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Changes in Nucleus Accumbens and Neostriatal c-Fos and DARPP-32 Immunoreactivity During Different Stages of Food-Reinforced Instrumental Training

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Abstract

Nucleus accumbens is involved in several aspects of instrumental behavior, motivation and learning. Recent studies showed that dopamine (DA) release in the accumbens shell was significantly increased on the first day of training on a fixed ratio (FR) 5 schedule (i.e. the transition from FR1 to FR5) compared to those rats that continued FR1 training, even though the rats on their first day of FR5 training received less food reinforcement than rats continuing on the FR1 schedule. Additionally, the second day of FR5 responding was marked by a significant increase in DA release in accumbens core. The present studies employed immunohistochemical methods to characterize the changes in cellular markers of accumbens and neostriatal neural activity that occur during various stages of food-reinforced FR5 training. c-Fos and DARPP-32 immunoreactivity in accumbens shell was significantly increased on the first day of FR5 training, while core c-Fos and DARPP-32 expression showed large increases on the second day of FR5 training. Additional studies showed that c-Fos and DARPP-32 expression in neostriatum increased after more extensive training. Double-labeling studies with immunofluorescence methods indicated that increases in accumbens c-Fos and DARPP-32 expression were primarily seen in substance-P positive neurons. These increases in accumbens c-Fos and DARPP-32 immunoreactivity seen during the initial phases of FR training may reflect several factors, including novelty, learning, stress, or the presentation of a work-related challenge to the organism. Moreover, it appears that the separate subregions of the striatal complex are differentially activated at distinct phases of instrumental training.

Keywords

striatum; learning; motivation; reinforcement; habit

Introduction

Within the last few years, there has been a considerable revision of ideas about the behavioral functions of nucleus accumbens. As the traditional emphasis on primary “reward” and hedonia has waned, it has given rise to multiple lines of research focusing on aspects of instrumental learning (Wise, 2004), pavlovian/ instrumental interactions (Robbins

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& Everitt, 2007), reward prediction (Schultz, 2010), incentive salience (Berridge & Robinson, 2008), and behavioral activation and effort-related processes (Salamone *et al.*, 2005, 2007, 2009, 2010; Robbins & Everitt, 2007; Kurniawan *et al.*, 2010, 2011). Microdialysis studies in rats have shown that accumbens DA release is increased during food-reinforced operant performance in well trained animals (e.g. Hernandez & Hoebel, 1988; McCullough *et al.*, 1993b; Salamone *et al.*, 1994; Sokolowski *et al.*, 1998; Cousins *et al.*, 1999; Doyon *et al.*, 2004; Stefani & Moghaddam, 2006; Hauber, 2010; Ostlund *et al.*, 2011). However, less is known about the characteristics of accumbens DA release during the initial learning of food-reinforced instrumental behavior (Cheng & Feenstra, 2006; Ahn & Phillips, 2007). Recent experiments (Segovia *et al.*, 2011) have focused upon the initial acquisition of operant performance by studying the transition from a fixed ratio (FR) 1 schedule to another operant schedule with a higher ratio requirement (FR5). Microdialysis sessions were conducted in rats that were tested on either the FR1 schedule, the first, second or third day of FR5 training, or after weeks of FR5 training. On the first day of FR5 training, there was a substantial increase in DA release in nucleus accumbens shell (i.e., approximately 300% of baseline). In contrast, accumbens core DA release was greatest on the second day of FR5 training.

The present studies focused upon the initial transition from FR1 to FR5 performance, using two immunohistochemical markers of signal transduction activity in accumbens neurons: c-Fos (Le Moine *et al.*, 1997; Kaczmarek, 2002; Farrar *et al.*, 2010) and DARPP-32 (Borgkvist & Fisone, 2007, 2008; Danielli *et al.*, 2010; Li *et al.*, 2011; Yger & Girault, 2011). Double-labeling experiments were conducted to determine if increases in accumbens shell c-Fos and DARPP-32 expression were occurring in neurons that co-express substance P or enkephalin, which are two common markers of distinct populations of striatal and accumbens neurons. Because of previous research suggesting that different parts of the striatal complex are engaged at different stages of the learning process (Porrino *et al.*, 2004; Yin *et al.*, 2004, 2005; Belin & Everitt, 2008), and that neostriatal mechanisms are particularly important under conditions of extended training (Everitt *et al.*, 2008), the present experiments obtained cell counts not only from nucleus accumbens shell and core, but also from overlying neostriatum. Based upon the microdialysis results described above (Segovia *et al.*, 2011), it was hypothesized that increases in c-Fos and DARPP-32 immunoreactivity in nucleus accumbens shell and core would be maximal early in training. In addition, because stimulation of DA D_1 receptors tends to increase striatal immunoreactivity of c-Fos (Graybiel *et al.*, 1990; Keefe & Gerfen, 1996) and DARPP-32 at the Thr^{34} site (Nishi *et al.*, 2000; Kuroiwa *et al.*, 2008; Bateup *et al.*, 2008), and because D1 receptors are most likely to be co-localized with markers of substance P (Le Moine & Bloch, 1995), it was hypothesized that the increased expression of c-Fos and DARPP-32 immunoreactivity seen after the first day of FR5 lever pressing in nucleus accumbens shell would be largely localized in neurons that also contained markers of substance P. Finally, based upon studies showing that neostriatal mechanisms are particularly important for mediating the effects of extended instrumental training (Everitt *et al.*, 2008), it was expected that neostriatal areas would show their largest increases in expression after extended periods of training.

Materials and methods

Subjects

A total of 68 male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing between 300–325 g at the beginning of the study, were housed in a colony maintained at 23°C with a 12h light/dark cycle (lights on at 08:00h). Rats subjected to operant testing were food restricted to 85% of their free-feeding body weight for initial training and then allowed modest weight growth (i.e., an additional 15–30 grams) during the experiments. Water was available *ad libitum* in the home cages. All animal procedures were

approved by the University of Connecticut Institutional Animal Care and Use Committee, and the studies have been conducted according to National Institute of Health Guide for the care and use of animals.

Behavioral Procedures

After two days of magazine training (presentation of a single 45 mg food pellet every 30 sec), all rats were initially trained in 30-min sessions to press the lever on a continuous reinforcement schedule (CRF or FR1), in which every lever press is reinforced with a 45-mg food pellet (Bioserv, Frenchtown, NJ). After the initial acquisition of lever pressing, all rats received 5 additional days of training on the FR1 schedule. This training ensured that all the animals were experienced with the FR1 schedule prior to the final stages of training; this level of training and session duration are consistent with the amount of FR1 training that rats normally receive in our laboratory before being shifted to the FR5 schedule (e.g. Farrar *et al.*, 2010; Segovia *et al.*, 2011). After the initial acquisition and FR1 training, rats were assigned to different training conditions (based upon Segovia *et al.*, 2011). The rats in the FR1 condition had 5 additional days of FR1 training, and were tested again on the FR1 schedule on the final test day when tissue was collected. The rats in the Day 1 FR5 condition had 5 additional days of FR1 training, and then received one day of FR5 training, after which tissue samples were collected. The rats in the Day 2 FR5 condition had 4 additional days of FR1 training, followed by 2 days of FR5 training, and tissue was collected after the second day of FR5 training. The rats in the Day 3 FR5 condition had 3 additional days of FR1 training, followed by 3 days of FR5 training, and tissue was collected after the third day of FR5 training. Finally, the rats in the trained FR5 condition had 10 days of FR5 training, and tissue was collected after the last day of FR5 training. This training procedure was designed to give all the groups except for the well trained group the same number of training days. The day of the testing the rats were placed in the chambers around 8:00 am, with lights on and lever removed from the chambers. Levers were then attached around 12:00 pm, with lights off for the 30 min lever pressing session, after which the lights were turned on with the lever removed, and the rats remained in the chambers for an additional 90 minutes. For the control group, all animals were randomly assigned to either a food-restricted condition or a non-food deprived condition, and all the animals were handled 7 min for ten consecutive days. The food-deprived animals were restricted to 85% of their free-feeding body weight and received 15–18g of regular chow in the home cage mid-morning for the ten consecutive days. c-Fos and DARPP-32 expression data from these two control conditions were similar, and thus were pooled for the statistical analyses.

Experiments

Experiments 1 and 2—Experiment 1 studied the effects of various FR training conditions on c-Fos expression in nucleus accumbens core and shell (N = 34 (n=6 per training condition, n=4 for the control condition)). Experiment 2 studied the effects of the different FR training conditions on DARPP-32 immunoreactivity in core and shell (N = 34 (n=6 per training condition, n=4 for the control condition)).

Experiments 3 and 4—Brain sections through nucleus accumbens core and shell were assessed for double labeling of c-Fos and DARPP-32 with substance P (experiment 3) and enkephalin (experiment 4) immunoreactivity, using the 6 animals from the first day FR5 training condition (i.e., adjacent sections acquired from animals in experiments 1&2).

Experiments 5 and 6—Experiment 5 studied the effects of various FR training conditions on c-Fos expression in dorsomedial and dorsolateral neostriatum. Experiment 6 studied the effects of the different FR training conditions on DARPP-32 immunoreactivity in the same

neostriatal regions. (These experiments used sections of neostriatal tissue from the same animals used in experiments 1&2).

Tissue preparation—Ninety min following the experimental testing days, the animals were anesthetized with CO_2 and transcardially perfused with 0.9% physiological saline for 10 min followed by perfusion with 3.7% paraformadelhyde for 10 min. Brains were fixed for 24 hours by immersion in ice-cold 4% paraformadelhyde and then transferred into a 30% cryoprotectant solution for 48 hours 4°C prior to brain sectioning.

Immunohistochemical Methods

Free floating coronal sections of brains (50 μm) were serially cut using a microtome cryostat (Weymouth, MA, USA), rinsed in 0.01 M phosphate buffer (PBS) (Dulbecco's phosphate buffered saline; pH 7.4; Sigma Chemical Co) and incubated in 0.3% hydrogen peroxide (H_2O_2) for 30 min to block endogenous peroxidase staining. c-Fos was visualized with a rabbit polyclonal anti-cFos (1:5000; Calbiochem, Germany) containing 1% BSA/PBS for a 48 h incubation on a rotating shaker at 4° C. Following the primary antibody treatment, the sections were rinsed in PBS (3× for 5min) and incubated in the secondary, anti-rabbit Horseraddish Peroxidase (HRP)- conjugate envision plus (DAKO, Denmark) for 2 h on a rotating shaker at room temperature. Thereafter, sections were washed and rinsed for 1–3 min in 3,3' diaminobenzidine chromagen (DAB) (brown).

Another series of tissue sections rinsed in 0.01 M phosphate buffer (PBS) (Dulbecco's phosphate buffered saline; pH 7.4; Sigma Chemical Co) and incubated in 0.3% hydrogen peroxide (H_2O_2) for 30 min to block endogenous peroxidase staining. Thereafter, non-specific binding sites were blocked in a solution containing 1% solution of bovine serum albumin (BSA, Upstate) with 0.1% Triton X-100 in PBS for 30 min at room temperature on a rotating platform prior to primary antibody incubation. DARPP-32 was visualized with a rabbit polyclonal anti-DARPP-32 (1:1000; Santa Cruz Biotechnology, CA, USA) containing 1% BSA/PBS for a 48 h incubation on a rotating shaker at 4° C. Following the primary antibody treatment, the sections were rinsed in PBS (3× for 5min) and incubated in the secondary, anti-rabbit Horseraddish Peroxidase (HRP)- conjugate envision plus (DAKO, Denmark) for 2 h on a rotating shaker at room temperature. Thereafter, sections were washed and rinsed for 1–3 min in 3,3' diaminobenzidine chromagen (DAB) (brown).

All the sections were mounted to gelatin-coated slides, air dried and cover-slipped using Cytoseal 60 (Thermo Scientific) as a mounting medium. The tissue sections were then examined by light microscopy.

Quantification of the number of c-Fos and DARPP-32-labeled cells

Quantification of the number of c-Fos and DARPP-32-labeled cell body profiles in striatal sections was achieved by taking photomicrographs with a 20× (0.125mm^2/field) objective (Nikon Eclipse E600; Melville, NY, USA) upright microscope equipped with an Insight Spot digital camera (Diagnostic Instruments, Inc.; see Figure 1 for regions of interest in nucleus accumbens and neostriatum). A counting grid was then superimposed on each 20× captured photomicrograph, and for consistency the size of the counting grid was kept the same for each structure studied. The total number of c-Fos and DARPP-32-labeled cells was counted by an observer who was blind to the experimental treatments; three adjacent coronal sections and a minimum of three fields were counted per section in the control and experimental groups.

Immunofluorescence double labeling studies of c-Fos, DARPP-32, and Substance P

A series of cryostat sections were prepared for immunofluorescence by incubating in a blocking solution for 45 min of 5% normal donkey serum (Jackson ImmunoResearch, USA) and 0.1 % Triton X-100 (Sigma, St. Louis, MO, USA) in a 0.1 M phosphate buffer, pH 7.4. The sections were next incubated for 24 hr in a cocktail of an affinity-purified rabbit anti-cFos polyclonal antisera (1:2000; Calbiochem, Germany), goat anti-substance P polyclonal antisera (1:400; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), Inc) (used to label striatonigral neurons), rabbit anti-DARPP-32 (Thr^{34}) polyclonal antisera (1:500; Santa Cruz Biotechnology, Inc), goat anti-substance P polyclonal antisera (1:400; Santa Cruz Biotechnology, Inc), rabbit anti-cFos polyclonal antisera (1:2000; Calbiochem, Germany), and an affinity-purified goat anti-DARPP-32 (Thr^{34}) polyclonal antisera (1:500; Santa Cruz Biotechnology, CA, USA) containing 5% NDS and 0.1 % Triton X-100 in 0.1 M phosphate buffer, pH 7.4 over night at 4° C with gentle agitation on a rotating shaker. Thereafter, sections were washed 3 × 5min in PBS and transferred to fluorescent-marker conjugated secondary antisera. Sections were incubated in the dark for 2 h at room temperature in a diluted mixture of fluorescein-isothiocyanate (FITC) conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology, Inc) or tetramethyl rhodamine isothiocyanate (TRITC) conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Inc) in 5% NDS, 0.1% Triton-X-100-PBS. In some cases the substance P labeled sections were counterstained with 4,6-diamidino-2-phenylindol dihydrochloride (DAPI) (Vector Laboratories, Burlingame, CA, USA) or Topro (Invitrogen) for fluorescent detection of nuclei morphology. To achieve a better separation of fluorescent labeling and less fading, the secondary Alexa 488-conjugated donkey anti-rabbit IgG (1:100) was used to label c-Fos and DARPP-32 immunoreactivity. The secondary Alexa 594- conjugated donkey anti-goat IgG (1:100) was used to label substance P and DARPP-32 immunoreactivity. All sections were thereafter mounted on gelatin-coated slides and coverslipped with ProLong1 antifade medium (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The Alexa dyes are brighter and more fade resistant than are fluorescein or rhodamine (Panchuk-Voloshina et al., 1999). The ProLong1 antifade medium is more effective at preventing fading of fluorescence than the coverslipping media use in prior studies (Ono et al., 2001).

Double labeling Immunocytochemical studies for c-Fos, DARPP-32 and enkephalin

The final series of cryostat sections were processed for immunofluorescence double labeling with either c-Fos and enkephalin or DARPP-32 and enkephalin. Free-floating sections were permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked in 5% donkey serum in PBS/0.1% Triton X-100 for 45 min, and incubated with the following primary antibodies: rabbit anti-cFos polyclonal antisera (1:2000; Calbiochem, Germany); mouse monoclonal antiserum Leu5-enkephalin (1:400; Clone NOC1, Chemicon, Millipore Corp, Billerica, MA, USA); rabbit anti-DARPP-32 (Thr^{34}) (1:500; Santa Cruz Biotechnology, CA, USA). After washing three times in PBS, the sections were transferred to the appropriate Alexa Fluor dye conjugated secondary antibodies or the appropriate secondary fluorphores. The anti-c-Fos and anti-enkepahlin sections were incubated in a secondary antisera mixture that contained an Alexa 488-conjugated donkey anti-rabbit IgG and conjugated rhodamine donkey anti-mouse IgG (1:200) (Jackson ImmunoResearch, USA), while the anti-DARPP-32 and anti-enkepahlin sections were incubated in a secondary antisera mixture that contained mixture of fluorescein-isothiocyanate (FITC) conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology, Inc) and conjugated rhodamine donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, USA) containing of 5% NDS and 0.1 % Triton X-100 in a 0.1 M phosphate buffer, pH 7.4 in the dark at room temperature for 2 h. Thereafter, the sections were washed in PBS. All sections were mounted on gelatin-coated slides and coverslipped with ProLong1 antifade medium (Molecular Probes, Invitrogen, Carlsbad, CA, USA). In some cases, the slides were counterstained with DAPI to confirm nuclear staining of

enkephalin-positive neurons. Immunofluorescence staining was visualized for high resolution observation on a Zeiss Axioskop 2 upright fluorescent microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY), and photographed with a Retiga 1300 EX digital camera (Q-Imaging, Burnaby, BC, Canada) with Improvision Openlab 5.5.1 software (Lexington, MA, USA).

Controls for double-labeling immunocytochemistry

Control trials were performed to screen for non-specific binding of the reagents used in the immunofluorescence protocol. Primary antibodies were omitted from the immunofluorescence procedure as a negative control to confirm that background binding of reagents would not contribute to immunoreactivity. Tissue sections were incubated overnight in a solution that contained 5% NDS, 0.1% Triton-X-100 and PBS. Another control trial examined the cross-reactivity of the secondary antibodies within the procedure. Briefly, both primary antibodies were applied in the above protocols, but only one secondary antibody was applied and tris buffer solution (TBS) was substituted in lieu of the other secondary antibody. Similar immunocytochemical control procedures have been extensively characterized by other laboratories (Langely et al., 1997; Burry, 2000).

Statistical Analysis

For both the c-Fos and the DARPP-32 experiments, data were expressed as number of cells/mm^2. An average of the cell counts from 3 successive coronal sections was determined for each animal, such that each rat had a single number (mean number of cells/mm^2) for each brain area. Then, these cell count numbers were analyzed for the whole group of animals in each experiment. Factorial ANOVA (2 brain regions × 6 behavioral conditions) was used to determine overall significance of the c-Fos and DARPP-32 experiments. Tukey post-hoc comparisons were used also used to determine differences between the control group and experimental conditions. The behavioral data for both lever pressing and gram quantity of food intake for all groups were analyzed by ANOVA. A probability of $p < 0.05$ was considered to be significant. Data analysis was performed using SPSS (Chicago, IL) 12.0 statistical software.

Results

Experiments 1–2: c-Fos and DARPP-32 expression in nucleus accumbens shell and core during various stages of FR5 training

c-Fos immunoreactivity was assessed during the transition from the FR1 to the FR5 lever pressing task in the nucleus accumbens. Representative photomicrographs of sections from rats in the FR1 1st day FR5 training groups are shown in Figure 2A–F, and the cell count data for all training groups are shown in Figure 2G. FR1 lever pressing induced very little c-Fos expression in the accumbens shell and core (Fig. 2A–C). In contrast, the first day of FR5 lever pressing induced robust c-Fos expression in the accumbens shell (Fig. 2D–F; see 2F for a high magnification image of the accumbens shell). Figure 2G depicts the number of c-Fos containing neurons in the accumbens shell and core for the control group and all training days. Analysis with a 2 × 6 factorial ANOVA showed that there was a significant main effect of brain region ($F_{1,56} = 53.46$, $p < 0.001$) and a significant main effect of training condition ($F_{5,56} = 17.68$, $p < 0.001$) on c-Fos expression. Furthermore, there was a significant brain region × training condition interaction ($F_{5,56} = 24.59$, $p < 0.001$). Because there was a significant interaction, analysis of simple effects performed separately on the shell and core data revealed a significant effect of training condition in the accumbens shell ($F_{5,28} = 22.62$, $p < 0.001$), and the accumbens core ($F_{5,28} = 17.54$, $p < 0.001$). Tukey multiple comparisons of the shell data showed that the first day of FR5 responding significantly increased c-Fos expression ($p < 0.05$) relative to the control and FR1 groups, as

well as all other groups. In contrast, Tukey multiple comparisons of the accumbens core data revealed that there was a significant increase in c-Fos expression on the second day of FR5 responding ($p < 0.05$) compared to the control and FR1 groups, as well as all other training conditions. The behavioral data for animals tested in the five lever pressing training groups in experiment 1 are shown in Table 1. ANOVA revealed a significant overall treatment effect on lever pressing ($F_{4,25} = 30.65$, $p < 0.001$), and post-hoc comparisons with the Tukey test showed that all FR5 training groups showed significantly higher levels of lever pressing compared to the FR1 group. ANOVA also demonstrated that there was a significant main effect on consumption of the reinforcement pellets ($F_{4,25} = 29.28$, $p < 0.001$); Tukey multiple comparisons showed that all FR5 training groups had significantly lower levels of consumption of the reinforcement pellets compared to the FR1 group.

The first day of FR5 responding also was accompanied by a pronounced increase in DARPP-32 immunoreactivity in the shell of the nucleus accumbens (Fig. 3C) relative to control (Fig. 3A) and to the FR1 condition (Fig. 3B). Figure 3F depicts the number of DARPP-32 containing neurons in the accumbens shell and core for the control group and all training days. Analysis by means of a 2 × 6 factorial ANOVA showed an overall significant effect of brain region ($F_{1,56} = 6.76$, $p < 0.012$), a significant effect of training condition ($F_{5,56} = 9.66$, $p < 0.001$), and a significant brain region × training condition interaction ($F_{5,56} = 7.65$, $p < 0.001$). Because of the significant interaction, shell and core data were then analyzed separately. There was a significant effect of training condition in the accumbens shell ($F_{5,28} = 10.74$, $p < 0.001$). Post hoc tests revealed that the first day of FR5 responding resulted in a significant and substantial increase in DARPP-32-labeled cell density ($p < 0.05$) relative to control, FR1, and all other training days ($p < 0.05$). Analysis of simple effects also revealed a significant effect of FR training condition on DARPP-32 expression in the accumbens core ($F_{5,28} = 3.61$, $p < 0.01$). Post hoc tests indicated that the first day of FR5 responding was not accompanied by enhanced DARPP-32 expression in the accumbens core, but rather, the second day of FR5 responding resulted in a significant increase in DARPP-32 expression relative to the control and FR1 conditions ($p < 0.05$). The behavioral data for animals tested in the five lever pressing training groups in experiment 2 are shown in Table 2. ANOVA revealed a significant overall treatment effect on lever pressing ($F_{4,25} = 90.86$, $p < 0.001$), and post-hoc comparisons with the Tukey test showed that all FR5 training groups showed significantly higher levels of lever pressing compared to the FR1 group. ANOVA also demonstrated that there was a significant main effect on consumption of the reinforcement pellets ($F_{4,25} = 83.2$, $p < 0.001$); Tukey multiple comparisons showed that all FR5 training groups had significantly lower levels of consumption of reinforcement pellets compared to the FR1 group.

Experiments 3–4: c-Fos and DARPP-32 co-expression with Substance P but not enkephalin in nucleus accumbens shell and core neurons

It was hypothesized that the expression of c-Fos immunoreactivity seen after the first day of FR5 lever pressing in nucleus accumbens shell would be largely localized in neurons that also contained markers of substance P. Thus, we stained with both the c-Fos and substance P antibodies, and used immunofluorescent methods to detect double labeling in sections obtained from rats that had their first exposure to a FR5 lever pressing task. The first day of lever pressing on a FR5 schedule coincided with robust expression of c-Fos in accumbens shell (Fig. 4A), and there also was substantial substance P immunoreactivity (Fig. 4B). Counterstaining with DAPI demonstrated the presence of nuclei in the substance P immunoreactive cells (Fig. 4C) in the shell. Moreover, there was substantial double labeling of c-Fos and substance P immunoreactivity (Fig. 4D). Additionally, the accumbens core expressed some c-Fos immunoreactivity (Fig. 4E) that was co-localized with substance P immunoreactivity (Figs. 4F–G). Thus, c-Fos expression appears co-localized with substance

P immunoreactivity in nucleus accumbens of animals stained after the first day of FR5 training. There were no examples of separate labeling of the c-Fos positive neurons with substance P immunoreactivity.

DARPP-32 expression was also hypothesized to be co-localized with substance P immunoreactivity in sections obtained after the first day of FR5 training. The first exposure to the FR5 schedule of reinforcement coincided with augmented expression of DARPP-32 (Fig. 5A), and substance P immunoreactivity also was expressed (Fig. 5B). In addition, there was substantial double-labeling of DARPP-32 and substance P immunoreactivity (see Figs. 5C–E). The accumbens core also expressed some DARPP-32 and substance P immunoreactivity that was co-localized (Figs. 5F–G). Furthermore, we stained with the c-Fos and DARPP-32 antisera to detect co-localization of these markers of signal transduction activity in animals exposed to the first day of FR5 training. In these sections, there was substantial double labeling of c-Fos and DARPP-32 expression in neurons in the accumbens shell and core (Figs. 5H–M). There were no examples of separate labeling of the DARPP-32 positive neurons with substance P immunoreactivity.

We also stained with an enkephalin antibody to determine if there was co-localization of c-Fos and DARPP-32 immunoreactivity with enkephalin immunoreactivity in the accumbens (Figs. 6). Figure 6A–C shows that the c-Fos and ekephalin immunoreactivity are largely present in different neurons in sections of nucleus accumbens shell of rats responding on the first day of FR5 lever pressing. Similarly, in the accumbens core, there was little evidence of colocalization of c-Fos and enkephalin immunoreactivity (Figs. 6D–F). In addition, because there were no examples of double labeling with c-Fos and DARPP-32 with enkephalin, there was little apparent co-localization of DARPP-32 and enkephalin immunoreactivity in the accumbens shell and core (Fig. 6G–L) in these sections from animals trained on the first day of FR5 responding.

Experiments 5–6: c-Fos and DARPP-32 expression in dorsomedial and dorsolateral neostriatum during various stages of FR5 training

Figure 7 shows the number of c-Fos-labeled cells in the two dorsal striatal subregions studied across the different control and FR training groups. Analysis with a 2 × 6 factorial ANOVA showed that there was an overall significant effect of FR training days ($F_{5,56}$ = 8.945, $p<0.001$), but there was no significant effect of striatal subregion ($F_{1,56}$ = 1.769, n.s), and no significant training group by subregion interaction ($F_{5,56}$ = 1.065, n.s). Because of the lack of interaction, post-hoc effects were analyzed by collapsing across striatal areas, in order to identify differences across training condition. Post-hoc analyses with the Tukey test showed that the day 3 FR5 and well trained FR5 groups significantly differed from the other four groups (control, FR1, day 1 and day 2 FR5; $p < 0.05$). The number of neostriatal cells expressing DARPP-32 immunoreactivity also was quantified (see figure 8). Factorial ANOVA showed that even though there was not an overall significant effect of striatal region ($F_{1,56}$ = 0.607 n.s) or a significant interaction ($F_{5,56}$ = 0.499, n.s), there was an overall significant effect of FR training condition ($F_{5,56}$ = 10.230, $p<0.001$). As with the c-Fos experiment, the lack of interaction necessitated that post-hoc analyses were performed by collapsing across striatal areas. Tukey test analyses demonstrated that the day 3 FR5 and well trained FR5 groups significantly differed from the control and FR1 groups ($p < 0.05$).

Discussion

The first two experiments employed immunohistochemical methods to label intracellular signaling cascades related to accumbens neuron activity (i.e. c-Fos and DARPP-32) under the same behavioral conditions that were used in recent microdialysis experiments (Segovia *et al.*, 2011). In the Segovia *et al.* (2011) study, it was observed that nucleus accumbens shell

DA release showed a very large increase on the first day of FR5 training, while core DA release showed its greatest increase on the second day of FR5 training. In the present work, it was found that c-Fos and DARPP-32 expression in the accumbens shell were significantly elevated relative to control following the first day of FR5 training. In contrast, c-Fos and DARPP-32 immunoreactivity in the core was significantly increased relative to control after the second day of FR5 training. Thus, the temporal pattern of results observed in the present experiments, which focused upon c-Fos and DARPP-32 immunoreactivity, was the same as that reported in recent microdialysis experiments (Segovia *et al.*, 2011). The present results are consistent with the study of Cheng & Feenstra (2006), which showed that DA release in the nucleus accumbens shell was more responsive than in the core during the first day of lever press acquisition training, and also with Danielli *et al.*, (2010), who demonstrated that DARPP-32 showed increased expression in nucleus accumbens shell during the first exposure to a novel food, but then showed rapid habituation. Furthermore, the c-Fos and DARPP-32 results described above are consistent with previous research demonstrating that core and shell subregions of nucleus accumbens have different behavioral functions (Kelly, 1999; Smith-Roe & Kelly, 2000; Cardinal *et al.*, 2002: Di Chiara, 2002; Cheng *et al.*, 2003: Cheng & Feenstra, 2006; Saddoris *et al.*, 2011), and that accumbens DA release in these subregions shows different patterns of responsiveness over repeated exposure to food-related conditions (Bassareo & Di Chiara, 1999; Segovia *et al.*, 2011).

Increases in c-Fos and DARPP-32 immunoreactivity in nucleus accumbens do not appear to be related in a simple or direct manner to the total amount of food reinforcement presented and consumed. The FR1 schedule generates a much higher density of food presentation than the first few days of FR5 training (see Tables 1 and 2), but FR1 responding was not accompanied by a significant increase in c-Fos or DARPP-32 expression in nucleus accumbens compared to the control group. Furthermore, it is unlikely that the marked increases in c-Fos and DARPP-32 expression in accumbens shell during the first day of FR5 training results from an increased net reward value, or a condition that is better than expected based upon previous training, because the FR5 schedule actually yielded a much lower density of food presentation than the FR1 (Tables 1 and 2), and therefore the transition from FR1 to FR5 yields a reinforcement density that is lower than expected based upon previous experience. Moreover, the increases in c-Fos and DARPP-32 expression in nucleus accumbens were not related in any simple way to the number of lever presses emitted across training days (see also Ostlund *et al.*, 2011). On the one hand, response rate increases during the initial transition from FR1 to FR5 training, as does c-Fos and DARPP-32 expression. Yet, it also is true that response rates were highest in well trained animals, despite the fact that this condition was not associated with significant increases in c-Fos or DARPP-32 immunoreactivity in nucleus accumbens. In view of the present results, it is possible that the increases in accumbens shell c-Fos and DARPP-32 expression seen on the first day of FR5 responding are related to novelty (De Leonibus *et al.*, 2006), stress (Salamone, 1994, 1996; Salamone *et al.*, 2007), uncertainty about reward delivery (Stefani & Moghaddam, 2006; D'Souza & Duvauchelle, 2008), an increased work requirement (Salamone *et al.*, 2007), or the behavioral flexibility requirements of shifting to a new schedule (Stefani & Moghaddam 2006; Haluk & Floresco, 2009). These neural responses also could be related to aspects of pavlovian or instrumental learning that are involved in the shift from FR1 to FR5 responding (Dickinson *et al.*, 1995; Balleine & Dickinson, 1998; Smith-Roe & Kelly, 2000; Baldwin *et al.*, 2002; Cheng *et al.*, 2003; Dalley *et al.*, 2005; Cheng & Feenstra, 2006; Ahn & Phillips, 2007; Flagel *et al.*, 2011; Saddoris *et al.*, 2011).

Together with microdialysis and electrophysiology data, the present results indicate that neurochemical and cellular markers of nucleus accumbens activity can show increased responsiveness under a diverse set of motivational conditions. Electrophysiology studies have shown that nucleus accumbens neurons respond to conditioned stimulus cues (Nicola

et al., 2004; Day *et al.*, 2011; Saddoris *et al.*, 2011), and some shell neurons respond specifically to conditions involving pavlovian-to-instrumental transfer (Saddoris et al., 2011). Subgroups on accumbens neurons also respond to effort discounting, response initiation, completion of high effort requirements, reward delay, and reward delivery (Day *et al.*, 2011). Although the present studies involved positive reinforcement, accumbens DA transmission or neural activity also is increased in response to various aversive or stressful conditions, as indicated by studies using neurochemical markers of DA release (Salamone, 1994, 1996; McCullough & Salamone 1992; McCullough *et al.*, 1993a; Tidey & Miczek, 1996; Salamone *et al.*, 1997; Datla *et al.*, 2002; Young, 2004; Marinelli *et al.*, 2005), electrophysiological recording of ventral tegmental area DA neurons (Anstrom & Woodward, 2005; Joshua *et al.*, 2008; Brischoux *et al.*, 2009; Bromberg-Martin *et al.*, 2010), and human imaging methods (Liberzon *et al.*, 1999; Jensen *et al.*, 2003; Pavic, 2003; Phan *et al.*, 2004; Preussner *et al.*, 2004; Scott *et al.*, 2006, 2007; Levita *et al.*, 2009; Delgado *et al.*, 2011). Stressful pharmacological or behavioral conditions have also been reported to increase nucleus accumbens c-Fos and CREB expression (Barrot *et al.*, 2002; Sink *et al.*, 2010).

Based upon previous research it was hypothesized that the increases in c-Fos and DARPP-32 expression associated with the transition from FR1 to FR5 responding should largely be present in substance-P positive neurons. This hypothesis was based upon several lines of evidence. First, while DA D_2 receptor antagonism generally increases c-Fos expression in striatal areas (Robertson & Fibiger, 1992; Robertson *et al.*, 1992; Betz *et al.*, 2009; Farrar *et al.*, 2010), it is stimulation of DA D_1 receptors that tends to increase striatal c-Fos immunoreactivity (Graybiel *et al.*, 1990; Keefe & Gerfen, 1996). Also, considerable evidence indicates that DARPP-32 phosphorylation at the threonine residue (Thr^{34}) site is differentially regulated in striatal medium spiny neurons that contain D_1 vs D_2 receptors (Svenningsson *et al.*, 2000; Kuroiwa *et al.*, 2008; Bateup *et al.*, 2008; Yger & Girault, 2011). DA acting through the D_1 receptor and the G proteins (G_s/G_{olf}) activates adenylate cyclase activity, thereby stimulating PKA-mediated phosphorylation of DARPP-32 at the Thr^{34} site (Nishi *et al.*, 2000; Kuroiwa *et al.*, 2008; Bateup *et al.*, 2008; Yger & Girault 2011). Furthermore, medium spiny neurons that predominantly express D_1 receptors co-express markers of substance P, while neurons that express mainly D_2 receptors express markers of enkephalin (Le Moine & Bloch, 1995; Lu *et al.*, 1998). In the present studies, immunofluorescence double-labeling techniques demonstrated a marked co-localization of c-Fos expression and DARPP-32 expression in substance-P positive neurons in the accumbens shell during the first day of FR5 training. In contrast, there was minimal co-localization of c-Fos and DARPP-32 immunoreactivity in enkephalin-positive neurons. Taken together, these data are consistent with the hypothesis that during the transition from FR1 to FR5 training, increased expression of c-Fos and DARPP-32 immunoreactive neurons reflects, at least in part, the impact of increased DA release (Segovia *et al.*, 2011) on D_1 receptors contained on substance-P positive neurons. Of course, this is not to say that DA D_2 receptors are not being stimulated under these conditions of heightened DA release; rather, it is the case that the cellular markers of signal transduction mechanisms that were used in the present studies offer a better reflection of D_1 receptor stimulation, rather than D_2 receptor stimulation.

For the experiments focusing on dorsomedial and dorsolateral neostriatum (experiments 5 and 6), there were no significant increases in c-Fos and DARPP-32 expression in the first or second day FR5 training groups relative to the control or FR1 groups. Rather, it was demonstrated that neostriatal c-Fos and DARPP-32 immunoreactivity were increased relative to the control and FR1 conditions only in the 3^{rd} day FR5 and well trained FR5 groups. This pattern of effects shown with neostriatal c-Fos and DARPP-32 expression is in marked contrast to that shown in the nucleus accumbens shell and core. Even though there

was minimal induction of c-Fos and DARPP-32 expression in the dorsal striatal subregions during the initial FR training days, the core and shell subregions of the nucleus accumbens demonstrated a robust induction of c-Fos and DARPP-32 expression after the initial FR training days (i.e., shell on day 1, core on day 2). In addition, increases in neostriatal c-Fos and DARPP-32 expression are highest after 3 days or more of training, which is also when the response rate was highest. Taken together, these data are consistent with the concept that different parts of the striatal complex are engaged at different stages of instrumental learning (Porrino *et al.*, 2004; Yin *et al.*, 2004, 2005; Corbit & Baleine, 2005; Everitt et al. 2008; Ashby et al., 2010). It has been suggested that the nucleus accumbens participates in pavlovian and instrumental learning processes relating to motivation and behavioral activation (Mogenson *et al.*, 1980; Corbit *et al.*, 2001; de Borchgrave *et al.*, 2002; Cardinal *et al.*, 2002; Robbins & Everitt, 2007; Salamone *et al.*, 2005, 2007, 2010), which are particularly relevant early in the training process. In contrast, it has been suggested that plasticity in the dorsal striatum is involved in later stages of instrumental learning (McDonald & White, 1993; Graybiel *et al.*, 1998; Packard & Knowlton, 2002; Yin *et al.*, 2005; Yin & Knowlton, 2006), and is critical for the process of habit formation. In view of the present studies showing that the neostriatal c-Fos and DARPP-32 are highly activated after continued exposure to instrumental training on the FR5 schedule, and that the different parts of the striatal complex are engaged at different stages of the instrumental learning, the results from experiments 1–2 and 5–6 lend support to the hypothesis of a transition from ventral to dorsal striatal regulation (i.e., the “ventral to dorsal striatum shift”, Belin *et al.*, 2009) during instrumental responding for natural reinforcers. Several researchers have suggested that the nucleus accumbens (i.e., ventral striatum) is preferentially involved in earlier stages of instrumental learning, while neostriatum (i.e., dorsal striatum) is preferentially engaged at later stages of training (Haber et al., 2000; Porrino *et al.*, 2004; Ferrario et al., 2005; Everitt & Robbins, 2005; Volkow *et al.*, 2006; Ikemoto, 2007; Belin & Everitt, 2008). Although this principle is often cited in relation to studies involving drug reinforcers (e.g. Porrino *et al.*, 2004; Everitt & Robbins 2005; Belin *et al.*, 2009), it also appears to be important for studies involving food reinforcement. Thus, the c-Fos and DARPP-32 immunoreactivity results from the present experiments are consistent with the general principle that there is a gradual shift from ventral to dorsal striatal involvement during different stages of instrumental learning (Everitt & Robbins, 2005; Everitt *et al.*, 2008; Belin *et al.*, 2009; Ashby et al., 2010).

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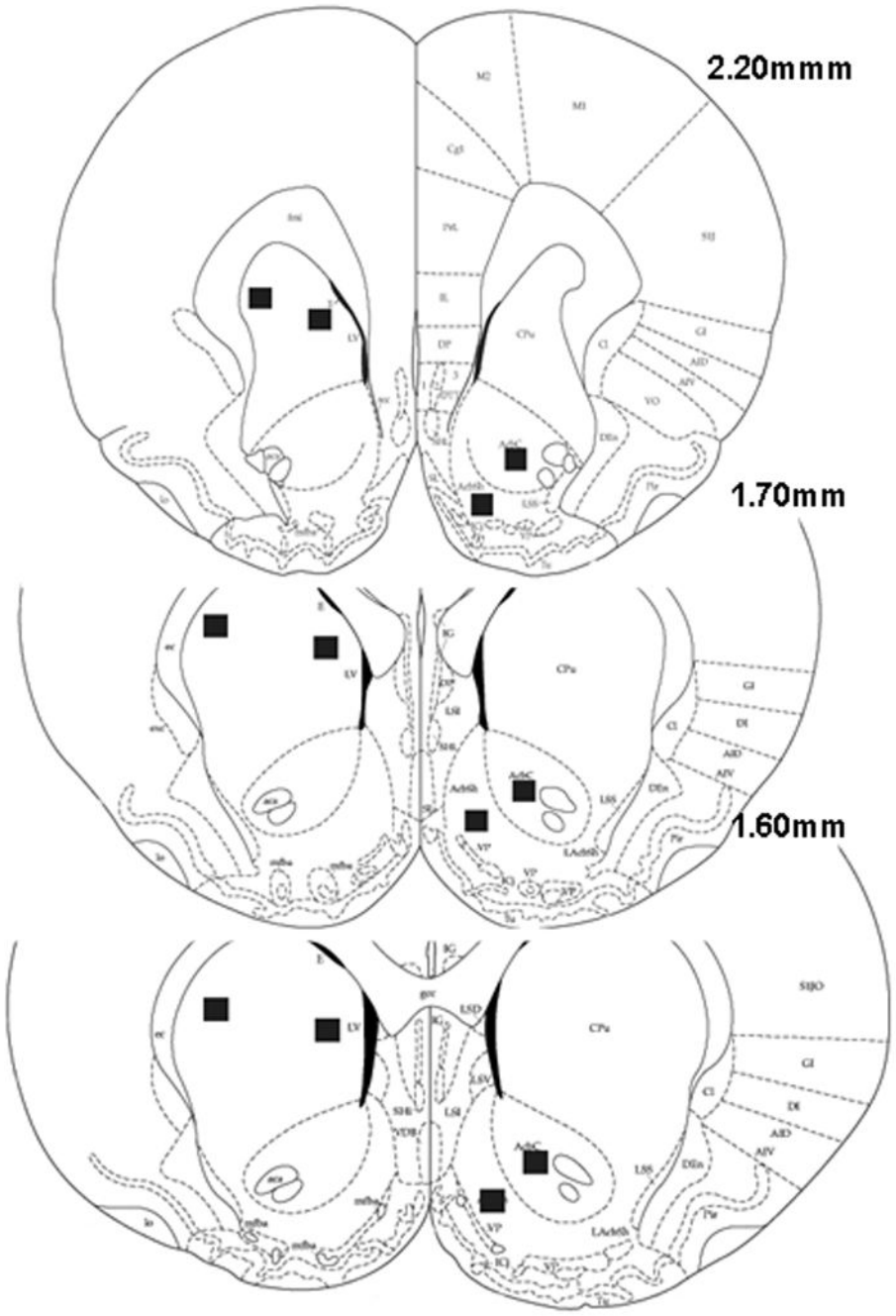

Figure 1.

Representative schematic showing regions of interest for photomicrographs in the nucleus accumbens core and shell, and the dorsomedial and dorsolateral striatum (for clarity, regions are marked on only one side of the brain). The numbers indicated are relative to bregma for each brain level (Figure is modified from Paxinos and Watson, 1998).

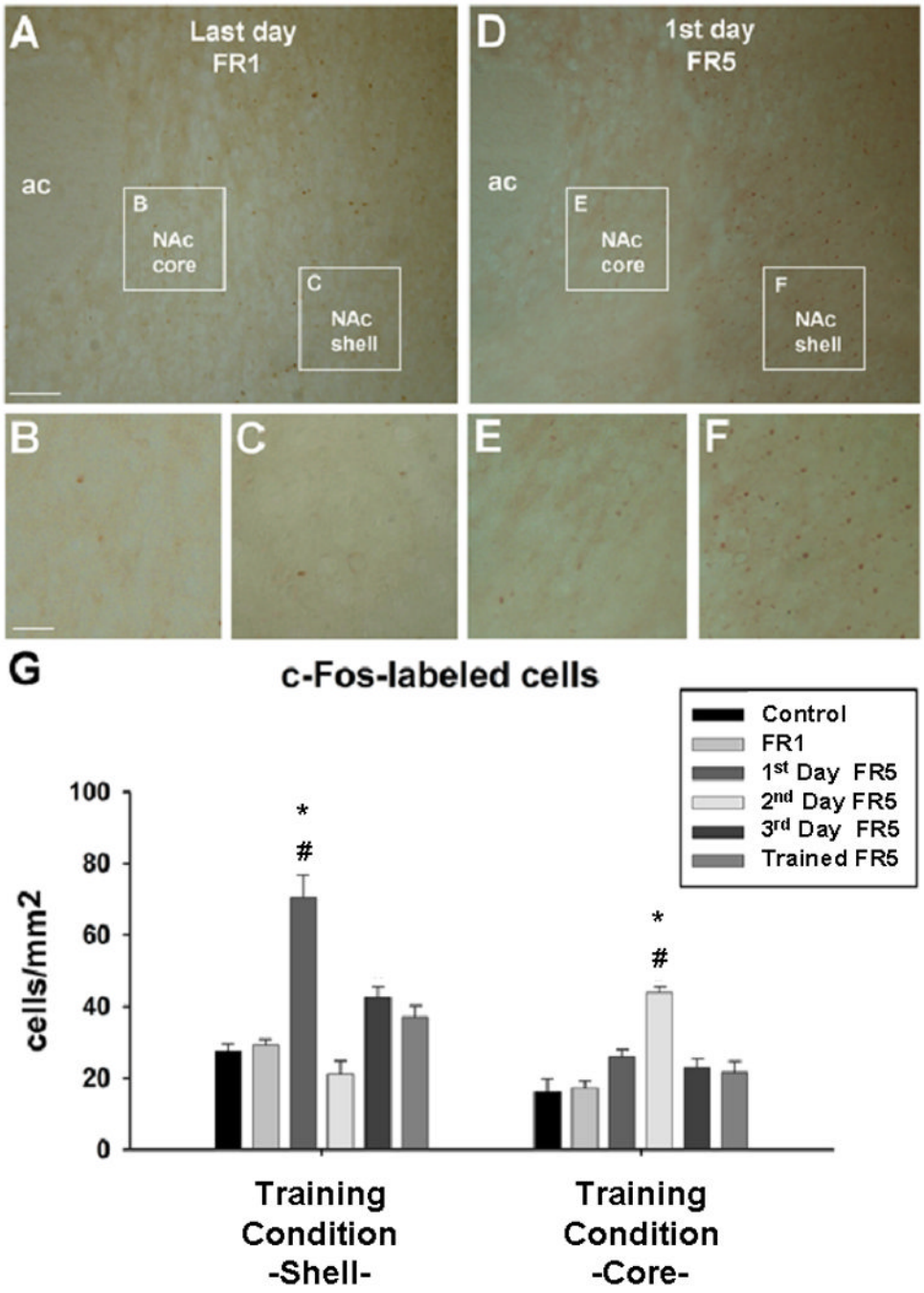

Figure 2.

Expression of c-Fos immunoreactivity in nucleus accumbens under control conditions and during the transition from FR1 to FR5 lever pressing. **A–C.** Photomicrographs from a representative rat in the FR1 group. **A**. Low power photomicrograph of c-Fos expression showing regions of interests in the core (B) and shell (C) that were quantified. **B–C**. High power images of the accumbens core (B) and shell (C) showing minimal c-Fos expression. **D–F.** Photomicrographs from a representative rat in the first day FR5 group. **D**. Low power photomicrograph of c-Fos expression showing regions of interests in the core (B) and shell (C) that were quantified. **E**. High power image of the accumbens core showing minimal c-

Fos expression. **F**. High power image showing the robust expression of c-Fos containing neurons in the accumbens shell. **G**. Quantification of number of c-Fos positive cells in the accumbens shell and core across the different training conditions. *$p < 0.05$, different from control and FR1 groups #$p < 0.05$, different from all other groups. Abbreviations: ac= anterior commissure, NAc= nucleus accumbens. Scale bars: **A, D** = 200 μm; **B, C, E, F** = 75 μm

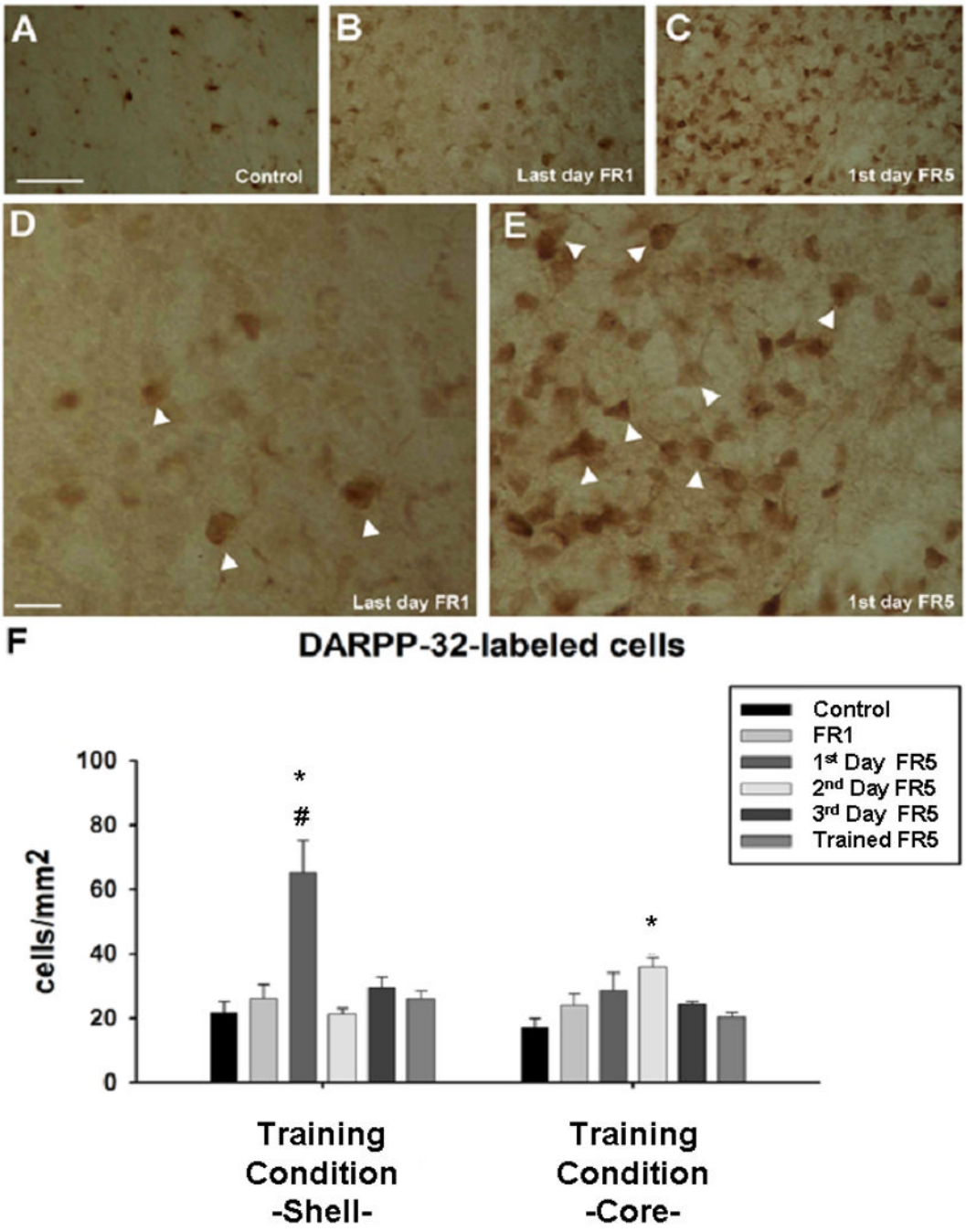

Figure 3.

Expression of DARPP-32 immunoreactivity in nucleus accumbens under control conditions and during the transition from FR1 to FR5 lever pressing. **A–C**. Low-power images of the accumbens shell showing DARPP-32 immunoreactivity in a representative animal from the control group (A), the FR1 group (B), and the first day FR5 condition (C). **D–E**. Higher-power images showing DARPP-32-labeled cells (arrowheads) in more detail. Note that the DARPP-32-labeled cells on the first day of FR5 training appear to be more morphologically reactive in appearance with more ramified processes (E). **F.** Quantification of number of DARPP-32 positive cells in the accumbens shell and core across the different training

conditions. *$p < 0.05$, different from control and FR1 groups #$p < 0.05$, different from all other groups. Scale bars: A–C =100μm; D–E= 50μm.

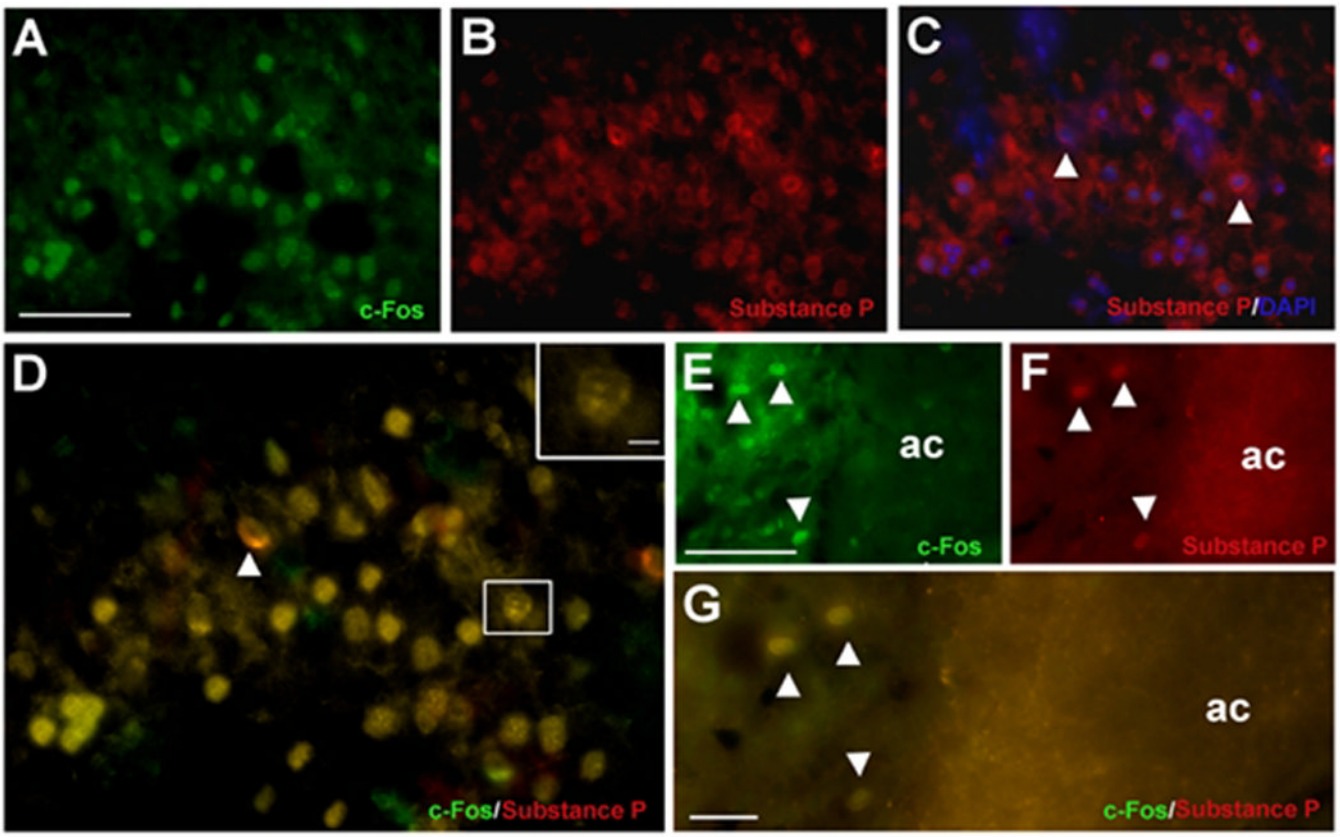

Figure 4.

Double labeling of c-Fos and substance P immunoreactivity in nucleus accumbens on the first day of FR5 training. **A**. A low-power image of the shell in which many cells are c-Fos positive (green). **B**. A low-power image of many cells showing substance P immunoreactivity (red). **C**. A low-power image of substance P cells (red) showing pyknotic DAPI-labeled nuclei (blue); note the substance P cells with clear nuclei (arrowheads). **D**. Higher-power image showing numerous double-labeled cells expressing both substance P and c-Fos imunoreactivity (yellow, from the merging of the two separate images). An intact cell clearly demonstrates co-localization (arrowhead). **D**. Inset, A high power image shows the double-labeled cells. **E**. Low-power image of the accumbens core showing few c-Fos expressing cells (arrowheads). **F**. Low-power image of the accumbens core showing few substance P-positive neurons (arrowheads). **G**. A higher power image showing co-localization of c-Fos expression in substance P containing neurons (arrowheads) in the accumbens core. Abbreviations: ac, anterior commissure, Scale bars: **A-C**. 100 μm; **D.** 50 μm (inset), 25 μm; **E–F**. 100 μm; **G**. 75μm.

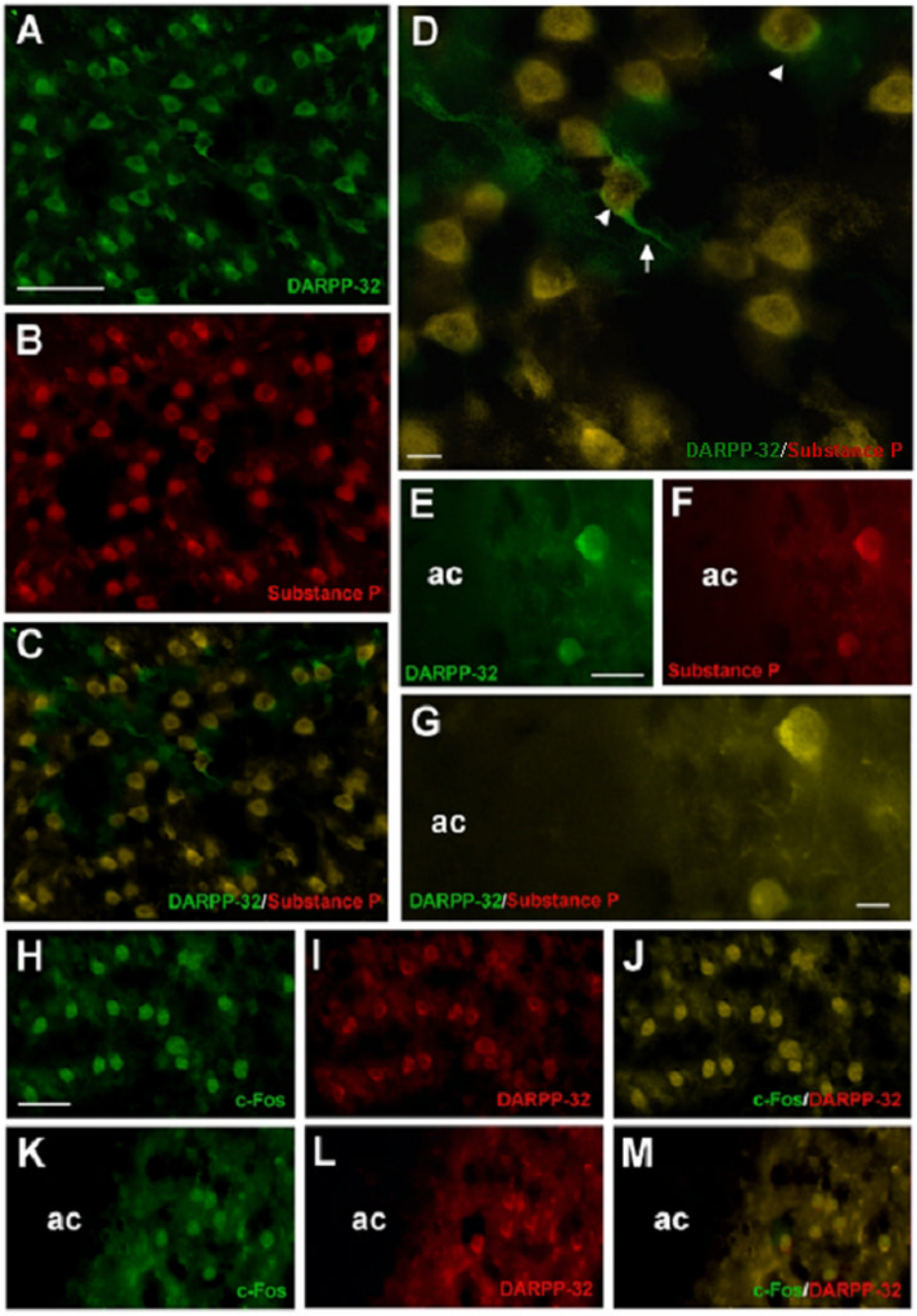

Figure 5.

Double labeling of DARPP-32 and substance P immunoreactivity in nucleus accumbens on the first day of FR5 training. **A–B**. The shell of the nucleus accumbens showing a large number of DARPP-32-positive cells (A; green) and substance P containing cells (B; red). **C**. Merged low-power image showing the co-localization of DARPP-32 and substance P containing neurons (yellow). **D**. High-power image showing numerous double-labeled cells with both substance P and DARPP-32 immunoreactivity; some neurons appear to have DARPP-32, but not substance P immunoreactivity in the dendrites (arrows). **E–G**. Photomicrograph of nucleus accumbens core, showing a few DARPP-32 positive cells (E;

green) and substance P positive cells (F; red) that are co-localized (G; yellow). **H–J**. Low-power image showing cells in the accumbens shell containing c-Fos (H; green) and DARPP-32 (I; red) immunoreactivity, and their co-localization in the merged image (J; yellow). **K–M**. Low-power image showing cells in the accumbens core containing some c-Fos (K; red) and DARPP-32 (L; red) immunoreactivity, and their co-localization in the merged image (M; yellow). Abbreviations: ac, anterior commissure, Scale bars: **A–C**. 100 μm; **D**. 50 μm; 25 μm, (inset); **E–F**. 75 μm; **G**. 50 μm; **H–M**. 100 μm.

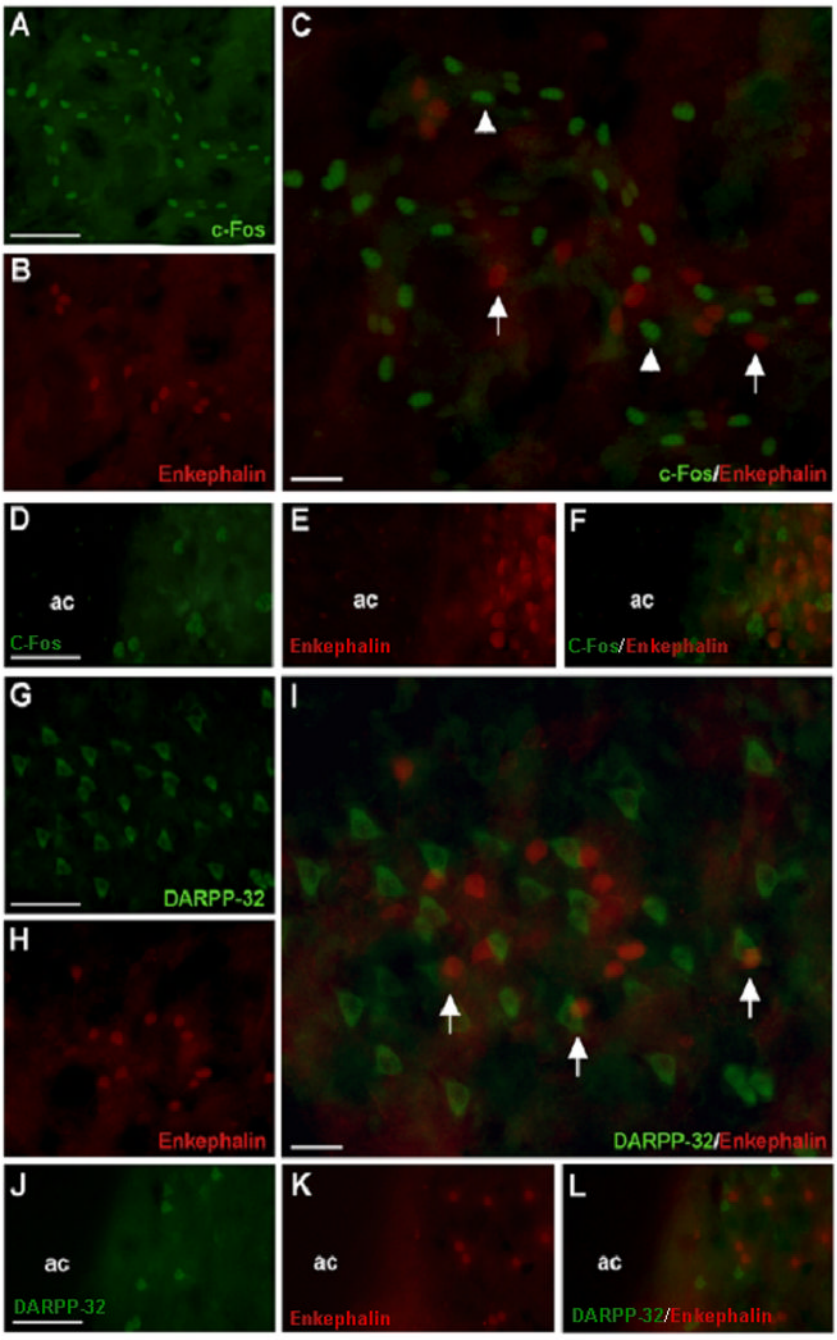

Figure 6.

Double labeling of c-Fos and DARPP-32 with enkephalin immunoreactivity on the first day of FR5 training. **A–B**. Photomicrographs of accumbens shell, showing c-Fos (A; green) and enkephalin (B; red) immunoreactivity. **C**. High-power view of the merged image, showing a separate localization of c-Fos expression (green, arrowheads) and enkephalin (red, arrows) immunoreactivity. **D–F**. Low-power photomicrograph of the accumbens core, showing c-Fos (D; green) and enkephalin (E; red) immunoreactivity; merged image (F) shows separate labeling of cells (i.e., separate red and green cells). **G–H**. Photomicrographs of accumbens shell, depicting DARPP-32 (G; green) and enkephalin (H; red) immunoreactivity. **I**. Merged

high-power image shows separate DARPP-32 (green) and enkephalin (red) immunoreactive cells in the shell. **J–L**. DARPP-32 immunoreactivity (J; green), and enkephalin immunoreactivity (K; red), and their separate localization (L; separate green and red labeled cells) in the accumbens core. Abbreviations: ac, anterior commissure, Scale bars: **A–B**, 100 μm; **C**, 50 μm **D–F**, 100 μm; **G–H**, 100 μm; **I**, 50 μm, **J–L**, 100 μm.

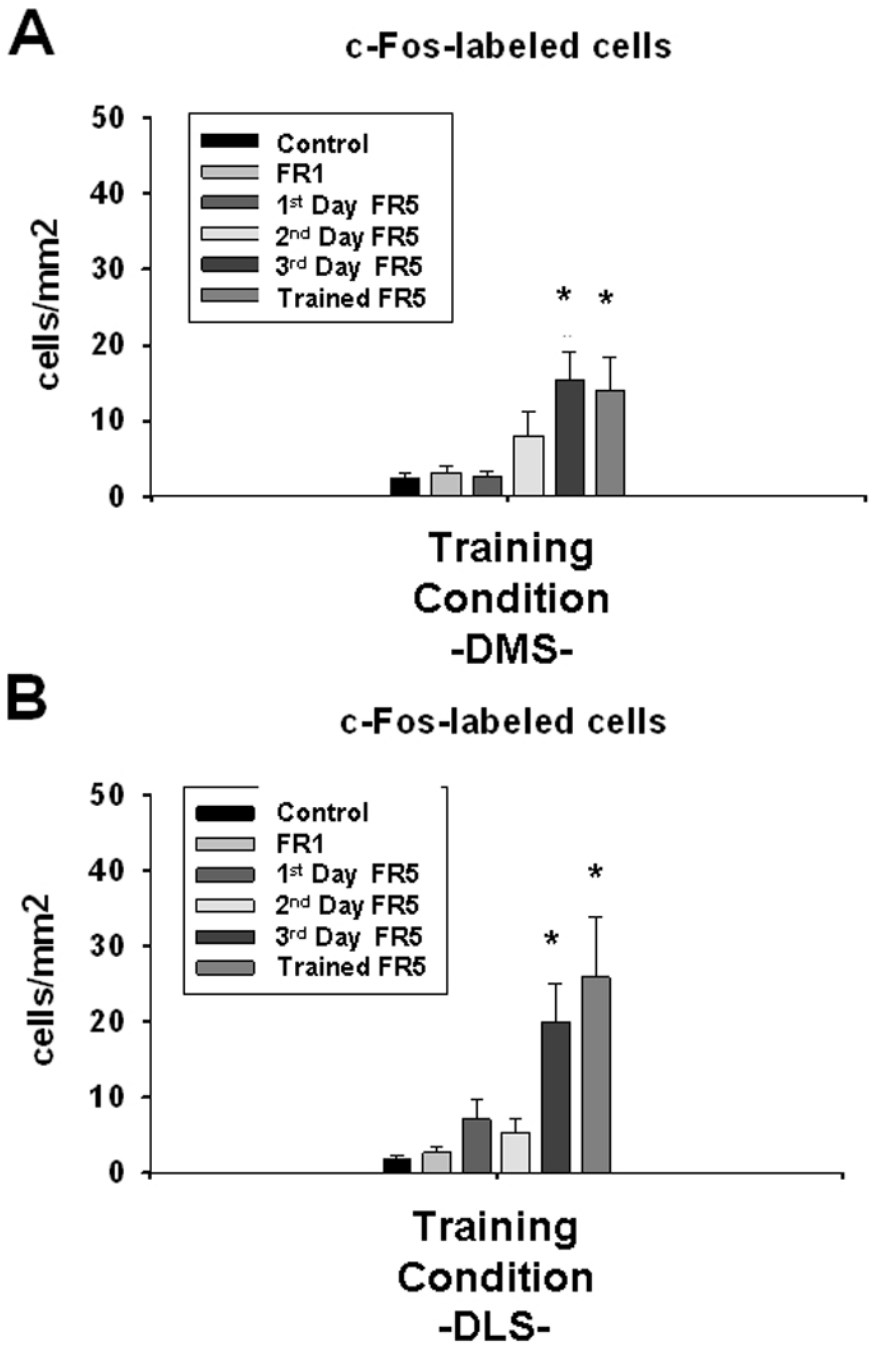

Figure 7.

Expression of c-Fos immunoreactivity in neostriatum under control conditions and during the transition from FR1 to FR5 lever pressing. **A–B**, Quantification of c-Fos density in the dorsomedial neostriatum (A; DMS) and dorsolateral neostriatum (B; DLS) after control and the different FR lever pressing training days (means ± SEM). *p< 0.05, different from control and FR1 training groups, collapsed across both DMS and DLS

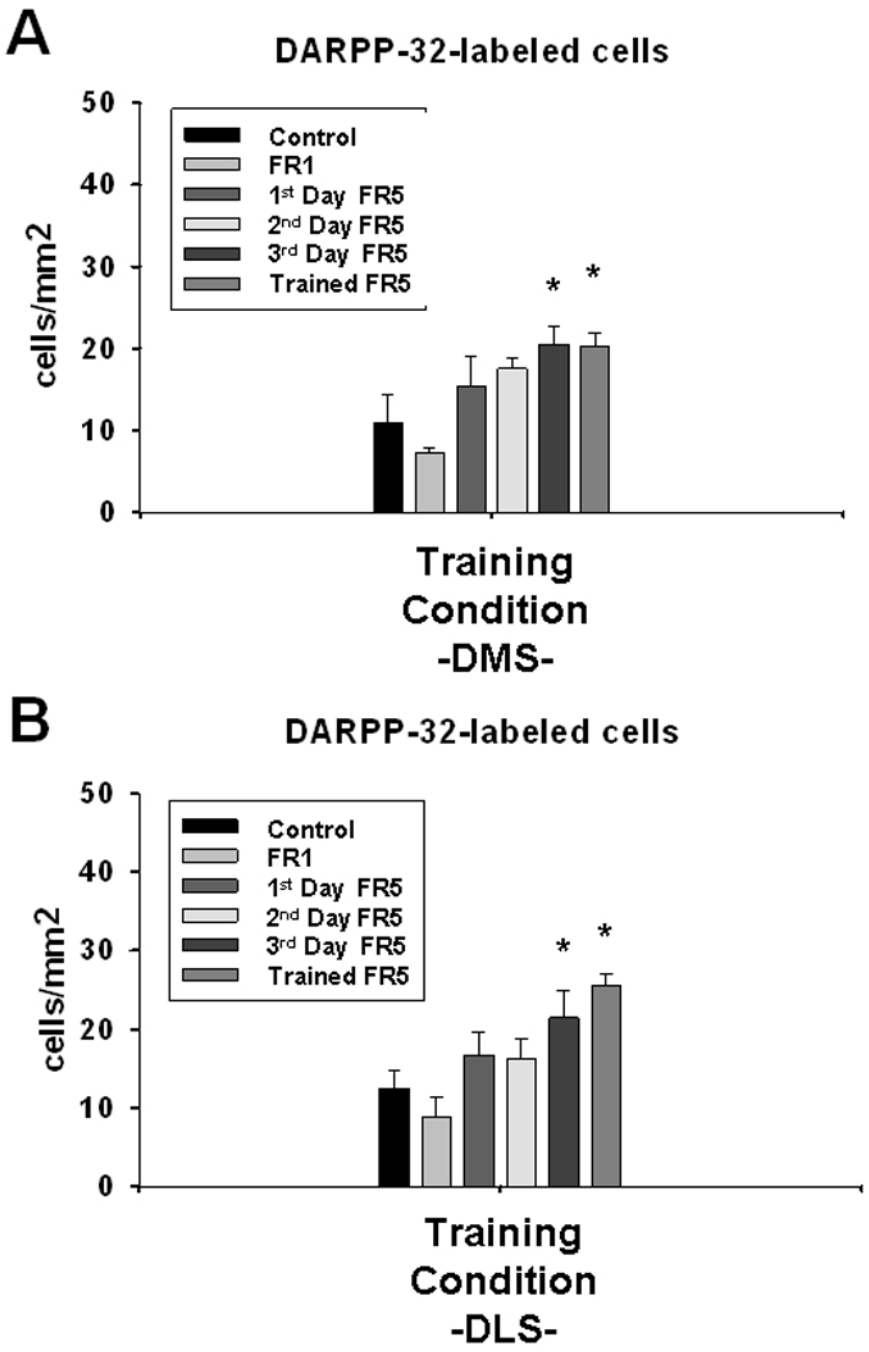

Figure 8.

Expression of DARPP-32 immunoreactivity in neostriatum under control conditions and during the transition from FR1 to FR5 lever pressing. **A–B**, Quantification of DARPP-32-labeled cells in the dorsomedial neostriatum (A; DMS) and dorsolateral neostriatum (B; DLS) after control and the different FR lever pressing training days (means ± SEM). *p< 0.05, different from control and FR1 training groups, collapsed across both DMS and DLS

Table 1

Lever pressing and food consumption data in rats tested in the c-Fos experiment. Data are the mean number of lever presses and food intake (grams) (± SEM).

FR TRAINING DAYS	LEVER PRESSES	± SEM	FOOD CONSUMED (grams)	± SEM
FR1	351.0	32.5	15.8	1.6
1st Day FR5	702.0 *	44.0	7.07 **	0.87
2nd Day FR5	762.0 *	81.0	6.8 **	0.7
3rd Day FR5	961.0 **	50.5	7.56 **	1.03
Trained FR5	1412.0 **	140.0	12.53 *	1.16

* $p<0.05$,

** *p<0.01* from FR1 training group.

Table 2

Lever pressing and food consumption data in rats tested in the DARPP-32 experiment. Data are the mean number of lever presses and food intake (grams) (± SEM).

FR TRAINING DAYS	LEVER PRESSES	± SEM	FOOD CONSUMED (grams)	± SEM
FR1	273.0	13.0	12.24	0.05
1st Day FR5	455.0 *	38.0	3.09 **	0.49
2nd Day FR5	566.0 *	69.0	4.99 **	0.62
3rd Day FR5	895.0 **	87.0	8.1 *	0.82
Trained FR5	1049.0 **	40.0	9.21 *	0.55

* $p<0.05$,

** *p<0.01* from FR1 training group.