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Neuronal pentraxins modulate cocaine-induced neuroadaptations

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Abbreviations: AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR- AMPA receptor; GluR- glutamate receptor subunit; KO- knock out; mGluR- group I metabotropic glutamate receptor; DHPG- (S)-3,5-dihydroxyphenylglycine; Narp- neuronal activity regulated pentraxin; NP- neuronal pentraxin; NP1- neuronal Pentraxin 1; NPR- neuronal pentraxin receptor; WT- wild type

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ABSTRACT

Neuronal pentraxins (NP) function in the extracellular matrix to bind AMPA receptors. Three NPs have been described, Narp which is regulated as an immediate early gene, NP1 and NPR. Narp and NP1 enhance synaptogenesis and glutamate signaling by clustering AMPA receptors, while NPR contributes to removing AMPA receptors during mGluR-dependent long-term depression. Here, we examine mice with genetic deletions (KO) of each NP to assess their contributions to cocaine-induced neuroplasticity. Consistent with a shared AMPA receptor clustering function for Narp and NP1, deletion of either NP caused similar behavioral alterations. Thus, while both Narp and NP1 deletion promoted cocaine-induced place preference, NPR deletion was without effect. Also, while Narp and NP1 KO showed reduced time in the center of a novel environment, NPR KO mice spent more time in the center. Finally, while Narp and NP1 KO mice showed blunted locomotion following AMPA microinjection into the accumbens 3 weeks after discontinuing repeated cocaine injections, the AMPA response was augmented in NPR KO. Similarly, endogenous glutamate release elicited less motor activity in Narp KO mice. Consistent with reduced AMPA responsiveness after chronic cocaine in Narp KO mice, GluR1 was reduced in the PSD fraction of Narp KO mice withdrawn from cocaine. These data indicate that NPs differentially contribute to cocaine-induced plasticity in a manner that parallels their actions in synaptic plasticity.

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INTRODUCTION

Neuronal Pentraxins (NPs) are extracellular matrix proteins that are enriched in excitatory synapses where they aggregate AMPA receptors (AMPAR) (O'Brien et al., 1999; 2002). Three different NPs have been described: Narp (neuronal activity regulated pentraxin, also termed NP2), NP1 (neuronal pentraxin 1) and NPR (neuronal pentraxin receptor) (Kirkpatrick et al., 2000). Narp is an immediate early gene that is induced by synaptic activity, while NP1 and NPR are constitutively expressed (Xu et al., 2003). Narp and NP1 multimerize; thereby, enhancing the clustering and synaptogenic activity of the individual proteins (Xu *et al.*, 2003). Since Narp expression is dynamically regulated by synaptic activity (Berke et al., 1998; Reti and Baraban, 2000), it is proposed that the synergistic effect of forming multimers with NP1 gives neurons a mechanism to tune the degree of AMPA R clustering and synaptogenesis in response to synaptic activity (Xu *et al.*, 2003).

In contrast to Narp and NP1, which are secreted proteins, NPR possesses a single transmembrane domain that confers a unique function. Group 1 metabotropic receptors (mGluR1/5) activate the extracellular metalloprotease ADAM 17 (TACE) which cleaves and releases NPR to promote endosome-dependent removal AMPAR from the synapse. This regulated cleavage of NPR is essential for mGluR-induced long-term depression (LTD) (Cho *et al.*, 2008). Thus, NPs appear to have nearly opposing effects on AMPAR aggregation at synapses, with Narp and NP1 acting to enhance AMPAR clustering and NPR acting to remove AMPAR.

Cocaine induces changes in glutamate neurotransmission in nucleus accumbens that are linked to behavioral sensitization and addiction (Kalivas and O'Brien, 2007). For example, withdrawal from chronic cocaine increases the expression of the GluR1 subunit of AMPAR in accumbens (Boudreau and Wolf, 2005; Conrad et al., 2008), and correspondingly increases the ratio of AMPA to NMDA currents in accumbens medium spiny neurons (Kourrich et al., 2007). Also, AMPA injected into the accumbens demonstrates behavioral cross sensitization with cocaine and induces cocaine-seeking behavior in

cocaine-experienced rats, while AMPA antagonists block cocaine-induced behavioral sensitization and seeking behavior (Pierce et al., 1996; Cornish and Kalivas, 2000; Di Ciano and Everitt, 2001).

The expression of Narp mRNA is transiently increased in striatum after acute cocaine (Berke *et al.*, 1998), combined with Narp's AMPAR clustering properties and the relevance of AMPAR in cocaine-induced neuroadaptations, identifies the Narp-NP1 complex as a candidate for regulating cocaine-induced neuroplasticity. Since previous studies failed to find changes in Narp in the accumbens after chronic cocaine administration (Lu *et al.*, 2002; Reti *et al.*, 2002), we made use of mice harboring deletions of individual NP genes to study the role of the NPs in behavioral adaptations produced by chronic cocaine administration, as well as the capacity of cocaine to affect the response to AMPAR stimulation and the levels of glutamate receptors and subunits.

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METHODS

Experimental subjects. The generation of NP KO and WT adult mice (8–10 weeks age; 129sv X C57BL/6 background) is described elsewhere (Bjartmar *et al.*, 2006). Mice were bred from heterozygous mating pairs, and genotyped at 4-5 weeks old. Narp KO mice were derived from one background, while the NPR and NP1 KO were derived from another. Mice were group housed in an AAALAC-approved animal facility (lights on, 07:00 h; 23 °C) with ad libitum food and water. All experiments were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and conducted in accordance with the National Institutes of Health (NIH) Principles of Laboratory Animal Care (1985). The data from male and female were pooled for final analysis because no significant gender effect was found.

Surgical Procedures. One week after cocaine treatment, mice were anesthetized with ketamine (120 mg/kg ip) and xylazine (6 mg/kg ip), and mounted in a Kopf stereotax apparatus equipped with a Cunningham mouse adapter. Bilateral guide cannula (10mm, 20 gauge, Small Parts) were positioned over the nucleus accumbens and secured with a light-cure dental resin (Kerr Corporation, Orange, CA). The stereotaxic coordinates employed were (in mm): AP +1.1, DL ± 1 , DV -2.2 (Franklin and Paxinos, 1997). The mice were allowed to recover for 1 week.

Behavioral tests

Cocaine place conditioning: The cocaine place conditioning procedure was described previously (Pacchioni *et al.*, 2007). The place preference apparatus was a plexiglass activity chamber (22x43x33 cm; Accuscan, Columbus, OH) with a black plastic enclosure (11x43x33 Harvard apparatus) that had black walls and ceiling, dividing the chamber into a black “closed” and a transparent “open” side. The procedure consisted of 4 phases: Habituation, Pre-Test, Conditioning and Post-Test. Habituation and Pre- and Post-Tests were 15 min sessions where the mice had free access to both sides of the chamber. According to the time spent in each side of the chamber during Pre-Test, mice were assigned to receive

cocaine in the non-preferred side and saline in preferred side during the Conditioning phase. There were 8 daily 15 min conditioning sessions (4 saline and 4 cocaine), where the mouse was restricted to the respective side immediately after an ip injection. A separate group of animals received a saline injection before being restricted in each side of the chamber, to facilitate the interpretation of the biased place conditioning paradigm results such as habituation to the non preferred side aversiveness (Bardo and Bevins, 2000). The Post-Test was conducted the day after the last conditioning session. Place conditioning was quantified as an increase in the time spent in the drug paired side during Post-Test compared to Pre-Test and referred to as occupancy time (sec).

Motor activity: all animals were habituated to Accuscan activity boxes (22x43x33 cm; Columbus, OH) for 1h. The locomotor activity was recorded during habituation and for 2 hrs immediately after the injection, and estimated by distance traveled (cm).

a) Cocaine Behavioral Sensitization Paradigm: Consisted of 7 daily injections (1 x 15mg/kg ip, 5 x 30mg/kg ip, 1 x 15mg/kg ip). The motor activity was recorded after first and last injection of cocaine (15/mg/kg ip) or saline, and at 3 weeks after finishing treatment. .

b) Locomotor response to acute caffeine: Each animal received all three injections (saline, and caffeine at 10 and 30 mg/kg ip) separated by 7 days.

c) Microinjection of AMPA or DHPG: An injection needle (33 gauge) was introduced into each guide cannula, and extended 1.5 mm below the tip. Bilateral infusions of 0.25 μl/side were made over 90 seconds, and the injectors were removed 60 seconds later. Saline plus two doses of AMPA (0.1, and 0.3 nmol/side) (Bell and Kalivas, 1996) or (S)-3,5-dihydroxyphenylglycine (DHPG, 1 and 2.5 nmoles/side) (Swanson *et al.*, 2001) were infused into the accumbens. Separate groups of mice were used for the AMPA and DHPG studies. All mice randomly received all doses in a counterbalanced order. Each injection was separated by at least 3 days.

In vivo microdialysis study. A microdialysis probe (24 gauge; 1-1.5 mm of active membrane) was lowered into the guide cannula and perfused with aCSF (5 mM glucose, 2.7 mM KCl, 140 mM NaCl, 1.4

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mM CaCl2, 1.2 mM MgCl2, 0.15% PBS [pH= 7.4]) at 2 µl/min. After four hours, dialysis samples were collected every 20 min. Increasing concentrations of DHPG (0, 3 and 30 µM/side; Tocris Cookson, Ballwin, MO) were infused into the accumbens through the probe for 60 min, as previously described for mice (Szumlinski *et al.*, 2004). The last two samples of each drug concentration were averaged and used in statistical evaluation of the data.

Glutamate measurement by HPLC with fluorescent detection. A precolumn derivatization of amino acids with o-phthalaldehyde was performed using a Gilson 231 XL autosampler (Gilson Medical Electronics, Middleton, WI). Glutamate was separated via a RP column (10 cm, 3 µm ODS; Bioanalytical Systems, West Lafayette, IN) using the following mobile phase: 11% acetonitrile (v/v), 100 mM NaH_2PO_4, 0.1 mM EDTA, pH 6.0. Glutamate was detected using a Shimadzu 10RF-A fluorescence detector (excitation wavelength: 340 nm, emission wavelength: 450 nm). For quantification, peak height was compared to an external standard curve.

Subcellular fractionation. After three weeks withdrawal from chronic cocaine or saline treatment mice were decapitated. The ventral striatum was dissected from the brain as described elsewhere (Szumlinski *et al.*, 2004), and subcellular fractionation was performed as described previously with minor modifications (Toda *et al.*, 2006). Briefly, fresh brain tissues were homogenized in cold buffer containing 0.32 M sucrose and 10 mM HEPES, pH 7.4. Homogenates were cleared two times at 1000 g for 10 min to remove nuclei and large debris (P1). The resulting supernatants were concentrated at 12 000 g for 20 min to obtain a crude membrane fraction (P2), which was rinsed twice (4 mM HEPES, 1 mM EDTA, pH=7.4; 20 min at 12 000g). Then, it was incubated (20 mM HEPES, 100 mM NaCl, 0.5% triton X, pH= 7.2) for 15 min and centrifuged at 12 000 g for 20 min to pellet the synaptosomal membrane fraction (LP1). The supernatant was considered the non-postsynaptic density membrane fraction (non-PSD), sometimes referred to as the triton soluble fraction. The pellet was then solubilized (20 mM HEPES, 0.15 mM NaCl,

1% triton X100, 1% deoxycholic acid, 1% SDS, pH= 7.5) for 1 h and centrifuged at 10 000 for 15min. The supernatant contained the postsynaptic density fraction (PSD) or triton insoluble fraction. The integrity of non-PSD and PSD fractions was verified by immunoblotting for PSD-95 which was enriched in PSD fraction, and synaptophysin which was enriched in non-PSD fraction (Supplemental Figure 1). All buffers were supplemented with protease inhibitors cocktail (Complete mini tablets, Roche). Protein concentration was measured using the Bradford assay (Pierce).

Western blotting. Western blotting for different subcellular fractions was conducted as described in detail elsewhere (Toda *et al.*, 2006). Samples (5 µg per lane) were run in 10% Tris–acrylimide gels (Invitrogen), and transferred to PVDF membranes. Overnight incubation with each primary antibody (Glur2, 1:250 BDbiosciences; Glur1, 1:500 Chemicon; mGluR5, 1:2000 Upstate; Narp, 1:2000 provided by coauthor, Dr Paul Worley) was followed by a secondary HRP conjugated antibody (1:10000 or 1:20000 Upstate), and the reactivity was detected using Supersignal West Pico (Pierce). Densitometric analysis was performed with Image J program (version 1.36b). Band intensities were measured by taking the pixels integrated density. Every gel has one lane loaded with an internal standard (accumbens P2 fraction from WT mice) in order to compare densities between gels. After normalizing to the internal standard, data were normalized to percent change from respective controls. For the PSD subfraction, a control protein within each band, such as actin, was not used to normalize each lane. As described elsewhere, the PSD is very dynamic making it difficult to insure the stability of a protein in this subfraction after gene deletion or pharmacological treatments (Toda et al., 2006).

Histology. After concluding the microinjection or microdialysis experiments, mice were deeply anesthetized with pentobarbital (70 mg/kg ip), and perfused intracardially with 10% formalin. The brain was removed and stored in 10% formalin for a 1 week. Coronal sections (60 µm) were mounted and stained with cresyl violet. Injection sites and probe placements were determined according to Franklin and Paxinos (1997) (Supplemental Figure 2).

Data Analysis. Because of the different genetic backgrounds Narp KO mice were compared to their WT littermates, while NP1 KO, NPR KO and their WT littermates were analyzed separately. The data were statistically evaluated using 2-, 3-, or 4-way ANOVAs with main factors being genotype, drug treatment (saline or cocaine), time and/or dose. When significant effects or interactions were identified, data were appropriately partitioned and evaluated using 1 or 2-way ANOVAs followed by Bonferroni or least significant difference post hoc analyses to identify specific genotypic differences. Statistical comparisons of protein levels were made on data that were normalized to the chronic saline group in each genotype and comparisons made using an ANOVA or Student's t-test.

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RESULTS

Deletion of NP genes differentially affects cocaine place preference.

Figure 1A shows that in the Narp KO, 10, 20 and 30 mg/kg of cocaine induced place conditioning relative to saline control group (see Supplemental Table 1 for pre- and post-test scores). A two-way ANOVA revealed a significant effect of dose ($F_{(1,100)}$= 12.75, p= 0.006), but no effect of genotype or interaction. Because of the significant effect of dose, each genotype was examined separately with a one-way ANOVA to determine which doses differed significantly from saline. Only in Narp KO was a significant effect of cocaine versus saline observed ($F_{(4,48)}$= 3.75, p= 0.017). Although 30 mg/kg cocaine in the WT appeared to induce place conditioning in comparison with saline control group, the one-way ANOVA did not reveal statistical significance. These data support a greater tendency for cocaine to induced place preference in KO than in WT mice.

Akin to Narp KO, NP1 KO mice showed place preference to cocaine (20 mg/kg, ip), while NPR KO mice showed no place preference to cocaine (Figure 1B). The WT for the NP1 and NPR KO mice showed near significant place conditioning to cocaine relative to saline group (p= 0.071). A two-way ANOVA revealed a significant effect of drug ($F_{(1,57)}$= 15.83, p= 0.0002), and genotype ($F_{(2,57)}$= 3.272, p= 0.045) but no interaction. A Bonferroni post hoc test revealed a significant effect of cocaine versus saline only in NP1 KO (p< 0.001).

NP gene deletion alters open field behavior. Mice were placed into a novel environment (photocell apparatus) and behavior estimated for 60 min, including locomotion (distance traveled) and the amount of time spent in the center of the box. Figure 2A shows that locomotor activity in response to a novel environment was not altered in Narp KO relative to WT. Except during the first 10 min NP1 KO were also not different from WT. However, NPR KO showed elevated locomotor activity in response to novelty across the first 50 min. Two-way ANOVAs with repeated measures over time revealed an effect of time for Narp ($F_{(5,305)}$ = 121.20, p< 0.001) and NP1/NPR ($F_{(5,175)}$ = 83.90, p< 0.001), but only the

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NP1/NPR experiment showed a significant effect of genotype ($F_{(2,175)} = 8.90$, $p < 0.001$) and an interaction between genotype and time ($F_{(10,175)} = 7.21$, $p < 0.001$).

The amount of time spent in the center of the box is a measure of the level of anxiety (Prut and Belzung, 2003). The time spent in the center of the box was reduced in the Narp and NP1 KOs, and elevated in the NPR KO relative to their respective WT (Figure 2B); suggesting elevated anxiety levels in the Narp and NP1 KOs, while NPR KO were less anxious than WT. Two-way ANOVAs with repeated measures over time revealed an effect of genotype and time for Narp (genotype $F_{(1,225)} = 5.74$, $p = 0.021$; time($F_{(5,225)} = 4.16$, $p = 0.001$) and NP1/NPR (genotype $F_{(2,175)} = 9.11$, $p < 0.001$; time ($F_{(5,175)} = 6.01$, $p < 0.001$), but only the NP1/NPR experiment showed a significant interaction between genotype and time ($F_{(10,175)} = 2.07$, $p = 0.029$).

Deletion of NP genes and cocaine-induced locomotion and behavioral sensitization. Locomotor activity induced by acute cocaine and the development of behavioral sensitization to repeated cocaine was examined in the NPs KO and their respective WT mice by assessing the motor stimulant effect of cocaine on the first and last day of 7 daily cocaine injections (Days 1 and 7), and after a 3 wk withdrawal period (Day 28). Figure 3A shows that Narp gene deletion significantly reduced the acute motor response to cocaine relative to WT (e.g. Day 1). Seven days of daily cocaine resulted in sensitized motor activity in both genotypes. However, in Narp KO the response to cocaine on day 7 was lower than after 21 days of withdrawal (e.g. day 28), while the responses on Day 7 and 28 were equivalent in WT mice. The majority of differences between treatment groups occurred during the first 20 min after cocaine injections. In contrast to cocaine, no significant differences in the response to saline injection were observed on any day or between genotypes. A 4-way ANOVA revealed significant main effects of time ($F_{(17,1989)} = 94.65$, $p < 0.001$), genotype ($F_{(1,117)} = 19.94$, $p < 0.001$), injection day ($F_{(2117)} = 9.16$, $p < 0.001$), and drug ($F_{(1,117)} = 107.76$, $p < 0.001$). Also, all between subjects main effects showed significant interactions with time [genotype ($F_{(17,1989)} = 3.86$, $p < 0.001$); injection day ($F_{(34,1989)} = 12.94$, $p < 0.001$); drug ($F_{(17,1989)} = 83.50$,

$p < 0.001$)]. Therefore, the data were evaluated using multiple 2-way ANOVAs with repeated measures over time followed by a least significant difference test to determine time points where the genotypes differed when a significant interaction between main effects was found.

To determine whether the lower response to acute cocaine in Narp KO generalized to all psychomotor stimulants, locomotor activity was elicited by two different doses of caffeine (10 and 30 mg/kg ip). Figure 3B shows that Narp KO and WT showed equal motor stimulation at both doses of caffeine (time course data are shown in Supplemental Figure 3). A two-way ANOVA with repeated measures over dose revealed a significant effect of dose ($F_{(2,39)} = 31.40$, $p < 0.001$), but no effect of genotype or interaction.

Figure 3C shows that NP1/NPR WT, NP1 KO and NPR KO mice all developed behavioral sensitization by 3 wks of withdrawal from repeated cocaine compared to the first injection day. However, akin to the Narp KO, the NP1 KO showed less sensitization on the Day 7 than on Day 28, while the sensitized WT and NPR KO the motor responses were equivalent between Days 7 and 28. No genotypic differences were identified in the motor response elicited by the first cocaine injection, and daily saline did not induce sensitization in the WT mice. A 3-way ANOVA was conducted on the cocaine treatment data and revealed significant main effects of time ($F_{(17,1292)} = 172.45$, $p < 0.001$), genotype ($F_{(2,76)} = 11.74$, $p < 0.001$), and injection day ($F_{(2,76)} = 4.39$, $p = 0.016$). Also, all between subjects main effects showed significant interactions with time [genotype ($F_{(34,1292)} = 2.91$, $p = 0.001$); injection day ($F_{(34,1292)} = 18.69$, $p < 0.001$)]. Therefore, the data were evaluated using multiple 2-way ANOVA with repeated measures over time followed by a least significant difference test to determine time points where the genotypes differed. The fact that Narp and NP1 KO mice showed a full expression of sensitization on Day 28 but not on Day 7 while NPR KO and both WT mice showed it on Day 7, suggest a delayed development of sensitization in Narp and NP1 KO compared to their WT.

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Deletion of NP genes differentially affect the motor response to AMPA injected into the accumbens. Figure 4A shows that akin to previous reports in rats (Bell and Kalivas, 1996; Pierce et al., 1996) , the motor stimulant response elicited by intra-accumbens administration of AMPA was augmented in mice pretreated 3 weeks earlier with daily cocaine relative to daily saline. Thus, neither dose of AMPA (0.1 and 0.3 nmol/side) elicited a motor stimulant response in WT or Narp KO pretreated with daily saline (Supplemental Table 3). While both WT and Narp KO mice withdrawn from repeated cocaine showed enhanced motor activity at the highest dose of AMPA, only the WT showed elevated motor activity at the lower dose. This differential effect was especially apparent in the time course analysis (Figure 4B). A 3-way ANOVA showed significant main effects of AMPA dose $F_{(2,46)}$= 4.20, p= 0.021 and pretreatment $F_{(1,23)}$=14.92, p<0.001. Thus, saline and cocaine pretreated animals were evaluated by separate 2-way ANOVAs with repeated measures over dose to identify genotypic differences, and in cocaine pretreated mice there were significant main effects of genotype $F_{(1,13)}$= 5.38, p= 0.037 and dose $F_{(2,26)}$=6.19, p<0.01. A 2-way ANOVA at each dose revealed a significant main effect of genotype only at 0.1 nmol AMPA $F_{(1,143)}$= 5.09, p= 0.042; as expected Saline infusions did not induce locomotor activity under any condition (See Supplemental Figure 4A for temporal course of Saline infusion in Narp KO and WT pretreated with daily cocaine).

Figure 4C shows that after withdrawal from cocaine the highest dose of AMPA elevated motor activity in both the NP1/NPR WT and NP1 KO, but that the response in the NP1 KO was significantly blunted compared to the WT. In contrast, both doses of AMPA elicited motor activity in the NPR KO, indicating that at the lower dose NPR deletion potentiated AMPA relative to WT. Also, the motor response seen after acute microinjection of saline was blunted in NP1 KO relative to WT or NPR KO. The genotypic distinctions in AMPA-induced locomotion were also seen in the time course analysis (Figure 4D), as expected Saline infusions did not induce locomotor activity under any condition (See Supplemental Figure 4B for temporal course of Saline infusion in WT, NP1 and NPR KO pretreated with daily cocaine). A 2-way ANOVA at each dose revealed significant main effects of genotype $F_{(2,62)}$= 7.66, p=

0.002 and dose $F_{(2,62)}$= 13.49, p< 0.001. Two-way ANOVAs at each dose revealed significant effects of time at both doses of AMPA, a main effect of genotype at 0.1 AMPA $F_{(2,330)}$= 26.57, p= 0.001, and a significant interaction between time and genotype at 0.3 AMPA $F_{(22,319)}$= 4.94, p< 0.001.

The motor response to endogenously released glutamate is blunted in Narp KO. It was previously shown that stimulation of mGluR1 receptors releases synaptic glutamate (Schrader and Tasker, 1997; Swanson et al., 2001), and mGluR1-induced glutamate release into the accumbens promotes locomotor activity by stimulating AMPAR (Swanson *et al.*, 2001). To determine if, akin to AMPA, the motor response induced by endogenously released glutamate is also blunted by Narp gene deletion, the mGluR1/5 agonist DHPG was microinjected into the accumbens of WT and Narp KO mice. Figure 5A shows that DHPG produced a dose-dependent increase in motor activity in WT, but had no significant effect in Narp KO mice. A two-way ANOVA revealed a significant effect of genotype $F_{(1,36)}$=4.62, p= 0.041 and dose $F_{(2,36)}$=26.84, p< 0.001, as well as an interaction between dose x genotype $F_{(2,36)}$=13.4, p= 0.006. This genotypic difference is clearly reflected in the time course of DHPG-induced motor activity (Figure 5B). A three-way ANOVA revealed a significant effect of dose $F_{(2,54)}$=10.1, p= 0.001, and significant interactions of genotype x dose $F_{(2,54)}$= 4.61, p= 0.001; and time x genotype x dose $F_{(22,594)}$= 3.21, p< 0.001. Importantly, the genotypic distinction in DHPG induced motor activity was not the result of a difference in the ability of DHPG to release glutamate. Thus, the dose-dependent increase in extracellular glutamate elicited by reverse dialysis of DHPG into the accumbens was equivalent in both genotypes (Figure 5C). A two-way ANOVA revealed only a significant main effect of dose $F_{(2,11)}$=31.06, p< 0.001. The time course of DHPG induced changes in extracellular glutamate. A two-way ANOVA revealed only a significant effect of time $F_{(1,8)}$=33.95, p< 0.001 (figure 5D).

One mechanism whereby glutamate can regulate the clustering and internalization of NPs and AMPAR is via stimulation of mGluR5 (Cho *et al.*, 2008). Therefore, the ventral striatum (including the nucleus accumbens) of WT and Narp KO was subfractionated and the level of mGluR5 measured by

immunoblotting to determine if alterations in mGluR5 could have influenced the actions of DHPG, which is an agonist at both mGluR1 and mGluR5 . No genotypic difference was found between WT and Narp KO in the levels of mGluR5 monomer or dimmer in the PSD or non-PSD subfraction (Supplemental Figure 5). The location of dialysis and injection cannulae in the nucleus accumbens is shown in Supplemental Figure 2.

Since the stimulation of mGluR in the NAcc by DHPG induced the same glutamate release in both genotypes, the decreased locomotor response induced by intra-accumbens DHPG in Narp KO suggests a deficit in AMPAR signaling. However, when AMPA is injected, the deficit in AMPA signaling is evident only after a cocaine treatment but not after saline treatment.

Cocaine decreases the expression of GluR1 in PSD of Narp KO. Narp clusters AMPAR by interacting with subunits GluR1-4, and consistent with previous reports (Lu *et al.*, 2002; Reti *et al.*, 2002), withdrawal from chronic cocaine did not affect the level of Narp in the PSD or non-PSD subfractions of WT mice (Supplemental Figure 6). The levels of GluR1 and GluR2 were also measured in WT and Narp KO at 3 wks of withdrawal from daily cocaine or saline administration. No differences between genotypes were measured in chronic saline pretreated mice, and chronic cocaine had no effect on GluR1 or GluR2 measured in the PSD of WT mice (Figures 6A and Supplemental Table 2). However, figure 6A shows that withdrawal from chronic cocaine significantly reduced the levels of GluR1 in the PSD subfraction of Narp KO, but not WT mice ($t_{(13)} = 2.58$, $p = 0.022$). In contrast, chronic cocaine did not affect the level of GluR1 in the non-PSD subfraction of either genotype. No effects of cocaine were found on the levels of GluR2 in the PSD or non-PSD subfractions (Supplemental Table 2). Figure 6B illustrates representative immunoblots showing the reduction in GluR1 in the PSD subfraction of Narp KO after withdrawal from chronic cocaine. Note that only in the Narp KO pretreated with chronic cocaine did the densities in the PSD fraction approximate those in the non-PSD fraction. Otherwise, in all other treatment groups there is substantially more GluR1 in the PSD than the non-PSD fraction.

DISCUSSION

Our findings suggest opposing actions of Narp and NP1 versus NPR in cocaine-induced plasticity that parallel their differential actions in synaptogenesis and excitatory transmission. Narp and NP1 are able to multimerize and promote clustering of AMPAR (O'Brien et al., 2002; Xu et al., 2003), while NPR is thought to bind to the Narp/NP1/AMPA clusters and promote internalization of the clustered receptors (Cho *et al.*, 2008). Consistent with a shared AMPAR clustering function for Narp and NP1, deletion of either NP caused similar alterations in spontaneous and cocaine-induced behavior and desensitized the motor response elicited by stimulating AMPAR in the nucleus accumbens. Thus, while both Narp and NP1 deletion promoted cocaine-induced place preference, NPR deletion was without effect, and while Narp or NP1 deletion inhibited AMPA induced locomotion, NPR deletion potentiated the motor effect of AMPA. Also, while Narp and NP1 KO showed reduced time in the center of a novel environment, NPR KO mice spent more time in the center. As reported previously, withdrawal from chronic cocaine potentiates the motor stimulant effect of intra-accumbens AMPA (Pierce *et al.*, 1996), and the fact that this potentiation was inhibited in Narp KO is consistent with the marked down-regulation of GluR1 in the ventral striatal PSD in Narp KO mice withdrawn from chronic cocaine administration. Taken together, these data implicate the NPs as potential regulators of the behavioral and cellular adaptations produced by chronic cocaine.

Neuronal pentraxins and cocaine-induced behavioral adaptations. Cocaine administration initiates reward learning causing animals to develop a preference for the location where cocaine is administered relative to a location where a more neutral saline injection is given (Kelley, 2004; Tzschentke, 2007). The ability to make this association was potentiated in Narp and NP1 KO mice. Previous work revealed that Narp KO mice were unimpaired in acquiring conditioned reinforcement and Pavlovian-instrumental transfer in food-rewarded learning (Johnson *et al.*, 2007), and morphine place conditioning (Crombag et al., 2008). By showing augmented cocaine-rewarded learning, the present findings are consistent with the lack of impairment by Narp gene deletion on reward learning and indicate interactions with cocaine-

specific learning. A specific interaction with cocaine was indicated by the fact that Narp KO showed an impaired locomotor response to acute cocaine administration, but normal caffeine-induced locomotion. Interestingly, although the acute response was impaired, the capacity to develop sensitized motor behavior with repeated administration appeared largely intact. However, akin to the similarities between the Narp and NP1 KO genotypes in cocaine place preference, both genotypes showed delayed development of sensitized motor behavior.

The co-occurrence of augmented place preference but delayed sensitization was surprising given the literature supporting the role of sensitization of incentive learning in chronic cocaine treated animals (Robinson and Berridge, 2001). One possible explanation for this apparent dissociation may be the inability of Narp or NP1 KO to regulate AMPAR in response to cocaine. Thus, the capacity of AMPAR stimulation to induce behavioral activation was markedly reduced in the Narp and NP1 KO mice, and some studies have indicated that decreased GluR1 in the nucleus accumbens is associated with increased cocaine reward (Sutton et al., 2003), while others show that blocking AMPAR inhibits cocaine-induced behavioral sensitization (Vanderschuren and Kalivas, 2000). Furthermore, the overexpression of WT GluR1 in accumbens reduced cocaine induced place conditioning (Kelz *et al.*, 1999), and recently Bachtell et al (2008) showed that overexpressing a WT-GluR1 in accumbens increases AMPA induced locomotion after cocaine treatment, but decreases cocaine sensitization and reinstatement. Also, the overexpression of GluR1 holding a single point mutation (Q582E) in the pore region that reduces synaptic current through AMPAR decreases AMPA induced locomotion after a cocaine treatment, but increases cocaine sensitization and reinstatement. In the present study, Narp and NP1 KO showed similar responses to the overexpression of the mutant GluR1 in the Batchtell et al (2008) study, suggesting that in the absence of pentraxins the function of AMPAR may be decreased; which is also suggests by the lower DHPG induced activity in Narp KO and by the reduced AMPA induced activity in Narp/NP1 KO.

It should be noted that other studies examining the rewarding effects of cocaine using conditioned place preference have yielded mixed results regarding the involvement of AMPAR. Furthermore, studies using

AMPAR antagonists showed inhibition (Maldonado et al., 2007) or no effect (Cervo and Samanin, 1995) on cocaine place conditioning. Similar discrepancies were found using GluR1 KO mice where Dong et al (2004) showed blunted cocaine induced CPP in KO mice, but Mead et al (2005) showed no differences in CPP between KO and WT mice. Notwithstanding it is important to acknowledge that because the gene deletions were constitutive KO it is not possible to rule out possible developmental influences of the missing protein that may underlie the differential effects on cocaine-induced neurodaptations.

It is also important to note the possibility that the different levels of anxiety in the Narp, NP1 and NPR KO compared to WT may interfere with the interpretation of the biased place conditioning procedure. Thus, it is possible that habituation to the anxiety provoking effects of the non-preferred side may contribute to the increase in time spent in the non-preferred side associated with repeated cocaine administration. However, exposure of the Narp and NP1 KO mice to the non-preferred side after saline injections increased occupancy time similar to WT, and this increase was significantly lower than cocaine induced increase in occupancy time in the Narp and NP1 KO genotypes.

Neuronal pentraxins and AMPA receptor plasticity. Although not quantified for all genotypes the reduction in GluR1 in the PSD subfraction from the ventral striatum of Narp KO only after withdrawal from cocaine reveals an interesting interaction between cocaine regulation of AMPAR and Narp. Previous studies show an increase in GluR1 after withdrawal from chronic cocaine that is associated with the development of behavioral sensitization (Boudreau and Wolf, 2005). While behavioral correlations were not evaluated in the present study, the absence of Narp altered the effect of cocaine on AMPAR expression in the PSD fraction. Thus, only in the absence of Narp were the levels of GluR1 significantly reduced in the PSD subfraction after chronic cocaine; while in WT mice the decrease after cocaine is just a trend. At present it is not clear if the presumed reduced capacity to cluster AMPAR in Narp KO results in less GluR1 after chronic cocaine due to a change in the trafficking of GluR1 into or out of the PSD

subfraction, or a change in synthesis of AMPAR subunits. However, the lack of change in GluR1 in the non-PSD fraction of either genotype indicates that major changes in synthesis were not present.

It is tempting to speculate that the functional segregation of NPs in synaptogenesis and excitatory transmission may underlie the differential effects of individual NP gene deletion on AMPA-induced motor activity, cocaine-induced place preference and behavioral sensitization. Thus, the presumed deficit in AMPAR clustering associated with Narp or NP1 gene deletion would be predicted to impair AMPAR signaling. Conversely, NPR KO mice show reduced mGluR5-dependent LTD due to reduced internalization in NPR KO (Cho *et al.*, 2008),which would be predicted to potentiate AMPA-induced responses. Correspondingly, Narp and NP1 deletions inhibited AMPA-induced locomotion, and NPR deletion augmented the motor response. Also, cocaine place preference was enhanced and the development of behavioral sensitization retarded in Narp and NP1 KO, while place preference and behavioral sensitization were either inhibited or not affected in NPR KO. As discussed above, this segregation of cocaine phenotypes to specific genotypes is consistent with portions of the behavioral literature regarding a role for AMPAR in differentially regulating cocaine-induced reward and locomotion.

Conclusions. This study demonstrates a differential interaction between the various NPs and cocaine. The behavioral distinctions between the NPs correspond to previously reported functional categories in that deletion of the NP genes responsible for clustering AMPAR, Narp and NP1, resulted in a behavioral phenotype distinct from deletion of NPR, which is responsible for the internalization of clustered AMPAR. Moreover, it was shown that Narp may contribute to the regulation of AMPAR by cocaine since GluR1 levels in the PSD were reduced by a combination of withdrawal from chronic cocaine and Narp gene deletion. These data support the possibility that the NPs influence the excitatory neuroplasticity produced in the accumbens by chronic cocaine administration that is thought to contribute to addiction.

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Footnotes

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b) Reprint request should be made to: Dr. Alejandra M. Pacchioni, Department of Neurosciences, Medical University of South Carolina, 173 Ashley Avenue, BSB 403, Charleston, SC 29425,USA. Phone: (843) 792-4400 Fax: (843) 792-4423 Email: pacchio@musc.edu

LEGENDS FOR FIGURES

Figure 1. Narp and NP1 KO mice show enhanced cocaine-induced conditioned place preference. A) Cocaine induces place conditioning in Narp KO but not in WT mice. Cocaine place conditioning is expressed as mean ± sem occupancy difference in sec (Post test time – Pre test time). The number of determinations is shown in the bars. **B)** Cocaine (20 mg/kg, ip) elicited place preference in NP1 KO, but not NPR KO or the NP1/NPR WT.

*p< 0.05 comparing saline to cocaine-treated animals within each genotype using a Dunnett's (panel A) or Bonferroni post hoc test (panel B).

Figure 2. The deletion of NP genes altered spontaneous behavior in a novel open field. A) Spontaneous locomotor activity, estimated by distance traveled, was not affected by Narp gene deletion, was greatly elevated in NPR KO, and elevated during the first 10 min in NP1 KO. **B)** The amount of time spent in the center of the open field was reduced in Narp and NP1 KO, and increased in NPR KO. Data are shown as mean ± sem and the number of determinations in each group is shown in parenthesis.

*p< 0.05, comparing KO to respective WT using a Bonferroni post hoc or main effect of genotype (Narp KO vs WT).

Figure 3. The deletion of NP genes altered the development, but not the expression of cocaine-induced behavioral sensitization. Mice were injected once a day for 7 days with either saline or cocaine (1 x 15 mg/kg, 5 x 30mg/kg, 1 x 15mg/kg). **A)** Narp deletion reduced the acute response to cocaine, and delayed the development of full sensitization relative to WT by showing reduced motor activity on day 7 versus 28. The number of determinations in each group were WT/saline= 10, Narp-KO/saline= 10, WT/cocaine= 11, Narp-KO/cocaine= 12. **B)** Lack of effect by Narp gene deletion on caffeine-induced motor activity. N= 9 for Narp-KO and N= 6 for WT. **C)** NP1 deletion reduced the development of sensitization to daily cocaine relative to WT by showing reduced motor activity on day 7 versus day 28.

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The number of determinations in each group were WT/saline= 8, WT/cocaine= 10, NP1-KO/cocaine= 10, NPR-KO/cocaine= 9. All data are expressed as mean ± sem distance traveled (cm).

*p< 0.05 comparing saline to cocaine- or caffeine-treated animals within each genotype

+p< 0.05 comparing KO to corresponding WT

#p< 0.05, comparing genotypes on Day 1.

Figure 4. Narp and NP1 gene deletion inhibit AMPA-induced motor activity. A) AMPA microinjection into the nucleus accumbens produced a dose-dependent increase in motor activity only in mice withdrawn from daily cocaine, and Narp KO show reduced locomotor activity to the lowest dose of AMPA. The data are shown as mean ± sem total distance traveled (cm) during 2 hour after AMPA. **B)** Time course for AMPA-induced locomotion in WT and Narp KO. **C)** NP1 KO inhibits locomotor responding to AMPA relative to WT, while NPR KO shows enhanced responding. **D)** Time course for AMPA-induced locomotion in WT, NP1 KO and NPR KO. *p< 0.05 compared to saline infusion within each genotype using a 1-way ANOVA followed by a Bonferroni post hoc test (panels A and C). Main effect of KO vs WT (panels B and D)

+p< 0.05 comparing KO to corresponding WT using a one way ANOVA followed by a Dunnetts post hoc (panels A and C) or a least significant difference post hoc (panel D).

Figure 5. Narp KO show deficits in the motor response elicited by glutamate released endogenously after DHPG injection into the accumbens. A) The dose-dependent induction of motor activity by DHPG in WT in absent in Narp KO mice. **B)** Time course of DHPG-induced (2.5 nmol) locomotor activity revealed a marked genotypic difference. **C)** DHPG induced an equivalent dose-dependent release of glutamate between WT and Narp KO when reverse dialyzed into the accumbens. **D)** Time course of DHPG induced changes in extracellular glutamate.*p< 0.05, comparing between genotypes.

+p< 0.05, comparing to baseline or saline injection within genotypes.

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Figure 6. Withdrawal from chronic cocaine reduces GluR1 in the Narp KO, but not WT mice. **A)** The levels of GluR1 are reduced after withdrawal from chronic cocaine in the PSD subfraction of Narp KO, but not WT mice. GluR1 was not reduced by cocaine in the non-PSD subfraction. **B)** Representative immunoblots of GluR1. C- internal standard control (see methods, 1- non-PSD, 2- PSD, Sa- chronic saline, Co- chronic cocaine, M- protein standard, WS- WT/saline, WC- WT/cocaine, KS- KO/saline, KC- KO/cocaine

*$p < 0.05$, comparing chronic cocaine to saline treatments within each genotype

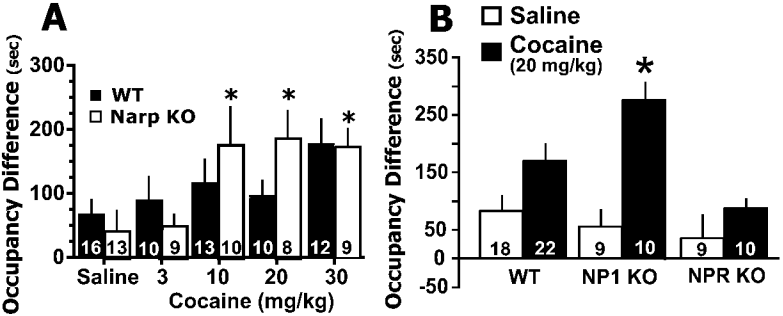
A
Occupancy Difference (sec)
300
200
100
0
WT
Narp KO
*
*
*
16
13
10
9
13
10
10
8
12
9
Saline
3
10
20
30
Cocaine (mg/kg)
B
Saline
Cocaine
(20 mg/kg)
Occupancy Difference (sec)
350
250
150
50
0
-50
*
18
22
9
10
9
10
WT
NP1 KO
NPR KO

Figure 1

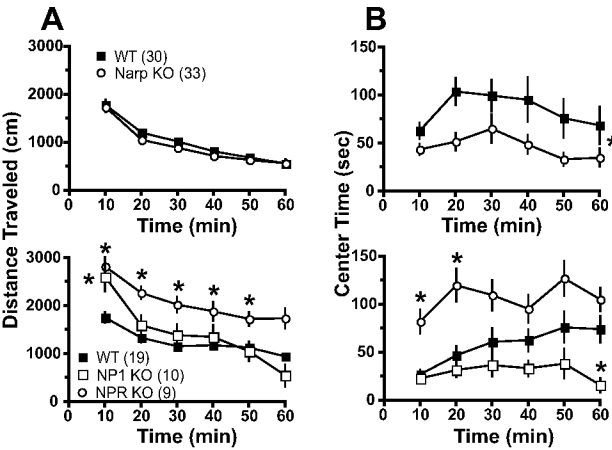
A
B
WT (30)
Narp KO (33)
Distance Traveled (cm)
Time (min)
Center Time (sec)
WT (19)
NP1 KO (10)
NPR KO (9)

Figure 2

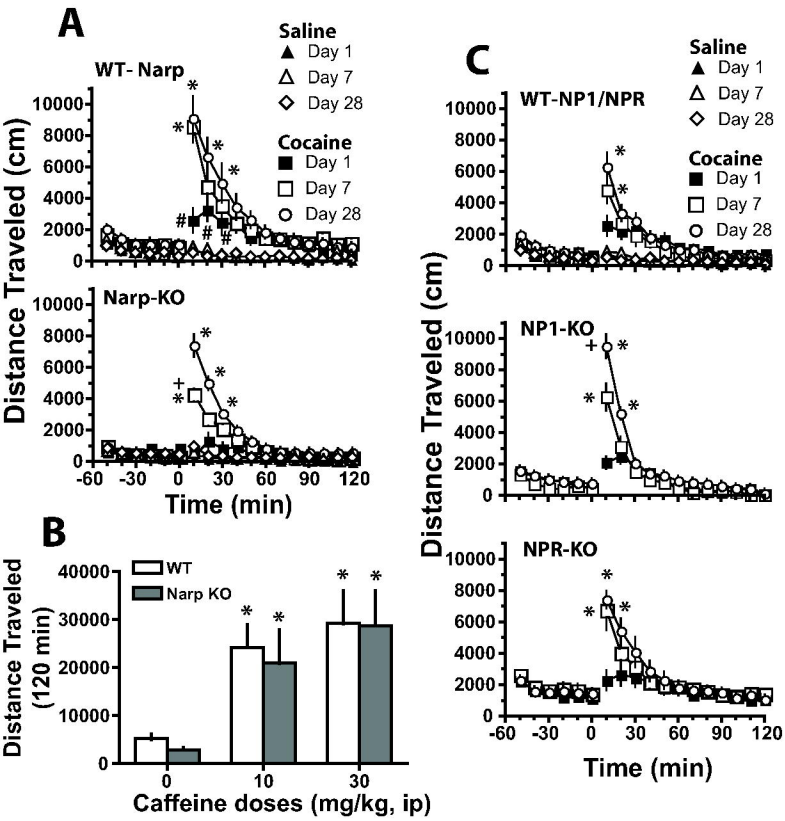
A
WT- Narp
Saline
Day 1
Day 7
Day 28
Cocaine
Day 1
Day 7
Day 28
Distance Traveled (cm)
Narp-KO
Time (min)
B
WT
Narp KO
Distance Traveled (120 min)
Caffeine doses (mg/kg, ip)
C
WT-NP1/NPR
NP1-KO
NPR-KO
Distance Traveled (cm)
Time (min)

Figure 3

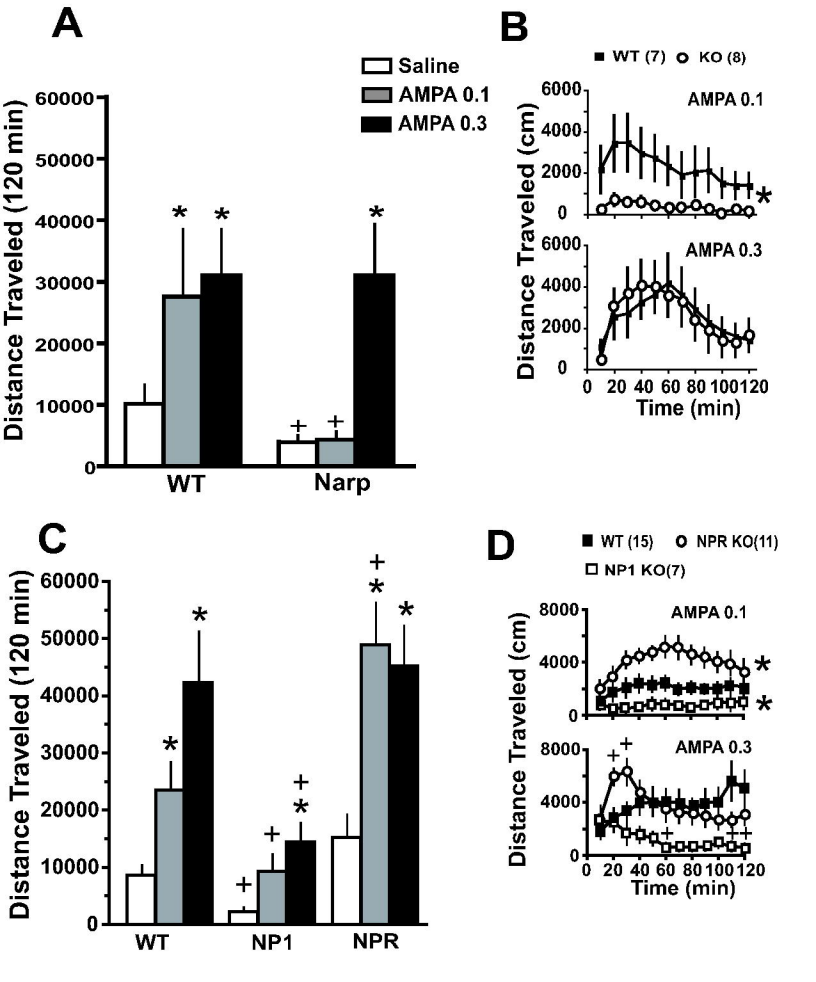
A
Saline
AMPA 0.1
AMPA 0.3
Distance Traveled (120 min)
60000
50000
40000
30000
20000
10000
0
WT
Narp
B
WT (7)
KO (8)
AMPA 0.1
AMPA 0.3
Distance Traveled (cm)
6000
4000
2000
0
0 20 40 60 80 100 120
Time (min)
C
Distance Traveled (120 min)
60000
50000
40000
30000
20000
10000
0
WT
NP1
NPR
D
WT (15)
NPR KO(11)
NP1 KO(7)
AMPA 0.1
AMPA 0.3
Distance Traveled (cm)
8000
4000
0
0 20 40 60 80 100 120
Time (min)

Figure 4

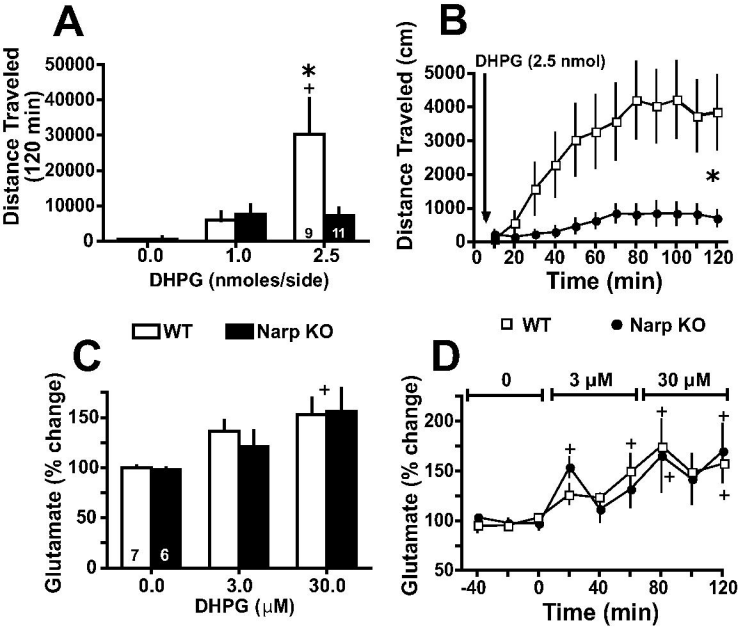
A
Distance Traveled (120 min)
50000
40000
30000
20000
10000
0
*
+
9
11
0.0
1.0
2.5
DHPG (nmoles/side)
B
Distance Traveled (cm)
DHPG (2.5 nmol)
5000
4000
3000
2000
1000
0
*
0
20
40
60
80
100
120
Time (min)
WT
Narp KO
C
Glutamate (% change)
150
100
50
0
7
6
+
0.0
3.0
30.0
DHPG (μM)
WT
Narp KO
D
Glutamate (% change)
0
3 μM
30 μM
200
150
100
50
+
-40
0
40
80
120
Time (min)

Figure 5

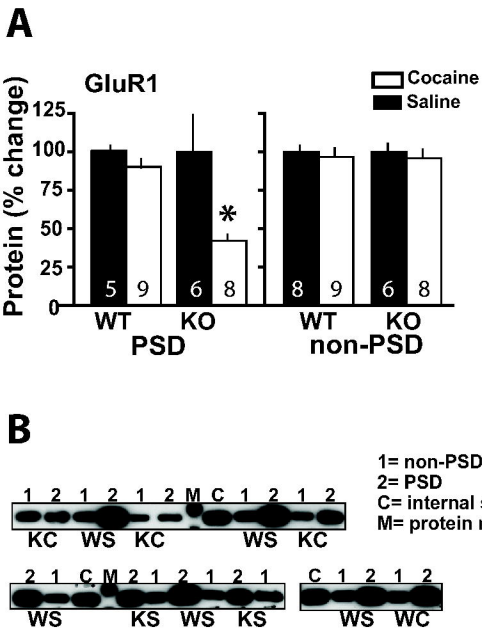
A
GluR1
Cocaine
Saline
Protein (% change)
125
100
75
50
25
0
*
5
9
6
8
8
9
6
8
WT
KO
WT
KO
PSD
non-PSD
B
1= non-PSD
2= PSD
C= internal standard
M= protein marker
1 2 1 2 1 2 M C 1 2 1 2
KC WS KC WS KC
2 1 C M 2 1 2 1 2 1
WS KS WS KS
C 1 2 1 2
WS WC

Figure 6