Lifespan of neurons is uncoupled from organismal lifespan

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Neurons in mammals do not undergo replicative aging, and, in absence of pathologic conditions, their lifespan is limited only by the maximum lifespan of the organism. Whether neuronal lifespan is determined by the strain-specific lifetime or can be extended beyond this limit is unknown. Here, we transplanted embryonic mouse cerebellar precursors into the developing brain of the longer-living Wistar rats. The donor cells integrated into the rat cerebellum developing into mature neurons while retaining mouse-specific morphometric traits. In their new environment, the grafted mouse neurons did not die at or before the maximum lifespan of their strain of origin but survived as long as 36 mo, doubling the average lifespan of the donor mice. Thus, the lifespan of neurons is not limited by the maximum lifespan of the donor organism, but continues when transplanted in a longer-living host.

Results

An outline of the experiment is presented in Fig. 1.

Recipient Rats Survived as Long as Two Times the Average Survival of Donor Mice. With the exception of animals electively perfused within 18 mo of life, the remaining graft recipient rats (n = 59; 86%) were let survive until moribund and unlikely to outlive longer than 48 h. Maximum survival of live-born recipient rats was 36 mo, whereas the maximum survival of siblings of parents of donor mice embryos was 26 mo. Median survival for recipient rats that were not intentionally killed was 30 mo (minimum, 20 mo; maximum, 36 mo; n = 59 rats), whereas median survival of siblings of the parents of donor embryos was 18 mo (minimum, 6 mo; maximum, 26 mo; n = 41 mice). Survival curves of those mice and the recipient rats (Fig. 2 B and E–G) and glia (Fig. 3 E) were analyzed by the Kaplan–Meier procedure with log-rank tests and found to differ significantly (P < 0.0001). Maximum lifespan of host rats and mice was also found to differ significantly (P = 0.018) by using the Fisher exact test applied to the proportion of animals alive in each group when 90% of the pooled populations had died (11).

Grafted Cells Developed into Cerebellar Neurons and Integrated into Host. Among recipient rats, 20 had EGFP-positive cells in the CNS, and 12 survived longer than 18 mo: the median survival of donor mice. Frequency, distribution and phenotypes of donor cells in the host brain (Figs. 2 and 3) were consistent with previous observations at shorter survival times (12, 13). Integration of transplanted cells was not limited to the cerebellum; graft-derived cells were also found in the whole brainstem and, rarely, in the hemispheres. Ectopic and heterotopic locations of the transplanted cells are typical of the fetal grafting procedure and well documented at shorter survival times (12, 13). At all ages, EGFP-positive cells ectopically and orthotopically displayed typical features of different categories of mature cerebellar neurons (Figs. 2 B, C, and E–G) and glia (Fig. 3 E). When integrated orthotopically in the host cerebella, the donor cells always occupied characteristic positions and established morphologically appropriate relationships within the host tissue. At all ages, mouse PCs engrafted in the rat cerebellum showed the typical laminar position, polarity, and orientation of their rat counterparts (Fig. 2 B and E–G). This was true even for granule cells that, after grafting, proliferated extensively, formed parallel fibers in the molecular layer, and settled in the internal granular layer (Figs. 2 B and 3 A).

Grafted PC Number Does Not Decrease with Aging. As we were studying if maximum survival of grafted mouse neurons corresponded to that of the donor mice or increased according to the host one, we focused our quantitative analyses on 13 rat brains containing multiple EGFP-positive PCs in the cerebellum. Under normal conditions, PCs would die with aging in a higher proportion (~40%) in mice (5, 14) than in rats (~11%) (15). Excluding from computations granule cells that proliferate after transplantation (12), the median number of EGFP-positive neurons

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The area of the soma of mouse PCs was, on average, 245.56 μm² (±78.19; n = 160; Fig. 3D) whereas that of host rat cells was 354.59 (±74.16; n = 308; Figs. 2D and 4C). The span of the dendritic arbor of grafted mouse PCs measured perpendicularly to the cell body was 87.82 μm (±42.98; n = 193; Fig. 2E), whereas dendrites of the surrounding rat PCs reached 178.76 μm (±45.95; n = 308; Figs. 2E and 4D). When located in extracerebellar sites, EGFP-positive cells maintained cerebellar-specific traits that allowed their reliable identification. Ectopic PCs (Fig. 2C) also retained mouse morphometric traits, with an average soma area of 225.41 μm² (±66.46; n = 198) and dendritic span of 60.88 μm (±26.42; n = 213). Means and medians of areas and dendritic span of grafted mouse PCs were significantly different (P < 0.0001, unpaired t test and Mann–Whitney test) from those of the rat hosts, whereas the dimensions of grafted mouse PCs were similar to those reported for PCs in B6 mice, one of the parental strains of our mice (16). To investigate if the different dimensions reflected species-specific features or were induced by xenotransplantation, we grafted mouse and rat cerebellar precursors into conspecific embryos in utero. One month after grafting, the average soma area and the average dendritic span of transplanted rat PCs were 346.64 μm² (±89.36; n = 22) and 162.07 μm (±35.64; n = 26), respectively, values not significantly different from the respective values of 321.89 μm² (±56.52; n = 43) and 160.41 μm (±15.98; n = 27) of the host rat PCs. Contrary to grafted mouse PCs (Fig. 4B), the dendritic arbor of grafted rat PCs reached the top of the molecular layer (Fig. 4A) and was morphometrically undistinguishable from those of the surrounding host PC. Similarly, 1 mo after transplantation of E12 mouse PCs into E15 Friend leukemia Virus 1B (FVB) mouse embryos, the area of their soma and their dendritic span were 257.38 μm² (±34.20; n = 11) and 102.48 μm (±38.05; n = 10), respectively, values significantly smaller than those of the rat cells reported earlier (unpaired t test and Mann–Whitney test, P < 0.0003) but not significantly different from those of the surrounding mouse PCs.

**Grafted PCs Lose Dendritic Spines with Aging.** Although the number of surviving PCs did not change with aging, we found a significant decrease in spine density on their dendrites, as previously described in aging rats (17). The median number of spines every 5 μm of dendritic length of the grafted PCs decreased from 14 (range, 10–20; n = 134 samples from six different PCs) at 1 mo to 9 (range, 5–15; n = 269 samples from nine different PCs) at 18 mo and down to 6 (range, 4–11; n = 233 samples from 13 different PCs) in rats surviving 36 mo (Fig. 4E and F).

**Discussion**

Our results suggest that mouse cerebellar neural and glial precursors, xenotransplanted into the rat CNS, integrate into the host tissue and differentiate, maintaining species-specific morphometric traits, but survive as long as the surrounding host rat neurons, doubling their expected average survival in the mouse. Alternative explanations that might posit the generation of stable mouse–rat heterokaryons after cell fusion as described for PCs after bone marrow grafting (18, 19), are unlikely because EGFP-labeled PCs maintained mouse morphometric traits, were uncultured (Fig. S1), and showed appropriate developmental stages in rats killed a few days after grafting (Fig. S2). Moreover, at all ages, we also found fluorescent PCs ectopically scattered along the brainstem. In normal rats, ectopic PCs are extremely rare and restricted to the dorsal cochlear nucleus (20, 21), whereas ectopic PCs are common after in utero transplantation of PC precursors (12), further indicating that the EGFP-labeled PCs did not result from fusion of grafted cells with host PCs. Direct transfer of EGFP to host PCs from microglial cells loaded with fluorescent cellular debris generated by the transplantation procedure, as described by Ackman et al. (22) in pyramidal neurons during...
acute experiments involving retroviruses, is also not an acceptable explanation for the lifelong presence of EGFP-containing PCs, as EGFP after transfer persists less than 2 wk (22). All the aforementioned considerations indicate that EGFP-containing PCs found at all ages in the transplanted rats are mouse PCs integrated into the host CNS. Contrary to the long-term results of xenotransplantation of quail neural primordia into chicken embryos, which developed, after a few months, an immunemediated demyelinating disease (23), we did not detect any sign of rejection of the grafted mouse cells. In our experiment, the absence of grafted cells outside the recipient CNS, where they would have induced an immune response (24), and species-specific differences in the immune system may explain the undisturbed survival of our cells. Aging in CNS is accompanied by reduced synaptic connectivity, but not by generalized neuronal loss (25). PCs are an exception, with a documented age-related loss in several mammalian species (5, 6, 15). PC loss may be as much as 40% in mice (5, 14) but is lower in rats (10%) (15) or is even absent in specific cerebellar lobules (26). In our transplants, the number of mouse PCs did not decrease in older rats, further suggesting that lifespan of the transplanted neurons was not dictated by their mouse genetic background, but by the rat microenvironment. In mice, age-dependent loss of PCs has been related to a decrease in concentration of circulating sex steroid hormones (27). However, the increased survival of mouse PCs after xenotransplantation is unlikely to depend on differences in sex steroid, as levels and oscillations of those hormones are similar in aging mice and rats (28, 29).

The maximum survival of transplant-bearing rats fed ad libitum and maintained under clean but not “pathogen-free” conditions doubled the mean lifespan of transplanted mouse neurons, increasing their maximum lifespan by 38%. Those increases are larger than the relative increases in organismal lifespan induced in mice by dietary (30), pharmacologic (31), and most genetic manipulations (32), even though, rarely, individual mice of other strains and lines may reach lifespans comparable to those of our longest-surviving rats (9, 33). Despite the increase in PC survival induced by xenotransplantation, the expected age-related loss of connectivity (25), as shown by the progressive decrease in the number of dendritic spines (17), did not cease. This further indicates that the transplanted mouse PCs are well integrated in the rat cerebellum and undergo the same changes induced by aging in their rat counterpart.

Fig. 2. Developing mouse cerebellar neurons transplanted in utero into the developing rat CNS integrate and survive to the rat host maximum lifespan, doubling the average lifespan of the neurons in the donor mice strain. (A) Kaplan–Meier plot showing lifespan of siblings of donor mice and rat host. Rats perfused electively were excluded from the survival curve. Each red dot indicates a transplanted rat in which we found mouse cells. (B) Parasagittal section from confocal microscopy of a 20-mo-old rat cerebellum. Fluoroesences are as follows: EGFP (green), anti-calbindin antibody (red), overlapping fluorescence (yellow), and DAPI nuclear staining (blue). Two grafted mouse PCs are integrated in the host PC and molecular layers, and granular cells of mouse origin are visible in the granular layer (asterisks). (Scale bar: 25 μm.) (C) Same as Fig. 1A, but coronal section of the brainstem and fourth ventricle of a 36-mo-old graft, in which six ectopic calbindin/EGFP-positive PCs are visible in the brainstem outside the dorsal cochlear nucleus. Interrupted line indicates the ventricle. (Scale bar: 50 μm.) (D) Plot of the number of PCs found in the transplanted animals at different ages (<19 mo, rats perfused before 19 mo of age; >18 mo, rats perfused after 18 mo to 36 mo of age). Each square represents a single animal: <19 mo, n = 6; >18 mo, n = 7. Differences in mean and median number of PCs in the two groups were not significant (P = 0.5968, unpaired Student t test; P = 0.5338, Mann–Whitney test). Horizontal line indicates the median; central box encloses values from the lower to upper quartile; vertical line extends from the minimum to the maximum value, excluding outside and far out values. (E–G) Parasagittal sections as in Fig. 1A but without DAPI. Rat cerebellum at 3, 20, and 36 mo of age showing mouse-derived PCs. The span of mouse PC dendrite did not reach the top of the host molecular layer and remained approximately the same at all survival times. (Scale bar: 25 μm.)
Our results suggest that neuronal survival and aging are co-
incidental but separable processes, thus increasing our hope that
extending organismal lifespan by dietary, behavioral, and phar-
macologic interventions will not necessarily result in a neuronally
depleted brain.

**Materials and Methods**

**Animals.** Animals were maintained and handled, and all experimental ma-
 nipulation performed, in agreement with the institutional guidelines of our
institutions, the University of Pavia and the University of Turin. Our in-
stitutional guidelines comply with the National Institutes of Health guidelines,
the requirements of the European Communities Council Directive (86/609/
EEC), and the Italian law for care and use of experimental animals (DL116/92).

The colony of fluorescent mice was generated in 1999 by crossing a female
TgN(beta-act-EGFP)01Obs (34) and a male B6.129S7-Gtrosa26 (35). Initially,
the offspring were individually checked for EGFP expression, and only the
siblings with fluorescence were crossed again. Selection of siblings and
 crossing was repeated at each generation until all siblings showed
fluorescence; thereafter, we maintained the colony by sibling crossing without
further need to select the offspring with fluorescence (10). During the entire
length of the experiment (2007–2011), siblings of the female mice crossed to
obtain the embryos dissected for harvesting cells for transplantation were
allowed to survive until death or euthanasia when moribund and unlikely to
survive for more than 48 h, and a survival curve was obtained. E14 transgenic
rat embryos overexpressing EGFP under the control of the human CMV
enhancer and β-actin promoter were obtained from a colony maintained in
our laboratory (founders were a gift from M. Okabe, Osaka University,
Osaka, Japan) (36). Time pregnant Wistar rats and FVB mice were obtained
from Harlan. Mice and rat colonies were maintained at 21 °C to 23 °C in four
of six mice or one of three rats per cage without microbiological barriers in
a clean animal house environment, with access restricted to authorized
personnel only. Mouse and rats colonies were routinely (twice per year)
monitored for common pathogens; cages and bedding were changed every
week for mice and two times per week for rats. Mice had ad libitum access to
water and standard complete pelleted diet (Harlan).

**Transplantation.** All surgical procedures were carried out under deep general
anesthesia obtained by i.p. injection of ketamine (100 mg/kg; Ketavet; Bayer)
supplemented by diazepam (2.5 mg/kg; Roche).

**Heterospecific transplantations: Mouse to rat.** In utero, into the CNS of E15 Wistar
rat fetuses, we transplanted 10⁵ cells obtained by mechanical dissection of
the cerebellar primordia of E12 mice embryos of mixed 86;129sv back-
ground expressing EGFP in all neurons (10). Transplantation techniques and
dissociation procedures were as previously described (12, 37). An outline of
the experiment is presented in Fig. 1. A total of 69 live-born graft recipient
Wistar rats entered the experiment; of these, 10 were perfused before 18
mo and 59 were perfused after 18 mo of life. Data obtained from four of the
rats perfused before 18 mo were also included in an unrelated study.

**Fig. 3.** Together with PCs, all other neural and glial phenotypes derived from transplanted mouse cerebellar precursors survived, as long as the host rat did.
All of the following examples are from rats surviving beyond 20 mo. Confocal images of sagittal sections of brainstem and cerebellum of transplant-bearing
rats. (A) EGFP-positive granule cells engrafted in the recipient internal granular layer. The axons of these neurons (parallel fibers) can be seen in the overlying
molecular layer. Green indicates EGFP; red indicates calbindin. (Scale bar: 25 μm.) (B) Unipolar brush cells in the host granular layer displaying the typical
dendritic structure. Green indicates EGFP; blue indicates DAPI nuclear staining. (Scale bar: 10 μm.) (C) Molecular layer interneurons and basket and stellate
cells located in the recipient molecular layer of the cerebellar cortex. Green indicates EGFP; red indicates parvalbumin; blue indicates DAPI nuclear staining.
(Scale bar: 10 μm.) (D) Neurons in the deep nuclei show multipolar morphology and express NeuN. Green indicates EGFP; red indicates NeuN; blue indicates
DAPI nuclear staining. (Scale bar: 10 μm.) (E) Astrocytes scattered through the cerebellar white matter. Green indicates EGFP; blue indicates DAPI nuclear
staining. (Scale bar: 10 μm.)
Isospecific transplantations. Mouse to mouse. A total of $10^4$ of the same cells used for mouse-to-rat transplantations were transplanted in utero into E15 FVB mouse fetuses at a developmental stage equivalent to E15 in the rat. After transplantation, we obtained 11 live-born graft recipient FVB mice, and they were all perfused before 18 mo.

Rat to rat. The same number of cells described in the two previous experimental paradigms, but derived from E14 transgenic rat embryos (36)—a developmental stage equivalent to E12 in the mouse—were grafted in utero into the cerebellum of E15 Wistar rat fetuses. We obtained 37 live-born graft recipient Wistar rats; of these, three were perfused before 18 mo and 34 were perfused after 18 mo of life.

Histology and Data Collection. Harvesting and processing of the tissues. Animals were killed by transcardiac perfusion of 500 mL of 4% (v/v) paraformaldehyde.
After perfusions, the brains were removed, stored overnight in the same fixative agent at 4°C, and finally transferred in 30% sucrose in 0.12 M phosphate buffer. The fixed cerebella were placed in 30% sucrose in PBS solution overnight, embedded in Tissue-Tek optimum cutting temperature compound (VWR International), and serially cut on a cryostat at 30 μm. Cryostat sections were immunohistochemically stained with anti-calbinbin antibodies in label PCs (1:1500; monoclonal or polyclonal; Swant), anti-parvalbumin (1:150; monoclonal; Swant) for molecular layer interneurons, anti-NeuN (1:500; monoclonal; Chemicon) for deep nuclei interneurons, and anti-GFP antibodies (1:700; polyclonal or monoclonal; Life Technologies) to enhance the GFP fluorescent signal of transplant-derived cells. Incubation of cerebellar slices with primary antibodies was made overnight at room temperature in PBS solution with 1.5% normal serum and 0.25% Triton X-100. The sections were then exposed for 1 h at room temperature to secondary biotinylated antibodies followed by a solution of streptavidin Texas red conjugate (1:200; Invitrogen) or fluoresceinated secondary antibody (1:200; Vector Laboratories). Nuclei were counterstained with DAPI (Sigma-Aldrich). The sections were mounted on microscope slides with Tris-glyceral supplemented with 10% Mowiol (Calbiochem) to reduce fading of fluorescence.

**Microscopical analysis.** By systematic inspection of all serial sections under a Zeiss Axiohot light microscope equipped with a Nikon DS-SM digital camera, or a Fluoview 300 microscope confocal microscope (Olympus), we traced and counted all EGFP-positive cells present in the cerebellum and brainstem of the transplanted animals. Positive cells were classified on the basis of their specific phenotype and antigenic reactivity. Quantitative and morphometric evaluations were made using the NeuroLucida software (MicroBrightField) connected to an E-800 microscope (Nikon) via a color CCD camera. Photoshop 8.0 (Adobe Systems) was used to adjust image contrast and assemble the final plates.

**Measurement of PC dendritic span.** We measured the maximum dendritic span in sagittal sections by measuring, in Neuroulucida software, the perpendicular distance between the plane of origin of the principal dendrite from the PC soma and the end of the dendritic arbor in the molecular layer.

**Spine counts.** In a randomly selected subset of mouse PCs found after 1, 18, and 36 mo of survival after heterospecific grafting, we analyzed dendritic spines present in the entire dendritic arbor by confocal optical sectioning. Spine numbers were obtained by counting all spines present in 134, 269, and 223 randomly selected S-μm segments of mouse PC dendrites, respectively, in 6, 9, and 13 different PCs. Spine counts were performed in at least three animals for the different survival times.

**Statistical Analysis.** Survival was calculated by using the date each mouse was found dead or moribund. For rats surviving >18 mo, we used the date of perfusion that corresponded to the day when the rat was considered unable to survive for more than 2 d. Statistical significance was assessed by parametric (unpaired Student t test) and nonparametric (Mann-Whitney test) methods when two groups were compared. Statistical analyses and survival curves were calculated by using MedCalc software.

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