ELSEVIER

Int. J. Devl Neuroscience 25 (2007) 299–307

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

www.elsevier.com/locate/ijdevneu

Generation and survival of midbrain dopaminergic neurons in *weaver* mice

Joaquín Martí [a,*], M.C. Santa-Cruz [a], Shirley A. Bayer [b], Bernardino Ghetti [c], José P. Hervás [a]

[a] *Unidad de Citología e Histología, Facultad de Ciencias, Universidad Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

[b] *Laboratory of Developmental Neurobiology, Indianapolis, IN 46278, USA*

[c] *Department of Pathology and Laboratory Medicine, Indiana School of Medicine, Indianapolis, IN 46202, USA*

Received 15 December 2006; received in revised form 2 May 2007; accepted 9 May 2007

Abstract

Generation and survival of midbrain dopaminergic (DA) neurons were investigated using tyrosine hydroxylase (TH) immunocytochemistry combined with tritiated thymidine autoradiography at appropriate anatomical levels throughout the anteroposterior (A/P) axes of the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). The wild-type (+/+) and homozygous *weaver* (*wv*/*wv*) mice used here were the offspring of pregnant dams injected with the radioactive precursor when the mesencephalic neurons were being produced (gestational days 11–15). Data reveal that, at postnatal day 90, depletion of TH-stained cells in the *wv*/*wv* presented an A/P pattern of increasing severity and, therefore, the DA cells located in posterior parts of the SNc or the VTA appear to be more vulnerable than the settled anterior neurons. When the time of neuron origin is inferred for each level of these cell groups, it is found that the neurogenesis span is similar for both experimental groups, although significant deficits in the frequency of *wv*/*wv* late-generated neurons were observed in any level considered. On the other hand, it has been found that TH-positive neurons were settled along the extent of the SNc and the VTA following precise and differential neurogenetic gradients. Thus, the acute rostrocaudal increase in the proportion of late-generated neurons detected in both +/+ DA-cell groups is disturbed in the *weaver* homozygotes due to the indicated A/P depletion.

Keywords: *Weaver* mice; Midbrain; Dopaminergic neurons; Neurogenesis; Autoradiography; Immunocytochemistry

1. Introduction

Weaver is a spontaneous mutation identified as a single base-pair substitution in a gene coding for a G-protein-activated inward rectifying potassium channel, *Girk 2* (Patil et al., 1995). At the level of the central nervous system, homozygous *weaver* mice (*wv*/*wv*) present structural anomalies in the olfactory bulb (Schein et al., 1998) and hippocampus (Sekigucchi et al., 1995), as well as neuronal loss in the pontine nuclei (Ozaki et al., 2002) and cerebellum (Rakic and Sidman, 1973; Smeyne and Goldowitz, 1989; Maricich et al., 1997); the latter following a lateral to medial gradient of increasing severity (Herrup and Trenkner, 1987; Eisenman et al., 1998; Martí et al., 2001).

In the *wv*/*wv* midbrain, the stage of neuronal vulnerability to gene mutation progresses with age, depending on the analyzed region. Depletion starts after postnatal (P) day 8, it is advanced by P16 (Verney et al., 1995) and at P20 a major wave of substantia nigra pars compacta (SNc) degeneration occurs (Triarhou et al., 1988; Bayer et al., 1995). In the ventral tegmental area (VTA), on the other hand, cell deficit is evident at P90 (Triarhou et al., 1988). The lack of dopaminergic (DA) cells in these *weaver* midbrain populations is accompanied by abnormally low dopamine content in the neostriatum (Schmidt et al., 1982), whereas, it is unchanged in the nucleus accumbens (Roffler-Tarlov and Graybiel, 1987).

Deficit of midbrain DA cells is an important feature of Parkinson's disease (PD) in which neurodegeneration is variable among the different DA-cell groups. Data obtained from postmortem studies consistently revealed that the SNc is the mesencephalic region most severely affected in the disorder (Agid et al., 1993; Burke, 1999). In addition, it has been reported that cell depletion varies significantly along the rostrocaudal extent of this motor nucleus (Damier et al., 1999).

Abbreviations: [^{3}H]TdR, tritiated thymidine; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VTA, ventral tegmental area

* Corresponding author. Tel.: +34 935811666; fax: +34 935813357.
E-mail address: joaquim.marti.clua@uab.es (J. Martí).

0736-5748/$30.00
doi:10.1016/j.ijdevneu.2007.05.002

Our objective in this report is to determine whether a similar pattern of vulnerability also occurs in the SNc of the *weaver* homozygotes. In this regard, the number of SNc DA cells is compared between wild type (+/+) and *wv*/*wv* to establish whether the neurons' viability in the *weaver* group varies along the anterior to posterior (A/P) axis of the SNc. To infer the neurogenetic timetables of SNc neurons within selected anatomical levels, we have used a combination of tritiated thymidine ([^{3}H]TdR) injections at the embryonic period with the corresponding autoradiography and the immunocytochemical stain for tyrosine hydroxylase (TH) at 90 days of postnatal life. Since VTA DA cells are also affected in Parkinsonian brains (Uhl et al., 1985; Hirsch et al., 1988; German et al., 1989) a similar analysis was performed in this region of the *wv*/*wv* group. Thus, neurogenetic gradients in both SNc and VTA reveal that survival of *weaver* DA neurons is closely linked to the proper age of such neurons, irrespective of the A/P level analyzed.

2. Materials and methods

2.1. Animals

All mice used in this study were obtained from the colony of control (+/+) and *weaver* (*wv*/*wv*) mice at Indiana University School of Medicine maintained on the B6CBA-A$^{w\text{-}j}$/A hybrid stock. The parents of the +/+ and *wv*/*wv* offspring used in the present research were either carrier *wv*/+ or *wv*/*wv* females mated to *wv*/+ males. No *wv*/+ were collected. During experimental procedures, dams and litters were maintained in a quiet room under controlled conditions (12 h light/dark cycle, 22 ± 2 °C food and water *ad libitum*). All procedures were approved by the animal care and use Committee of the Indiana University School of Medicine.

2.2. Experimental design

Since the birth of midbrain dopaminergic neurons is an event occurring in prenatal life, pregnant females were injected subcutaneously on two successive days in an overlapping series with [^{3}H]TdR (5 μCi/g of body weight, New England Nuclear #NET-027) according to the following time windows: embryonic day (E)11–12, E12–13, E13–14 and E14–15. Injections were always delivered between 8 and 9 a.m. After administration, the dams gave birth normally and pups were weaned at P20; male and females were housed separately. Eight to nine animals were used in each experimental group (+/+ and *wv*/*wv*).

2.3. Tissue processing

At P90, pups were deeply anesthetized with sodium pentobarbital (50 mg/kg of body weight) and perfused through the heart with 10% neutral buffered formalin. Brains were rapidly removed, dissected and placed in the same fixative overnight. The block containing the midbrain was dehydrated, paraffin-embedded and sectioned serially at 10 μm in the coronal plane. Care was taken to maintain the encephalon axis parallel to block side in order to obtain the best anatomically matched sections. Only one of every fifth section was placed on microscopic slides previously coated with poly-(L-lysine). The *wv*/*wv* were identified, taking their motor abnormalities into account, and were confirmed *post hoc* by microscopic examination of the cerebellum, which is smaller and has disorganized cytoarchitecture in consequence of the important depletion of several neuron populations.

2.4. Tyrosine hydroxylase immunocytochemistry and tritiated thymidine autoradiography

In order to identify the dopamine neurons of the SNc and the VTA as well as to infer their developmental timetables, we have sequentially combined TH immunocytochemistry – to define the dopaminergic phenotype – with [^{3}H]TdR autoradiography for determining the post-labeling progeny of S-phase neuroblasts. Procedures were carried out following a previously described protocol (Martí et al., 2002). Basically, this consisted of incubated sections with rabbit anti-TH antiserum (Eugene Tech International, Allendale, NJ) 1:1600 in TBS. After the first antibody incubation, a second with goat anti-rabbit IgG (Amershan) 1:20 was done followed by a further incubation with rabbit PAP complex. Finally, sections were reacted with 3,3′-diaminobenzidine-H_2O_2. Control sections were incubated with normal rabbit serum. After immunocytochemistry was finished, [^{3}H]TdR autoradiography was performed in the same sections. These slides, coated with liquid photographic emulsion, were stored in light-tight boxes at 4 °C for an exposure time of 12 weeks. The autoradiograms were developed in Kodak D-19 and were then lightly post-stained with hematoxylin, dehydrated and cover-slipped with Permount. [^{3}H]TdR-labeled neurons, including TH-positive neurons, are identified by the cluster of reduced silver grains over their nuclei (Fig. 1).

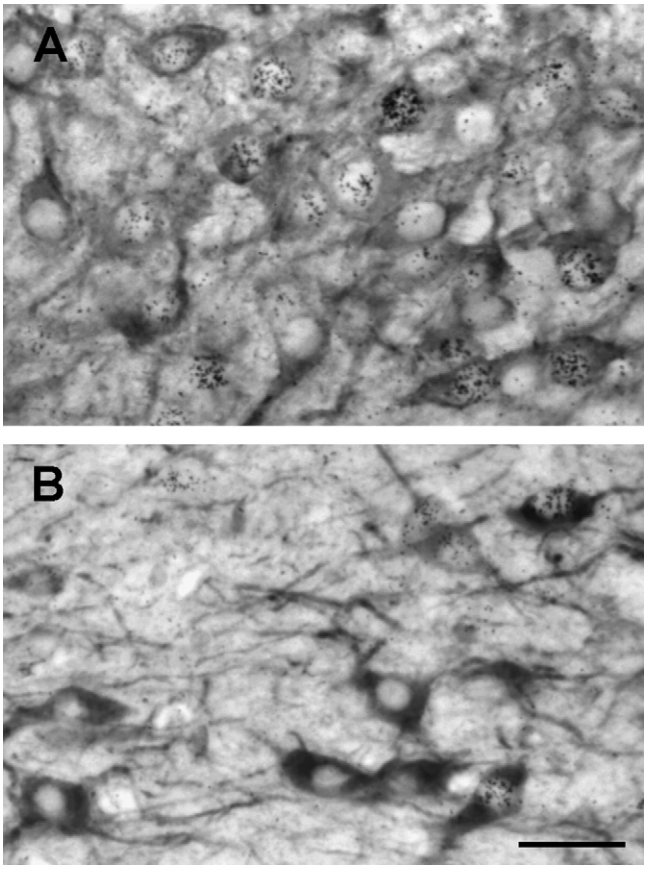

Fig. 1. Comparative autoradiograms of TH-immunostained sections through the midbrain of wild type (A) and mutant *weaver* (B) mice collected at P90. Both fields correspond to the substantia nigra pars compacta. Note the relative decrease in cell density in the *weaver* image as well as a less dense neuropil. Scale bar: 30 μm.

2.5. Quantitative analyses

Two +/+ and *wv*/*wv* midbrain regions were separately studied in the present paper, the SNc and the VTA. Labeled and unlabeled TH-positive neurons with a recognizable nucleus were recorded at four coronal anatomical levels (L1 to L4) along the A/P extent of both DA-cell groups. Fig. 2 shows the chosen levels, which are based on the levels L1, L2, L3 and L4 from the atlas of Sidman et al. (1971). Despite differences between *weaver* and control DA-cell groups, midbrain coronal sections were matched as closely as possible. Both sides of the brain were used for analyses. Cell