The retrograde tracer fluoro-gold interferes with the expression of fos-related antigens

Teresa R. Franklin a, Jonathan P. Druhan b,*

a Center for Neurobiology, MCP Hahnemann University, 3300 Henry Ave., Philadelphia, PA 19129, USA
b Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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Abstract

The retrograde tracer, fluoro-gold (FG) has been used in combination with immediate early gene (IEG) immunohistochemistry to identify neural circuits activated by pharmacological, physiological or behavioral manipulations. However, since FG has been shown to be toxic to cell bodies, axons and terminals at the injection site, the question arises as to whether FG alters the detection of IEG products. To examine this question, FG was microiontophoresed unilaterally into the nucleus accumbens (NAc) of rats and Fos-related antigens (FRAs) were examined in both hemispheres 12 days later. Approximately half as many FRA-positive nuclei were observed in the tracer-injected NAc as were found in the contralateral NAc. Similar results were observed in the ventral subiculum of the hippocampus and the basolateral and central amygdaloid nuclei, but not in the lateral septum or lateral habenula. These results suggest that FG microiontophoresed into the NAc interferes with the expression of FRAs at the injection site and also at other ipsilateral limbic sites. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Immunohistochemical localization of immediate early gene products (IEGs) has been widely accepted as a procedure to identify neurons within the CNS activated by pharmacological, physiological and behavioral stimuli (Dragunow and Faull, 1989; Morgan and Curran, 1991; Campeau et al., 1997). This genomic mapping technique is advantageous in that it allows cell groups activated by such stimuli to be distinguished. However, this technique by itself, does not indicate whether immunohistochemically labeled cell groups are interconnected. To obtain such information, investigators have combined IEG immunohistochemistry and retrograde tracing techniques to delineate the circuits mediating effects of specific manipulations.

Fluoro-gold (FG) has been used extensively as a retrograde axonal tracer to determine neuroanatomical connectivity (Schmued and Fallon, 1986; Pierbone and Aston-Jones, 1988; Schmued and Heimer, 1990) and has recently been used in conjunction with immunohistochemistry for fos-related antigens (FRAs) to identify the nuclei and pathways involved in the response to various pharmacological (Page and Everitt, 1993; Jasmin et al., 1994), physiological (Senba et al., 1993; Kelly and Watt, 1996) and behavioral (Cullinan et al., 1996; Dinardo and Travers, 1997) manipulations. However, a potential pitfall of combining FRA immunohistochemistry and FG tract-tracing techniques is that FRA expression might be affected by intracranial infusions of FG. Pressure injections of FG at concentrations as low as 2.5% have been shown to produce degeneration of neuronal cells, terminals and axons at the injection site (Schmued and Fallon, 1986; Schmued et al., 1993). Such toxic effects of FG could alter the activity of cells within regions functionally connected to the injection site and influence FRA expression in those regions. In fact, a previous study found a significant reduction in FRA-positive cells in the ipsilateral paraventricular nucleus of the thalamus following nucleus accumbens (NAc) or amygdala infusions of FG (Peng...
et al., 1995). Although the reasons for the decreased FRA expression could not be determined in that study, it is possible that the reductions were caused by functional changes associated with the FG injection. This interpretation would be consistent with other findings that FG can interfere with neuroanatomical labeling associated with the infection and spread of herpes simplex virus (LaVail et al., 1993).

The possibility that FG may interfere with FRA expression was investigated in this study by measuring bilateral FRA expression in the NAc and in limbic regions of rats unilaterally injected with FG into the NAc. The NAc receives afferents from several limbic structures, and limbic-acumbens circuits would therefore be useful for studying retrograde tracer-induced alterations in neuronal function (Heimer et al., 1991). Moreover, previous studies have shown that FRA expression is induced within the NAc and associated limbic regions of rats following environmental stimulation (Brown et al., 1992; Franklin et al., 1996). Accordingly, quantifying FRA-positive cells in the NAc and limbic regions of each hemisphere after unilateral injections of FG into the NAc could show whether or not FRA expression is altered by the tracer injection.

2. Materials and methods

2.1. Subjects

Ten male Wistar rats (Charles River, Wilmington, MA) weighing 250–300 g were used for the present study. The animals were housed in clear plastic cages (two rats per cage) in an AAALAC-approved animal-care facility maintained at 21°C with a 12 h light/dark cycle (lights on at 07:00 h). Food and water were available ad libitum throughout the experiment. All experimental procedures were approved by an Institutional Animal Care and Use Committee in accordance with NIH guidelines.

2.2. Surgery and microiontophoresis

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and positioned in a stereotaxic apparatus. Glass micropipettes (10–15 μm) were inserted unilaterally into the right NAc at a 15° angle lateral to the sagittal plane (thus avoiding contact with the ventricle) using skull-surface coordinates of 1.6 mm anterior to bregma, 3.0 mm lateral to midline, and 6.6 ventral to the skull surface according to the atlas of Paxinos and Watson (1998). These coordinates, together with the inward trajectory of the angled pipettes, resulted in tip placements within the dorsal portion of the NAc, near the border of the core and shell regions (see Fig. 1A Fig. 2A). FG (Fluorochrome, Englewood, CA; 2% solution in saline) was injected iontophoretically into the NAc (n = 6) using a pulsed-positive current of 2 μA (4 s on, 4 s off) for 10 min. A second group of non-injected control rats (n = 4) was subjected to the same surgical procedures but no injection was given. All rats were allowed to recover for 12 days before being euthanized and perfused for tissue recovery.

2.3. Environmental exposures

This study was originally designed to assess the feasibility of combining FG and FRA immunohistochemical techniques with behavioral manipulations wherein rats would be exposed repeatedly to a distinctive environment under different drug treatment conditions. In order to approximate the experimental conditions under which animals would be tested in those planned experiments, the rats were given daily hour-long exposures to a distinctive environment on 7 consecutive days (starting 5 days after the FG injection). For each exposure, the rats were placed individually into separate Plexiglas chambers (40 × 40 × 40 cm³; San Diego Instruments San Diego, CA) located in a sound attenuated, dimly lit room. Subjects were euthanized and perfused immediately following the seventh exposure and the brains were processed for immunohistochemical localization of FRA-positive nuclei.

2.4. Dual immunohistochemistry

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) immediately after the seventh exposure to the distinctive environment and perfused transcardially with 100 ml of saline followed by 800 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer). The brains were removed from the cranial cavity and post-fixed for 90 min at 4°C before being transferred to a 20% sucrose solution and stored at 4°C. Subsequently, each brain was sliced into 40 μm coronal sections and collected in 0.1 M phosphate buffered saline (PBS). Immunohistochemical procedures for localization of FRAs were performed prior to FG immunohistochemistry. The sections were incubated for 10 min in 0.1 M PBS with 2% H₂O₂, and rinsed twice in PBS (10 min/ rinse) before being stored overnight in PBS with 0.1% sodium azide (PBS-Az). The sections were then incubated overnight at 4°C, in a solution containing 2% normal donkey serum (NDS) in PBS-Az with 0.3% Triton-X (PBS-Az-Tx), followed by 48 h with primary antibody (rabbit anti-Fos antiserum at 1:50 000 dilution, Oncogene Sciences, Cambridge, MA) in PBS-Az-Tx with 2% NDS at 4°C. The tissue was incubated for 90 min in biotinylated donkey anti-rabbit (1:500, Jack
son) in PBS-Tx at room temperature, and then transferred to Avidin-Biotin Complex (ABC 1:500, Jackson Immunoresearch Laboratories, West Grove, PA) in PBS-Tx for 90 min at room temperature. FRA-positive nuclei were visualized as dark elliptical spheres, by placing the tissue in 3,3′-diaminobenzidine (DAB, 0.02%, Sigma, St. Louis, MO) with 0.0002% peroxide and 0.6% nickel ammonium sulfate in 0.01 M Tris buffer for 3.5 min. The reaction was arrested by immediate transfer into 0.05 M Tris buffer (pH = 7.6). The tissue was rinsed three times (10 min/rinse) in PBS-Tx between each incubation phase, and given an additional rinse in 0.05 M Tris buffer prior to the DAB reaction. Following the DAB reaction, the sections were rinsed once in PBS, washed in PBS with 2% H₂O₂ to terminate the reaction, and rinsed twice more in PBS.

FG immunohistochemistry was subsequently performed on the FRA-labeled tissue by incubating the sections overnight at room temperature with primary antibody (rabbit anti-Fluoro-gold polyclonal at 1:1000, Chemicon, Temecula, CA) in PBS-Az-Tx. FG labeled cells were visualized using immunohistochemical procedures similar to those described above, except that nickel ammonium sulfate was omitted from the DAB solution and the reaction proceeded for 4 min. This visualization reaction produced a light orange color within the cytoplasm of retrogradely-labeled cells. The sections were given two final rinses in PBS following arrest of the DAB reaction with 0.05 Tris buffer. The sections were mounted on gelatin-coated slides, dehydrated, defatted and cover slipped for subsequent examination.

2.5. Statistical analysis

Camera-lucida drawings were constructed from representative coronal sections of the NAc (1.6 mm anterior to bregma), the basolateral and central nuclei of the amygdala (2.6 mm posterior to bregma), the ventral subiculum of the hippocampus (5.0 mm posterior to bregma), the lateral septum (1.6 mm anterior to bregma), and the lateral habenula (2.6 mm posterior to bregma) viewed at 10× magnification. The regions chosen were those in which qualitative assessments revealed the presence of FRA-positive nuclei. A representative level was chosen for each structure and the sections most similar to that level in each animal were used for quantification. FRA-positive cells were easily identified by the presence of dense immunohistochemical staining within the nuclei. The presence of light background staining provided sufficient landmarks to allow the boundaries of individual structures to be determined. All regions that were labeled with both FG and FRA antibodies are displayed in Fig. 1. The atlas of Paxinos and Watson (1998) was used as a guide to ensure accurate delineation of the structures. FRA-pos-
Fig. 2. Photomicrographs of sections from the NAc and the VSb. (A) Coronal section showing the extent of FG diffusion in the NAc following a unilateral microiontophoretic injection (1.6 × magnification). (B) Nissl-stained coronal section (from the same brain as shown in ‘A’) showing the extent of damage typically produced by infusion of FG into the NAc (5 × magnification). The arrow indicates a region of cell shrinkage surrounding the pipette tip. Damage at the injection site appeared to be minor, and was not evident at all in control rats (not shown). (C) Coronal section showing cells within the VSb that were labeled by antibodies against only FRA, only FG, or both labels (taken at 10 × magnification). Arrows point to cells both retrogradely labeled with FG and expressing FRA-positive nuclei, confirming that dual immunohistochemistry was performed properly. (D) Higher-power view (16 × magnification) of the section from ‘C’ above showing cells double labeled for FG and FRA. Calibration bars: A = 700 μm; B = 350 μm; C = 230 μm; and D = 140. Abbreviations: ac, anterior commissure; CPu, caudate putamen; LS, lateral septum; NAc, nucleus accumbens; v, lateral ventricle.
Fig. 3 displays the mean numbers of FRA-positive nuclei observed within each hemisphere for the NAc, the basolateral amygdala, the central nucleus of the amygdala, the ventral subiculum of the hippocampus, the lateral septum, and the lateral habenula of FG and sham-operated rats. Significantly fewer FRA-positive nuclei were observed in the NAc (t(5) = 3.39; P < 0.01), the basolateral amygdala (t(4) = 3.58; P < 0.025), the central nucleus of the amygdala (t(5) = 2.00; P < 0.05) and the ventral subiculum (t(5) = 3.72; P < 0.01) ipsilateral to the injection site relative to the numbers observed in the contralateral structures of FG-treated rats. In contrast, interhemispheric differences in FRA expression were not observed in the lateral septum or lateral habenula. Interhemispheric differences also were not observed in any region examined in sham-operated rats, indicating that the ipsilateral reductions in FRA expression in FG-treated rats were not a result of mechanical damage due to surgery or insertion of the micropipette. Necrosis, as determined by increased gliosis at the injection site, appeared to be minor in Nissl-stained sections of FG-injected rats and was not evident in control rats (see Fig. 2B). Examination of the FG/FRA stained sections revealed the presence of regions labeled with FG only, regions labeled with FRA-positive nuclei only, and several dually labeled regions in which both FG-labeled and FRA-labeled cells could be distinguished (see Fig. 2C, D). Regions that were immunohistochemically labeled with both the retrograde tracer and the IEG product, included the orbitofrontal cortex, cingulate cortex, claustrum, paraventricular nucleus of the thalamus, lateral habenula, basolateral amygdala, and ventral subiculum of the hippocampus. The paraventricular nucleus of the thalamus and cortical regions were excluded from FRA quantification because FG label was observed bilaterally in these regions, making it impossible to draw comparisons between the two hemispheres.

3. Results

Necrosis, as determined by increased gliosis at the injection site, appeared to be minor in Nissl-stained sections of FG-injected rats and was not evident in control rats (see Fig. 2B). Examination of the FG/FRA stained sections revealed the presence of regions labeled with FG only, regions labeled with FRA-positive nuclei only, and several dually labeled regions in which both FG-labeled and FRA-labeled cells could be distinguished (see Fig. 2C, D). Regions that were immunohistochemically labeled with both the retrograde tracer and the IEG product, included the orbitofrontal cortex, cingulate cortex, claustrum, paraventricular nucleus of the thalamus, lateral habenula, basolateral amygdala, and ventral subiculum of the hippocampus. The paraventricular nucleus of the thalamus and cortical regions were excluded from FRA quantification because FG label was observed bilaterally in these regions, making it impossible to draw comparisons between the two hemispheres.
4. Discussion

The results of this study show that microiontophoretic injections of FG into the NAc reduce FRA expression at the injection site and in certain CNS regions ipsilateral to the injection site. These latter regions include the basolateral and central nuclei of the amygdala and the ventral subiculum of the hippocampus. The reduction in FRA expression seen here is consistent with the observations of an earlier study that combined the techniques of FRA immunohistochemistry with retrograde labeling (Peng et al., 1995) in which reductions in FRA-positive nuclei were observed in the ipsilateral paraventricular nucleus of the thalamus following injections of FG into either the amygdala or the NAc. However, Peng et al. did not use metal intensification to enhance FRA immunostaining and it was unclear as to whether the reduced number of FRA-positive cells was due to decreased FRA expression or to inadequate visualization of the FRA-positive nuclei. The present study clarified this issue by demonstrating FG-induced decreases in FRA expression even when metal intensification procedures were used to visualize FRA in the cell nucleus.

The reduction of FRA expression in the hemisphere ipsilateral to the FG injection was not related to technical problems such as cross-reactivity between the antibodies or the physical blockade of FRA visualization by the FG reaction. Had cross-reactivity between the two antibodies occurred, FG labeling would have been observed bilaterally within all regions expressing FRAs, rather than localized to specific regions that are known to project to the NAc, as was observed in this study. Physical blockade of FRA visualization by the FG reaction was also avoided in this study by performing FRA immunohistochemistry prior to FG immunohistochemistry. Moreover, nickel ammonium sulfate was added to the DAB reaction during FRA immunohistochemical procedures which intensifies the visualization of the IEG product. In this manner, FRA-positive nuclei were prominently visible through the light colored cytoplasmic FG label.

Representative limbic regions were chosen to quantify FRA-positive nuclei based on their connectivity with the NAc. The basolateral nucleus of the amygdala and ventral subiculum of the hippocampus send substantial projections to the NAc (Mogenson, 1987; Groenewegen et al., 1996) and therefore may be affected by a FG injection into the NAc. The lateral habenula also projects to the NAc, albeit rather weakly, and the lateral septum shares reciprocal connections with the NAc (Groenewegen et al., 1996). The central nucleus of the amygdala, which is not directly connected to the NAc (Brog et al., 1993; Groenewegen et al., 1996), was chosen in order to examine FRA changes that may occur in circuitously connected regions. Examination of the effects of FG on FRA expression was not possible in certain limbic regions such as the prefrontal cortex and paraventricular nucleus of the thalamus because of extensive bilateral retrograde labeling.

Presently, the mechanisms underlying the decrease in FRA-positive nuclei seen in ipsilateral brain regions following unilateral FG injections are not known. However, several hypotheses may be suggested. One possibility is that FRA induction was asymmetric under the conditions of this study. It has been shown previously that CNS activation may vary between hemispheres. Indeed, regional cerebral blood flow showed significant lateralization during a conditioned eyelink response in humans (Schreurs et al., 1997) and lateralization of dopamine function has been observed in mouse strains displaying differing paw preferences (Nielson et al., 1997). However, we did not observe interhemispheric differences in FRA expression in control rats that received only sham injections into the NAc. Interhemispheric differences in FRA expression were also not seen in a prior study that examined conditioned FRA expression in the absence of tracer (Franklin et al., 1996). Such findings suggest that the lower number of FRA-positive nuclei on the ipsilateral side seen in the present study was not a general characteristic of the procedure employed, but rather it appeared to be related to the unilateral injection of FG into the NAc.

Another hypothesis is that FG alters FRA expression specifically in NAc afferents that accumulate FG through their axons. This alteration in FRA expression could be the result of neuronal death or altered functioning of the affected cells. However, other studies have shown that FG is not toxic to retrogradely labeled cells even after extended survival times (Divac and Mogenson, 1990; Schmued et al., 1993 although see Dado et al., 1990). Moreover, decreases in FRA expression were seen in the central nucleus of the amygdala in which FG was not directly taken up. These considerations point to a more subtle mechanism for the FG-induced decrease in FRA expression.

The reduced FRA expression in regions ipsilateral to the FG injection may be an indirect result of necrosis or reduced cellular activity at the injection site. Infusions of FG at concentrations as low as 2.5% have been shown to produce detectable degeneration of neuronal cells, terminals and axons at the injection site (Schmued and Fallon, 1986; Schmued et al., 1993). Conceivably, FG-induced changes in cellular function may have altered neuronal activity in limbic regions that receive direct or indirect feedback from the NAc. For example, reduced activity of GABA-ergic projections from the NAc to the VTA (Groenewegen and Russchen, 1984; Mogenson, 1987; Groenewegen et al., 1996) could result in greater dopaminergic activity and a consequent
increase in inhibition of cells within mesolimbic terminal fields. Such changes in the activity of NAc-related forebrain circuits could reduce the expression of IEG products in a variety of CNS regions. Although examination of the injection site in this study revealed only minor damage, it is possible that even minimally detectable damage might produce changes in NAc cell function that could substantially affect neuronal activity within regions such as the ventral subiculum, and the central and basolateral amygdaloid nuclei. In contrast, the lack of change in FRA expression within the lateral septum or lateral habenula could be due to an absence of direct or indirect feedback regulation of these sites by the affected areas within the NAc.

While the present findings indicate that intra-cerebral FG injections can reduce FRA expression in the ipsilateral hemisphere they do not suggest that FG will always interfere with FRA expression. The present study examined FRA expression that was near or at the level of constitutively expressed FRAs. This level may be susceptible to inhibition by the FG injection whereas inhibition may be masked at a higher level of neuronal activation that results in extremely elevated FRA levels. In fact, studies involving more intense pharmacological or behavioral manipulations have shown substantial FRA expression ipsilateral to a FG injection site (Jasmin et al., 1994; Kelly and Watt, 1996; Dinardo and Travers, 1997). In such experiments in which FRA expression is more robust, dual labeling procedures appear to be sufficient to define the major neuronal pathways involved in the particular manipulation (Wang et al., 1994; Cullinan et al., 1996). However, the present results indicate that FG may elevate the threshold for FRA expression so that its measurement cannot be combined with FG tracer techniques in less intense manipulations.

Although the mechanisms behind the reduction in FRA expression in regions ipsilateral to the FG injection site are as of yet unknown, the results of this study strongly suggest that FG interferes with FRA expression. Therefore, caution must be exercised when interpreting the results of studies in which FG is used in conjunction with the expression of IEG products such as FRAs.

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