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Locomotor sensitization to cocaine is associated with distinct pattern of glutamate receptor trafficking to the postsynaptic density in prefrontal cortex: Early versus late withdrawal effects

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ABSTRACT

Glutamatergic neurotransmission plays an important role in the behavioral and molecular plasticity observed in cocaine mediated locomotor sensitization. Recent studies show that glutamatergic signaling is regulated by receptor trafficking, synaptic localization, and association with scaffolding proteins. The trafficking of the glutamate receptors was investigated in the dorsal and ventral prefrontal cortex at 1 and 21 days after repeated cocaine administration which produced robust locomotor sensitization. A subcellular fractionation technique was used to isolate the cellular synaptosomal fraction containing the postsynaptic density. At early withdrawal, the prefrontal cortex displayed a reduction in the synaptosomal content of the AMPA and NMDA receptor subunits. In contrast, after extended withdrawal, there was a significant increase in the trafficking of the receptors into the synaptosomal compartment. These changes were accompanied by corresponding trafficking of the postsynaptic glutamatergic scaffolding proteins. Thus, enhanced trafficking of glutamate receptors from cytosolic to synaptosomal compartment is associated with prolonged withdrawal from repeated exposure to cocaine and may have functional consequences for the synaptic and behavioral plasticity.

1. Introduction

Repeated cocaine administration produce enduring molecular, cellular and behavioral plasticity that resemble some addiction-related features in humans (Vanderschuren and Kalivas, 2000; Kalivas and Volkow, 2005; Everitt and Wolf, 2002; Robinson and Berridge, 2003; Everitt and Vanderschuren, 2005). In rodents, repeated intermittent exposure to cocaine leads to locomotor sensitization defined as progressive augmentation in locomotion and stereotypy in response to a dose of cocaine (Henry and White, 1995; Vanderschuren and Kalivas, 2000). Suto et al., (2004) showed that withdrawal from repeated amphetamine administration leads to an augmented cocaine self-administration and drug-seeking behavior in locomotor sensitized animals. These observations suggest that this animal model might be useful in elucidating the cellular and molecular mechanisms responsible for the enduring behavioral plasticity. Despite a wealth of information in the literature, the molecular mechanisms underlying the enduring molecular, cellular and behavioral plasticity after repeated exposure to cocaine is not clear. However, experimental data suggest that modulation of excitatory neurotransmission and signaling in a brain circuit comprising prefrontal cortex, nucleus accumbens, and ventral tegmental area plays an important role in the cocaine-mediated effects (Wolf, 1998; Vanderschuren and Kalivas, 2000; Everitt and Wolf, 2002; Li et al., 2004).

Prefrontal cortex, a component of the motive circuit of the brain, has been implicated in the behavioral and molecular changes after repeated exposure to cocaine (Steketee, 2003; McFarland and Kalivas, 2001; McFarland et al., 2004). The pyramidal projection neurons of the prefrontal cortex innervate subcortical regions, such as nucleus accumbens and ventral tegmental area, and provide glutamatergic neurotransmission (Sesack et al., 1989, Sesack and Pickel, 1992; Groenewegen et al., 1990). The medial prefrontal cortex in the rat is a heterogeneous structure that can be subdivided into dorsal and ventral compartments based on the functional and anatomical distinctions (Heidbreder and Groenewegen, 2003). The dorsal prefrontal cortex consists of the cingulate cortex area 1 and the dorsal portion of the prelimbic cortex and preferentially innervates the dorsal striatum and nucleus accumbens core regions (Sesack et al., 1989; Groenewegen et al., 1990; Berendse et al., 1992). The ventral prefrontal cortex consists of the ventral portion of the prelimbic and the infralimbic cortex and preferentially innervates the nucleus accumbens shell (Sesack et al., 1989; Hurley et al., 1991; Gorelova

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and Yang, 1997). The mPFC is innervated by the dopaminergic afferents from the ventral tegmental area with the highest innervation received in the infralimbic and prelimbic regions (Conde et al., 1995; Lindval et all., 1978; Thierry et al., 1973). In turn, prefrontal cortex innervates the ventral tegmental area with prelimbic cortex exhibiting a larger innervation than cingulate or infralimbic regions (Sesack et al., 1989; Sesack and Pickel, 1992; Hurley et al., 1991; Beckstead, 1979).

Several studies have suggested the involvement of the prefrontal cortex in the locomotor sensitization to cocaine. Lesion of the prelimbic, but not cingulate or infralimbic regions, prevented the development of locomotor sensitization to cocaine (Tzschentke and Schmidt, 1998, 2000). Lesion of dorsal prefrontal cortex, but not ventral PFC, inhibited the expression of locomotor sensitization to cocaine (Pierce et al., 1998). Locomotor sensitization to cocaine leads to augmented glutamate release in PFC after a cocaine challenge administration at 1 and 7 days of withdrawal but not after 30 days of withdrawal (Williams and Steketee, 2004). However, the basal levels of extracellular glutamate is not changed after early (1 or 7 days) or extended (21 or 30 days) withdrawal period (Baker at al., 2003; Williams and Steketee, 2004). Further, recent studies suggest an important role for mPFC in cocaine self-administration. For example, inhibition of dPFC, but not vPFC, prevented cocaine and footshock mediated reinstatement of drug seeking (McFarland and Kalivas, 2001; McFarland et al., 2004).

Similar to other membrane bound receptors, postsynaptic glutamate receptor signaling is partly regulated by the presence of functional receptor on the cell membrane and receptor-mediated intracellular signaling (Malinow and Malenka, 2002; Kennedy, 2000; Kennedy and Ehlers, 2006; Derbach et al., 2007). Therefore, glutamate receptor gene activation, protein synthesis, membrane trafficking, synaptic localization, and intracellular signaling are important candidates as possible mechanisms for cocaine-mediated plasticity. In support of these observations, we have recently shown that locomotor sensitization to cocaine is associated with an extensive trafficking and redistribution of glutamate receptors in the nucleus accumbens (Ghasemzadeh et al., 2008). Furthermore, neuroimaging of addicts suggest abnormalities in frontal cortical neuronal activity during withdrawal as well as craving and drug use (Goldstein and Volkow, 2002; Kalivas and Volkow, 2005). Two recent reports using primary prefrontal cortex neuronal cultures, have indicated that brief D1 dopamine receptor stimulation leads to augmented trafficking and membrane expression of AMPA and NMDA receptor subunits on the pyramidal neurons (Sun et al., 2005; Gao and Wolf, 2008). These studies suggest that plasticity in the glutamate receptors in the prefrontal cortex may contribute to the addiction-related behaviors. However, the nature and mechanisms of the glutamate receptor neuroadaptations in the prefrontal cortex after locomotor sensitization to cocaine is not clear. This information is critical since it provides a framework to enhance our understanding of the role of the glutamate signaling in addiction.

In the current study, in an attempt to address some of these issues, we investigated the redistribution of the glutamate receptors in the medial prefrontal cortex and anterior cingulate cortex, brain nuclei associated with addiction-related behaviors, after locomotor sensitization to cocaine and withdrawal. The glutamate receptor proteins were monitored by utilizing a biochemical subcellular fractionation procedure which isolates the cellular synaptosomal membrane fraction containing the postsynaptic density (Xiao et al., 1998; Lin et al., 2003; Wyszynski et al., 1998; Dunah and Standaert, 2001; Toda et al., 2003). This study is the first to report a significant redistribution of the three subtypes of the glutamate receptors in the synaptosomal membrane fraction associated with the postsynaptic density in PFC after withdrawal from repeated exposure to cocaine. Furthermore, the time course of the receptor redistribution suggest a role for augmented glutamate receptor signaling in withdrawal-mediated increase in drug seeking and other addiction-related behaviors.

2. Materials and Methods

2.1. Animal housing

Male Sprague Dawley rats weighing 275–300 g were obtained from Harlan Laboratories (Indianapolis, IN) and housed two per cage with water and food available *ad libitum*. All housing and experimental procedures were conducted in AAALAC approved facilities according to guidelines established by the National Institutes of Health. A 12 h light/dark cycle was used with the lights on at 7:00 AM. Animals were acclimated to the housing facility for one week before starting experiments. Behavioral experiments and cocaine injections were performed during the light cycle.

2.2. Repeated cocaine administration

Rats were assigned to saline or cocaine treatment groups and habituated for 4 days in photocell cages (AccuScan, Columbus, OH), each receiving a saline injection (1 ml/kg, i.p.) 1 h into the three hour session. On treatment day 1, during a three hour session, all animals were habituated to the photocell cages for 1 h. Subsequently, cocaine (10 mg/kg i.p.) or saline (1 ml/kg, i.p.) was administered and locomotor activity was recorded for 2 h. Over the next 7 days, rats were treated in home cages with cocaine (30 mg/kg, i.p.) or saline (1 ml/kg, i.p.). On day 9, rats were placed in photocell cages for 1 h of habituation; subsequently, the saline and cocaine groups received saline (1 ml/kg, ip) or cocaine (10 mg/kg, ip) and behavior was monitored for 2 h. The rats spent 1 or 21 days in their home cages without treatment and were then sacrificed (Ghasemzadeh et al., 2003).

2.3. Anatomical dissection and biochemical subcellular fractionation

Rats were decapitated and brains quickly removed from the skull and placed in ice-cold saline for 1 min. The tissue was blocked and coronal slices which contained areas of interest were cut using a rat brain matrix (ASI, Warren, MI). The medial prefrontal cortex (PFC) was blocked between plates 6 and 11 (Bregma 5.16–3.00 mm) (Paxinos and Watson, 2005). The dorsal and ventral PFC border was set at the middle of prelimbic cortex based on previous anatomical and functional studies (Heidbreder and Groenewegen, 2003) and was hand dissected. The anterior cingulated cortex (areas 1 and 2) was blocked between plates 12–24 (Bregma 2.76–1.08 mm) and was hand dissected. All tissue samples were immediately frozen on dry ice and stored at −80 °C. The membrane preparation and biochemical subcellular fractionation were based on the methods by Dunah and Standaert (2001) and Toda et al., (2003). A schematic of the membrane purification procedure, adopted from Dunah and Standaert (2001), is presented in Fig. 1. The tissue was homogenized in ice-cold TEVP buffer containing (in mM) 10 Tris–HCl, pH 7.4, 5 NaF, 1 Na_3VO4, 1 EDTA, 1 EGTA, 320 sucrose, using a Dounce glass homogenizer. An aliquot of the whole tissue Dounce homogenate (H fraction) was collected, and the remainder was centrifuged at 1000 g to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10,000 *g* to obtain a crude synaptosomal fraction (P2) and subsequently was lysed hypo-osmotically for 30 min at 4 °C and centrifuged at 25,000 *g* to pellet a synaptosomal membrane fraction (LP1), which is enriched in postsynaptic density. All centrifugations were carried out at 4 °C. LP1 pellets were resuspended in TEVP buffer. The H and LP1 fractions were solubilized with the addition of SDS to 1% (v/v) and used for protein concentration measurement and Western blot analysis. All fractions were saved and stored at −80 °C.

2.4. Western blot analysis

Protein concentration was determined by the Lowry based DC protein assay (Bio-Rad, Hercules, CA). Protein samples were resolved

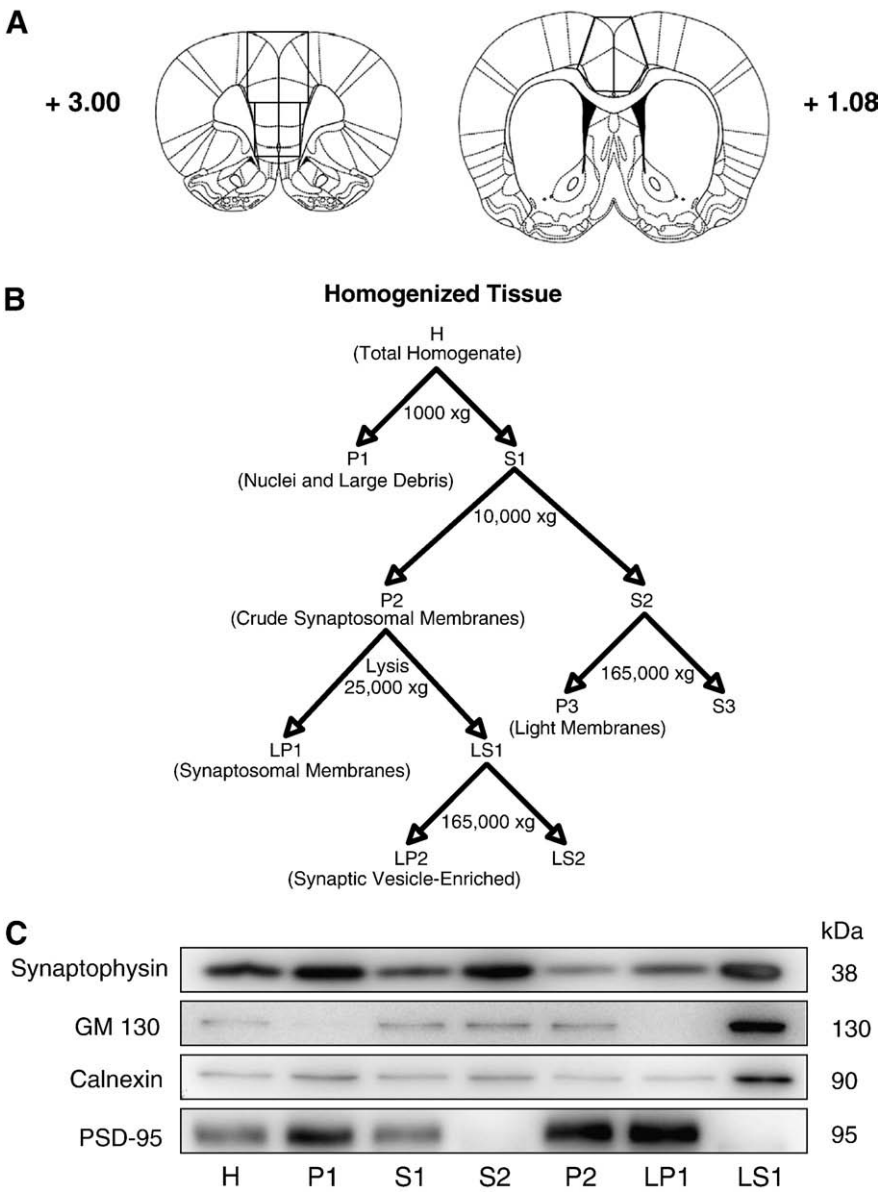

Fig. 1. Brain tissue dissection and subcellular fractionation analysis. Panel A. The two coronal brain sections show the brain regions dissected for dorsal and ventral prefrontal cortex and the anterior cingulated cortex. The numbers are distance from Bregma. The caudal surfaces of the coronal brain sections are shown. Panel B. A schematic of the procedure as described in Materials and Methods is shown (Adopted from Dunah and Standaert, 2001). The fractionation procedure was carried out only far enough to obtain synaptosomal membranes (LP1 and LS1 fractions). H and LP1 fractions were used for Western blot analysis of proteins. Panel C. A representative immunoblot is shown to characterize the content of each fraction. The membrane was probed with markers for presynaptic vesicles (synaptophysin), Golgi apparatus (GM 130), endoplasmic reticulum (calnexin), and postsynaptic density (PSD 95). The H fraction contains all of the probed subcellular compartments while the LP1 fraction is highly enriched in PSD95, contains lower amounts of synaptophysin, trace amounts calnexin, and is devoid of GM 130. Based on these markers, we were able to conclude that the LP1 fraction represents synaptosomal membranes containing the postsynaptic density with few contaminating subcellular compartments.

by SDS-PAGE according to the method described by Ghasemzadeh et al. (2003). Briefly, proteins were transferred from 7.5% SDS-PAGE gel to a polyvinyldine fluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked for 2 h at room temperature (Tris-buffered saline containing 5% non-fat dry milk) and incubated with primary antibody in antibody buffer (blocking buffer containing Tween-20, 50 μl/100 ml) overnight at 4 °C, washed with antibody buffer, and incubated with HRP-conjugated secondary antibody for 1.5 h at room temperature. Membranes were washed and immunolabeling was visualized with enhanced chemiluminescence (ECL method). Chemiluminescent images were captured using a Kodak Image Station 4000MM. Band density was measured using Kodak Molecular Imaging Software v4.0. The following primary antibodies were used in this study: mouse NMDAR1 (Millipore, Cat. # 05-432, 1:3000 dilution), rabbit NMDAR2A (Millipore, Cat. # 07-632, 1:3000 dilution), mouse NMDAR2B (Antibodies Inc., Cat. # 75-097, 1:3000 dilution), rabbit GluR1 (Millipore, Cat. # 07-660, 1:10,000 dilution), mouse GluR2 (Antibodies Inc., Cat. # 75-002, 1:3000 dilution), rabbit mGluR5 (Millipore, Cat. # 06-451, 1:10,000 dilution), rabbit mGluR2/3 (Millipore, Cat. # 06-676, 1:3000 dilution), mouse PSD95 (Antibodies Inc., Cat. # 75-028, 1:10,000 dilution), mouse PICK1 (Antibodies Inc., Cat. # 75-040, 1:3000 dilution), rabbit Homer1b/c (Santa Cruz Biotech, Cat. # sc-20807, 1:3000 dilution), goat Actin (Santa Cruz Biotech, Cat. # sc-1615, 1:10,000 dilution). The following HRP-conjugated secondary antibodies were used: goat anti-rabbit IgG (Millipore, Cat. # 12-348, 1:20,000 dilution), goat anti-mouse IgG (Millipore, Cat. # 12-349, 1:20,000 dilution).

2.5. Statistics

The locomotor responses on the first and last days of cocaine administrations were compared using two-way ANOVA with treatment and days as the main factors. The differences between individual groups were determined using a *post hoc* Fisher PLSD test. The immunoreactivity of protein samples from cocaine treated rats were normalized against saline controls and tested for significant differences using Student's *t*-test. The statistical significance was set at $p<0.05$.

3. Results

3.1. Cocaine treatment and locomotor sensitization

Male Sprague Dawley rats (275–300 g) rats were treated with daily administration of saline (1 mg/kg, ip) or cocaine for 9 days (2×10 mg/kg $+7\times30$ mg/kg, ip). Four groups of 16 rats were treated with either saline ($n=8$) or cocaine ($n=8$) at separate times using identical procedure. The locomotor response was monitored for 120 min after the first (Day 1) and last (Day 9) administration of saline or cocaine. Two groups of animals were killed 1 day after the last saline or cocaine administration (1 day withdrawal) and two groups were killed 21 days after the last saline or cocaine administration (21 days withdrawal). Brains were quickly removed, dissected for regions of interest, as described in methods section, frozen on dry ice and kept at −80 °C (Fig. 1). The protein analysis for the anterior cingulate cortex (21 days withdrawal groups) indicated a higher level of variability than other tissues examined. Therefore, two additional groups of animals were treated with repeated cocaine or saline in an identical manner at a separate time followed by 21 days of withdrawal and included in protein analysis in order to increase the sample size.

Animals treated with daily cocaine administration showed a clear locomotor sensitization when comparing the locomotor response to an acute challenge injection of cocaine (10 mg/kg, ip) on day 9 and day 1 [For 1 day withdrawal: treatment $F(1,60)=72.676$, $p<0.001$; days $F(1,60)=13.102$, $p<0.001$; treatment × days $F(1,60)=14.721$, $p<0.001$; For 21 days withdrawal: treatment $F(1,122)=48.875$, $p<0.001$; days $F(1,122)=13.025$, $p<0.001$; treatment × days $F(1,122)=10.701$, $p<0.001$]. Table 1 shows the horizontal bean breaks over 120 min after saline or cocaine administration on day 1 and day 9.

3.2. Biochemical fractionation of subcellular compartments

The biochemical fractionation protocol employed in this study was adopted from Dunah and Standaert (2001) and a schematic is presented in Fig. 1. The efficacy of the fractionation protocol to isolate various subcellular compartments was illustrated by Dunah and Standaert (2001) and others (Lin et al., 2003; Wyszynski et al., 1998; Xiao et al., 1998; Toda et al., 2003) and was reproduced in our laboratory. Equal amounts of protein (6 μg) from each cellular fraction was resolved on 7.5% SDS-PAGE to characterize its content. Synaptophysin, a presynaptic marker protein which participates in the fusion of vesicles to the presynaptic membrane (Wiedenmann and Franke, 1985), was separated from the synaptosomal fraction (P2 vs. S2) and, thus, was present in the synaptosomal membrane fraction at much lower levels (H vs. LP1). Similarly, GM130, a Golgi membrane protein (Seemann et al., 2000), and Calnexin, an endoplasmic reticulum protein (Rubio and Wenthold, 1999; Williams, 2006), were reduced in the synaptosomal membrane fraction while being concentrated in the synaptic vesicle fraction (LP1 vs. LS1). All three intracellular proteins were enriched in the synaptic vesicle-enriched fraction (LS1) and separated from the synaptosomal membrane fraction (LP1). In contrast, PSD-95, a scaffolding protein located at the postsynaptic density (Hunt et al., 1996), was highly enriched in the synaptosomal membrane fraction (H, LS1 vs. LP1). In agreement with previous reports, these analyses demonstrated that biochemical fractionation enriched the synaptosomal membrane fraction and separated it from the presynaptic and cytosolic compartments.

Table 1
Locomotor sensitization to cocaine.

Treatment	*n*	Day 1	Day 9
A. 1 day withdrawal			
Saline	16	2362.7 ± 168.2	1907.1 ± 186.4
Cocaine	16	12199.9 ± 2039.6*	27845.4 ± 3659.0*#
B. 21 days withdrawal			
Saline	32	2377.8 ± 202.2	2941.2 ± 339.9
Cocaine	31	8582.2 ± 1651.0*	20057.8 ± 2932.6*#

Six groups of rats (16 rats/group; 8 saline, 8 cocaine) were treated with repeated saline (1 ml/kg, ip) or cocaine (2 days × 10 mg/kg + 7 days × 30 mg/kg, ip) at separate times. Two groups were killed after 1 day of withdrawal and four groups after 21 days of withdrawal. The data are mean ± SEM of horizontal beam breaks over 120 min. * $p<0.05$ compared to saline on same day of treatment. # $p<0.05$ compared to cocaine group on day 1.

3.3. Locomotor sensitization modulates glutamate receptor trafficking in dorsal and ventral prefrontal cortex after 1 day withdrawal

3.3.1. The tissue level of proteins

Fig. 2 shows that repeated cocaine administration followed by 1 day of withdrawal did not change the tissue protein levels (H fraction, Fig. 1) of any of the glutamate receptors or scaffolding proteins in dorsal or ventral prefrontal cortex except for a near significant decrease in the tissue level of NR2A protein (saline 100 ± 2.0, cocaine 90.6 ± 5.4, $t(25)=1.78$, $p=0.087$) in the ventral PFC.

3.3.2. The synaptosomal membrane fraction proteins

3.3.2.1. Dorsal PFC. There were significant changes in the level of glutamate receptor and scaffolding proteins in the synaptosomal membrane fraction of dorsal PFC (LP1 fraction, Fig. 1). The ionotropic glutamate receptor proteins in dorsal prefrontal cortex showed a significant decrease in synaptosomal membrane fraction. The NMDA receptor subunits NR1 (saline 100 ± 6.3, cocaine 77.0 ± 7.9, $t(27)=2.30$, $p=0.029$), NR2A (saline 100 ± 2.5, cocaine 85.6 ± 4.9, $t(27)=2.66$, $p=0.013$), and NR2B (saline 100 ± 4.0, cocaine 81.3 ± 6.8, $t(28)=2.37$, $p=0.025$) showed a significant decrease in dorsal prefrontal cortex. While the AMPA receptor subunit GluR1 was not changed (saline 100 ± 4.1, cocaine 96.0 ± 1.0, $t(26)=0.395$, $p=0.70$), GluR2 receptor protein was significantly decreased (saline 100 ± 2.9, cocaine 86.3 ± 6.3, $t(27)=2.09$, $p=0.046$). In contrast to ionotropic receptors, the group1 metabotropic glutamate receptor mGluR5 dimer (but not monomer) protein was significantly increased in the dorsal prefrontal cortex (saline 100 ± 4.0, cocaine 120.2 ± 8.2, $t(27)=-2.15$, $p=0.040$). The group2 metabotropic glutamate receptor mGluR2/3 did not display any changes. The group1 and group2 metabotropic glutamate receptor proteins, mGluR5 and mGluR2/3, exist in monomer and dimer forms. Both monomer and dimer proteins were measured due to reports suggesting the presence of the protein in monomer and dimer forms in the brain tissue (Testa et al., 1998; Schaffhausen et al., 2000). Among the synaptic scaffolding proteins, PSD95 was significantly decreased (saline 100 ± 4.1, cocaine 88.5 ± 3.0, $t(28)=2.20$, $p=0.036$) while PICK1, Homer1b/c, and actin proteins did not indicate any changes.

3.3.2.2. Ventral PFC. Similar to dorsal PFC, the NR1 (saline 100 ± 4.4, cocaine 85.8 ± 4.9, $t(26)=2.15$, $p=0.041$) and GluR2 (saline 100 ± 3.0, cocaine 89.5 ± 2.5, $t(29)=2.67$, $p=0.012$) protein levels were significantly reduced in ventral PFC; while that of other ionotropic glutamate receptor subunits were not changed. There were no changes in the group1 (mGluR5) or group2 (mGluR2/3) metabotropic glutamate receptor proteins in the ventral PFC. Among the synaptic scaffolding proteins, PICK1 (saline 100 ± 5.1, cocaine 85.7 ± 6.2, $t(26)=$

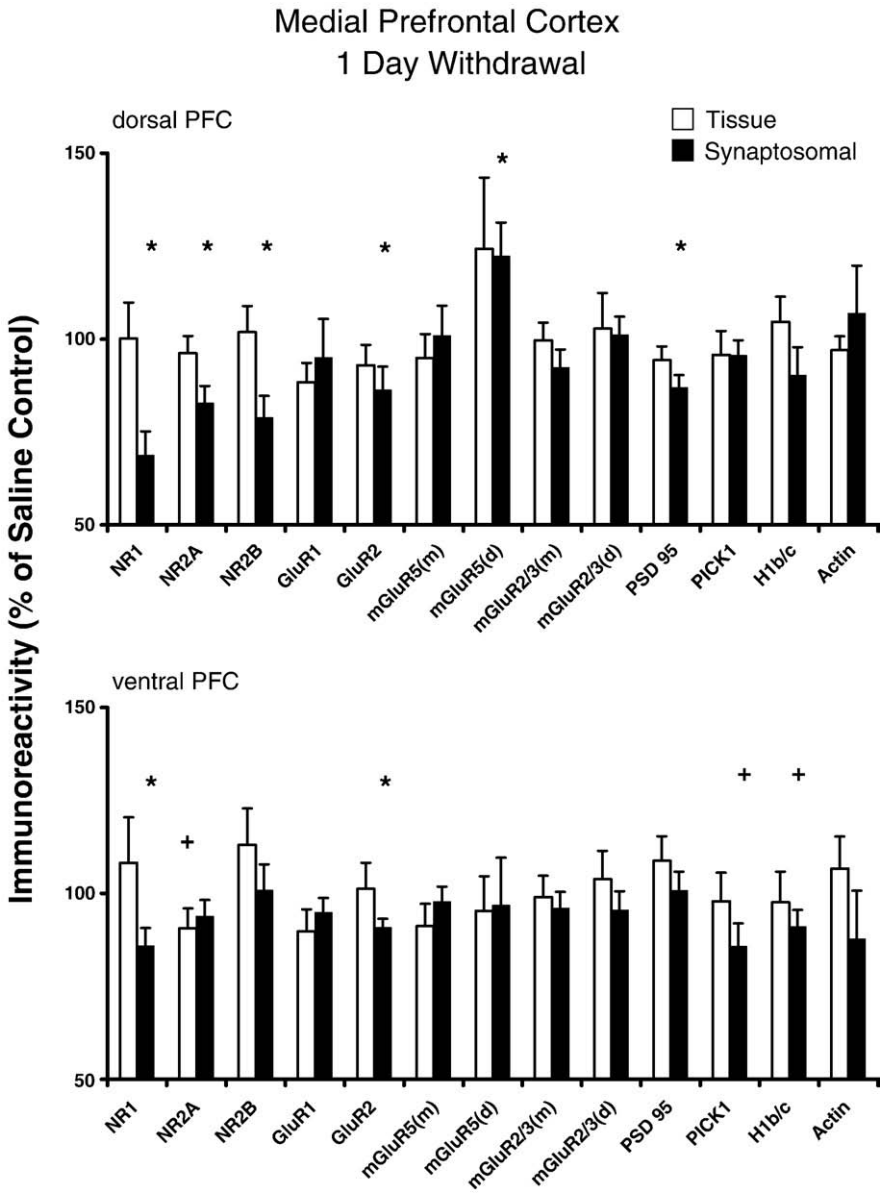

Fig. 2. Protein analysis results for dorsal and ventral prefrontal cortex after 1 day of withdrawal from locomotor sensitization to cocaine. The open bars represent proteins in the total tissue fraction (fraction H). The black bars represent proteins in the synaptosomal membrane fraction (LP1 fraction). The data (mean ± SEM) are normalized versus saline treated controls ($n = 10–16$/group) and presented as a percentage of saline treated group. The data represent two groups of saline or cocaine treated rats. The protein band intensities were compared using a two-tailed Student's *t*-test. *$p<0.05$ versus saline controls. $^{+}$Data shows near significant decrease. See text for details.

1.799, $p = 0.084$) and Homer1b/c proteins (saline 100 ± 2.6, cocaine 91.0 ± 4.6, $t(29) = 1.706$, $p = 0.099$) showed decreasing trends, while PSD95 and actin proteins were not modified.

3.4. Locomotor sensitization augments glutamate receptor trafficking in dorsal and ventral prefrontal cortex after 21 days withdrawal

3.4.1. The tissue level of proteins

The AMPA glutamate receptor subunit GluR2 showed a significant decrease in dorsal prefrontal cortex (saline 100 ± 1.8, cocaine 90.1 ± 3.5, $t(24) = 2.63$, $p = 0.015$), but other receptor proteins or the synaptic scaffolding proteins were not changed. There were no changes in the tissue level of proteins in the ventral prefrontal cortex (Fig. 3).

3.4.2. The synaptosomal membrane fraction proteins

3.4.2.1. Dorsal PFC. The glutamate receptor and scaffolding protein levels in the synaptosomal membrane fraction of dorsal PFC were significantly elevated after 21 days of withdrawal. (LP1 fraction, Fig. 1). The ionotropic glutamate receptor proteins showed a significant increase in the synaptosomal membrane fraction. The NMDA receptor subunits NR1 (saline 100 ± 8.6, cocaine 155.6 ± 21.1, $t(28) = -2.443$, $p = 0.021$) and NR2A (saline 100 ± 4.6, cocaine 136.9 ± 11.0, $t(30) = -3.09$, $p = 0.004$) displayed a significant increase while NR2B protein level was not changed (saline 100 ± 8.3, cocaine 109.7 ± 12.2, $t(25) = -0.648$, $p = 0.523$).

The AMPA receptor subunits GluR1 (saline 100 ± 3.1, cocaine 121.6 ± 6.8, $t(29) = -2.97$, $p = 0.006$) and GluR2 (saline 100 ± 5.0, cocaine 139.6 ± 13.1, $t(29) = -2.90$, $p = 0.007$) proteins were significantly increased in the synaptosomal membrane fraction.

The mGluR5 monomer (saline 100 ± 4.5, cocaine 123.4 ± 8.8, $t(29) = -2.41$, $p = 0.022$) and mGluR5 dimer (saline 100 ± 9.5, cocaine 183.4 ± 22.1, $t(27) = -3.55$, $p = 0.001$) proteins in dorsal PFC were significantly redistributed into the synaptosomal membrane fraction. Similarly, there were increased redistribution of mGluR2/3 monomer (saline 100 ± 7.2, cocaine 147.6 ± 17.4, $t(28) = -2.53$, $p = 0.018$) and mGluR2/3 dimer proteins (saline 100 ± 8.6, cocaine 149.9 ± 17.2, $t(30) = -2.59$, $p = 0.015$).

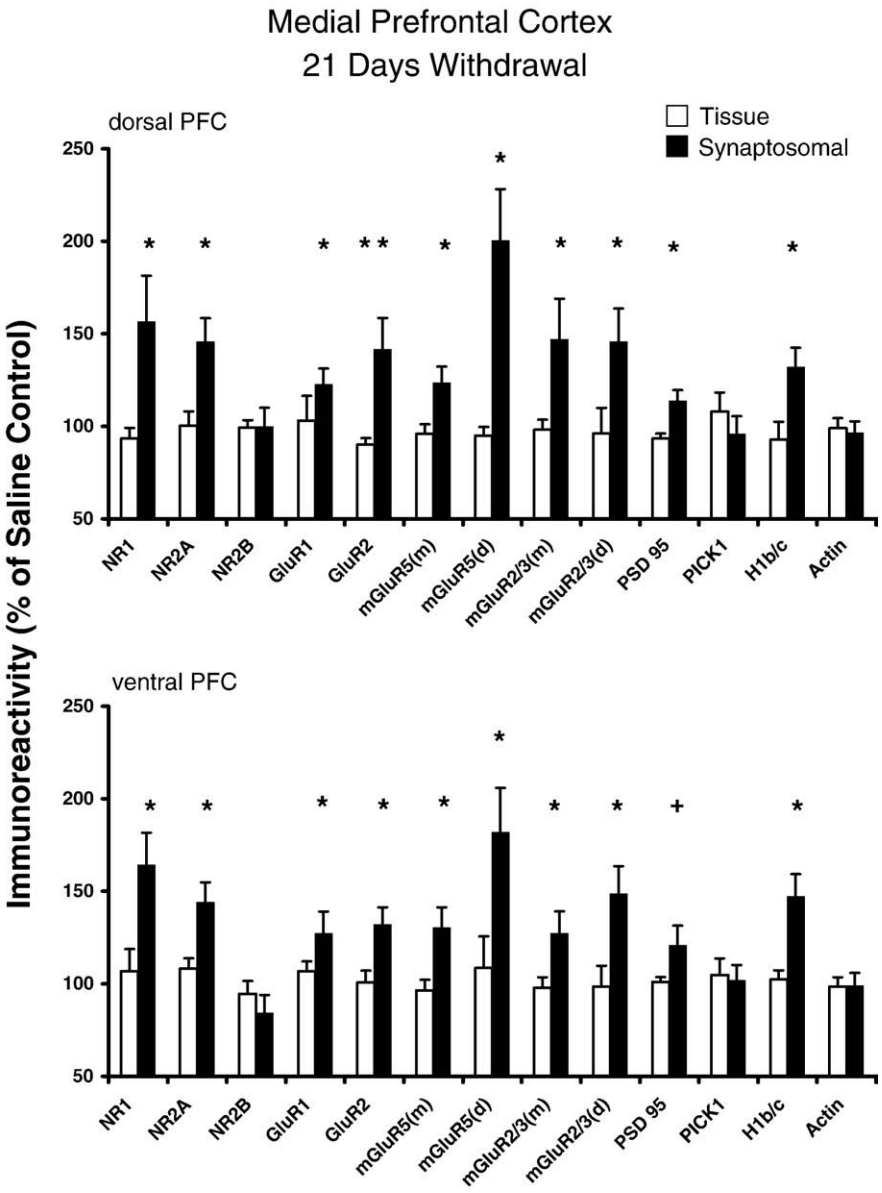

Fig. 3. Protein analysis results for dorsal and ventral prefrontal cortex after 21 days of withdrawal from locomotor sensitization to cocaine. The open bars represent proteins in the total tissue fraction (fraction H). The black bars represent proteins in the synaptosomal membrane fraction (LP1 fraction). The data (mean ± SEM) are normalized versus saline treated controls ($n = 10–16$/group) and presented as a percentage of saline treated group. The data represent two groups of saline or cocaine treated rats. The protein band intensities were compared using a two-tailed Student's *t*-test. *$p<0.05$ versus saline controls. $^{+}$Data shows near significant increase. See text for details.

Among the synaptic scaffolding proteins, PSD95 protein (saline 100 ± 3.7, cocaine 113.6 ± 5.9, $t(25) = -2.062$, $p = 0.049$) and Homer1b/c proteins (saline 100 ± 3.7, cocaine 126.7 ± 8.3, $t(28) = -2.959$, $p = 0.006$) were significantly redistributed into the synaptosomal membrane fraction in dorsal PFC. PICK1 and actin proteins were not modified. Representative protein bands are shown in Fig. 4.

3.4.2.2. Ventral PFC. The pattern of redistribution of glutamate receptors and scaffolding proteins into synaptosomal membrane fraction were similar in the dorsal and ventral compartments of the prefrontal cortex. Thus, similar to dPFC, the NMDA receptor subunits NR1 (saline 100 ± 6.5, cocaine 166.1 ± 14.2, $t(28) = -4.22$, $p = 0.0002$) and NR2A (saline 100 ± 3.9, cocaine 140.6 ± 8.6, $t(29) = -4.38$, $p = 0.0001$) were significantly redistributed into the synaptosomal membrane fraction while NR2B (saline 100 ± 6.0, cocaine 87.5 ± 7.6, $t(29) = 1.301$, $p = 0.203$) was not changed.

The AMPA receptor subunits GluR1 (saline 100 ± 5.7, cocaine 127.0 ± 11.9, $t(25) = -2.18$, $p = 0.039$) and GluR2 (saline 100 ± 3.2, cocaine 131.4 ± 7.4, $t(30) = -3.90$, $p = 0.0005$) were increased in the synaptosomal membrane fraction.

The mGluR5 monomer (saline 100 ± 6.4, cocaine 136.6 ± 10.2, $t(29) = -3.081$, $p = 0.005$) and mGluR5 dimer (saline 100 ± 9.9, cocaine 179.0 ± 18.7, $t(27) = -3.799$, $p = 0.001$) proteins in ventral PFC were significantly redistributed into the synaptosomal membrane fraction. Similarly, there were increased redistribution of mGluR2/3 monomer (saline 100 ± 4.8, cocaine 124.7 ± 10.1, $t(29) = -2.25$, $p = 0.032$) and mGluR2/3 dimer proteins (saline 100 ± 6.5, cocaine 155.2 ± 14.7, $t(29) = -3.511$, $p = 0.0015$).

Among the synaptic scaffolding proteins, PSD95 showed an increasing trend in ventral PFC (saline 100 ± 6.4, cocaine 120.5 ± 10.9, $t(30) = -1.626$, $p = 0.114$) and Homer1b/c proteins (saline 100 ± 7.4, cocaine 151.5 ± 10.7, $t(30) = -3.965$, $p = 0.0004$) was significantly redistributed into the synaptosomal membrane fraction in ventral PFC. Similar to dorsal PFC, PICK1 and actin proteins were not modified. Representative protein bands are shown in Fig. 4.

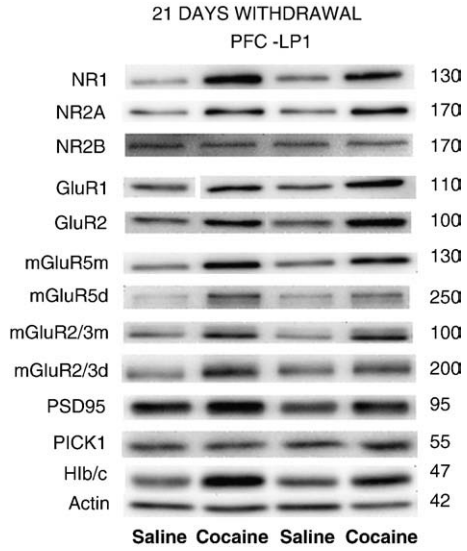

Fig. 4. Representative protein bands from prefrontal cortex of saline and cocaine treated animals at 21 days of withdrawal from last daily treatment. The protein bands shown represent protein levels in the synaptosomal membrane fraction (LP1 fraction). Similar pattern of protein band intensities were observed for both dorsal and ventral prefrontal cortex.

3.5. Glutamate receptor redistribution in the anterior cingulated cortex (ACC) after locomotor sensitization to cocaine

3.5.1. 1 day withdrawal

At 1 day of withdrawal, ACC showed an increasing trend in the tissue level of mGluR2/3 monomer protein (saline 100 ± 3.5, cocaine 112.7 ± 5.5, $t(28) = -1.947$, $p = 0.062$) but other proteins were unchanged. At the synaptosomal membrane level (LP1 fraction), the mGluR2/3 dimer protein was significantly increased (saline 100 ± 4.3, cocaine 116.3 ± 6.3, $t(26) = -2.165$, $p = 0.040$) but the redistribution of other proteins was unchanged (Fig. 5).

3.5.2. 21 days withdrawal

At the tissue level, mGluR2/3 dimer protein showed a near significant increase (saline 100 ± 5.0, cocaine 117.5 ± 7.3, $t(58) = -1.987$, $p = 0.052$) after 21 days of withdrawal; however, other glutamate receptors and scaffolding proteins were unchanged. At the synaptosomal membrane level (LP1 fraction), there were several glutamate receptors and scaffolding proteins that indicated changes in redistribution. The NMDA receptor subunits NR1 and NR2B proteins were significantly decreased in ACC (NR1: saline 100 ± 3.3, cocaine 84.4 ± 5.6, $t(30) = 2.469$, $p = 0.019$; NR2B: saline 100 ± 4.5, cocaine 86.7 ± 4.0, $t(49) = 2.199$, $p = 0.033$). The AMPA receptor subunit GluR2 also showed a significant decrease (saline 100 ± 2.3, cocaine 82.2 ± 3.2, $t(57) = 4.529$, $p < 0.0001$). The group2 metabotropic glutamate receptor mGluR2/3 dimer protein showed an increasing trend in redistribution into synaptosomal membrane fraction (saline 100 ± 3.4, cocaine 113.2 ± 7.0, $t(58) = -1.706$, $p = 0.093$). The scaffolding protein PICK1 was increased (saline 100 ± 3.2, cocaine 110.9 ± 4.8, $t(57) = -2.070$, $p = 0.043$) while Homer1b/c protein showed a near significant decrease (saline 100 ± 3.3, cocaine 91.0 ± 3.1, $t(57) = -1.994$, $p = 0.051$) in the synaptosomal membrane fraction (Fig. 5).

4. Discussion

This study demonstrates that locomotor sensitization to cocaine is accompanied by a complex pattern of redistribution of the glutamate receptor proteins in the synaptosomal compartment associated with the postsynaptic density in the prefrontal cortex. Furthermore, the redistribution of glutamate receptor proteins was not accompanied by changes in tissue protein levels suggesting a redistribution of the existing pool of cellular proteins. The protein redistribution was dependent on the withdrawal time from cocaine treatment, anatomical regions and compartments. The concurrent redistribution of synaptic scaffold proteins suggests that the redistribution of the glutamate receptors may have a functional impact on synaptic function. The results suggest that redistribution of glutamate receptors in the prefrontal cortex following withdrawal from repeated cocaine exposure may contribute to the locomotor sensitization to cocaine, but a causal link between cocaine-induced behavioral plasticity and receptor trafficking remains to be established.

4.1. Redistribution of glutamate proteins in the prefrontal cortex after locomotor sensitization to cocaine

Prefrontal cortex plays an important role in cocaine-mediated locomotor sensitization. The glutamatergic pyramidal neurons of dorsal and ventral prefrontal cortex innervate nucleus accumbens core and shell compartments in a topographically organized manner, respectively (see Introduction). Since these two compartments of the prefrontal cortex have been differentially implicated in locomotor sensitization and motivated behaviors including cocaine seeking and reward, we investigated the redistribution of the glutamate receptors in each compartment after withdrawal from locomotor sensitization to cocaine (Tzschentke and Schmidt, 1998; Vanderschuren and Kalivas, 2000; Pierce et al., 1998; McFarland and Kalivas, 2001; McFarland et al., 2004).

It is interesting to note that, for the most part, there were not any changes in the tissue level of glutamate receptor proteins in the dorsal and ventral PFC after 1 or 21 days of withdrawal, with the exception of the tissue level of NR2A protein that showed a reducing trend in the ventral PFC after 1 day withdrawal and the GluR2 receptor protein that was significantly reduced in dorsal PFC after 21 days of withdrawal from locomotor sensitization to cocaine. The lack of change in the cellular level for NMDA receptor subunits and GluR1 proteins is in agreement with previous studies in PFC neuronal culture where D1 dopamine receptor stimulation mediated an increase in the trafficking and membrane insertion of NR1, NR2B, and GluR1 receptor subunits in the absence of receptor protein synthesis (Sun et al., 2005, Gao and Wolf, 2008). However, in contrast to our current results, a recent study using a similar cocaine locomotor sensitization paradigm reported an increase in the tissue level of NR2A and NR2B proteins (Ary and Szumlinski, 2007). In light of the significant redistribution of the glutamate receptors, our observation suggests that locomotor sensitization to cocaine is associated with a significant intracellular signaling that mediates the trafficking of receptor proteins in the neurons of the prefrontal cortex in the absence of any protein synthesis or degradation. In agreement, previous studies using primary prefrontal cortex neuronal culture have suggested that D1 receptor mediated GluR1 trafficking to the cellular membrane is dependent on PKA activation, while the NMDA receptor subunits trafficking (NR1 and NR2B, but not NR2A subunits) is independent of PKA activity but depends on the tyrosine phosphorylation of NR2B subunit (Sun et al., 2005; Gao and Wolf, 2008). Similar findings on NMDA receptor trafficking have been reported in striatal neuronal culture (Hallett et al., 2006). However, D1 receptor stimulation of the striatal brain slices from adult rats showed a significant redistribution of all three NMDA receptor subunits into the synaptosomal compartment (Dunah and Standaert, 2001). The emerging data suggest that D1 receptor mediated signaling mediate concurrent increases in the trafficking of the AMPA and NMDA receptors in the prefrontal cortex pyramidal neurons through activation of two intracellular signaling pathways, the protein kinase A and the tyrosine kinase pathways. Furthermore, the augmented surface and synaptic NMDA receptor expression may contribute to the synaptic expression of AMPA receptors (Sun et al., 2005; Gao and Wolf, 2008).

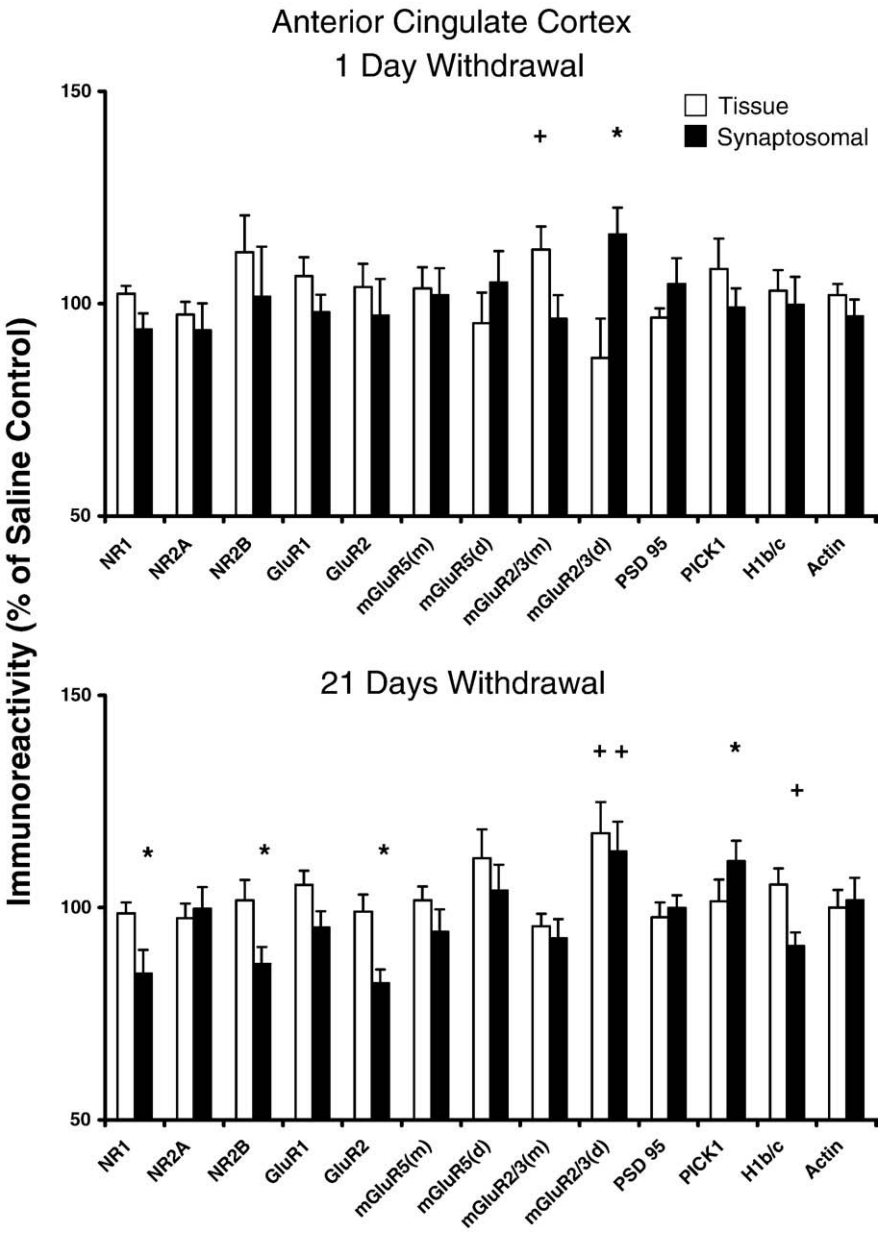

Fig. 5. Protein analysis results for anterior cingulate cortex after 1 and 21 days of withdrawal from locomotor sensitization to cocaine. The open bars represent protein in the total tissue fraction (fraction H). The black bars represent protein in the synaptosomal membrane fraction (LP1 fraction). The data (mean ± SEM) are normalized versus saline treated controls (1 day withdrawal $n = 8$–16/group; 21 days withdrawal $n = 15$–32/group) and presented as a percentage of saline treated group. The protein band intensities were compared using a two-tailed Student's *t*-test. *$p < 0.05$ versus saline controls. $^{+}$Data shows near significant increase/decrease. See text for details.

The overall changes in the synaptosomal content of the glutamate receptors suggest that there may be a general reduction in the glutamatergic signaling in the prefrontal cortex at 1 day withdrawal. The only exception was the significant increase in the synaptosomal mGluR5 dimer protein in the dorsal PFC. The reduction in the synaptosomal GluR2 protein in both dorsal and ventral PFC suggests that the early withdrawal may be associated with a change in the AMPA receptor subunit composition to include increased number of GluR1 compared to GluR2 subunits. Since GluR2 subunits determine the calcium-permeability of the receptor ion channel, there may be an increased calcium influx through the AMPA receptor (Hollmann et al., 1991). Moreover, the changes in the synaptosomal NMDA receptor subunits also suggest a possible decrease in the NMDA receptor activation in the prefrontal neurons at 1 day withdrawal. The decrease in the synaptic scaffolding proteins PSD95, PICK1 and Homer1b/c in the synaptosomal compartment of the dorsal and ventral prefrontal cortex at 1 day of withdrawal lends support to the functional implications of the glutamate receptor redistribution (Tu et al., 1999). It is noteworthy to mention that, similar to glutamate receptor proteins, while trafficking and redistribution of scaffolding proteins were affected by locomotor sensitization to cocaine, the tissue level of the four scaffolding proteins was not changed. In contrast to early withdrawal, there was a significant increase in the redistribution of the glutamate receptors into the synaptosomal compartment, containing the postsynaptic density, of both dorsal and ventral PFC after 21 days of withdrawal. NR1, the obligatory subunit of the NMDA receptor, and NR2A subunits were increase in both compartments of PFC while NR2B was unchanged. Similarly, the AMPA receptor subunits, GluR1 and GluR2, showed a significant redistribution into the synaptosomal fraction. The increase in the synaptic scaffolding protein PSD95 which binds and regulates the trafficking, synaptic localization, and function of the NR2 and GluR1 subunits suggests that the redistribution of the receptors may contribute to the synaptic plasticity in the prefrontal cortex (Schnell et al., 2002; Ehrlich and

Malinow, 2004). In contrast to early withdrawal, the GluR2 protein also demonstrated an increased trafficking into synaptosomal fraction. This observation is similar to that of nucleus accumbens core and shell which have been reported recently (Ghasemzadeh et al., 2008). The data suggest that there is an overall increase in the postsynaptic metabotropic glutamate receptor function since the synaptosomal localization of group1 and group2 receptors were increased at 21 days withdrawal. This notion is supported by the observation that the synaptic scaffolding protein Homer1b/c, which selectively binds group 1 mGluRs, was also increased in the synaptosomal fraction in both compartments of PFC (Xiao et al., 1998; Tu et al., 1999). Therefore, the overall change in the redistribution of the glutamate receptors after 21 days withdrawal points to a possible increase in excitatory drive from prefrontal cortex to the subcortical regions including the nucleus accumbens and ventral tegmental area. This is in agreement with the augmented glutamate neurotransmission in the nucleus accumbens and ventral tegmental area after long withdrawal (Pierce et al., 1996; Cornish and Kalivas, 2000; Kalivas and Duffy, 1993). A recent report indicated that withdrawal from repeated amphetamine administration augments cocaine self-administration and drug-seeking behavior in locomotor sensitized rats (Suto et al., 2004). This observation may be analogous to the incubation of craving where prolonged withdrawal from drug use augments relapse to drug-seeking behavior in both human addicts and an animal model of addiction (Gawin and Kleber, 1986; Neisewander et al., 2000; Lu et al., 2004). Brain imaging data from human addicts suggest that while there is a reduction in the frontal cortical activity during withdrawal from drug use, the cortical activity is increased during craving and drug use (Goldstein and Volkow, 2002; Kalivas and Volkow, 2005; Kalivas et al., 2005). Our data support these observations suggesting that there is a decrease in the synaptosomal or postsynaptic density content of glutamate receptors during early withdrawal from cocaine exposure. However, at later withdrawal times, the glutamate receptors are redistributed to the postsynaptic density suggesting an increase in synaptic activity of the prefrontal cortex at timepoints when the rewarding value of cocaine is augmented (Suto at al., 2004).

Our data indicate that locomotor sensitization to cocaine is associated with a complex pattern of withdrawal-dependent glutamate receptor redistribution in the postsynaptic density of prefrontal cortical neurons. The relationship of the glutamate receptor redistribution to the locomotor sensitization is not clear from our data. However, the observed timecourse of changes in glutamate receptor trafficking is consistent with increased drug intake at longer withdrawal times from repeated cocaine administration (Suto at al., 2004), suggesting a possible link that is deserving of further empirical investigation.

4.2. Redistribution of glutamate receptor proteins in the anterior cingulated cortex after locomotor sensitization to cocaine

In order to determine the specificity of the glutamate receptor redistribution in the prefrontal cortex after locomotor sensitization to cocaine, we examined the receptor distribution in the anterior cingulated cortex (ACC). The ACC was chosen for comparison because, similar to dorsal prefrontal cortex, it selectively innervates the nucleus accumbens core (Brog et al., 1993) but displays functional distinctions from the prefrontal cortex (Muir et al., 1996; Bussey et al., 1997a,b; Cardinal et al., 2002; Ng et al., 2007). The pattern of change in the glutamate receptor redistribution in ACC did not resemble that of prefrontal cortex compartments. At early withdrawal, there was an increase in the tissue level of mGluR2/3 protein, as well as, in the synaptosomal fraction. Unlike prefrontal cortex, at 21 days of withdrawal, there were decreases in NMDA and AMPA receptor subunits and Homer1b/c proteins. These observations suggest that glutamate receptor plasticity in the prefrontal cortex is anatomically specific and may contribute to the functional role of prefrontal cortex in the development and expression of locomotor sensitization to cocaine. An interesting observation in the ACC is that the changes in the NMDA and AMPA receptors redistributions suggest that there is an increase in AMPA receptor calcium permeability and an augmented AMPA/NMDA current ratio in ACC at 21 days of withdrawal. This suggestion is in agreement with recent studies in nucleus accumbens after extended withdrawal from cocaine treatment (Kourrich et al., 2007; Conrad et al., 2008).

Taken together, our data suggest that locomotor sensitization to cocaine is associated with a significant trafficking and redistribution of the glutamate receptors in the prefrontal cortex at both early and late withdrawal. The glutamate receptor plasticity suggests a hypoactivity in the prefrontal cortex signaling at early withdrawal which is transformed into a hyperactive state at later withdrawal time. This is in agreement with the notion that, at late withdrawal, activity of the prefrontal cortex is required for the expression of locomotor sensitization and relapse to drug-seeking behaviors.

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