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IHC and image analysis

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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.

ATTACHMENTS

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MATERIALS

Materials

- diaminobenzidine (DAB, Vector Laboratories)
- Cresyl violet
- 1% paraformaldehyde
- NH_4Cl
- 30% sucrose
- Normal Goat Serum
- Leica TCS SP8 confocal microscope (Leica, Germany)

IMMUNOHYSTOCHEMISTRY AND IMAGE ANALYSIS

8h 5m

1 For CNS analyses, each brain was cut in the coronal plane and serially-sectioned at 14 µm with a freezing sliding microtome (Leica). For the SNpc, every section from the rostral hippocampus to the anterior aspects of the cerebellar-midbrain junction was saved, coded and mounted, onto polilysine-coated slides (four sections per slide).

2 TH immunostaining was visualized using biotinylated secondary antibodies (Vector Laboratories) and diaminobenzidine (DAB, Vector Laboratories) as the developing agent. Cresyl violet was used to visualize Nissl substance. DAB-immunostained sections were analyzed by bright-field microscopy, using an Olympus microscope BX50F (Olympus optical CO.LTD, Japan).

3 Alternate sections were used for the dual stainings with TH and the described glial/immune markers revealed with fluorescent secondary antibodies and Dapi counterstaining. 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.

4 For double-staining experiments, brain sections were simultaneously incubated with 2 primary antibodies developed in different species

5 After overnight incubation, the sections were washed extensively and incubated in darkness for 2 h with fluorochrome (FITC, CY3, CY5)-conjugated species-specific secondary antibodies (1:200; JacksonImmuno Research, *Supplementary Table 1*). For DAPI staining, mounted brain sections were coverslipped using Fluoromount-G with DAPI (00-4959-52, Invitrogen). In all of these protocols, blanks were processed as for experimental samples except that the primary antibodies were replaced with PBS.

6 All fluorescent stainings were analyzed using a confocal laser scanning microscope LEICA TCS-NT (Version 2.5, Build 1227, Leica Microsystems GmBH, Heidelberg, Germany, equipped with image analysis software), with an argon/krypton laser using 10 X, 20 X, and 40 X and 100 X (oil) immersion objectives.

7 For immunohistochemistry with 3,3'-diaminobenzidine (DAB) and Nissl staining, brain sections were treated as above except for a quench for 30 min at room temperature with 0.3% H202 in methanol following antigen retrieval. Secondary antibodies (Jackson Immunologic), conjugated to biotin were used and sections treated with the ABC kit (Vector) followed by development with Impact DAB reagent (Vector).

8 Sections were dehydrated progressively in ethanol and then Histo-Clear (national Diagnostics)

and mounted to coverslipsw ith Permount reagent (Fisher).

9 Sections were rehydrated with immersion to water and then treated with cresyl violet solution (0.1% cresyl violet, 0.08% acetic acid) for one-minute in a microwave, and then rinsed in water and dehydrated with ethanol, and immersed back into Histo-clear and mounted onto slides.

10 Slides were analyzed by bright-field microscopy, using an Olympus BX50F microscope.

TH+ cell counts

11 For TH^+ cell counts, model-based stereological estimates were performed by sampling sections equally spaced covering the rostro-caudal planes from Bregma -2.92 to Bregma -3.8 mm[52]which included the majority of the dopaminergic neurons distributing in the medial-lateral aspects of the SNpc (rostral to caudal), as reported by Baquet *et al.*

12 A sampling interval of 112 µm was used. On average, 20 sections/brain were counted by an investigator blinded to the experiment identity.

13 After TH immunostaining visualized with the ABC method and subsequent Nissl counterstaining, the anterior to posterior extent of the SN was identified based on the mouse brain atlas of Franklin and Paxinos, using landmarks shown in Nelson et al. 1996. The rostral portion of the SNpc starts with the first TH^+ cells located near the end of the subthalamic nucleus and lateral to the TH^+stained fibers in the medial forebrain bundle. The caudal SNpc ends where the retrorubral field becomes visible. On the section to be counted (one section per slide, chosen randomly and then maintained throughout all sections), the SN was outlined and within this outline, all neurons including both TH-positive Nissl positive and a few $TH^-$and $Nissl^+$ cells were counted separately for the left and right side of the brain.

14 Once a section was counted, the TH^+ cell count was corrected for uncounted sections and corrected for split nuclei using the Abercrombie correction factor. The average thickness (t) was measured in representative sections, then used to estimate the correction factor to be applied to the total number of TH^+ neurons actually counted according to the equation: [N = n (t/t . x], where *N* equals the estimated cell number, *n* equals raw cells counts, t equals section

thickness measured for each brain, and x equals the empirically determined counting particle size. For each brain, the total number of TH^+ and of $Nissl^+$ neurons are indicated (Figure 1C and *Supplementary Figure 2C-D*).

Striatal dopaminergic innervation

15 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. 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.

Glial and immune cell counts

16 To estimate the number of glial and immune cells in the SNpc, the alternative slides used for model-based TH^+ cell counting covering the rostro-caudal extent of the SNpc were used. For astrocytes and microglia an average number of 8 sections and n= 5/6 mice/genotype/age/treatment group were used.

17 For all other cell counting, an average number of 4-8 sections and n= 4-5 mice/genotype/age/treatment group were used.

18 In some experiments, $CD4^+$ / $CD8^+$cells were counted. The VM sections were triple-stained for TH and the different glial/immune markers (TH/GFAP/DAPI; DAT/IBA1/DAPI or TH/MAC/CD11b/Dapi; TH/CD3/Dapi), and examined under 10x and 20x objectives.

19 For co-localization experiments, triple stainings were performed with CCR2/IBA1/DAPI and Gal3/IBA1/Dapi. For astrocytes and microglial cell counts, each VM 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.On average 8-10 pictures(4-5 fields right +4 -5 fieds left sides) were taken using 10X objective for $GFAP^+$ /Dapi+ astrocytes and $IBA1^+$/$Dapi^+$ microglia. Cell number in SNpc per unit of surface area was determined, cell counts averaged for each animal and the mean number of cells per mm^2 was estimated. CCR2+/IBA1- cells were counted in an average number of 6 fields (3R + 3L), in n= 5 mice/genotype/age/treatment group. CCR2 + cell counts averaged for each animal and the mean number of cells ± SEM /section are indicated. Gal3+/IBA1+/Dapi+ cells

were counted in an average number of 6 fields (3R + 3L), in n= 4 mice/genotype/age/treatment group and results expressed as the mean percentage (%) of $Gal3^+/IBA1^+$ cells over the total number of $IBA1^+$cells ± SEM /section presented. $CD3^+$cells were quantified in 6 fields (3R + 3L), and 4 mice/group with the mean number of cells per section is shown; some sections were immunostained for CD4 or CD8 in the 10 M age group in 3-4 mice/experimental group.

20 Fluorescence intensity (FI) assessments and colocalizations were carried out using 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. Immunofluorescent images were acquired by sequential scanning of 12–16 serial optical sections[74]. Three-dimensional reconstructions from *z*-series were used to verify colocalization in the *x*–*y*, *y*–*z*, and *x*–*z* planes. Serial fluorescent images were captured in randomly selected areas and the number of labeled cells per field was manually counted, and cell counts obtained averaged (mean ± SEM).

Intestine cell counts and fluorescence intensity analysisFor...

21 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.

22 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.

23 Samples were then incubated overnight with primary antibodies in 0.3% Triton X- in PBS and 1% of Normal Goat Serum

24 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).

25 After washing with PBS, samples were incubated for 5 minutes at room temperature with DAPI to stain nuclei (1:10000 in PBS).

26 Images were acquired using a Leica TCS SP8 confocal microscope (Leica, Germany) equipped with a 63 × /1.4 numerical aperture oil-immersion objective or with a ZEISS Axio Imager with ApoTome 2 (ZEISS) equipped with a 10 x / 0.3 numerical aperture and analyzed with ImageJ.

27 To normalize immune cell quantifications (CD4+, CD68+), areas of approximately 0.1 mm2 of epithelial-covered villus mucosa from 6-8 fields for each mouse were examined by an unbiased observer. The number of positive cells per villi section per mm was manually counted in at least 10 high power fields/sections. For HuC/D+ cell counts an unbiased investigator manually counted the number of cells in at least 8 random pictures across the entire large intestine from 3 distinct slides per animal.

28 The mean fluorescence intensity (MFI) of the aggregated a-synuclein specific signal was calculated using ImageJ software from data collected from 8-10 pictures taken with a 10x objective covering the entire gut section. A threshold was set where only the aggregate-specific signals were visible. The same threshold was applied to all images. The signals were normalized to the total surface area of each slide detected using the DAPI staining

29 Gut tissue sections were mounted onto slides and treated for 15 minutes at room temperature using PBS containing 10µg/ml proteinase-K (Promega, V302A).

30 The slides were then rinsed three times in PBS for ten minutes

31 Following the washes, the slides were blocked for 1 hour at room temperature in PBS with 0.3% Triton X- and 10% Normal Goat Serum and subsequently stained according to the immunofluorescence procedure described in the previous section.