the membrane under conditions where internalization and recycling are both occurring. In cycling assays, labeled cell surface AMPA receptors undergo endocytosis and either return to the membrane or are replaced by unlabeled receptors, resulting in a net decrease in cell surface labeling. A more robust decrease in labeling over a given time-period is indicative of a faster rate of cycling. Because constitutive cycling provides a measure of the fraction of the surface pool that is turning over, it is distinct from assays that measure the absolute amount of internalized receptor (e.g., Fig. 2). For the same reason, the rate of cycling determined with this assay is independent of the starting level of surface receptors. This is important in our experiments because we are comparing cycling rates under normal conditions, after scaling up, and after scaling down; starting levels of surface receptors differ substantially under these conditions (Fig. 4).

A prior study in cultured retinal ganglion neurons found that GluA2, but not GluA1, exhibited rapid cycling following a period of activity blockade with CNQX (Xia et al., 2007), suggesting that the level of prior synaptic activity can affect the subsequent rate of constitutive AMPA cycling in a subunit-specific fashion. To learn whether this is true for MSNs, we used an identical assay (Section 2.5) to compare constitutive cycling rates of CI-AMPA receptors and CP-AMPA receptors, and then determined if these rates are differentially affected following synaptic scaling. MSNs maintained in normal media (no synaptic scaling)

exhibited similar rates of constitutive cycling for GluA1, GluA2, CI-AMPA receptors and CP-AMPA receptors (Fig. 5a and b, open bars), indicating that the net effect of receptor internalization and re-insertion does not differ for these populations. We then performed the same experiment after incubation for 24 h with CNQX or bicuculline (these drugs were washed out before measuring cycling rates). Chronic activity blockade with CNQX (Fig. 5a, dark bars) increased cycling of GluA1, GluA2 and CI-AMPA receptors, but not CP-AMPA receptors (GluA1, $t_{(31)} = 3.30$, $**p < 0.01$; GluA2, $t_{(31)} = 3.50$, $**p < 0.01$; CI-AMPA receptors, $t_{(31)} = 2.97$, $**p < 0.01$; CP-AMPA receptors, $t_{(31)} = 0.87$, n.s.). In contrast, chronic stimulation of activity with bicuculline (Fig. 5b, dark bars) did not significantly alter cycling (GluA1, $t_{(39)} = 0.76$, n.s.; GluA2, $t_{(39)} = 1.89$, n.s.; CI-AMPA receptors, $t_{(39)} = 0.19$, n.s.; CP-AMPA receptors, $t_{(39)} = 0.25$, n.s.). Thus, scaling up increases cycling rates of CI-AMPA receptors but not CP-AMPA receptors, while scaling down has no effect.

4. Discussion

Expression of incubated cocaine craving after prolonged withdrawal depends upon the accumulation of CP-AMPA receptors in NAc MSN synapses (Conrad et al., 2008; Lee et al., 2013; Loweth et al., 2014; Ma et al., 2014; Mameli et al., 2009; Wolf, 2016). It is therefore important to understand mechanisms that selectively regulate CP-AMPA receptors versus the CI-AMPA receptors that normally predominate in these synapses. Here, we compared trafficking of CI-AMPA receptors and CP-AMPA receptors in MSNs using an NAc/PFC co-culture system (Fig. 1) that recapitulates one aspect of glutamate transmission in the NAc of animals that have undergone incubation of cocaine craving – high levels of surface-expressed homomeric GluA1 receptors (CP-AMPA receptors; Sun and Wolf, 2009). We measured constitutive internalization (Fig. 2), constitutive and PKA-stimulated insertion (Fig. 3), synaptic scaling (Fig. 4) and constitutive cycling (Fig. 5). In addition, we assessed the influence of prior synaptic scaling on the rate of cycling (Fig. 5). We found that: 1) CI-AMPA receptors show less constitutive internalization than CP-AMPA receptors, 2) GluA2-containing receptors (presumably GluA1A2) undergo constitutive insertion that is accelerated by PKA signaling, extending previous results obtained by measuring GluA1 insertion (see Section 3.3), 3) CI-AMPA receptors and CP-AMPA receptors constitutively cycle at similar rates, and 4) CI-AMPA receptors are preferentially involved in synaptic scaling and alterations in cycling that result from scaling. We acknowledge that electrophysiological recordings employing a selective blocker of CP-AMPA receptors will be required to functionally assess CP-AMPA receptors. Despite this caveat, we believe these findings add important information to the understanding of AMPAR trafficking in MSNs.

4.1. AMPAR subunit composition and trafficking

The importance of AMPAR trafficking for plasticity was first demonstrated by studies showing that trafficking of AMPARs in and out of synapses, through exocytosis and endocytosis, determines excitatory synaptic strength and contributes to long-term potentiation (LTP) and long-term depression (LTD; Malinow and Malenka, 2002), a concept expanded to include an important role for lateral diffusion in the plasma membrane (Choquet and Triller, 2013). The foundation of these processes is the cycling of AMPARs, i.e., their endocytosis, entry into the endosomal system and recycling back to the cell surface

(Henley et al., 2011; Shepherd and Huganir, 2007; van der Sluijs and Hoogenraad, 2011). Complex mechanisms regulate AMPAR recycling under different cellular conditions (e.g., Petrini et al., 2009; Zheng et al., 2015). During LTP, synaptic AMPAR levels increase through a two-step process involving AMPAR insertion into the membrane at extrasynaptic sites followed by translocation into the synapse. The first step is accelerated by PKA, via phosphorylation of GluA1 at serine 845, whereas the second step requires NMDAR stimulation. This two-step process has been demonstrated in multiple regions (Esteban et al., 2003; Gao et al., 2006; Man et al., 2007; Oh et al., 2006; Passafaro et al., 2001; Sun et al., 2005; Yudowski et al., 2007) including the NAc (Sun et al., 2008; see Wolf, 2010b for review). For many of the trafficking steps outlined above, it is unclear whether CP-AMPARs are selectively regulated or how this might occur, although it has been shown that they constitute a portion of the extrasynaptic storage pool that supplies the synapse in hippocampal neurons (Hanley, 2014; He et al., 2009; Yang et al., 2009), as well as the NAc (Ferrario et al., 2011a, 2011b). More generally, they participate in certain forms of LTP, LTD and synaptic scaling, as well as excitotoxicity associated with disorders such as epilepsy and Alzheimer's disease (Cull-Candy et al., 2006; Isaac et al., 2007; Lee, 2012; Liu and Zukin, 2007) and their selective trafficking has functional relevance for incubation of craving and other addiction models (Lüscher, 2013; Wolf, 2016; Wolf and Tseng, 2012) and for fear conditioning (Clem and Huganir, 2010).

4.2. Constitutive internalization

We found significant constitutive internalization of homomeric GluA1 CP-AMPARs over 30 min whereas very little internalization of GluA1A2 CI-AMPARs was observed (Fig. 2). While the basis for this is unclear, it may reflect looser coupling of homomeric GluA1 receptors to the postsynaptic density in the NAc (see Ferrario et al., 2011a). Furthermore, based on comparison of GluA1, GluA2, CI-AMPAR and CP-AMPAR internalization rates, it appears that GluA2-containing AMPARs that do not contain GluA1 (which are probably GluA2A3 receptors) undergo more rapid constitutive internalization than GluA1A2 receptors. GluA2A3 receptors were not studied here because our image analysis system can only analyze three fluorophores; a fourth would be necessary to detect GluA3 along with GluA1, GluA2 and the cyan fluorescence that identifies PFC neurons. However, we have previously demonstrated that GluA2A3 receptors exist in NAc MSNs (not studied here, but known to exist in NAc MSN; Conrad et al., 2008; Reimers et al., 2011). Some results indicate that AMPARs comprised of subunits with short C-termini (GluA2 or GluA3) undergo constitutive trafficking in and out of synapses, whereas AMPARs containing a subunit with a long C-terminus (e.g., GluA1) are inserted into synapses in an activity-dependent manner (Malinow, 2003; but see Lu et al., 2009). While our results may suggest more rapid constitutive internalization of GluA2A3 receptors than GluA1A2 receptors (above), the observation of constitutive internalization of homomeric GluA1 receptors, as well as our results on constitutive AMPAR cycling (Section 3.5), are not consistent with this scheme.

4.3. Constitutive receptor cycling

Receptor cycling represents the net effect of internalization and reinsertion. We found that GluA1A2 CI-AMPARs and homomeric GluA1 CP-AMPARs constitutively cycle at similar

rates over 60 min (Fig. 5). At first glance, this seems at odds with the observation that CP-AMPA receptors show more rapid constitutive internalization (Section 4.2), but the results can be reconciled if CP-AMPA receptors also recycle more efficiently than CI-AMPA receptors (i.e., internalized CP-AMPA receptors are more often returned to the membrane than CI-AMPA receptors). As noted above, we showed that CP-AMPA receptors in the adult rat NAc are enriched in extrasynaptic membrane fractions (Ferrario et al., 2011b), which is where insertion and internalization are believed to occur (Blanpied et al., 2002; Groc and Choquet, 2006; Sun et al., 2005). It is very likely that cultured MSNs also contain a pool of extrasynaptic CP-AMPA receptors (Sun and Wolf, 2009). We were not able to directly demonstrate the existence of this pool in our experiments, as four different fluorophores would be required to distinguish PFC cells from NAc cells (based on cyan fluorescence) and then assess colocalized and non-colocalized pools of GluA1, GluA2 and a synaptic marker.

4.4. Receptor insertion

We attempted to compare the rates of constitutive and PKA-stimulated insertion of GluA1A2 receptors and homomeric GluA1 receptors. We have previously shown that activation of PKA signaling in cultured NAc MSNs increases insertion of GluA1-containing AMPARs into extrasynaptic pools that supply the synapse (for review, see Wolf, 2010b). This was demonstrated by activating PKA directly (using SpcAMPS) or indirectly (using an agonist of the D1 dopamine receptor, which is positively coupled to PKA) (Chao et al., 2002a, 2002b; Ferrario et al., 2011b; Mangiavacchi and Wolf, 2004a; Sun et al., 2008). Unfortunately, the reagent previously employed to isolate newly inserted pools of GluA1 was no longer usable (see Results). However, we demonstrated constitutive and PKA-stimulated insertion of GluA2 into the plasma membrane of MSNs (Fig. 3). Since the effect of PKA activation on AMPAR surface insertion is mediated through GluA1 phosphorylation in MSNs (Chao et al., 2002a, 2002b) and other cell types (e.g., Esteban et al., 2003; Gao et al., 2006; Man et al., 2007; Oh et al., 2006; Sun et al., 2005), the increase in GluA2 insertion after PKA activation must reflect insertion of GluA1A2 AMPARs. Combined with prior results suggesting that homomeric GluA1 receptors are similarly regulated by PKA (Ferrario et al., 2011a, 2011b), these findings suggest that PKA activation increases insertion of both homomeric GluA1 receptors and GluA1A2 receptors. PKA phosphorylation of GluA1 also mediates scaling up of GluA1A2 receptors in hippocampal neurons (Diering et al., 2014).

4.5. CI-AMPA receptors undergo synaptic scaling and exhibit altered cycling after scaling up

Synaptic scaling is a form of homeostatic plasticity used by neuronal circuits to maintain stable function in response to destabilizing influences (Fernandes and Carvalho, 2016; Lee, 2012; Lee et al., 2014; Turrigiano, 2012; Turrigiano and Nelson, 2004; Vitureira et al., 2012). In neuronal cultures, scaling up of synaptic strength is typically elicited by long-term (1–2 days) activity blockade, while scaling down is elicited by long-term increases in activity. In many instances, scaling up is expressed via an increase in postsynaptic AMPAR levels, whereas scaling down is expressed via decreased AMPAR levels.

Synaptic scaling has been found to alter postsynaptic AMPAR expression differentially dependent on brain region. Scaling of both GluA1- and GluA2-containing receptors has been observed in cultured cortical neurons (Wierenga et al., 2005), spinal neurons (O'Brien et al.,

1998) and NAc MSNs (Sun and Wolf, 2009), whereas scaling up of GluA2-lacking receptors has been observed in hippocampal cultures (Ju et al., 2004; Sutton et al., 2006; Thiagarajan et al., 2005). Some studies suggest that the mechanism(s) by which activity is manipulated determines which AMPAR subtype(s) undergo scaling. When postsynaptic firing is blocked, using an AMPAR antagonist or tetrodotoxin, CI-AMPA receptors scale up, whereas CP-AMPA receptors scale up if this is combined with NMDA receptor blockade (Gainey et al., 2009; Sutton and Schuman, 2006; Turrigiano, 2008). However, this theory does not fit all results (Sun and Wolf, 2009; Sutton and Schuman, 2006; Thiagarajan et al., 2005).

We found that activity blockade (CNQX, 24 h) scaled up CI-AMPA receptors but had no effect on CP-AMPA receptors (Fig. 4b). Activity stimulation (bicuculline, 24 h) produced robust scaling down of CI-AMPA receptors but also had a small effect on CP-AMPA receptors (Fig. 4d). This is generally consistent with our previous study (Sun and Wolf, 2009), although in that study, chronic activity stimulation (bicuculline, 48 h) only produced a trend towards decreased surface CP-AMPA receptors in NAc MSN. This discrepancy is likely due to slightly different methods for analyzing surface expression (see Section 3.4). Together, these data suggest CI-AMPA receptors are preferentially involved in synaptic scaling in NAc MSNs. Thus, even though there is evidence that cocaine exposure and withdrawal lead to decreased metabolic activity in the NAc and in regions that provide glutamate inputs to the NAc, which could lead to scaling up in the NAc and thereby explain cocaine-induced increases in AMPAR levels (Wolf, 2010a), our results in cultured MSNs from young animals suggest that synaptic scaling alone is unlikely to account for selective upregulation of CP-AMPA receptors (Sun and Wolf, 2009 and present results). It remains possible, however, that other cocaine-induced adaptations alter the biochemical state of the MSN such that CP-AMPA receptors participate to a greater degree in synaptic scaling during the incubation of cocaine craving.

We also examined the effect of synaptic scaling on cycling rates, based on prior work showing that, following prolonged inactivity (CNQX), the cycling of GluA2-containing CI-AMPA receptors is selectively increased (Xia et al., 2007). Similarly, we found that CNQX treatment (24 h), which increased surface expression of CI-AMPA receptors (Fig. 4b), also increased cycling of CI-AMPA receptors but did not alter cycling of homomeric GluA1 CP-AMPA receptors (Fig. 5a). Scaling down, which decreased both CI-AMPA and CP-AMPA surface expression but had a much more pronounced effect on CI-AMPA receptors (Fig. 4d), had no effect on cycling rates (Fig. 5c). These data suggest that chronic decreases in excitatory transmission in the NAc will preferentially increase the cycling rate of CI-AMPA receptors, while chronic increases in excitatory transmission will not alter AMPAR cycling.

4.6. Conclusions

We found similarities and differences in the trafficking of CP-AMPA receptors and CI-AMPA receptors in cultured MSNs. Overall, CP-AMPA receptors are more labile under basal conditions, whereas CI-AMPA receptors are more important for synaptic scaling. These results provide fundamental information about AMPAR function in MSNs that can be used to develop and evaluate hypotheses for cocaine-induced plasticity. However, many factors complicate comparison of NAc/ PFC co-cultures to the NAc of rats that have undergone incubation of cocaine craving. One important factor is that the ages of the MSNs are very different. Rats that express

incubated cocaine craving following extended-access cocaine self-administration are >16 weeks old. In contrast, cultured MSNs are obtained on P1 and studied after 2–3 weeks *in vitro*. In other systems, young cultured neurons show higher expression of CP-AMPARs (Perkinton et al., 1999). It is possible that high CP-AMPAR levels in the NAc of “incubated rats” signal a return to a developmental-like state, as has been argued based on other neuroadaptations observed after cocaine exposure (Bellone and Lüscher, 2012; Dong and Nestler, 2014).

While the present studies focused on potential differences in CP-AMPAR and CI-AMPAR trafficking that could contribute to increased CP-AMPAR levels during incubation of cocaine craving, it is important to keep in mind that cocaine exposure alters a myriad of signaling pathways in NAc MSNs (Nestler, 2014; Russo et al., 2010). Signaling alterations may be superimposed on fundamental differences in CI-AMPAR versus CP-AMPAR trafficking to favor selective alterations in CP-AMPARs during incubation. For example, an increase in PKA phosphorylation of GluA1 may speed CP-AMPAR insertion (Ferrario et al., 2011b). Other types of contributing mechanisms have also been identified, including a decrease in mGluR1 surface expression during incubation that removes a braking influence on CP-AMPAR levels and thus enables their accumulation (Loweth et al., 2014). Intriguingly, a decrease in GluA2 RNA editing occurs in the NAc shell during cocaine abstinence that could increase the pool of GluA2-containing CP-AMPARs (Schmidt et al., 2015), although this cannot account for homomeric GluA1 CP-AMPAR accumulation in the NAc core. A challenge for future studies is to understand how these disparate mechanisms interact to regulate excitatory transmission in the NAc.

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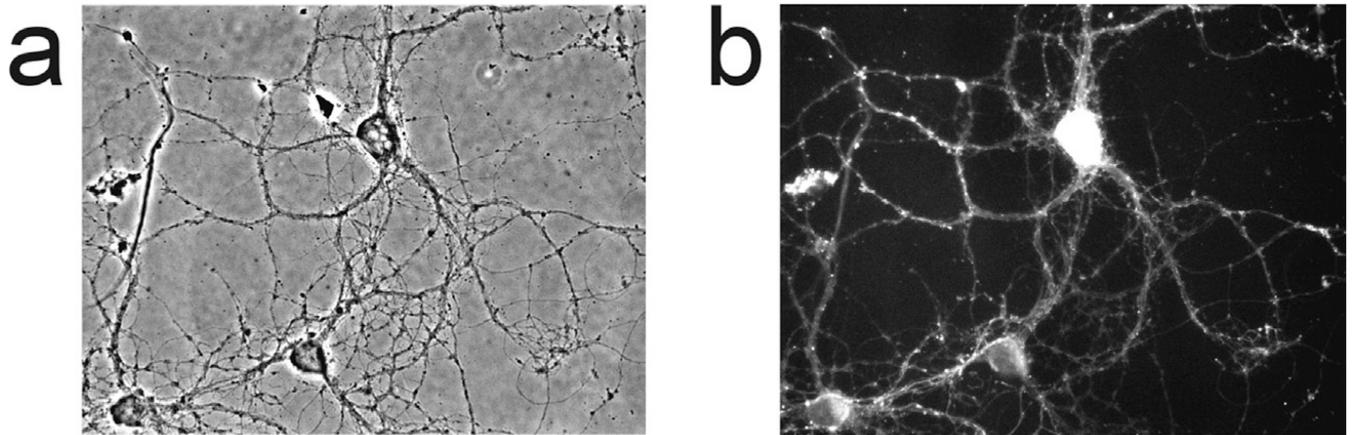


Fig. 1. NAc MSNs can be distinguished from PFC neurons in NAc/PFC co-cultures. (a) Phase contrast image. (b) Fluorescence microscopy image showing that PFC neurons (obtained from ECFP mice) exhibit cyan fluorescence, whereas NAc MSNs (obtained from rats) do not.

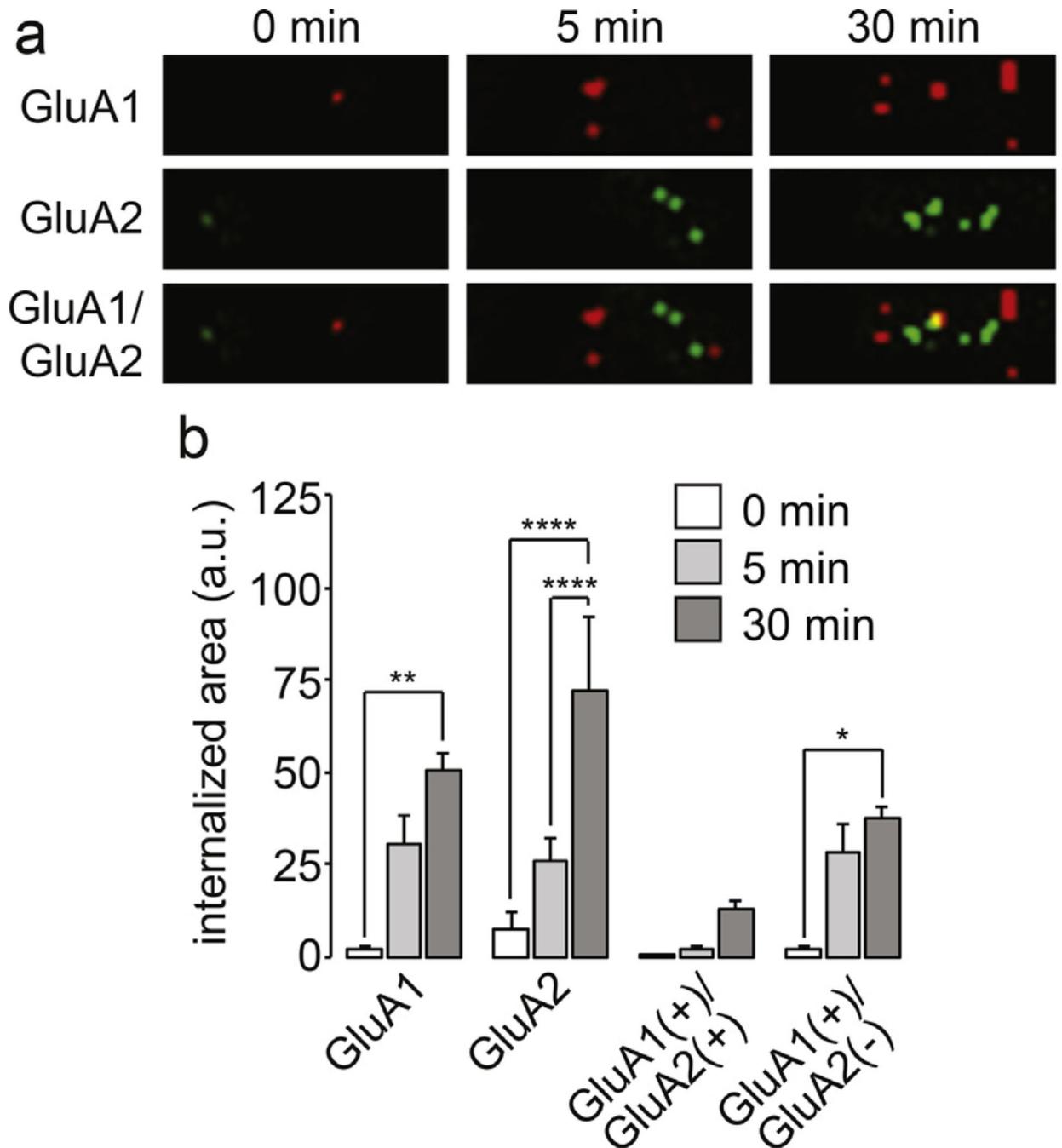


Fig. 2. CP-AMPARs exhibit more rapid constitutive internalization than CI-AMPARs. (a) Representative images of GluA1 and GluA2 co-immunostaining on medium spiny neuron dendrites in NAc/PFC co-cultures. (b) GluA1 showed significant internalization after 30 min, compared to the 0-min time-point. GluA2 showed significant internalization after 30 min, compared to both 0- and 5-min time-points. CI-AMPARs showed no significant internalization over 30 min. CP-AMPARs showed significant internalization after 30 min. Results are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, two-way

ANOVA followed by Tukey tests. $n = 13\text{--}20$ cells/group. In this and subsequent figures, we used the area of GluA1/GluA2 colocalization as a measure of CI-AMPA receptors [GluA1(+)/GluA2(+)] and the area of GluA1 staining that did not overlap with GluA2 staining [GluA1(+)/GluA2(-)] as a measure of homomeric GluA1 CP-AMPA receptors.

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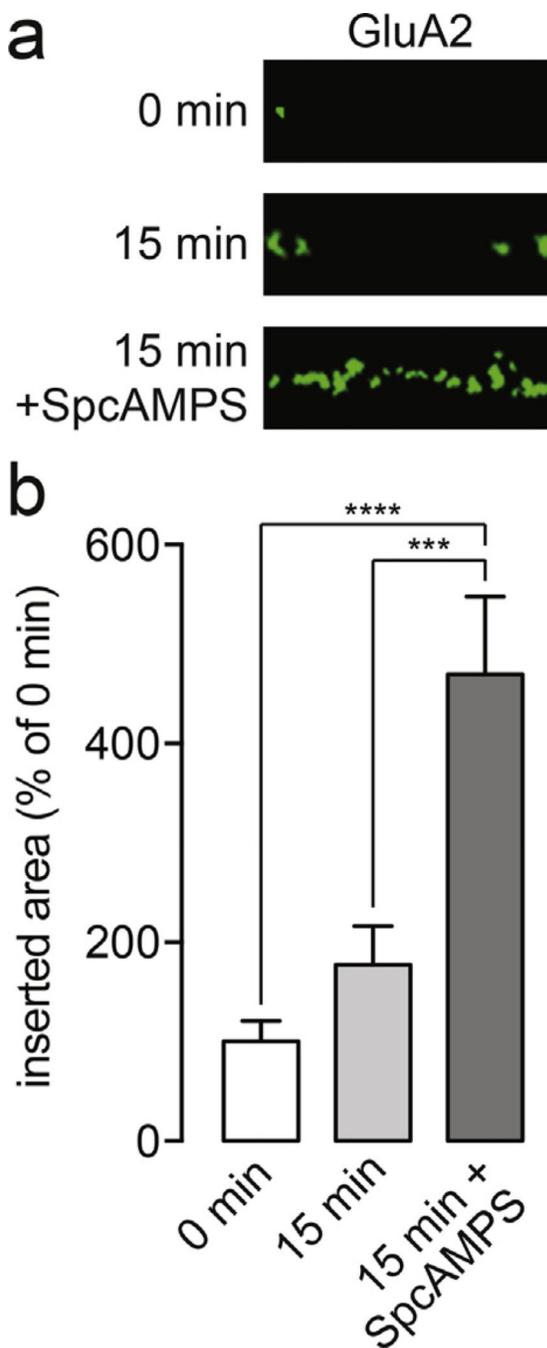
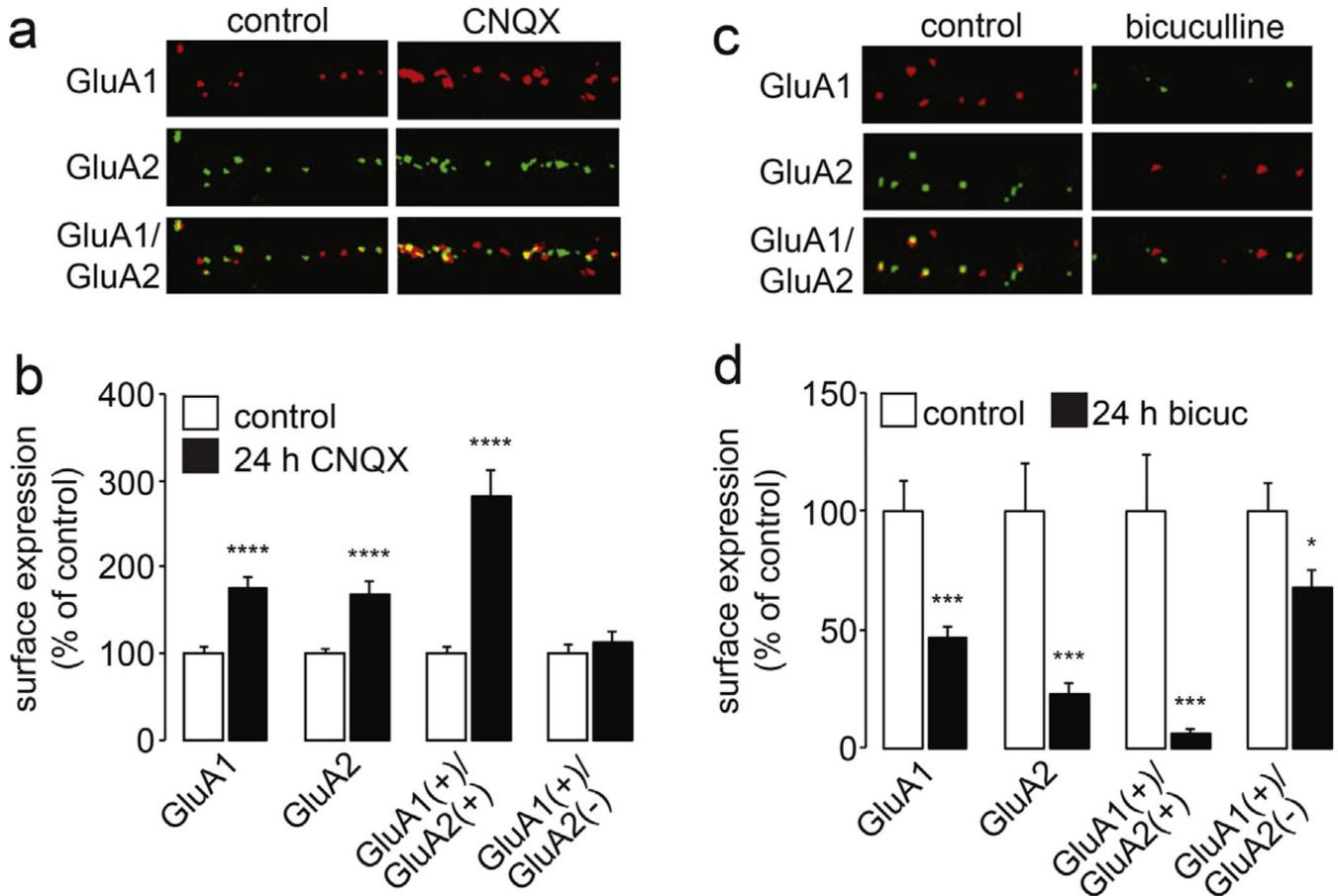


Fig. 3. Activation of PKA increases membrane insertion of GluA2-containing AMPARs. (a) Representative images of GluA2 surface staining on medium spiny neuron dendrites in NAc/PFC co-cultures. (b) Constitutive insertion of GluA2 occurs in control media but is significantly greater if the insertion experiment is conducted in the presence of the membrane permeable PKA activator SpcAMPS (15 min; 10 mM). Results are presented as mean \pm SEM. *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey tests. $n = 19$ – 23 cells/group.

**Fig. 4.**

Synaptic scaling in NAc MSNs is primarily mediated by changes in levels of CI-AMPA receptors.

(a) Representative images of GluA1 and GluA2 co-immunostaining on medium spiny neuron dendrites in NAc/PFC co-cultures from control and CNQX groups. (b) Blocking activity (CNQX; 20 μ M; 24 h) increased surface expression of GluA1, GluA2 and CI-AMPA receptors but not CP-AMPA receptors. (c) Representative images of GluA1 and GluA2 co-immunostaining on medium spiny neuron dendrites in NAc/PFC co-cultures from control and bicuculline groups. (d) Stimulating activity (bicuculline; 20 μ M; 24 h) decreased surface expression of GluA1, GluA2, CI-AMPA receptors and CP-AMPA receptors, with the magnitude of effects indicating a preferential reduction in CI-AMPA receptors. Results are presented as mean \pm SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, t tests. n = 13–22 cells/group.

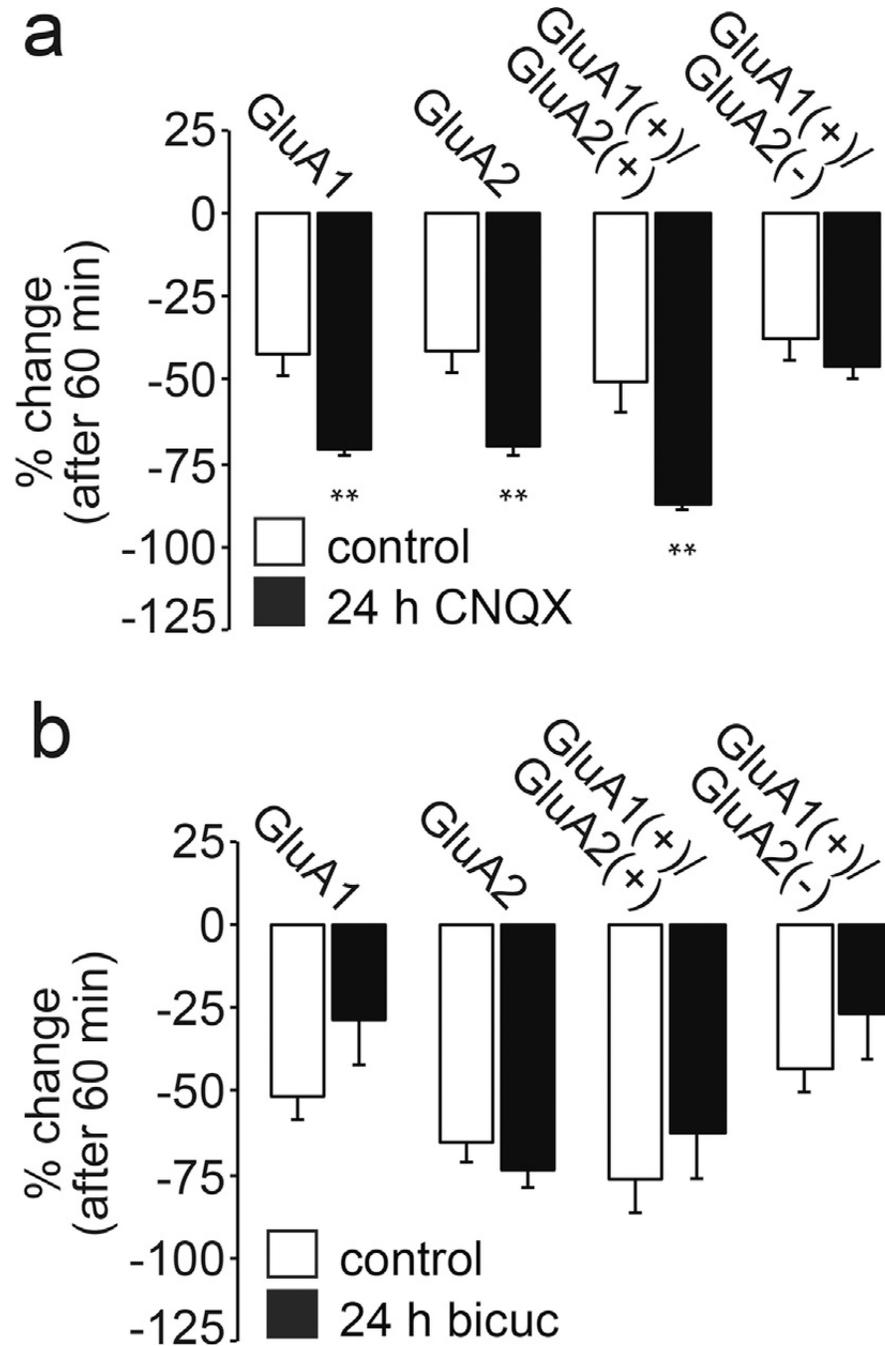


Fig. 5. Activity-dependent scaling up, but not scaling down, alters cycling rate of CI-AMPA receptors. **(a)** Blocking activity (CNQX; 20 μ M; 24 h) increased cycling of GluA1, GluA2 and CI-AMPA receptors but not CP-AMPA receptors. **(c)** Stimulating activity (bicuculline; 20 μ M; 24 h) did not change cycling. Results are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, t tests. $n = 13$ –22 cells/group.