**AUTHORS**

Michela Deleidi3,4, Bianca Marchetti1,2, Federico Bertoli3,4, Carmela Giachino1

1Neuropharmacology Laboratory, Oasi Research Institute-IRCCS, Troina, Italy

2 Biomedical and Biotechnological Sciences, Pharmacology Section, University of Catania-Italy

3 Mitochondria and Inflammation in Neurodegenerative Diseases, DZNE, Tübingen-Germany

4 Hertie Institute for Clinical Brain Research, University of Tübingen

**ABSTRACT**

A laboratory method that uses antibodies to check for certain antigens (markers) in a sample of tissue. The antibodies are usually linked to an enzyme or a fluorescent dye.

**PROTOCOL**

For brain tissue analysis, serial coronal sections (14 µm-thick), encompassing the striatum (Bregma 1.54 to bregma -0.46) and the SNpc (Bregma -2.92 to bregma -3.8 mm) according to Franklin and Paxinos (1997)were collected, mounted on poly-L-lysine-coated slides, and processed as previously described. TH immunoreactivity was also detected using biotinylated secondary antibodies (Vector Laboratories) and diaminobenzidine (DAB, Vector Laboratories) as the developing agent. Cresyl violet was used to visualize Nissl substance. DA neuronal counts were performed by serial section analysis of the total number of TH+ neurons in the right and left SNpc through the entire extent of the SNpc using DAPI or PI as nuclear markers, as previously described. TH+ cells were counted through the entire rostro-caudal axis of the murine SNpc (bregma coordinates: 2.92, 3.08, 3.16, 3.20, 3.40, and 3.52) according to Franklin and Paxinos (1997) as described. Striatal TH- and dopamine transporter (DAT)-immunofluorescent (IF) fiber staining was assessed in n = 3 coronal sections at three levels (bregma coordinates: + 0.5, + 0.86, and 1.1 mm, respectively) of caudate-putamen (CPu), in n 5-6 mice/group/time. Cell counts were obtained for IBA1+/Dapi+ or Mac-1+/Dapi+ reactive microglial cells and GFAP+/Dapi+ astrocytes, averaged for each animal and the mean number of cells per mm2 per animal was estimated. A comparable countable area ranging from 1.90 mm2 to 2.00 mm3 was analyzed in the different groups. Results are expressed as % of saline-injected controls.

For intestine analysis, the tissue was fixed in 1% paraformaldehyde for 2 hours, washed with 50 mmol/L NH4Cl, and cryoprotected in 30% sucrose (w/v) at 4°C overnight. Tissue was then embedded in OCT medium, snap-frozen, and stored at –80°C. Gut slices were rehydrated with PBS and blocked with 0.3% Triton X- in PBS and 10% of Normal Goat Serum for 1 h at room temperature. Samples were then incubated overnight with primary antibodies in 0.3% Triton X- in PBS and 1% of Normal Goat Serum. Samples were then washed with PBS and incubated for 1 h with phalloidin-iFluor-594 (1:500, Abcam) and with the appropriate secondary antibody (Invitrogen). After washing with PBS, samples were incubated for 5 minutes at room temperature with DAPI to stain nuclei (1:10000 in PBS). Images were acquired using a Leica TCS SP8 confocal microscope (Leica, Germany) equipped with a 63 × /1.4 numerical aperture oil-immersion objective and analyzed with ImageJ. For each mouse, 6–8 fields, each containing approximately 0.1 mm2 of epithelial-covered villus mucosa, were analyzed by a blinded observer. Number of positive stained cells per villi section per mm was calculated from at least 10 high power fields/section. For quantification of aggregated a-syn -specific signal, 8-10 pictures covering the whole gut section were taken using 10X objective, and a threshold was set where only the aggregate-specific signals were visible. The same threshold was applied to all images. The mean fluorescence intensity (MFI) of the selected aggregate signals was quantified using ImageJ software. Signals were normalized to the total surface area of each slice detected using the DAPI staining.

Dopaminergic neuronal counts were determined by serial section analysis of the total number of TH+ neurons in the right and left SNpc throught the entire extent of the SNpc using DAPI or PI as nuclear markers, as previously described.73,74 Briefly, at least three sections were obtained from each animal representing each of the five representative planes from -2.92 to -3.8 mm relative to bregma according to the stereotaxic coordinates.80.81 Total numbers of TH- and cresyl violet (CV)-stained neurons in adjacent tissue sections were estimated in parallel to validate TH+ neuron survival.74 A total of ≥ 5 mice/group/time-point was analyzed in a blind fashion. Each midbrain section was viewed at low power (X 10 objective) and the SNpc was outlined and delineated from the ventral tegmental area immunoreactive neurons by using the third nerve and cerebral peduncle as landmarks. For fluorescence microscopy, a confocal laser scanning microscope LEICA TCS-NT (Version 2.5, Build 1227, Leica Microsystems GmBH, Heidelberg, Germany, equiped with image analysis software), with an argon/krypton laser using 10 X, 20 X, and 40 X and 100 X (oil) immersion objectives, was used. TH immunoreactivity was also detected using biotinylated secondary antibodies and CV was used to visualize Nissl substance. Estimates of total TH+ and CV-stained neurons in the SNpc were calculated using Abercrombie correction.72-75,81,170 Briefly, the Abercrombie correction considers the number of sections collected, the interval between sections, and the thickness of each section to estimate the total number of neurons (cresyl violet) or TH+ cells within the entire SNpc. The Abercrombie values are calculated by multiplying the total neuronal count for each of these factors. The total number of TH+Nissl+ neurons was estimated at the different time-intervals studied170. Results of the right and left SN were thus added to generate a total TH+ SNpc neuron count. Treatment groups were averaged and represent the means ± SEM. Differences were analyzed by ANOVA followed by Tukey’s multiple comparison or Bonferroni-post hoc tests, and considered significant when *P* < 0.05.

Striatal TH- and dopamine transporter (DAT)-immunoreactive (IR) fiber staining was assessed in n = 3 coronal sections at three levels (bregma coordinates: + 0.5, + 0.86, and 1.1 mm, respectively) of caudate-putamen (CPu), in ≥ 5 mice/group/time. In all cases of immunohistochemical quantification, analyses were performed by an individual unaware of the experimental treatments. Fluorescence intensity (FI) of TH-staining above a fixed threshold using the corpus callosum for background subtraction;72,74,171,172 Measurements of FI were carried out by computer-assisted image analysis software (LEICA), and changes in average FI (mean ± SEM) expressed as percentage (%) of saline-injected controls. Cell counts were obtained for ameboid IBA1/Dapi+ or Mac-1+/Dapi+ reactive microglial72 cells and GFAP+ Dapi+ astrocytes, averaged for each animal and the mean number of cells per mm2 per animal was estimated. A comparable countable area ranging from 1.90 mm2 to 2.00 mm3 was analyzed in the different groups. Results are expressed as % of saline-injected controls.