The age and genomic integrity of neurons after cortical stroke in humans

Hagen B. Huttner[1,2,*], Olaf Bergmann[1,*], Mehran Salehpour[3], Attila Rácz[2], Emma Lindgren[1], Tamás Csonka[4], László Csiba[5], Tibor Hortobágyi[4], Gábor Méhes[4], Elisabet Englund[6], Beata Werne Solnestam[7], Sofia Zdunek[1], Christian Scharenberg[1], Lena Ström[1], Patrik Ståhl[1], Joakim Lundeberg[7], Andreas Dahl[8], Olle Lindvall[9], Stefan Schwab[2], Samuel Bernard[10], Göran Possnert[3] and Jonas Frisén[1]

* these authors contributed equally to this study

[1]Department of Cell and Molecular Biology, Karolinska Institute, SE-171 77 Stockholm, Sweden

[2]Department of Neurology, University of Erlangen-Nuremberg, Schwabachanlage 6, 91054 Erlangen, Germany

[3]Department of Physics and Astronomy, Ion Physics, Uppsala University, SE-751 20, Sweden,

[4]Institute of Pathology, University of Debrecen, Nagyerdei krt. 98, 4012 Debrecen, Hungary,

[5]Department of Neurology, University of Debrecen, Nagyerdei krt. 98, 4012 Debrecen, Hungary,

[6]Department of Pathology, University Hospital, S-221 85 Lund, Sweden

[7]Department of Gene Technology, Royal Institute of Technology, SciLifeLab, SE-106 91 Stockholm, Sweden

[8]Deep Sequencing Group, Center for Regenerative Therapies Dresden, Fetscherstraße 105, 01307 Dresden, Germany

[9]Department of Neurology, University Hospital, S-221 84 Lund, Sweden

[10]Department of Methematics, Institut Camille Jordan, Université de Lyon, 69622 Villeurbanne cedex, France

Correspondence to:

Jonas Frisén, Phone: +46852487562, Fax: +468324927, E-mail: jonas.frisen@ki.se

Acknowledgments

The study was supported by grants from the ERC, the Swedish Research Council, the Swedish Cancer Society, the Karolinska Institute, Tobias Stiftelsen, AFA Försäkringar, the Strategic Research Program in Stem Cells and Regenerative Medicine at Karolinska Institutet (StratRegen) and Knut och Alice Wallenbergs Stiftelse. H.B.H. was funded by a grant from the German Research Foundation (Hu 1961/1-1).

Contributions

H.B.H., O.B. and J.F. designed the study and wrote the manuscript. M.S. and G.P. performed the AMS measurements. A.R., T.C., L.C., T.H., G.M., E.E., O.L. and S.S. procured post-mortem material and obtained clinical and pathological data. B.W.S., J.L., P.S. and A.D. performed sequencing of RNA and DNA samples. S.B. and S.Z. performed mathematical modeling of cell turnover rates. H.B.H., O.B., E.L., C.S. and L.S. performed experiments and collected data. All authors approved the final version of the manuscript.

Conflict of interest

None of the authors has a scientific or financial conflict of interest to disclose.

Abstract

It has been unclear whether cortical ischemic stroke induces neurogenesis or neuronal DNA-rearrangements in humans. We show here that neither is the case, using immunohistochemistry, transcriptome-, genome- and ploidy-analyses, and determination of nuclear bomb test-derived ^{14}C in neuronal DNA. A large proportion of neurons display DNA-fragmentation and DNA-repair short time after stroke, whereas neurons at chronic stages after stroke show DNA-integrity, demonstrating the relevance of an intact genome for survival.

The generation of neocortical neurons is restricted to development and they are not exchanged in adulthood in healthy humans[1, 2]. However, cortical ischemic stroke in animal models and humans has been suggested to induce both neurogenesis[3-5] and neuronal DNA rearrangements[6], which could contribute to post-stroke recovery. We have assessed, in post-mortem tissue from individuals who survived stroke and died from non-neurological causes (supplementary Table 1), whether cortical cerebral ischemia induces neurogenesis, causes DNA rearrangement or triggers DNA-repair.

To characterize DNA content alterations in neurons after ischemic stroke, neuronal nuclei from human healthy and ischemic cortical stroke tissue were isolated by flow cytometry (Fig. 1a and supplementary Fig. 1 and supplementary Fig. 2). DNA staining established that the vast majority of neuronal nuclei were diploid. However, there was a small population (<2%) with a DNA content corresponding to a polyploid genome (Fig. 1b). FISH analysis of isolated neuronal nuclei with probes for the centrosome on chromosome 7 revealed, however, that all stroke tissue-derived neuronal nuclei (n=100) from either the diploid (Fig. 1c-e) or polyploid fraction (Fig. 1f-h) showed only two FISH signals. Hence, neuronal nuclei of the polyploid fraction represented aggregates of diploid nuclei. In addition, whole exome sequencing was performed to assess copy number variations (CNV) in the neuronal cell population of two different patients (#10 and #11) with ischemic stroke (Fig. 1i and supplementary Table 1). There were no significant CNV in the neuronal population of the penumbra adjacent to the ischemic core as compared to the contralateral cortex. There was no evidence for aneuploidy or DNA content alternations of cortical neurons after ischemic stroke in humans.

To analyze whether ischemia induces genomic DNA fragmentation in neurons, that might be counteracted by endogenous DNA-repair mechanisms[7], the degree of DNA breaks was determined by the Comet assay (Fig. 2a-c). FACS-isolated neuronal nuclei showed a significant higher level ($p<0.05$) of DNA fragmentation after acute/subacute ischemic cortical stroke as compared to healthy human cortex and chronic stroke lesions (Fig. 2d). Moreover, a substantial proportion (19-22%) of neurons in the penumbra 7-20 days after stroke showed an up-regulation of markers for DNA damage (double-strand breaks, Fig. 2e) and repair (base excision, Fig. 2f), which were not detected in healthy cortex tissue (transiently evident in mice[8]) nor in chronic stroke lesions (Fig. 2g). Of note, DNA repair-positive neuronal nuclei in three cases of subacute cortical ischemic stroke were negative for markers of apoptosis such as caspase3 and TUNEL (data not shown). In humans, ischemia-induced neuronal DNA repair has not been reported in non-apoptotic neurons and might be a prerequisite for neuronal survival.

Genomic rearrangements that may occur after neuronal DNA repair in response to fragmentation - similar to chromothripsis as described recently in tumor tissue[9] – can be detected by the presence of fused gene transcripts. Transcriptome analysis by RNA-sequencing provided no evidence for substantial gene fusion events in ischemic cortical stroke tissue as compared to healthy cortex (supplementary Table 2). Thus, cortical neurons surviving ischemic stroke in humans have intact genomes without significant genomic rearrangements or translocations.

To assess the age of cortical cells after stroke, we determined the radiocarbon (^{14}C) concentration in their genomes. Retrospective ^{14}C dating has previously been

established the birthdate of cells[1, 10] and to quantify the presence of neurogenesis[11], or the lack thereof[2, 12]. The ^{14}C concentration in the DNA of neurons of healthy occipital control cortex was not significantly different from atmospheric ^{14}C concentrations at the time of birth of the subjects (Fig. 3a and supplementary Table 3), indicating no postnatal neurogenesis, as described previously[1, 2]. The DNA of human cortical neurons surviving ischemic stroke for at least two years was as old as the subject (Fig. 3b). This establishes that ischemic cortical stroke does not elicit any detectable neurogenesis or extensive DNA synthesis within 2-13 years after the ischemic event.

Consistent with a lack of any sustainable neurogenesis, we could not find any neuron in the adult human cortex, even after stroke, devoid of the age pigment lipofuscin (supplementary Fig. 3), which postmitotic cells accumulate with age[13]. However, in the penumbra adjacent to the stroke lesion core, the proportion of neurons with the most abundant lipofuscin deposits was lower than in control tissue from the contralateral hemisphere, raising the possibility that high lipofuscin levels may represent a predisposing factor for ischemic neuronal death.

In contrast to the neuronal populations, the radiocarbon analyses revealed that the ^{14}C concentration in DNA from the non-neuronal cell populations in the stroke lesions, i.e. glia cells, vascular cells and other cells types involved in post-ischemic lesion consolidation, was in average higher compared to controls ($p<0.05$) (Fig. 3c), indicating, as expected, an increased cellular turnover after ischemic stroke. Regression analyses indicated that the addition of new (non-neuronal) cells might not only be restricted to the time of stroke but continues in the years after stroke.

The present data uncover that, contrary to some experimental studies in animal models[14, 15], the adult human neocortex is incapable of regenerating neurons, even in response to stroke. Moreover, although we found evidence for DNA fragmentation and repair in neurons in the penumbra of the stroke, their genomic DNA had a ^{14}C concentration corresponding to the time of birth of the individual. Together with analysis of ploidy and genomic integrity, this suggests that there is little plasticity both in terms of neurogenesis or genomic rearrangements in human cortical neurons. Thus, the neurons that remain close to a stroke were generated during development and only neurons without too extensive DNA damage survive long-term.

References

1. Spalding, K.L., Bhardwaj, R.D., Buchholz, B.A., Druid, H. & Frisen, J. *Cell* **122**, 133-143 (2005).
2. Bhardwaj, R.D.*, et al. Proc. Natl. Acad. Sci. U. S. A.* **103**, 12564-12568 (2006).
3. Jin, K.*, et al. Proc Natl Acad Sci U S A* **103**, 13198-13202 (2006).
4. Kreuzberg, M.*, et al. Exp. Neurol.* **226**, 90-99 (2010).
5. Leker, R.R.*, et al. Stroke* **38**, 153-161 (2007).
6. Li, W.*, et al. J Cereb Blood Flow Metab* **26**, 181-198 (2006).
7. Li, P.*, et al. Antioxid Redox Signal* **14**, 1905-1918 (2011).
8. Suberbielle, E.*, et al. Nat Neurosci* **16**, 613-621 (2013).
9. Rausch, T.*, et al. Cell* **148**, 59-71 (2012).
10. Bergmann, O.*, et al. Science* **324**, 98-102 (2009).
11. Spalding, K.L.*, et al. Cell* **153**, 1219-1227 (2013).
12. Bergmann, O.*, et al. Neuron* **74**, 634-639 (2012).
13. Benavides, S.H., Monserrat, A.J., Farina, S. & Porta, E.A. *Arch Gerontol Geriatr* **34**, 219-231 (2002).
14. Osman, A.M., Porritt, M.J., Nilsson, M. & Kuhn, H.G. *Stroke* **42**, 3559-3565 (2011).
15. Ohira, K.*, et al. Nat Neurosci* **13**, 173-179 (2010).

Figure 1.

DNA content in human neurons following cortical ischemic stroke.

(a) Isolation of neuronal nuclei from the cerebral cortex by FACS. (b) DNA content analysis showed a small fraction of sorted neuronal nuclei with a higher DNA content (< 2%). FISH analysis of the NeuN-positive diploid (c-e) and polyploid nuclei (f-h) revealed that all nuclei with higher DNA content are doublets or larger aggregates. The dashed line indicates the nuclei borders of the doublet. (i) Comparison of copy number variations (CNVs) of neurons in healthy control cortex and post-ischemic cortex of the same patient analyzed by whole-exome sequencing of DNA. There were no significant changes between lesion (upper panel) and healthy tissue (lower panel) and no evidence of CNVs in cortical neurons after ischemic stroke.

Figure 2.

Ischemic cortical stroke-induced DNA fragmentation is followed by DNA-repair in surviving neurons.

DNA-fragmentation assay (Comet) of neuronal nuclei graded as “intact nuclei” (a), “incomplete” (b) or “full” comet tail (c). DNA-fragmentation analysis in control cortex, subacute and chronic ischemic stroke tissue revealed a significantly higher proportion of full Comets in subacute stroke as compared to healthy and chronic stroke samples ($p<0.05$) (d). Baseline DNA-fragmentation in healthy controls and chronic stroke subjects reflected most likely post-mortem DNA degradation. DNA repair markers, γ-H2AX (e and g) and APE-1 (f and g) were up-regulated in the penumbra of cortical neurons (arrows) and non-neurons (arrowheads) 7-20 days after the ischemic lesion.

Figure 3.

^{14}C concentrations in DNA of cortical cells following ischemic stroke.

(a) Genomic ^{14}C concentrations in cortical neurons contralateral to the lesion were not significantly different from atmospheric ^{14}C concentrations at the time of birth of the subject, indicating no postnatal neurogenesis (subjects 1 to 11). (b) ^{14}C concentrations of neuronal DNA in ischemic cortical lesions were not significantly different from the control cortices, indicating that ischemic stroke did not lead to the generation and survival of new neurons. The individual time-point of the ischemic stroke is indicated with a vertical arrow. (c) Genomic ^{14}C concentrations of non-neuronal cells in the control cortex (light blue) and stroke lesion (orange); pairwise vertical plotting of both data points of each individual at the corresponding year of birth of the individuals (numbered as in a and b). (a-c) Error bars indicate two standard deviations in ^{14}C concentration in the respective DNA sample. Note, that all autopsies were performed in 2011 and 2012 (see supplemental table 1).

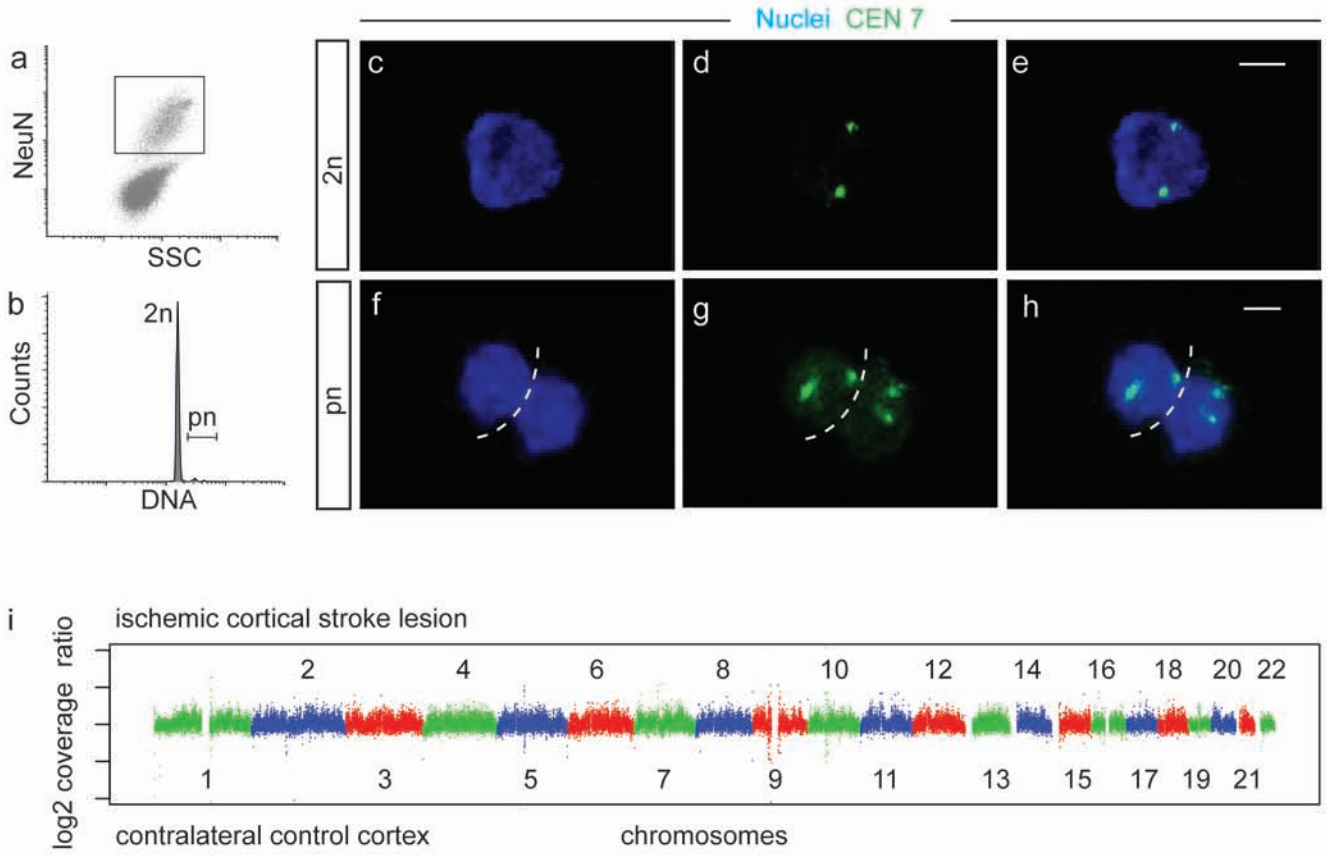
a
NeuN
SSC
b
Counts
2n
pn
DNA
Nuclei CEN 7
2n
pn
c
d
e
f
g
h
i
ischemic cortical stroke lesion
log2 coverage ratio
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
contralateral control cortex
chromosomes

Figure 1

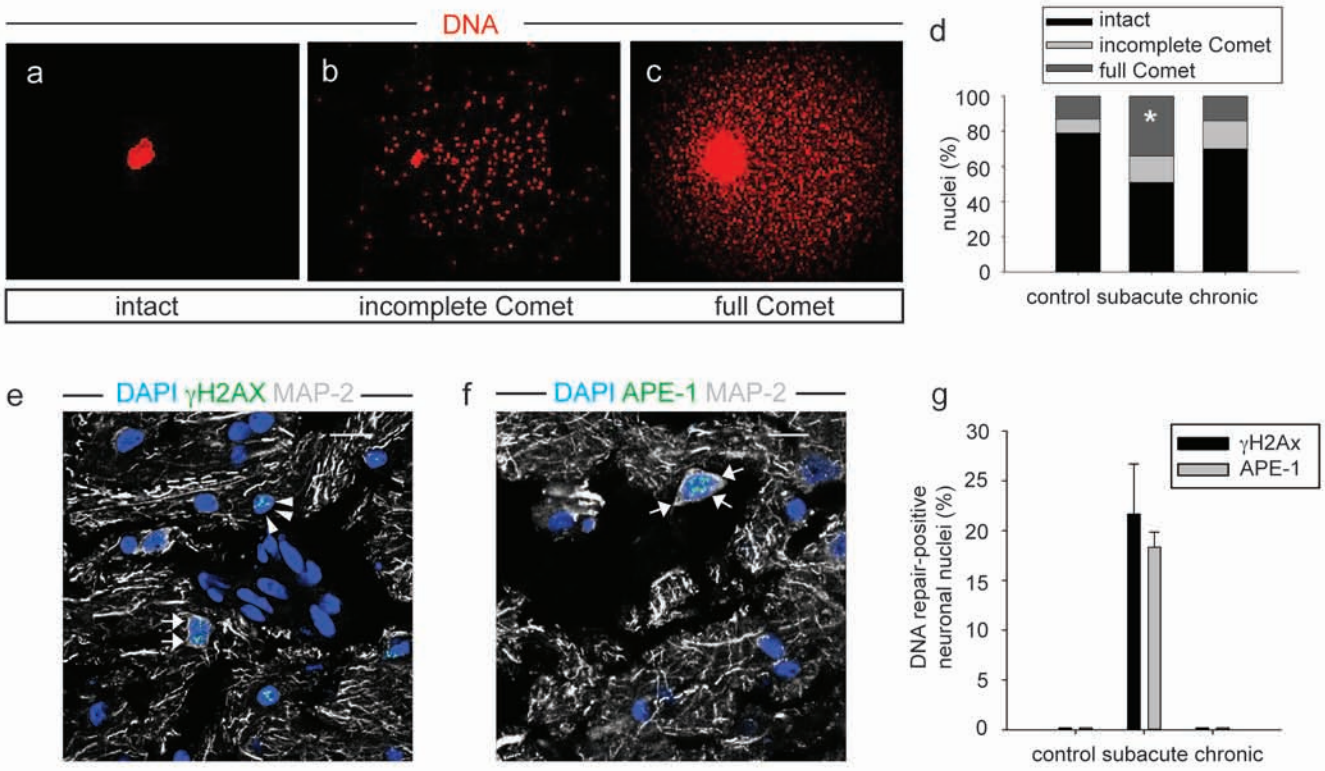
DNA
a
b
c
intact
incomplete Comet
full Comet
d
intact
incomplete Comet
full Comet
nuclei (%)
100
80
60
40
20
0
*
control subacute chronic
e
DAPI γH2AX MAP-2
f
DAPI APE-1 MAP-2
g
γH2Ax
APE-1
DNA repair-positive neuronal nuclei (%)
30
25
20
15
10
5
0
control subacute chronic

Figure 2

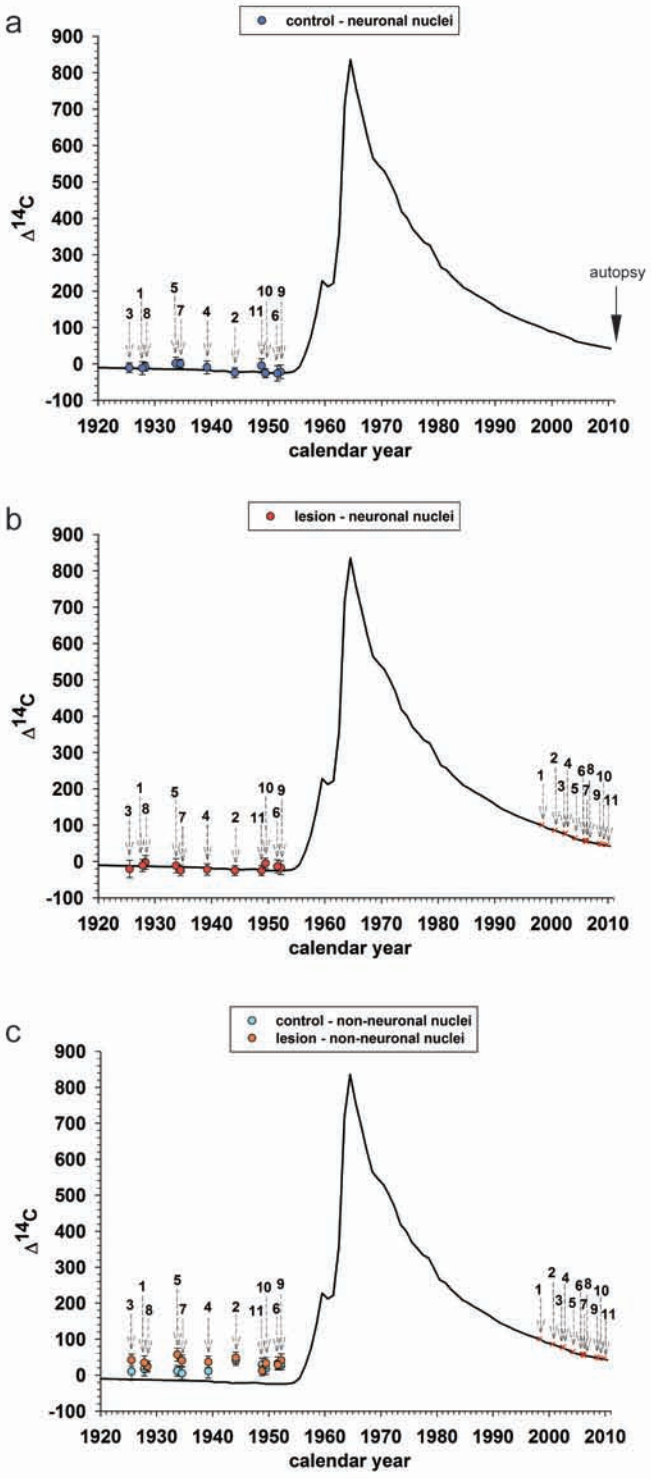
a
control - neuronal nuclei
Δ14C
calendar year
autopsy
b
lesion - neuronal nuclei
Δ14C
calendar year
c
control - non-neuronal nuclei
lesion - non-neuronal nuclei
Δ14C
calendar year

Figure 3

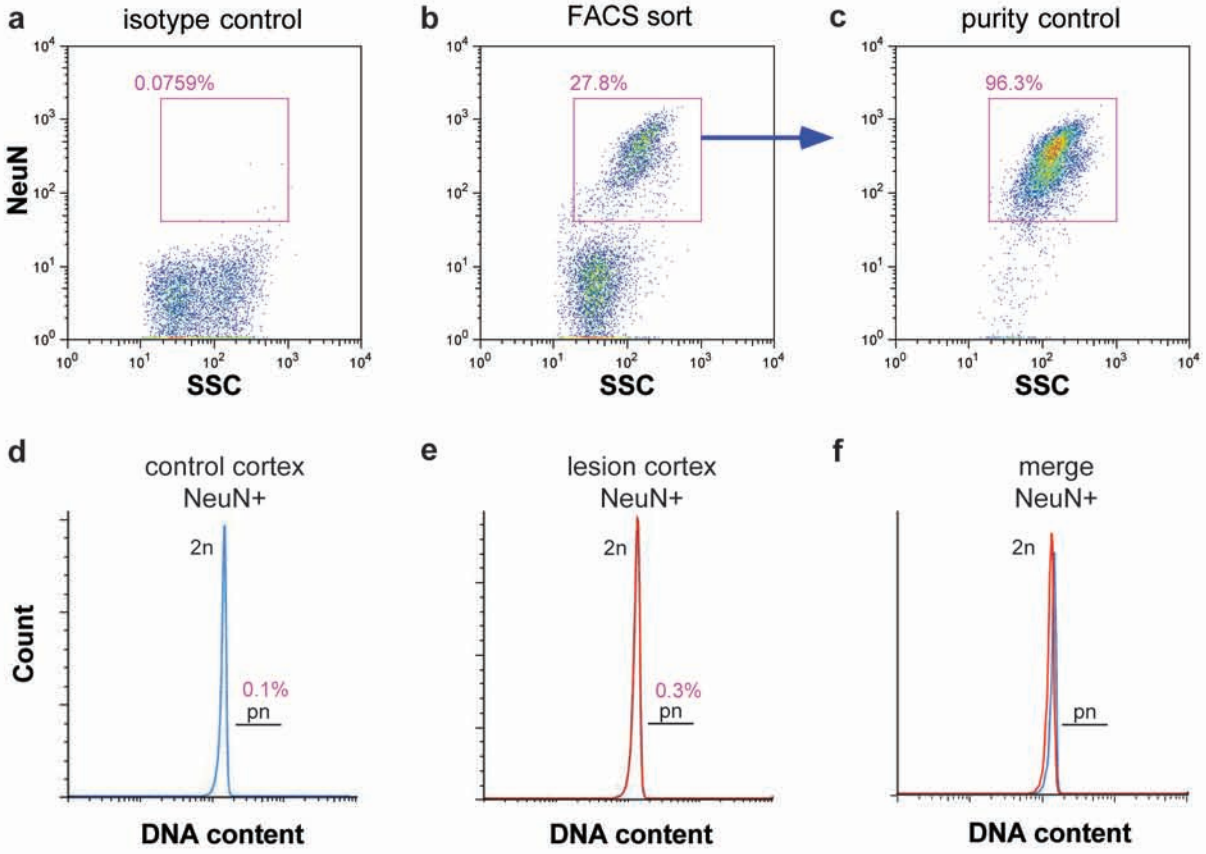
a
isotype control
0.0759%
NeuN
SSC
b
FACS sort
27.8%
SSC
c
purity control
96.3%
SSC
d
control cortex
NeuN+
2n
0.1%
pn
Count
DNA content
e
lesion cortex
NeuN+
2n
0.3%
pn
DNA content
f
merge
NeuN+
2n
pn
DNA content

Figure S1

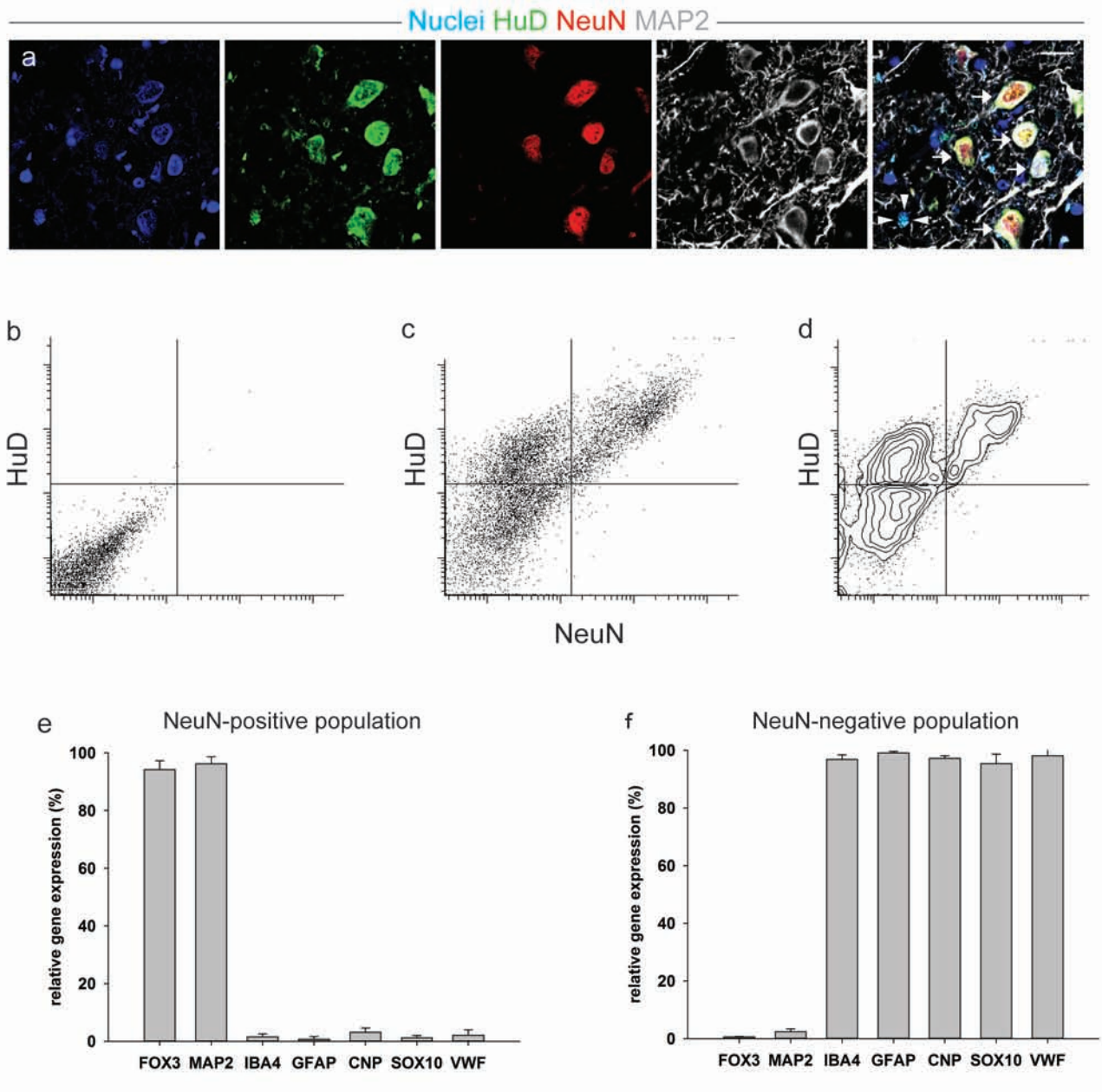
Nuclei HuD NeuN MAP2
a
b
c
d
HuD
HuD
HuD
NeuN
e
NeuN-positive population
relative gene expression (%)
100
80
60
40
20
0
FOX3 MAP2 IBA4 GFAP CNP SOX10 VWF
f
NeuN-negative population
relative gene expression (%)
100
80
60
40
20
0
FOX3 MAP2 IBA4 GFAP CNP SOX10 VWF

Figure S2

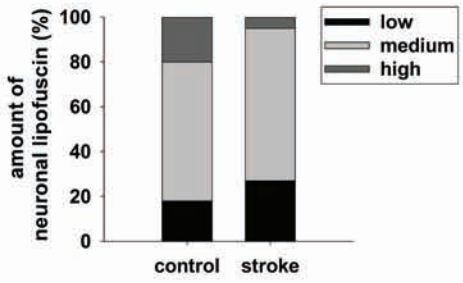
amount of neuronal lipofuscin (%)
100
80
60
40
20
0
control
stroke
low
medium
high

Figure S3