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Preferential relocation of the NMDA receptor NR1 subunit in nucleus accumbens neurons that contain dopamine D1 receptors in rats showing an apomorphine-induced sensorimotor gating deficit

Yuko Hara and Virginia M. Pickel

Abstract

Sensorimotor gating as measured by prepulse inhibition (PPI) to startle-evoking auditory stimulation (AS) is disrupted in schizophrenia and in rodents receiving systemic administration of apomorphine, a dopamine D1/D2 receptor agonist, or MK-801, an NMDA receptor antagonist. The functional analogies and our prior results showing apomorphine- and AS-induced relocation of the dopamine D1 receptor (D1R) in the nucleus accumbens (Acb) shell suggest that apomorphine and AS may affect the subcellular distribution of the NMDA receptor NR1 subunit, a protein that forms protein-protein interactions with the D1R. We quantitatively compared the electron microscopic immunogold labeling for NR1 in dendritic profiles distinguished with respect to presence of D1R immunoreactivity and location in the Acb shell or core of rats receiving a single subcutaneous injection of vehicle (VEH) or apomorphine (APO) alone, or combined with AS (VEH+AS, APO+AS). The rats in the APO+AS group were previously shown to have PPI deficits, whereas the rats in the VEH+AS group had normal PPI. A significantly higher percentage of plasmalemmal and a lower percentage of cytoplasmic NR1 immunogold particles were seen in D1R-labeled dendritic spines in the Acb shell of the APO+AS group compared with all other groups. D1R-containing small dendrites in the Acb shell of the APO+AS group also showed a significantly higher density of plasmalemmal and a lower density of cytoplasmic NR1 immunogold particles compared with VEH or APO groups. In the Acb core, the APO+AS group had significantly fewer dendritic spines co-expressing NR1 and D1R compared with VEH or VEH+AS groups. These results, together with our earlier findings, suggest that NMDA receptors are preferentially mobilized in D1R-containing Acb neurons of rats showing apomorphine-induced disruption of PPI in a paradigm using acoustic stimulation.

Keywords

accumbens shell; dendritic spine; immunogold; prepulse inhibition; receptor trafficking; schizophrenia

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INTRODUCTION

Prepulse inhibition (PPI) is an operational measure of sensorimotor gating, where the presence of a weak stimulus (prepulse) reduces the startle response to an immediately following intense auditory (AS) or other startling stimulus (Hoffman and Searle, 1965; Ison and Hammond, 1971). This PPI is disrupted in schizophrenia patients (Braff et al., 1978; Grillon et al., 1992), and also in rodents receiving systemic apomorphine, a dopamine D1/D2 receptor agonist (Swerdlow et al., 1986; Davis et al., 1990; Druhan et al., 1998; Geyer and Swerdlow, 1998; Kinney et al., 1999).

The PPI-disruptive effect of apomorphine is mediated in part by increased dopamine transmission in the nucleus accumbens (Acb; Swerdlow et al., 1986). The effective sites may include both the motivation-associated shell and the motor-associated core subregions of the Acb (Heimer et al., 1991; Zahm and Brog, 1992). These subregions are not only functionally distinct, but also differ in their major sources of dopamine and glutamate. The medial Acb shell receives exclusive dopaminergic input from the ventral tegmental area, whereas the core receives dopaminergic inputs from both the substantia nigra *pars compacta* and the ventral tegmental area (Brog et al., 1993; Groenewegen et al., 1999). The medial Acb shell also receives greater glutamatergic innervation from subcortical limbic regions compared to the core (Brog et al., 1993; Groenewegen et al., 1999). We have recently shown by electron microscopic immunogold labeling that these Acb subregions significantly differ in the extent to which apomorphine and AS affect dopamine D1 receptor (D1R) distribution in spiny dendrites receiving convergent dopaminergic and glutamatergic inputs (Hara and Pickel, 2007).

Glutamate NMDA receptors are also abundant in the Acb, and blockade of these receptors disrupts PPI in a manner similar to that produced by apomorphine (Reijmers et al., 1995). The essential NR1 and other subunits of the NMDA receptor, which were once thought to be stationary, are now known to undergo dynamically regulated surface expression, agonist-induced internalization, and rapid lateral movement to and from synaptic sites (Lan et al., 2001; Fong et al., 2002; Nong et al., 2003). In the dorsal striatum, activation of D1Rs induces trafficking of the NMDA receptor NR1 subunit between subcellular compartments (Dunah and Standaert, 2001) and conversely, NMDA receptor activation alters D1R subcellular distributions (Scott et al., 2002). Studies in cell lines and hippocampal neurons indicate that this interactive trafficking may be ascribed to physical protein-protein associations between the C-terminals of D1R and the NR1 subunit (Lee et al., 2002; Fiorentini et al., 2003; Pei et al., 2004).

Together, these observations suggest that neurons showing apomorphine and AS-induced trafficking of D1R in the Acb shell (Hara and Pickel, 2007) are likely to have NR1 distributions that are preferentially affected by these same experimental manipulations. To test this hypothesis, we examined the electron microscopic immunocytochemical labeling of the NR1 subunit in relation to the D1R in the Acb shell and core of rats receiving a single injection of vehicle (VEH) or apomorphine (APO) alone, or in combination of AS (VEH+AS, APO+AS). The rats in the APO+AS group used in this study were previously reported to have a significant disruption of PPI, whereas rats in the VEH+AS group had normal PPI (Lessard and Pickel, 2005; Hara and Pickel, 2007). The results from the current study provide the first ultrastructural evidence that redistribution of NMDA receptors in D1R-containing neurons occurs following apomorphine-induced disruption of PPI.

METHODS

Animal preparation

The animal protocols in this study strictly adhered to NIH guidelines concerning the Care and Use of Laboratory Animals in Research, and were approved by the Animal Care Committee at Weill Medical College of Cornell University. Every effort was made to minimize the number of animals used and their suffering. Adult male Sprague Dawley rats (280–350 g; Taconic Farms, Germantown, NY) arrived 14 days prior to their experimental use. All rats were housed two per cage under a 12 hour light/dark cycle. The level of background noise in the home cages was 68–70 dB. Food and water were available *ad libitum*.

Apomorphine (R(-)-Apomorphine-HCL, ICN Biomedicals Inc., Aurora, OH) was dissolved in the vehicle solution (0.9% saline and 0.1 mg/ml ascorbic acid) using a sonicator. The apomorphine solution was injected subcutaneously at 1 mg/kg, a dose previously shown to disrupt PPI to acoustic startle (Mansbach et al., 1988). The vehicle solution alone was used as a control.

Four groups of 3 rats/group were examined in this study: a single subcutaneous injection of 1) vehicle without AS (VEH), 2) apomorphine without AS (APO), 3) vehicle followed by AS used for testing PPI (VEH+AS), and 4) apomorphine followed by the same AS (APO+AS). Rats in VEH+AS and APO+AS groups received the vehicle or apomorphine injection five minutes before introduction to the acoustic startle chamber and exactly an hour before aortic arch perfusion. The rats in VEH and APO groups also received the injection an hour before perfusion.

Acoustic startle testing

All test sessions were performed in a one chamber SR-LAB startle apparatus with digitalized signal output (San Diego Instruments, San Diego, CA). The startle apparatus consisted of 1) a transparent acrylic cylinder 8.2 cm in diameter to hold the rat, 2) Radioshack Supertweeter (Radioshack Corporation, Fort Worth, TX, USA) mounted inside the isolation cabinet to generate the background noise and acoustic stimuli (prepulse and pulse), and 3) microcomputer and interface assembly (San Diego Instruments) to measure and record the startle magnitude as transduced by cylinder movement via a piezoelectric device mounted below a Plexiglass stand. The acoustic startle amplitude was defined as the peak of an average of 250 readings collected every 1 msec beginning at the onset of the acoustic stimulus. Sound levels were measured and calibrated with a Radioshack digital sound level meter with a resolution of 1 dB and accuracy of ± 2 dB. (Radioshack Corporation).

To reduce variation in behavioral results arising from handling stress, all rats were handled for 10 minutes daily starting on the day of shipment arrival until the day of acoustic startle testing two weeks later. All rats that underwent the acoustic startle evaluation were tested in the light cycle (Weiss et al., 1999). Seven days prior to acoustic startle testing, the rats were exposed to a brief matching startle session, a procedure that is commonly used to reduce group variability in responses (Geyer and Swerdlow, 1998; Pothuizen et al., 2005). For this, rats were placed in the startle chamber and exposed to 65 dB background noise for 5 minutes, followed by 11 pulses, each consisting of 40 msec bursts of 120 dB noise, with inter-trial intervals averaging 15 seconds (range 8 to 23 seconds). The peak and average responses from each rat on each of the 11 trials were collected, and the initial response value was recorded separately from the remaining ten responses for each rat, the latter of which were averaged together. Data from these sessions were used to divide the rats into two groups (VEH+AS and APO+AS) with similar mean and variance of basal startle amplitudes.

On the day of the acoustic startle and PPI evaluation, each rat received a subcutaneous vehicle or apomorphine injection. The rats in VEH+AS and APO+AS groups were introduced into the acrylic cylinder 5 minutes after the injection. The acoustic startle testing started with 5 minutes of 65 dB background noise to allow the rats to acclimate to the environment. The first six trials were pulse alone trials, which consisted of a 40 msec burst of 120 dB noise. Then a pseudorandom sequence of pulse alone trials and prepulse+pulse trials were presented at inter-trial intervals averaging 15 seconds (range 8 to 23 seconds). The pulse alone trial was presented ten times, and the three different prepulse+pulse trial types were each presented five times. These consisted of a 20 msec prepulse stimuli (3, 6 or 12 dB above the 65 dB background noise), which do not elicit startle responses, preceding the 120 dB pulse by 100 msec (onset-to-onset). The session concluded with an additional five pulse alone trials. The acoustic startle testing lasted approximately 15 minutes. The VEH and APO rats were returned to their home cages for one hour after the apomorphine or vehicle injection. The VEH+AS and APO+AS rats were returned to their home cages for approximately 40 minutes after the end of the acoustic startle testing.

The data showing apomorphine-induced disruption of PPI for the animals used in these ultrastructural studies was previously reported (Lessard and Pickel, 2005; Hara and Pickel, 2007).

Tissue preparation

All rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) an hour after vehicle or apomorphine injection. We chose this time point as one that would enable internalization, surface expression, and lateral movement of NR1 (Lan et al., 2001; Fong et al., 2002; Nong et al., 2003). A longer time point would be more likely to evoke changes in gene expression. The rats were perfused via the ascending aorta first with 10 ml of heparin (1000 U/ml), then with 60 ml of 3.75% acrolein and 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4), followed by 200 ml of 2% PFA in 0.1 M PB. The brains were removed and postfixed with 2% PFA in 0.1 M PB for 30 minutes at 4°C, and then cut coronally into 40 µm sections on a Vibratome (Leica, Deerfield, IL). Holes were punched at different locations within the cortex to distinguish between rats of different experimental groups. All Acb sections used for this study were collected at 1.60 mm anterior to Bregma (Paxinos and Watson, 1986) and contained both the shell and the core. All the sections from each of the four experimental groups were co-processed, and special care was taken to use identical sampling methods. These sections were placed in 1% sodium borohydride in 0.1 M PB for 30 minutes to remove excess aldehydes, and then rinsed thoroughly with 0.1 M PB. To enhance the penetration of immunoreagents, the sections were incubated in a cryoprotectant solution (25% sucrose and 3.5% glycerol in 0.05 M PB) for 15 minutes and rapidly freeze-thawed by immersing in liquid chlorodifluoromethane (Freon, Refron Inc., NY) followed by liquid nitrogen and finally in room temperature 0.1 M PB. The sections were then rinsed in 0.1 M Tris-buffered saline (TBS).

Antibody

A polyclonal rabbit antiserum raised against a 30 (909–938) amino acid peptide corresponding to the C-terminus of rat NR1 was affinity purified (AB1516, Chemicon International, Temecula, CA). This antiserum recognizes a single band of 120 kDa corresponding to the molecular weight of NR1, and has no cross-reactivities with other glutamate receptor subunits (Petralia et al., 1994). The specificity of this antiserum has also been confirmed by transfected cell immunoblots and peptide preadsorption controls for immunocytochemistry and Western blot (Petralia et al., 1994). In mice with a conditional deletion of NR1 in the lumbar spinal cord, there is an 80% reduction of immunocytochemical labeling with this antiserum in the Cre-injected side compared to the contralateral side (South et al., 2003). Furthermore, we have

previously reported that this antiserum has comparable labeling patterns with another commercially available NR1 antiserum using electron microscopic immunocytochemistry in the Acb shell and core (Hara and Pickel, 2005).

A rat monoclonal antibody raised against a 97 amino acid peptide corresponding to the C-terminus of the human D1R was obtained commercially (Clone 1-1-F11 S.E6, Sigma RBI, Saint Louis, MO). The D1R antibody has been well characterized and shown to specifically recognize the antigenic peptide by Western blot, immunoprecipitation and blocking control experiments used for immunocytochemistry (Levey et al., 1993). Immunofluorescence labeling for D1R was absent in D1R null mice using this antibody (Levine et al., 1996). This D1R antibody has also been localized by electron microscopic immunocytochemistry in the Acb shell and core (Hara and Pickel, 2005; Hara et al., 2006).

Immunocytochemical labeling

The free-floating sections were incubated in 0.5% bovine serum albumin (BSA) in 0.1 M TBS, pH 7.6, for 30 minutes to block non-specific binding of antisera. Then, the sections were incubated for 48 hours at 4°C in the primary antibody solution, which consisted of the rabbit polyclonal anti-NR1 (1:25 dilution) and rat monoclonal anti-D1R (1:100 dilution) with 0.1% BSA in 0.1 M TBS. Immunogold was chosen as a secondary marker for the NR1, because immunogold labeling is punctate and essential for determining quantitative differences in NR1 distributions on the plasma membrane or in the cytoplasm of individual neurons. Immunoperoxidase was chosen for the D1R, because immunoperoxidase labeling is both highly sensitive and diffuse, which facilitates detection of even the smaller distal dendrites and dendritic spines containing low abundance of D1Rs. This combination of secondary immunomarkers allowed for quantitative between-group comparisons of NR1 subcellular distributions in two populations of neurons (D1R-expressing versus those that do not contain D1R).

For immunoperoxidase labeling, the tissue sections were rinsed in 0.1 M TBS, incubated first in a 1:400 dilution of biotinylated donkey anti-rat (for D1R) IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 30 minutes, and then washed in 0.1 M TBS. For immunoperoxidase detection, the sections were incubated in avidin-biotin peroxidase complex (Vectastain Elite Kit, Vector Labs, Burlingame, CA) for 30 minutes, washed with 0.1 M TBS, and then placed in 0.022% 3,3'-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and 0.003% hydrogen peroxide in 0.1 M TBS for 6 minutes.

For immunogold-silver labeling, the sections were rinsed in 0.01 M PBS, pH 7.4, blocked in 0.8% BSA and 0.1% gelatin in PBS for 10 minutes, and subsequently incubated for 2 hours in a 1:50 dilution of goat anti-rabbit (for NR1) IgG conjugated with 1 nm colloidal gold (Amersham, Arlington Heights, IL). The sections were rinsed several times with 0.1 M TBS, then placed in 2% glutaraldehyde for 10 minutes to ensure adherence of the secondary gold-conjugated IgG to the sections (Chan et al., 1990). The gold particles were silver-enhanced using the IntenS-EM kit (Amersham) for 7 minutes at room temperature. The sections were fixed in 2% osmium tetroxide in 0.1 M PB for 60 minutes, followed by dehydration with a graded series of ethanols and propylene oxide. These sections were incubated overnight in 1:1 mixture of propylene oxide and Epon (Electron Microscopy Science, Fort Washington, PA). The sections were transferred to 100% Epon for 2–3 hours and flat-embedded between sheets of Aclar plastic. Flat-embedded sections were examined with a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) and images were captured using a Micropublisher 5.0RTV camera (QImaging, BC, Canada). Light microscopic images were adjusted for brightness and sharpness using Adobe Photoshop (version 7.0.1, Adobe Systems Inc., Mountain View, CA) and then imported into PowerPoint, Microsoft Office, 2003.

Areas containing both the Acb shell and core, immediately medial to the anterior commissure, were trimmed into trapezoids (Fig. 1) and cut into ultra-thin (60 nm) sections with a diamond knife (Diatome, Fort Washington, PA) using an ultratome (NOVA LKV-Productor AB, Bromma, Sweden). Thin sections from the outer surface of the tissue were collected on 400-mesh copper grids (Electron Microscopy Sciences). These sections were counterstained with 5% uranyl acetate for 20 minutes followed by Reynold's lead citrate (Reynolds, 1963) for 5 minutes. After washing and drying, the ultra-thin sections were examined at 60 kV with a Philips CM10 transmission electron microscope (FEI, Hillsboro, OR). The microscopic images were captured using AMT Advantage HR/HR-B CCD Camera System (Advanced Microscopy Techniques, Danvers, MA).

Electron microscopic analysis

Data analysis on NR1 and D1R immunolabeling was performed on ultra-thin sections collected near the surface of the tissue at the interface with the embedding material, where there was optimal penetration of primary and secondary antisera. All the sections from each of the four experimental groups were co-processed and the identical sampling methods were used, thus eliminating variables that could affect between-group comparisons. The profiles containing NR1 and/or D1R immunoreactivity were classified using descriptions from Peters et al. (1991). The dendritic profiles were characterized by abundant organelles such as mitochondria and endoplasmic reticulum and frequent contacts from vesicle-filled axon terminals. A profile was considered as a dendritic spine when smaller than 0.5 μm in diameter, largely devoid of organelles and microtubules and receptive to an axon terminal forming an asymmetric excitatory-type synapse.

Immunoperoxidase labeling was regarded as positive when the electron-dense reaction product in individual profiles was greater than that seen in other morphologically similar profiles in the neuropil. Gold particles and intense immunoperoxidase reaction product were distinguished by lowering the gray scale. Lowering the gray scale does not affect the black color of gold particles because of its color saturation, whereas peroxidase reaction products turn lighter. Profiles were considered to be selectively immunogold-labeled even when they contained only one gold particle of at least 40 nm in diameter to avoid false negative labeling of smaller profiles. The validity of this criterion was established by observations that virtually identical percentages of cellular profiles (dendrites, terminals, axons, somata, glia) were labeled using either the immunogold-silver or the highly sensitive immunoperoxidase method using the same NR1 antiserum (Gracy and Pickel, 1996; Hara and Pickel, 2005). The comparison was made with the inclusion of profiles containing a single NR1 gold particle, confirming that non-specific attachment of immunogold particles was unlikely to affect NR1 distributions. A minimal false positive identification of labeled profiles when using this criterion was further assured by examining the numbers of gold particles overlying myelin, which is not known to express NR1 subunits in adult rat Acb.

For each of the twelve rats, both the left and right Acb from the same or two different vibratome sections were examined. Prior to electron microscopic analysis, light micrographs were taken for reference of orientation and landmarks (blood vessels, bundles of myelinated axons, etc.) to enable collection of electron micrographs specifically from the shell or core subregion. For each coronal section through the Acb, microscopic images were taken from 18–20 grid squares illustrated by the small squares within the trapezoid that contained both the medial shell and core subregions (Fig. 1). None of these grid squares were adjacent to one another. This assumed the collection of a representative sample from a non-overlapping area of the medial Acb shell and core. A micrograph was captured when there were at least 2 transversely-cut NR1-labeled dendritic profiles within the camera field at 13,500X magnification (41.0 μm^2) to select areas where there was optimal antibody penetration. At least 90 micrographs at this magnification

were obtained from each rat and each subregion. At least 610 labeled dendritic profiles in each experimental group and each subregion were quantified (total of 5058 dendritic profiles in 90,651 μm^2 of neuropil area). NR1 immunogold particles were categorized with respect to whether or not they were 1) in contact with the plasma membrane or 2) present in profiles containing D1R immunoperoxidase reaction product. The gold particles were almost always inside the plasma membrane, consistent with the generation of the antibody against the intracellular C-terminus. When more than half of the gold particle was inside the plasma membrane of dendrites, the NR1 distribution was considered as plasmalemmal dendritic labeling. In rare cases, when more than 50% of the gold particle was outside the membrane, this particle was not counted as dendritic labeling. The number of NR1-labeled dendrites determined using these criteria parallels that seen with immunoperoxidase labeling in the Acb (Hara and Pickel, 2005). Two or more gold particles next to each other, but without defined boundaries, were counted as one particle. All microscopy and quantification of labeled profiles were applied equally in all sections and performed by an investigator blind to the experimental conditions.

MCID Elite software (version 6.0, Imaging Research Inc., Ontario, Canada) was used to measure the cross-sectional diameter, perimeter, surface area and form factor of all NR1-labeled dendritic profiles in the Acb shell and core. The diameter, perimeter and surface areas were all used as indirect measures of dendritic size. Only transversely-cut NR1-labeled dendrites with a form factor of over 0.50 were included in our data to exclude longitudinally-cut dendrites with high plasma membrane to surface area ratios. The parameters used for statistical comparisons were: 1) the percentage of plasmalemmal NR1 in D1R-labeled or -unlabeled dendritic spines, 2) the percentage of cytoplasmic NR1 in D1R-labeled or -unlabeled dendritic spines, 3) the number of NR1/D1R dual- or NR1 single-labeled spines/unit neuropil area, 4) the number of plasmalemmal NR1 gold particles on NR1/D1R dual- or NR1 single-labeled spines/unit neuropil area, 5) the total number of spines (NR1-labeled and unlabeled, combined)/unit neuropil area, 6) the number of plasmalemmal NR1 gold particles on a NR1/D1R dual- or NR1 single-labeled dendrite or spine/profile perimeter, 7) the number of cytoplasmic NR1 gold particles on a NR1/D1R dual- or NR1 single-labeled dendrite or spine/profile cross-sectional area, 8) the number of total NR1 gold particles in a NR1/D1R dual- or NR1 single-labeled dendrite or spine/profile cross-sectional area, and 9) the cross-sectional surface area of a NR1/D1R dual- or NR1 single-labeled dendritic profile.

Cluster analysis by dendritic diameter was performed to statistically divide all NR1/D1R dual- and NR1 single-labeled dendrites into small, medium and large subcategories using SPSS (SPSS Inc., Chicago, IL) in order to compare similar structures across groups. For statistical evaluation, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in SPSS (SPSS Inc.) was used for comparisons between the four experimental groups of rats. Observed power was calculated with each analysis to confirm that the sample size was sufficient to support the data.

The electron microscopic illustrations were prepared using the same software and methods described above for light microscopic images.

RESULTS

The electron microscopic immunolabeling of NR1 and D1R seen in the VEH group were similar to those previously reported in naïve rats of the same strain (Hara and Pickel, 2005). In both the Acb shell and core of control rats, NR1 immunogold particles were located predominantly near intracellular endomembranous structures in dendrites and dendritic spines, some of which also contained D1R immunoperoxidase reaction product. Less than 1% of myelinated axons within the surrounding neuropil showed a single NR1 gold particle over

myelin, where NR1 is not known to be expressed. The data showing disruption of PPI in the APO+AS group, but not in the VEH+AS group, was reported previously (Hara and Pickel, 2007). Quantitative comparisons of immunogold labeling showed significant region-specific and between-group differences in the subcellular distribution of NR1 in Acb dendritic profiles that co-expressed D1R.

In the Acb shell, apomorphine and AS alter the subcellular distribution of NR1 in D1R-containing dendritic profiles

D1R-containing dendritic spines and small dendrites in the Acb shell showed significant differences in NR1 distribution in the APO+AS group compared with other treatment groups. These are separately described.

NR1/D1R dendritic spine distribution—The dendritic spine distributions of NR1 immunogold and D1R immunoperoxidase labeling were compared between the four groups of rats. Of the NR1 immunogold-labeled dendritic spines, about half of them also contained D1R immunoperoxidase reaction product (97/183; 53.0%). These NR1/D1R dual-labeled dendritic spines received asymmetric excitatory-type synapses from unlabeled terminals (Fig 2A–D). In the APO+AS group, the NR1 immunogold was often present on the plasma membrane near an asymmetric synapse on dendritic spines containing the D1R (Fig 2D). Conversely, in the VEH, APO and VEH+AS groups, the NR1 immunogold particles were more prevalent in the cytoplasm, distant from the plasma membrane of D1R-labeled spines (Fig 2A, 2B, 2C). Quantitative analysis of D1R-containing spines in Acb shell showed that the APO+AS group had a significantly greater number of plasmalemmal NR1 gold particles per unit perimeter of spine compared with the APO group (Fig 2F; main group effect: $F(3, 95)=3.954$, $p=0.011$, observed power=0.818; Tukey post-hoc test: $p=0.021$), and a significantly lower number of cytoplasmic NR1 gold particles per cross-sectional area of spine compared with the VEH+AS group (Fig 2E; main group effect: $F(3,95)=3.513$, $p=0.018$, observed power=0.765; Tukey post-hoc test: $p=0.045$). No between-group differences were observed in the total number of NR1 gold particles per unit spine cross-sectional area ($p>0.05$). The APO+AS group ($69.7 \pm 8.1\%$) also had a significantly higher percentage of NR1 gold particles present on the plasma membrane compared to the VEH ($27.3 \pm 9.7\%$), APO ($27.3 \pm 9.7\%$), and VEH+AS ($27.8 \pm 10.9\%$) groups (main group effect: $F(3, 95)=6.048$, $p=0.001$, observed power=0.952; Tukey post-hoc test: $p=0.006$, 0.006 , and 0.013 , respectively). The APO+AS group ($30.3 \pm 8.1\%$) had a correspondingly lower percentage of NR1 in the cytoplasm compared to the VEH ($72.7 \pm 11.3\%$), APO ($72.7 \pm 9.7\%$) and VEH+AS ($72.2 \pm 10.1\%$) groups (main group effect: $F(3, 95)=6.709$, $p=0.000385$, observed power=0.970; Tukey post-hoc test: $p=0.003$, 0.008 , and 0.005 , respectively). When NR1 immunogold distribution was examined per unit neuropil area, APO+AS group also had a significantly greater plasmalemmal NR1 density in D1R immunoreactive spines compared to VEH, APO and VEH+AS groups (main group effect: $F(3, 1109)=8.571$, $p=0.00017$, observed power=0.994; Tukey post-hoc test: $p=0.00034$, 0.00017 , and 0.00017 , respectively), without a significant between-group difference in the total (plasmalemmal and cytoplasmic) NR1 density ($p>0.05$).

In the Acb shell, there were no between-group differences in the number of NR1/D1R dual-labeled spines per unit area ($p>0.05$), or in the sizes of NR1/D1R dual-labeled spines as measured by cross-sectional area and diameter ($p>0.05$). In addition, the Acb shell showed no between-group differences in the density per neuropil area of all spines (labeled and unlabeled combined; $p>0.05$), NR1-labeled spines without D1R immunoreactivity ($p>0.05$), or the plasmalemmal, cytosolic, or total density of NR1 immunogold particles in spines without D1R immunoreactivity ($p>0.05$).

NR1/D1R dendritic distribution—Of the NR1-labeled dendrites in our control VEH group, 53.6% also contained D1R immunoperoxidase reaction product, which was comparable to the percentage of colocalization in our prior study in naïve rats (56.0%; Hara and Pickel, 2005).

The average cross-sectional area of transversely-cut dendrites containing both NR1 and D1R was significantly smaller in the VEH+AS group compared with the VEH group (Fig 3A–C; main group effect: $F(3, 1311)=3.455$, $p=0.0004$, observed power=0.776; Tukey post-hoc test: $p=0.036$). The average diameter of these dendrites was also significantly smaller in the VEH+AS group compared to the VEH group (main group effect: $F(3, 1311)=6.099$, $p=0.0004$, observed power=0.961; Tukey post-hoc test: $p=0.031$). There were no statistically significant between-group differences in the average cross-sectional area or diameter of transversely-cut NR1-labeled dendrites without D1R immunoreactivity (Fig 3D; $p>0.05$).

Cluster analysis by dendritic diameter was performed to statistically divide NR1/D1R dual-labeled and NR1 single-labeled dendrites into small, medium and large subcategories in order to compare similar populations and structures across groups.

In small (diameters ranging from 0.09 to 0.62 μm ; $n=503$) D1R-labeled dendrites of the Acb shell, the APO+AS group had a significantly higher number of plasmalemmal NR1 gold particles per unit dendritic perimeter length compared to the VEH, APO and VEH+AS groups (Fig 4A–B, 4D; main group effect: $F(3, 503)=5.946$, $p=0.001$, observed power=0.956; Tukey post-hoc test: $p=0.004$, 0.005, and 0.007, respectively). Accordingly, the APO+AS group had a significantly lower cytoplasmic density of NR1 gold particles in these same dendrites compared with the VEH and APO groups (Fig 4C; main group effect: $F(3, 503)=3.837$, $p=0.010$, observed power=0.820; Tukey post-hoc test: $p=0.017$ and 0.037, respectively). In the APO+AS group, the plasmalemmal NR1 gold particles in these D1R-containing dendrites were often opposed by unlabeled axon terminals or glial profiles (Fig 4B). Cytoplasmic NR1 gold particles in the small D1R-labeled dendrites of the VEH, APO and VEH+AS groups were usually associated with endomembranous structures. There were no significant between-group differences in the total NR1 gold density in these dendrites ($p>0.05$).

D1R-immunoreactive dendrites of medium diameters ranging from 0.62 to 1.01 μm ($n=634$) in the Acb shell showed no significant between-group differences in the plasmalemmal, cytoplasmic, or total density of NR1 immunogold particles ($p>0.05$). In the D1R-containing large (diameters ranging from 1.01 to 2.40 μm ; $n=174$) dendrites, there were also no significant between-group differences in the NR1 cytoplasmic or total density, but the VEH+AS group had a significantly greater NR1 plasmalemmal density compared with the VEH group (Fig 4D; main group effect: $F(3, 174)=2.731$, $p=0.046$, observed power=0.654; Tukey post-hoc test: $p=0.039$).

There were no statistically significant between-group differences in the plasmalemmal, cytoplasmic, or total NR1 gold density in small (0.12 to 0.68 μm in diameter; $n=540$), medium (0.68 to 1.12 μm in diameter; $n=550$) or large (1.12 to 2.22 μm in diameter; $n=126$) dendrites without D1R immunoreactivity ($p>0.05$).

In the Acb core, apomorphine and AS affect only the number of NR1/D1R dual-labeled spines

In the Acb core, the APO+AS group had a significantly lower number of NR1/D1R dual-labeled spines per unit area compared to the VEH and VEH+AS groups (Fig 5A; main group effect: $F(3, 1102)=3.491$, $p=0.015$, observed power=0.781; Tukey post-hoc test: $p=0.026$ and 0.028, respectively). There were no statistically significant between-group differences in the number of NR1-labeled spines without D1R immunoreactivity or in the total NR1-labeled spines per unit area (Fig 5B, 5C; $p>0.05$). There were also no significant between-group

differences in the number of plasmalemmal or cytoplasmic NR1 gold particles in dendritic spines with or without D1R labeling ($p>0.05$).

Cluster analysis by dendritic diameter was used to statistically divide NR1/D1R dual-labeled and NR1 single-labeled dendrites in the Acb core into small, medium and large subcategories as performed in the Acb shell. No statistically significant between-group differences were observed in plasmalemmal, cytoplasmic or total NR1 gold density in small (0.08 to 0.57 μm in diameter; $n=518$), medium (0.58 to 0.94 μm in diameter; $n=752$) or large (0.95 to 2.27 μm in diameter; $n=178$) dendrites without D1R immunoreactivity ($p>0.05$). There were also no significant between-group differences in plasmalemmal, cytoplasmic or total NR1 gold density in small (0.11 to 0.56 μm in diameter; $n=354$), medium (0.56 to 0.89 μm in diameter; $n=572$) or large (0.90 to 2.07 μm in diameter; $n=157$) dendrites containing the D1R ($p>0.05$).

In the Acb core, there were no statistically significant between-group differences in the average cross-sectional area or diameter of dendrites containing NR1 alone, or together with D1R ($p>0.05$).

DISCUSSION

Our results provide the first ultrastructural evidence that in the APO+AS group, the NMDA receptor NR1 subunit relocates from the cytoplasm to the plasmalemma of D1R-containing dendritic spines and small dendrites in the rat Acb shell. This group of rats was previously reported to have PPI deficits, whereas the VEH+AS group had normal PPI (Lessard and Pickel, 2007; Hara and Pickel, 2007). The observed changes in NR1 distributions are summarized in Figure 6. These findings, together with our prior results showing that apomorphine and AS jointly affect D1R distribution preferentially in the Acb shell (Hara and Pickel, 2007), suggest that both NMDA receptors and D1Rs are mobilized in rats following apomorphine-induced disruption of PPI. In addition, the number of dendritic spines co-expressing NR1 and D1R is significantly reduced by apomorphine combined with AS in the Acb core. In this region, the decreased availability of the essential NR1 subunit may significantly decrease NMDA receptor-mediated currents in a manner comparable to that of NMDA receptor antagonists that are known to also produce sensorimotor gating deficits (Reijmers et al., 1995).

Methodological considerations

Both the NR1 and D1R antisera used in this study have been well characterized and shown to specifically recognize the antigenic peptide (Levey et al., 1993; Petralia et al., 1994). These NR1 and D1R antisera showed absence of labeling at the Cre-injection sites of NR1 floxed mice (South et al., 2003), and in D1R null mice (Levine et al., 1996), respectively. Moreover, the localization of NR1 and D1R in our control VEH group matched previous light and electron microscopic immunocytochemical studies in naïve rats using the same antisera (Hara and Pickel, 2005).

The immunogold quantification methods used for detecting apomorphine- and/or AS-induced changes in receptor/subunit distributions were the same as those used in previous studies (Lessard and Pickel, 2005; Hara and Pickel, 2007; Mengual et al., 2008). Profiles were considered as immunolabeled when they contained even one NR1 immunogold particle, because the numbers and types of labeled profiles identified using this criterion were virtually identical to those detected using the highly sensitive immunoperoxidase labeling (Gracy and Pickel, 1996; Hara and Pickel, 2005). Prior studies in the striatum have shown that even a single gold particle in a small profile can represent a greater density of labeling than multiple particles in a larger structure (Wang and Pickel, 2001, 2002). Any existent “background” immunogold would be expected to equally affect all experimental groups.

Our results and those of others (Aicher et al., 1997; Wang et al., 1999; Glass et al., 2004; Hara and Pickel, 2005) show that preembedding immunogold labeling for NR1 is detected within the asymmetric postsynaptic densities. Furthermore, the preembedding method allowed discrete subcellular localization of NR1 without the obstruction of spurious background labeling often seen in tissue processed after plastic embedding. We cannot exclude the possibility that additional undetected NR1 is expressed within postsynaptic densities that are less accessible with pre- compared with post-embedding immunocytochemistry (Baude et al., 1995; Nusser et al., 1995). This limitation, however, would be equally applicable to all experimental groups, and thus not likely to affect the between-group comparisons.

Apomorphine- and AS-induced changes in NR1 distribution in D1R-containing neurons of the Acb shell

In the Acb shell, the APO+AS group had a higher density of plasmalemmal NR1 in D1R-containing dendritic spines and small dendrites compared with VEH, APO or VEH+AS group. This plasmalemmal increase in the APO+AS group was paralleled by a correspondingly lower density of cytoplasmic NR1 in D1R-immunoreactive spines and small dendrites compared to other groups. No between-group differences were observed in the total NR1 gold density, indicating that apomorphine and/or AS did not affect NR1 protein expression. Together, these results suggest that the combination of apomorphine and AS preferentially induces movement of cytoplasmic NR1 to the plasma membrane in dendritic spines and small dendrites containing D1Rs in the Acb shell. Alternatively, this experimental condition may decrease the rate at which NR1 is removed from the plasma membrane to the cytoplasm in these dendritic spines and small dendrites.

A possible mechanism for this specific effect on the subcellular location of NR1 in D1R-containing neurons may be a consequence of convergent dopamine and/or glutamate release onto shared postsynaptic dendrites (Sesack and Pickel, 1990; Joyce and Rayport, 2000; Pinto et al., 2003). We have previously shown that apomorphine and AS together also increase the plasmalemmal density of D1R in dendritic spines and small dendrites in the Acb shell (Hara and Pickel, 2007). These sites are strategic locations for activation of D1R, since dopaminergic inputs preferentially terminate on the necks of dendritic spines (Sesack and Pickel, 1990; Groves et al., 1994). Dopaminergic and glutamatergic inputs can converge on the same dendritic spine (Sesack and Pickel, 1990; Pinto et al., 2003), and prefrontal cortical glutamatergic inputs form asymmetric synapses on spine heads (Sesack et al., 2003). The similarities in NR1 and D1R distributions after apomorphine and AS, and the specificity of NR1 redistribution in D1R-containing neurons suggest that NR1 and D1R may be co-trafficked to the plasma membrane, followed by lateral movement to respective functional sites in dendritic spines and small dendrites of the Acb shell. These and other equally plausible explanations require further study.

The specificity of NR1 redistribution in small dendrites and dendritic spines, and not in larger dendrites, is significant given that the changes occurred in parts of the neurons contacted preferentially by glutamatergic and dopaminergic inputs (Sesack and Pickel, 1992; Johnson et al., 1994). In contrast, GABAergic inputs often terminate on large, proximal dendrites (Bennett and Bolam, 1994), where we observed minimal changes in NR1 distribution.

In addition, apomorphine binding to presynaptic dopamine D2 receptor (D2R) may contribute to parallels in NR1/D1R trafficking. Startle-evoking high intensity AS activates the basolateral amygdala, which sends glutamatergic projections to the Acb (Johnson et al., 1994; Ebert and Koch, 1997). D2R activation reduces glutamate release via a mechanism involving retrograde endocannabinoid signaling (Yin and Lovinger, 2006). Interestingly, this D2R-mediated inhibition of glutamate release occurs only when the glutamatergic neurons are stimulated at high frequency (Bamford et al., 2004; Yin and Lovinger, 2006), a condition that may exist in

the APO+AS, but not in the APO group. This presumed decrease in glutamate release in the APO+AS group may induce NR1s to move towards the plasma membrane of distal dendrites and dendritic spines, where an increase in the number of functional NMDA receptors may compensate for decreased ligand availability. This hypothesis requires further study.

AS-induced changes in dendritic NR1 distribution in the Acb shell

In the Acb shell, the VEH+AS group had smaller NR1/D1R dual-labeled dendrites compared to the VEH group. Although the differences were not robust, our data suggests that AS induces movement of NR1 to smaller, more distal dendrites that contain D1R. Our previous study also showed that D1R is present in smaller dendrites after AS in the Acb (Hara and Pickel, 2007). The commonalities in NR1 and D1R distributions after AS suggest that D1Rs and NMDA receptors are similarly mobilized from medium to smaller, more distal dendrites and dendritic spines. High intensity AS is known to decrease extracellular dopamine levels in the Acb (Humby et al., 1996), and may have a similar effect on glutamate release. As alluded to previously, these similarities may be a compensatory response to decreased ligand availability at distal dendrites, where dopaminergic and glutamatergic inputs terminate (Sesack and Pickel, 1990; Groves et al., 1994; Johnson et al., 1994).

Apomorphine- and AS-induced changes in the number of NR1/D1R dual-labeled spines in the Acb core

Between-group differences in plasmalemmal density of NR1 were not observed in the Acb core, regardless of whether the dendrite or spine contained D1R. Therefore, activation of ventral prefrontal cortex, hippocampus, or other limbic glutamatergic inputs preferentially targeting the Acb shell, not the core (Groenewegen et al., 1999; Zahm, 2000), could account for the observed regional specificity in NR1 trafficking.

In the Acb core, the APO+AS group had fewer dendritic spines that contained both NR1 and D1R, compared to the VEH or VEH+AS groups. There were no between-group differences in the number of NR1-labeled dendritic spines without D1R immunoreactivity or the number of total NR1-labeled spines. The decrease in the number of NR1/D1R dual-labeled spines in the APO+AS group may indicate decreased co-trafficking and delivery of NR1 and D1R to the dendritic spines in the Acb core. Alternatively, the combination of apomorphine and AS may induce dissociation of the protein-protein interactions between NR1 and D1R (Lee et al., 2002; Fiorentini et al., 2003; Pei et al., 2004) in such a way that we detected less colocalization of NR1 and D1R in Acb core dendritic spines. These hypotheses require further study. The movement of the NR1 away from functional sites may be a compensatory response to overactivation of dorsal and ventral prelimbic cortices, which send glutamatergic projections preferentially to the Acb core (Brog et al., 1993).

Implications

Our results provide the first ultrastructural evidence that D1Rs may play a significant role in apomorphine- and AS-induced redistribution of postsynaptic NMDA receptors preferentially in the Acb shell, but also occurring in the core. The specificity of changes in NR1 distribution in D1R-expressing dendrites and dendritic spines of the Acb shell is interesting given that these spiny neurons are those that contain the aversion-associated dynorphin peptides, also implicated in disruption of PPI (Curran and Watson, 1995; Bortolato et al., 2005; Hara et al., 2006). The apomorphine- and AS-evoked redistribution of NR1 in the Acb, together with our previous findings on D1R redistribution (Hara and Pickel, 2007), suggest NMDA receptor-dependent plasticity in a subset of Acb neurons containing D1Rs in rats following apomorphine-induced disruption of PPI. There is no animal model for a complex psychiatric illness such as schizophrenia, but apomorphine-induced disruption of PPI in rats provides a model of sensorimotor gating deficits seen in schizophrenia patients (Braff et al., 1978;

Swerdlow et al., 1986; Geyer et al., 2001; van den Buuse et al., 2005). Thus our results have important implications for development of novel treatments for schizophrenia without the deleterious motor side-effects of existing antipsychotics that block the D2R (Lieberman et al., 2005; Missale et al., 2006).

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ABBREVIATIONS

Acb, nucleus accumbens
 ANOVA, analysis of variance
 APO, apomorphine-injected
 APO+AS, apomorphine injection followed by auditory stimulation
 AS, auditory stimulation
 BSA, bovine serum albumin
 D1R, dopamine D1 receptor
 D2R, dopamine D2 receptor
 dB, decibel
 IgG, immunoglobulin G
 i.p., intraperitoneal
 NMDA, N-methyl-D-aspartate
 NR1, NMDA receptor NR1 subunit
 P, probability value
 PB, phosphate buffer
 PBS, phosphate-buffered saline
 PFA, paraformaldehyde
 PPI, prepulse inhibition
 TBS, Tris-buffered saline
 U, unit
 VEH, vehicle-injected
 VEH+AS, vehicle injection followed by auditory stimulation

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Figure 1.

Medial Acb shell and core subregions sampled for electron microscopy are shown in a plastic-embedded vibratome hemisection of a rat brain. The trapezoid indicates the location from which the ultrathin sections were collected for electron microscopic image analysis. The trapezoid includes both the Acb shell (sh) and core (co), directly medial to the anterior commissure (ac) at the level 1.6 mm anterior to Bregma (Paxinos and Watson, 1986). Microscopic images were taken from 18–20 grid squares per Acb, illustrated by the small squares, within the trapezoid. None of these grid squares are adjacent to one another in order to collect a representative sample from a vast area of the medial Acb shell and core. A hole

punch (pu) within the cortex (ctx) is used to distinguish between rats in different experimental groups. cc, corpus callosum; cpn, caudate-putamen nucleus; lv, lateral ventricle.

Figure 2.

Apomorphine and AS together increase plasmalemmal NR1 in D1R-labeled spines in the Acb shell. (A–D) Electron micrographs showing NR1/D1R dual-labeled spine heads (Sp), receiving asymmetric excitatory-type synapses from unlabeled terminals (ut). In VEH (A), APO (B), and VEH+AS (C) groups, the NR1 immunogold particles are present in the cytoplasm (Cy), while in the APO+AS (D) group, the NR1 immunogold particle is contacting the plasma membrane (P) near the asymmetric excitatory-type synapse. The D1R immunoperoxidase reaction product is diffusely distributed throughout the dual-labeled spine heads in all experimental groups. In (B), the dual-labeled spine originates from a dendrite that has another protrusion, likely to be a spine neck labeled with D1R (D1-Ne). In (D), the dual-labeled spine

originates from a D1R-labeled dendrite (D1-De). Scale bars=0.5 μm . D1-s, D1R single-labeled spine. **(E, F)** Bar graphs showing the numbers of cytoplasmic NR1 gold particles per spine cross-sectional area (100 μm^2 ; E) and plasmalemmal NR1 gold particles per spine perimeter (100 μm ; F) in NR1/D1R dual-labeled dendritic spines. A one-way ANOVA followed by Tukey's post hoc test was used for statistical comparisons between the following groups: vehicle-injected (VEH), apomorphine-injected (APO), vehicle injection followed by auditory stimulation (VEH+AS) and apomorphine injection followed by auditory stimulation (APO+AS). * $p < 0.05$; $n = 3$ rats/each of the four experimental groups.

Figure 3.

AS targets NR1 to smaller NR1/D1R dual-labeled dendrites (NR1&D1 De) in the Acb shell. (A, B) Electron micrographs showing a large NR1/D1R dual-labeled dendrite in the VEH group (A) and a smaller NR1/D1R dual-labeled dendrite in the VEH+AS group (B). The NR1 gold particles are present in the cytoplasm (Cy), one of which is associated with an endomembranous structure (em). The D1R immunoperoxidase reaction product is diffusely distributed throughout the dual-labeled dendrites in both groups. In (B), a dendritic spine (D1-Sp) intensely labeled with D1R originates from the dual-labeled dendrite. Scale bars=0.5 μm. ud, unlabeled dendrite. (C, D) Bar graphs showing the average dendritic cross-sectional areas of NR1/D1R dual-labeled dendrites (C) and NR1 single-labeled dendrites (D) in the Acb shell. A one-way

ANOVA followed by Tukey's post hoc test was used for statistical comparisons between the following groups: vehicle-injected (VEH), apomorphine-injected (APO), vehicle injection followed by auditory stimulation (VEH+AS) and apomorphine injection followed by auditory stimulation (APO+AS). * $p < 0.05$; $n = 3$ rats/each of the four experimental groups.

Figure 4.

Apomorphine and AS together increase plasmalemmal NR1 immunogold and decrease cytoplasmic NR1 immunogold in D1R-labeled small dendrites in the Acb shell. (**A, B**) Electron micrographs showing NR1/D1R dual-labeled small dendrites (NR1&D1-De) in APO (**A**) and APO+AS (**B**) groups. The NR1 immunogold particles in the APO (**A**) group are present in the cytoplasm (Cy), distant from the plasma membrane. In the APO+AS (**B**) group, two NR1 gold particles are in contact with the dendritic plasma membrane (P) and one NR1 gold particle is in close proximity to the plasma membrane (Cy). Scale bars=0.5 μm . D1-De, D1R-labeled dendrite; ud, unlabeled dendrite. (**C, D**) Bar graphs showing the numbers of cytoplasmic NR1 gold particles (**C**) per dendritic cross-sectional area (100 μm^2) and plasmalemmal NR1 gold particles (**D**) per dendritic perimeter (100 μm) in NR1/D1R dual-labeled dendrites by size. A one-way ANOVA followed by Tukey's post hoc test was used for statistical comparisons between the following groups: vehicle-injected (VEH), apomorphine-injected (APO), vehicle injection followed by auditory stimulation (VEH+AS) and apomorphine injection followed by auditory stimulation (APO+AS). * $p < 0.05$; ** $p < 0.01$; $n = 3$ rats/each of the four experimental groups.

Figure 5.

Apomorphine and AS together decrease the number of NR1/D1R dual-labeled spines in the Acb core. Bar graphs showing the numbers of NR1/D1R dual-labeled spines (A), NR1 single-labeled spines (B) and total NR1-labeled spines (C) per 4100 μm^2 of Acb core tissue. A one-way ANOVA followed by Tukey's post hoc test was used for statistical comparisons between the following groups: vehicle-injected (VEH), apomorphine-injected (APO), vehicle injection followed by auditory stimulation (VEH+AS) and apomorphine injection followed by auditory stimulation (APO+AS). * $p < 0.05$; $n = 3$ rats/each of the four experimental groups.

Figure 6.

A schematic diagram showing the soma and dendrites of a D1R-containing spiny neuron in the Acb shell. The relative plasmalemmal/cytoplasmic densities of NR1 gold particles in dendritic compartments (dendritic spine, small (Sm)-, medium (Med)-, and large (Lg)-sized dendrites) are shown. The neuron is artificially divided (dotted line) so as to compare experimental groups (VEH, APO, VEH+AS and APO+AS). In D1R-containing dendritic spines, APO+AS group has a significantly higher percentage of plasmalemmal NR1, and a significantly lower percentage of cytoplasmic NR1 compared to VEH, APO and VEH+AS groups. Also, in D1R-containing small dendrites, APO+AS group has a significantly higher

plasmalemmal NR1 density and a correspondingly lower cytoplasmic NR1 density compared to VEH, APO and VEH+AS groups.