ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2009, 69: 198–206

Regional differences in the vulnerability of substantia nigra dopaminergic neurons in *weaver* mice

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Vulnerability of midbrain dopaminergic (DA) neurons in the *weaver* mouse was studied at postnatal (P) days 8 and 90, in chosen coronal levels throughout the anteroposterior (AP) extent of the substantia nigra pars compacta (SNc). Wild-type (+/+) and homozygous *weaver* (*wv/wv*) mice used were the offspring of pregnant dams injected in several cases with tritiated thymidine on embryonic days 11–15. DA neurons were identified for their tyrosine hydroxylase immunoreactivity. Data reveal that at P8, the frequency of both +/+ and *wv/wv* late-generated DA cells increases from rostral to caudal SNc. No apparent DA-cell loss was observed at P8 in the mutant genotype, irrespective of the AP level considered. However, throughout the AP, there was a significant reduction in the number of these neurons at any level in 90-day-old *weavers*. Comparison of P8 and P90 +/+ SNc suggests that cell death is not a major aspect in the developmental regulation of normal DA neurons, although numerical cell depletion in the postnatal development of *weaver* SNc probably results from the amplification of a basal cell-death process, which affected all the coronal levels studied.

Key words: homozygous *weaver* mice, substantia nigra pars compacta, tyrosine hydroxylase, tritiated thymidine, autoradiography, dopaminergic neurons

INTRODUCTION

During development of the Central Nervous System, many neuronal populations are produced in high numbers throughout the generative period. Supernumerary neurons subsequently undergo a naturally occurring death that determines the final cell number in adults. This regressive event serves as a junction for critical developmental processes (Sastry and Rao 2000). The midbrain dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc) may be subjected to this sculpting process. In rats, the event seems to begin in embryonic times and continues into postnatal life following a biphasic fashion (Janec and Burke 1993, Burke 2004). In mice, data are contradictory: it has been reported that midbrain DA neurons do not undergo programmed cell death (Lieb et al. 1996, Blum 1998), but results from Jackson-Lewis and colleagues (2000) provide evidence that apoptosis occurs in the SNc.

The *weaver* mutant mouse has been proposed as an animal model for Parkinson's disease (PD) because it represents a natural and non-invasive example of dopamine deficiency in the brain. *Weaver* cellular disorders bear many similarities to those described for PD patients. In both cases, there is a close correspondence between dopamine loss and DA fiber degeneration (Kish et al. 1988, Graybiel et al. 1990). Neurodegeneration of SNc in PD is progressive with age (Fearnley and Lees 1991, Damier et al. 1999), and neu-

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Received 24 July 2008, accepted 16 March 2009

ronal loss in the *weaver* condition is already detected at postnatal (P) day 14 (Verney et al. 1995), increases by P20 (Bayer et al. 1995), while by P90 surviving DA cells only represent a little over one-third of the control cell values (Martí et al. 2000).

Previous tritiated thymidine ([^{3}H]TdR) autoradiographic studies have shown that, in both adult normal mice (Bayer et al. 1995) and in *weaver* homozygotes (Martí et al. 2006), SNc DA neurons are generated according to precise timetables. In this paper, we determine the neurogenetic timetables for wild-type (+/+) and *weaver* (*wv/wv*) DA neurons, within four selected coronal anatomically matched levels along the rostrocaudal extent of the SNc in early postnatal life (P8). Moreover, some analyses were undertaken at two different ages (P8 and P90) in order to answer several questions. Are there spatial differences in DA-cell loss within the *wv/wv* SNc? If so, can they be linked to the anteroposterior (AP) arrangement of DA neurons? As cell death may also occur in normal postnatal development, will the presumed lack of +/+ DA cells be concentrated in any particular coronal level?

METHODS

Animals and experimental design

All experiments were carried out in accordance with the requirements of the Committee for Institutional Animal Care and Use. Animals were obtained from the colony of control (+/+) and homozygous *weaver* (*wv/wv*) mice at Indiana University School of Medicine, maintained on the B6CBA-A$^{w\text{-}j}$/A hybrid stock. Pregnant dams (*wv*/+ or *wv/wv*), previously mated with *wv*/+ males, 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 E11–12, E12–13, E13–14 and E14–15. E1 is the morning after mating. Injections were always delivered between 8 AM and 9 AM. After administration, the dams gave birth normally and pups were weaned at P20; males and females were housed separately. During experimental procedures, dams and litters were maintained in a quiet room under controlled conditions (12 hour light/dark cycle, 22 ± 2ºC food and water *ad libitum*). Four animals were used for the experimental group (+/+ and *wv/wv*) and data time point.

Tissue processing

At P8 and P90, pups were perfused transcardially with 10% neutral buffered formalin. Brains were removed, dissected and placed in the same fixative overnight. The blocks containing the midbrain were dehydrated, paraffin-embedded and sectioned serially at 10 μm in the coronal plane and mounted on polylysine-coated slide. As, in this study, the medium diameter observed for DA neuronal nucleus was approximately 11 μm, only the first section of every three was used and thus, sections were separated by 20 μm.

The cerebellum was sectioned and microscopically examined to confirm the *wv/wv* phenotype.

Tyrosine hydroxylase immunohistochemistry and [^{3}H] thymidine autoradiography

Tyrosine hydroxylase (TH) immunocytochemistry (to detect DA neurons) was applied to brain sections from animals killed at P8 and P90, following a previously described procedure (Martí et al. 2002). Basically, this consisted of incubated sections with rabbit anti-TH antiserum (Eugene Tech International, Allendale, New Jersey, USA) 1:1 600 in TBS. After the first antibody incubation, a second one with a goat anti-rabbit IgG was carried out (Amersham, UK) at 1:20, followed by a further incubation with rabbit PAP complex. Finally, sections were reacted with 3,3'-diaminobenzidine-H_2O_2.

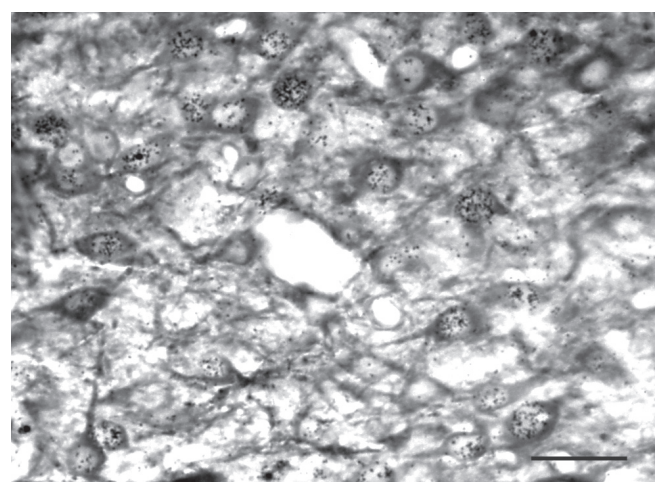

Fig. 1. High-magnification autoradiogram of a TH-immunostained section through the midbrain of a *weaver* mouse that was injected with [^{3}H]TdR at E12-13 and sacrificed at P8. This field corresponds to the substantia nigra pars compacta. Scale bar is 30 μm.

Control sections were prepared by replacing the primary antibody with tris-buffered saline. TH-reactive neurons were defined as those displaying a brown reaction product in their cytoplasms.

Only in those animals sacrificed at P8, when immunocytochemistry had been completed, was [^{3}H]TdR autoradiography undertaken in the same sections using the corresponding protocol (Martí et al. 2002). Briefly, slides, coated with liquid photographic emulsion, were stored in light-tight boxes at 4°C for an exposure time of 12 weeks. Subsequently, they were lightly post-stained with haematoxylin, dehydrated and cover-slipped with Permount. All slices were simultaneously processed to avoid variations between groups in different batches. [^{3}H]TdR-labeled neurons, including TH-positive neurons, were identified by the cluster of reduced silver grains over their nuclei (Fig. 1).

Quantitative analyses

[^{3}H]TdR-labeled and unlabeled TH-positive neurons with recognizable nuclei and cytoplasms were separately scored at four AP coronal levels (L1 to L4) along +/+ and *wv/wv* SNc. Fig. 2 shows the chosen anatomical levels, which are based on plates 46, 50, 54 and 60 from the atlas of Sidman and coauthors (1971). Only three consecutive sections per level and experimental mouse, collected as indicated above, were used for each data time point. Data from a complete series of sections were added in order to obtain a mean per animal/level/time point. With this information, means and SEM were calculated for each experimental group. Special consideration was given to accuracy when comparing the different anatomical levels of control and *weaver* mice. Each +/+ and *wv/wv* level was only considered to be matched if some anatomical landmarks were identified (Fig. 2). Hence, the SNc was assigned to L1, where this nucleus lies anterior to the ventral tegmental area (VTA). L2 was designated by the VTA, whereas L3 and L4 were recognized not only by the presence of VTA, but also by the interfascicular nucleus. Interpeduncular and pontine nuclei were only detected in L4.

All counts were carried out at 400× using a Leitz microscope with a 10 × 10 reticule in one of the eyepieces. The frequency of [^{3}H]TdR-labeled neurons was calculated as a percentage, by dividing the number of TH neurons labeled with the total number of TH neurons scored (Fig. 3). Neurogenetic timetables were

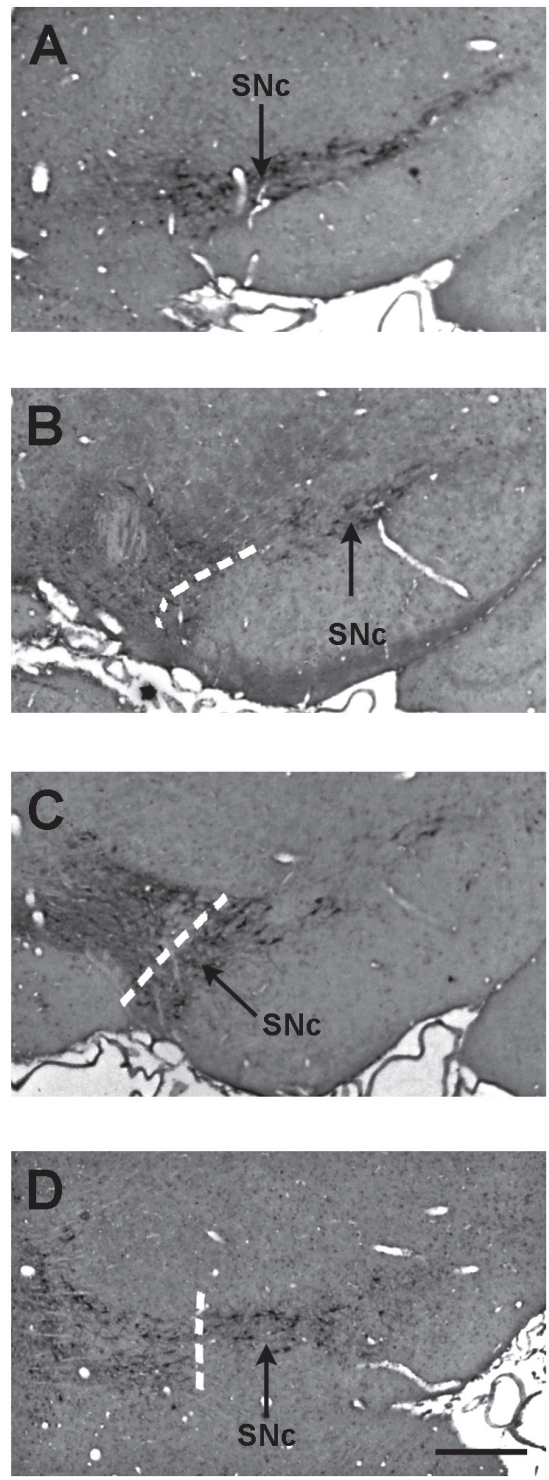

Fig. 2. Representative low-magnification view of four coronal sections used for cell counting in the SNc from *weaver* mice that survived until P90. Sections (A) to (D) correspond to L1–L4 anatomical levels and they show the right side of the ventral midbrain. As the SNc and ventral tegmental area do not have well-defined borders, white broken lines delimit the region studied. Scale bar is 300 μm.

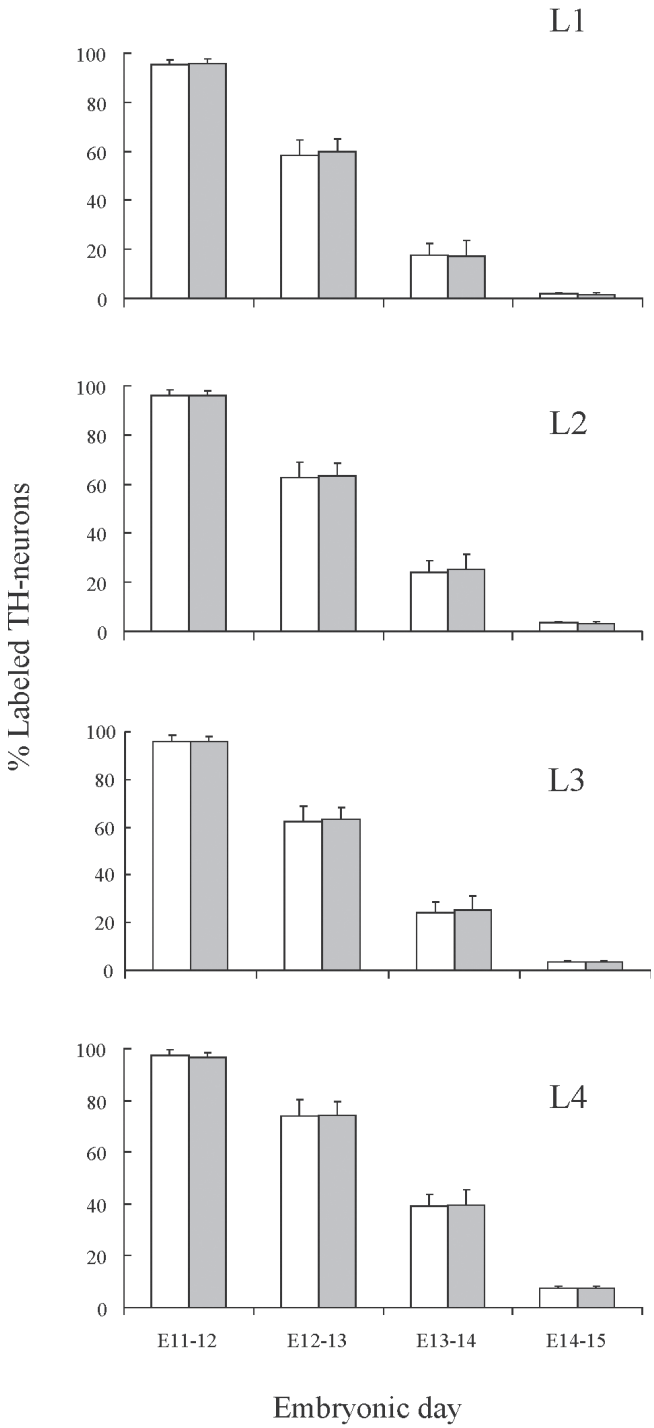

Fig. 3. Comparison of [^{3}H]TdR-labeling patterns between wild-type and *weaver* DA cells along the AP axis of the SNc: L1 to L4 are the anatomical levels considered. Frequency histograms of TH neurons labeled with [^{3}H]TdR on two successive days of the embryonic period (abscissa) and survival until P8. Empty and shared columns represent +/+ and *wv/wv*, respectively; percentages are expressed as mean ± SEM.

inferred by subtracting the percentage of labeled neurons in a given embryonic time-window from the percentage of the immediately previous [^{3}H]TdR injection window, in order to obtain the fraction of neurons generated between the two injection series (Fig. 4). As pilot data from injections on E10–11 indicated that nearly all +/+ or *wv/wv* TH neurons are labeled with [^{3}H]TdR, no mice were tested during this time-window. A value of 100% was assumed for both genotypes. In contrast, at E15–16, the percentage of labeled TH-positive neurons was very low; again, no animals were injected at this time-window and a value of 0% was established for both +/+ and *wv/wv*.

Statistical analysis

Differences in the neurogenetic timetables of TH cells between +/+ and *wv/wv* at P8 were assessed using the Student's *t*-test (*t*-test) or the Mann-Whitney *U*-test (*U*-test), for each anatomical level studied. The Fisher-Snedecor test was used to determine the homogeneity of variances, whereas two-way ANOVA was used, with genotype (+/+ and *wv/wv*) and age (P8 and P90) as main variables, to analyze variations in the number of TH-positive neurons along the AP extent of the SNc. Either the *t*-test or the *U*-test were used to determine any further statistical significance for inter-genotype or inter-age differences. A *P* value of less than 0.05 was considered to be statistically significant.

Photographic material

Pictures presented in this study were digitally captured by a CCD-IRIS color video camera (Sony, Japan) coupled to the microscope. The digitized images were processed with Adobe Photoshop software.

RESULTS

Developmental timetables of DA neurons (TH-immunoreactive neurons) at P8 were inferred at the four, previously indicated, anatomical levels through the AP axis of the SNc. Initially, the frequency of tagged DA neurons was estimated by sequential [^{3}H]TdR-labeling embryonic windows in each coronal level, as graphically displayed in Fig. 3. Statistical analyses of the results (*t*-test or *U*-test) showed that at any given time within this generation period, and both for the wild-type and

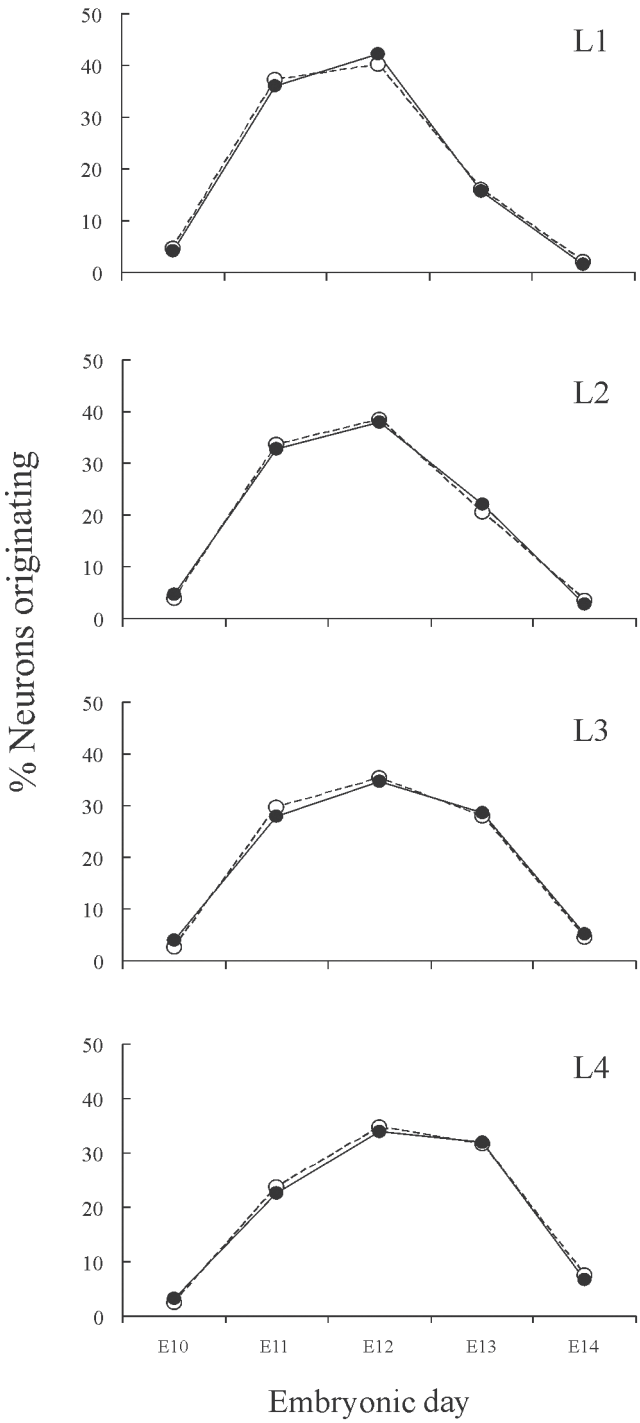

Fig. 4. Generative profiles at P8 for wild-type and *weaver* DA neurons within each SNc coronal level examined: L1 to L4. The inferred frequencies (ordinate) of newly generated DA neurons for +/+ (open circles and broken line) and *wv/wv* (closed circles and solid line) are plotted against the relative embryonic time-period (abscissa). Percentages of newborn neurons were determined by the rate of decline in [^{3}H]TdR-labeled cells at that time point.

mutant genotype, the percentage of labeled DA cells remains constant within each level studied. In a subsequent analysis, profiles were constructed of DA-cell origin. In Fig. 4, the frequencies of newly generated DA neurons at each selected level are plotted against embryonic time. Data analyses indicated that the birth sequences of postmitotic neurons for both genotypes (+/+ and *wv/wv*) within each SNc level are closely similar.

An important consideration in SNc development is to ascertain if the P8 location of TH-reactive neurons at the selected levels has any relationship with the time of cell origin. Existing examination timetables allowed us to differentiate between neurons born from E10 to E11 and those originating from E13 onwards, early- and late-generated DA neurons respectively. In Table I, the proportions of both population fractions are compared for each anatomical level and for normal and mutant SNc. As in the preceding section, similar data were obtained for both experimental groups. Interestingly, a trend became apparent in every genotype, namely that, the proportion of early-formed neurons declined from the anterior to the posterior anatomical levels, with a consequent increase in late-generated neurons.

To assess any possible differential survival of *weaver* DA neurons settled in distinct AP levels of the SNc, counts of these cells per section were compared between P8 and adulthood (P90). Figure 5 shows the number of TH-reactive neurons in each

Table I

Frequency of generated DA neurons*

Levels	Older (E10–E11)		Younger (E13–E14)	
	+/+	*wv/wv*	+/+	*wv/wv*
L1	41.9	40.3	17.9	17.4
L2	37.5	36.8	24.1	25.3
L3	32.2	31.8	32.4	33.5
L4	26.2	25.9	39.1	39.5

*Percentage of early and late-generated TH-reactive cells scored in each P8 SNc level. DA neurons formed from E10 to E11 are considered early formed, whereas those produced from E13 to E14 are late generated. Values were obtained from Fig. 4.

Table II

Two-way ANOVA results of dopaminergic neurons counts in the substantia nigra pars compacta

Factor	Level 1	Level 2	Level 3	Level 4
Age (A)	$F_{1,28}$=5.7 (P<0.024)	$F_{1,28}$=37.3 (P<0.0001)	$F_{1,28}$=47.3 (P<0.0001)	$F_{1,28}$=19.1 (P<0.001)
Genotype (G)	$F_{1,28}$=6.0 (P<0.021)	$F_{1,28}$=33.3 (P<0.0001)	$F_{1,28}$=47.0 (P<0.0001)	$F_{1,28}$=19.7 (P<0.001)
A × G	$F_{1,28}$=5.8 (P<0.023)	$F_{1,28}$=36.1 (P<0.0001)	$F_{1,28}$=50.9 (P<0.0001)	$F_{1,28}$=20.3 (P<0.001)

anatomically matched level of the +/+ and *wv/wv* SNc. A basic point emerges from these histograms: numerical depletion of DA neurons is only apparent at every P90 *wv/wv* level. Table II shows the results of the two-way ANOVA analysis of the selected levels, which demonstrated the significant effects of genotype and age at each SNc level, while the interaction genotype by age was also shown to be significant. Post hoc comparisons of means (Fig. 5) revealed no differences between P8 and P90 control mice and, at P8, no loss of DA neurons was detectable at any *wv/wv* studied level. However, when the P90 *weavers* were considered, there was a statistically significant decrease in DA-cell numbers within each level, with clear differences throughout the AP axis. Surviving DA neurons in L1 represent 42.8% of the control group (t_{14}=11.73, P<0.001), a percentage that decreases in L2 and L3 (38.9%; t_{14}=8.48, P<0.001 and 34.5%; t_{14}=8.54, P<0.001, respectively) and reaches the lowest level in L4 (23.3%; t_{14}=20.19, P<0.001). In other words, the lethal effect of *weaver* mutation on SNc DA neurons seems to follow an AP gradient of increasing severity.

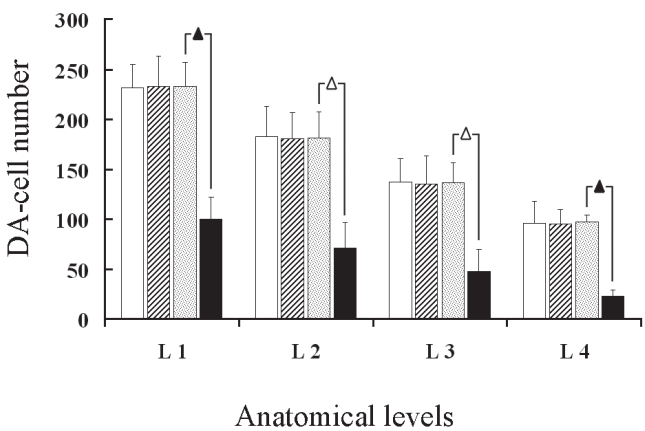

Fig. 5. Comparison of DA-cell number per section between wild-type and *weaver* SNc on two survival ages, P8 and P90. Cell counts were separately determined for each anatomically matched level, L1 to L4, plotted in the abscissa. Empty and dotted columns represent control mice at P8 and P90 respectively, whereas shaded and solid columns correspond to *weavers* at the same survival times as mentioned for the +/+. Solid symbols indicate a significant level of P<0.001. Values are expressed as mean ± SEM.

DISCUSSION

The current study demonstrates that, in P8 mice, +/+ and *wv/wv* DA cells are spatially distributed throughout the AP extent of the SNc following similar neurogenetic gradients. Therefore, in both genotypes, the frequency of late-generated TH neurons always increases from the most rostral (L1) to the most caudal (L4) coronal level considered, which in turn has the lowest proportion of early-formed TH cells. This neuronal age-distribution may arise from an AP sequence of postmitotic differentiation along the neuroepithelial domains (Smits et al. 2006). In any event, it seems likely that the *weaver* mutation does not interfere with the proliferative behavior of SNc DA-neuron precursors or with some early steps of the neuronal development, such as migration and settling throughout this mesencephalic region.

Our results also illustrate a substantial loss of DA cells between P8 and P90 *wv/wv* SNc at every selected level. This represents an extension of other studies in which cell counts were undertaken along the mediolateral axis of the developing nigral complex (Roffler-Tarlov et al. 1996), or in the largest coronal area of the SNc (Martí et al. 2006). Indeed, in a long-survival [^{3}H] TdR autoradiographic study (Martí et al. 2000), which considered the SNc as a whole, it was shown that late-generated DA neurons were preferentially

targeted by the *weaver* gene. Our experiments partly retrace this previous work. However, they are justified by their greater topographical sensitivity, as four consecutive anatomically matched levels (L1–L4) along the AP axis of the SNc are separately examined, constituting a numerical approach to the survival variability of DA neurons within this midbrain motor nucleus. This leads on to the second statement of this study: DA neurons at the posterior parts of the SNc seem to be more susceptible to the lethal effect of *weaver* mutation than those located at the anterior ones.

Evidence of patterns of AP cell vulnerability within the *weaver* SNc adds further resemblance to those described in idiopathic PD. Indeed, the mature DA neurons located rostrally in the human SNc are more resistant to cell death than those located caudally (Matzuk and Saper 1985, Damier et al. 1999). However, the *weaver* condition presents a generational feature: the greatest cell loss occurs in those AP anatomical levels that contain the highest proportion of late-generated DA neurons.

It is likely that the neurodegenerative process of *weaver* SNc stems from a variety of causes, including some that might depend on defaults in the availability of trophic factors during the synaptic period in the growing striatum (Kholodilov et al. 2004, Oo et al. 2005, Peng et al. 2006). In this context, it is well documented that glial cell line-derived neurotrophic factors (GDNFs) have potent survival ability on DA neurons in animal models of PD (Kordower et al. 2000, Grondin et al. 2002), as well as in *weaver* and human clinical trials (Broome et al. 1999, Love et al. 2005). The GDNFs bind to specific GDNF family α receptors, all of which form receptor complexes and signal through the receptor tyrosine kinase (RET) (Manié et al. 2001, Airaksinen and Saarma 2002). The subunit RET is another candidate as a potential cause of the neurodegenerative process, because it is linked to the survival of nigral cells, which is required for postnatal development of DA neurons and for their striatal target innervation (Sariola and Saarma 2003, Mijatovic et al. 2007). Most of the mature nigrostriatal projecting neurons express RET (He et al. 2008) and this protein persists in surviving DA neurons of substantia nigra in PD (Walker et al. 1998). Nevertheless, we still do not know how much of the decrease in DA-cell number in *weaver* SNc can be attributed to GDNF alterations, for example, in local concentration of such ligands, or if the decrease results from the dysfunction of GDNF receptors in specific sets of neurons.

Finally, our study also shows that in wild-type mice, between P8 and P90, there was no apparent loss of SNc DA cells at any selected AP level. This finding appears to differ from previous studies reporting cell death in rodent postnatal development (see Introduction). One possible explanation is that new DA cells would be added in adulthood, although the potential of neurogenesis in the adult SNc is also a matter of debate (Borta and Höglinger 2007). Alternatively, it is worth stressing that TH expression does not reach optimal immunoreactivity until the 4th week of age (Coyle 1977). Therefore, at P8, several neurons might not be categorized as dopaminergic, due to their weak TH sensitivity, and consequently cell counts could be underestimated. Subsequently, the increasing number of newly identified TH cells could mask any numerical depletion of DA neurons that should be noticeable by P90.

From the observations outlined above, we can hypothesize that DA-cell loss during the postnatal development of *weaver* SNc could be due to the inappropriate amplification of a basal physiological cell-death process. The *weaver*-induced decline in either limiting neurotrophic factors or their neuronal receptors, following a sort of AP spatial course, could trigger an intrinsic genetic program, balanced by the expression of pro- and anti-apoptotic genes (Merry and Korsmeyer 1997, Kuan et al. 2000, Oppenheim and Johnson 2003), which would lead to the death of some DA cells.

CONCLUSIONS

Working with four coronal anatomically matched levels along the AP extent of the mouse SNc, we have demonstrated that *weaver* mutation does not affect the proliferative behavior of DA-neuron precursors at every anatomical level studied.

Although the mechanism underlying neuronal degeneration between P8 and P90 *weaver* SNc is still not resolved, what has become evident is that cell loss increases following an AP gradient. We suggest that *weaver*-induced cellular demise could arise from the amplification of a naturally occurring process of developmental cell death.

ACKNOWLEDGEMENT

This research was partially supported by NIH (NS27613).

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