NEUROBIOLOGY OF DISEASE (2004) 17-1:108-113.

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Neuronal RNA Oxidation Is a Prominent Feature of Familial Alzheimer Disease

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Abbreviated Title: RNA oxidation in familial Alzheimer disease

Number of text pages: 20 Figures: 3 Tables: 0

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Abstract

An *in situ* approach was used to identify the oxidized RNA nucleoside, 8-hydroxyguanosine (8OHG) in the frontal cortex of familial Alzheimer disease (FAD) with a mutation in presenilin-1 (PS-1) or amyloid β protein precursor (AβPP) gene (n = 13, age 47-81 y). Neurons with marked 8OHG immunoreaction in the cytoplasm were widely distributed in the superior/middle frontal gyrus of FAD. Relative intensity measurements of neuronal 8OHG immunoreactivity showed that there was a significant increase in FAD compared with controls (n = 15, age 59-81 y), while there was no difference in relative 8OHG between the PS-1 and the AβPP FAD. Interestingly, a presymptomatic case carrying a PS-1 mutation showed a considerable level of relative 8OHG, and the increased levels of neuronal 8OHG in FAD were more prominent in cases with lower % area of Aβ42 burden. These results suggest that oxidative stress is an early event involved in the pathological cascade of FAD.

Key words: amyloid β protein precursor, familial Alzheimer disease; 8-hydroxyguanosine; oxidative stress; presenilin; RNA

Numerous studies have now established the association of neuronal oxidative stress with major neurodegenerative disorders such as Alzheimer disease (AD) (reviewed in Perry et al., 1998; Markesbery and Carney, 1999; Smith et al., 2000) and Parkinson disease (reviewed in Jenner, 1998; Zhang et al., 2000). We have reported RNA oxidation in vulnerable neurons of sporadic type of AD (Nunomura et al., 1999; 2001), Parkinson disease (Zhang et al., 1999), dementia with Lewy bodies (Nunomura et al., 2002) as well as Down syndrome (Nunomura et al., 2000), which suggests links between oxidative stress and not only age-associated degenerative diseases but also neurodegeneration due to genetic factors.

In this study, we used an *in situ* approach to identify the oxidized RNA nucleoside, 8-hydroxyguanosine (8OHG) in the cerebral cortex of familial AD (FAD) with a mutation in presenilin-1 (PS-1) or amyloid β protein precursor (AβPP) gene. Neurons with marked 8OHG immunoreaction in the cytoplasm were widely distributed in the cerebral cortex of FAD. Importantly, a presymptomatic case carrying a PS-1 mutation (Lippa et al., 1998) showed a considerable level of neuronal 8OHG. Moreover, semiquantitative analysis showed that the increased levels of neuronal 8OHG in FAD were more prominent in cases with lower amount of amyloid β (Aβ) which was immunolabeled with an end-specific antibody for the Aβ1-42 (Aβ42), as we showed in individuals with Down syndrome (Nunomura et al., 2000). These results suggest that oxidative stress is involved in the pathological cascade of FAD especially as an early stage event of the cascade.

Materials and methods

Tissue.

Brain tissue was obtained at autopsy from 13 clinically- and pathologically- confirmed cases of FAD according to the CERAD criteria (2 males and 11 females; ages 47-81 years, average 59). Eleven females of the FAD group (ages 47-81 years, average 59) were members of families possessing a PS-1 gene mutation and the other 2 males of the FAD group (ages 57 and 58 years) were members of families possessing an AβPP gene mutation. Mutations in PS-1 gene in these subjects were found on M146L (n = 1), A246E (n = 4), L286V (n = 1) and C410Y (n = 5), and mutations in AβPP gene were found on KM670, 671NL (Swedish mutation; n = 1) and A692G (Flemish mutation; n = 1). All these FAD subjects died from pneumonia except for a case whose information about cause of death was not available. Another 51 years old male was a member of a family with a PS-1 gene mutation (A246E), who died from myocardial infarction and had shown no clinical symptoms of dementia immediately before death (Lippa et al., 1998). As for the controls, we investigated a consecutive series of 15 subjects without dementia (7 males and 8 females; ages 59-81 years, average 66). Causes of death of these controls were internal malignancy (n = 9), leukemia (n = 2), cardiac failure (n = 3), and unknown (n = 1). Postmortem intervals prior to fixation were 2-19 h (average 8) in the FAD group, 14 h in the presymptomatic case with PS-1 gene mutation, and 3-20 h (average 9) in controls. Duration of dementia was known from clinical records in 12 cases of FAD as 5-25 years (average 11). Slices of the frontal cortex (the superior/middle frontal gyrus) and/or the temporal cortex (the inferior temporal/occipitotemporal gyrus) from all the subjects were fixed in neutral formalin, dehydrated through graded ethanol followed by xylene, and embedded in paraffin. Six-micron thick sections were cut, and mounted on Silane® (Sigma, St. Louis, MO)-coated glass slides.

Immunocytochemistry and Antibodies.

Following deparaffinization with xylene, sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30 min incubation with 3% H_2O_2 in methanol and non-specific binding sites were blocked in a 30 min incubation with 10% normal goat serum in Tris-buffered saline (150 mM Tris-HCl, 150 mM NaCl, pH 7.6). To detect oxidized nucleosides, we used a mouse monoclonal antibody against 8OHG, 1F7 (Yin et al., 1995) (1:30; Trevigen, Gaithersburg, MD), after treatment with 10 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) in phosphate buffered saline (pH = 7.4) for 40 min at 37°C. Immunostaining was developed by the peroxidase-antiperoxidase procedure (Sternberger, 1986) by using 0.75 mg/ml 3,3'-diaminobenzidine cosubstrate in 0.015% H_2O_2, 50mM Tris-HCl, pH 7.6 for exactly 10 min. The specificity of 1F7 to 8OHG was confirmed by primary antibody omission or by absorption with purified 8OHG (Cayman Chemical, Ann Arbor, MI) (Nunomura et al., 1999). Although 1F7 recognizes RNA-derived 8OHG as well as DNA-derived 8-hydroxydeoxyguanosine with similar binding affinities (Yin et al., 1995), we have confirmed that 1F7 immunolabeling in neurons in sporadic AD is predominantly in RNA by the pretreatment with DNase or RNase (Nunomura et al., 1999) as well as by immunoelectronmicroscopy, which showed most 8OHG is present in the endoplasmic reticulum (Nunomura et al., 2001). For FAD cases, additional sections were pretreated with RNase-free DNase I (10 U/μl for 2 h at 37°C; Roche, Mannheim, Germany) or DNase-free RNase (0.5 μg/μl for 2 h at 37°C; Boehringer Mannheim) after the proteinase-K treatment. For the detection of Aβ deposition in FAD cases, we used either of mouse monoclonal antibody, BC05 (1:1000; gift of Fukumoto, H., Takeda Chemical Industries, Osaka, Japan) specific for the carboxyl terminus of

Aβ1-42 (Aβ42), or BA27 (1:3,500; gift of Fukumoto, H.) specific for the carboxyl terminus of Aβ1-40 (Aβ40), with a 5 min pretreatment of 70% formic acid.

Relative scale of 8OHG and Aβ deposition

All measurements were performed in the layer III of the cerebral cortex (the superior/middle frontal gyrus and/or the inferior temporal/occipitotemporal gyrus) using a Q500IW-EX Image Processing and Analysis System (Leica) linked to a SONY CCD Camera (XC-75CE) mounted on a Nikon MICROPHOT-FX microscope. The intensity of immunoreaction with 1F7 was evaluated by measuring the average optical density in an area comprising the cytoplasm and nucleus, as we described previously (Nunomura et al., 1999). Three adjacent fields (each field = 460 μm × 428 μm) were selected, and in each field of the video camera, 5 pyramidal neurons sectioned near their equator, based on a section plane that included the nucleolus, were selected and outlined manually so that of the area of the nucleus to cytoplasm was rather constant. The nucleus was included because damage to RNA was nuclear as well as cytoplasmic. The average optical density measurement was obtained for each of the 3 fields and averaged. Finally, the optical density value was corrected for background by subtracting the optical density of the white matter on the same section. The superior/middle frontal gyrus was available in all 13 FAD cases, while the inferior temporal/occipitotemporal gyrus was available in all 19 controls. Both brain regions were available in two controls and an additional centenarian without dementia, in which levels of the relative 8OHG were similar in both brain regions. In these cases, the ratio of relative 8OHG in the frontal cortex to that in the temporal cortex was 0.78, 0.84 and 1.13 (average 0.92), which meant that the regional difference in relative neuronal 8OHG between the frontal and temporal cortices were virtually negligible in controls. Therefore, we used data from the

superior/middle frontal gyrus of FAD cases and the inferior temporal/occipitotemporal gyrus of controls for comparison.

For the measurement of the extent of Aβ42 or Aβ40 deposition in FAD cases, three adjacent fields (each field = 624 μm × 580 μm) were selected to include the same area used to measure 1F7 immunoreactivity in an adjacent serial section. The area of Aβ42 or Aβ40 deposits was determined with gray scale thresholding according to the methods described previously (Hyman et al., 1993). The sum of the areas of Aβ42 or Aβ40 deposits was divided by the total area to give the %Aβ42 or % Aβ40 burden.

All measurements were done under the same optical and light conditions as well as using an electronic shading correction to compensate for any unevenness that might be present in the illumination. Statistical analysis was performed with Mann-Whitney U-test and linear regression analysis, using StatView 5.0 program (Abacus Concepts, Berkeley, CA).

Results

In cases of FAD, 8OHG immunoreactivity was prominent in the neuronal cytoplasm in the superior/middle frontal gyrus (Figs. 1A-C). Neuronal 8OHG immunoreaction was widely distributed throughout the cortical layers (Fig. 1A) while in controls, staining was very low (Fig. 1E). Pyramidal neurons of larger size in the superior/middle frontal gyrus tended to show higher immunointensity of 8OHG in each case of FAD, although individual variation of immunointensity among FAD cases was observed. Moderately positive immunoreaction of neuronal 8OHG was observed in a presymptomatic case carrying a PS-1 gene mutation (Fig. 1D).

To investigate whether the immunoreaction with the 1F7 antibody was derived from oxidized RNA or oxidized DNA or both, we performed nuclease treatment before the immunostaining with 1F7. The immunoreaction in the sections of FAD was diminished greatly by the DNase free-RNase pretreatment (Figs. 2A, B) but only slightly by the RNase free-DNase pretreatment (Figs. 2C, D), as we demonstrated in the sections of sporadic AD and DLB (Nunomura et al., 1999; 2002). Therefore, not only in sporadic AD and DLB but also in FAD, RNA is a major site of nucleic acid oxidation.

Relative scale measurements of the 8OHG immunoreactivity using a computer-assisted image analysis system demonstrated that the increase was significant in FAD when compared with a control group (Fig. 3A). Because the 8OHG immunoreactivities tend to show an age-dependent increase in non-demented individuals (Nunomura et al., 1999), the significant increase in the 8OHG immunoreactivity in FAD cases (the mean age, 59 years) compared with controls (the mean age, 66 years) cannot be explained by the difference in age of subjects between the groups. Neither, these results cannot be explained by neuronal shrinkage, because

the average cell profile area remained unchanged between FAD cases (161 μm^2) and controls (148 μm^2). Similar levels of relative 8OHG were demonstrated between the PS-1 and the AβPP FAD [the average of the relative 8OHG = 10.2 (arbitrary units) and 9.6, respectively]. Interestingly, a presymptomatic case carrying a PS-1 gene mutation showed a considerable level of relative 8OHG (Fig. 3A). Levels of the relative 8OHG immunoreactivity were not related to postmortem intervals among FAD cases ($p > 0.9$ by linear regression analysis) as well as among controls ($p > 0.9$). Furthermore, an agonal state before death also failed to alter the relative 8OHG immunoreactivity. We found similar average values for the relative 8OHG immunoreactivity in controls who died from internal malignancy (n = 9, relative 8OHG = 4.7), leukemia (n = 2, relative 8OHG = 7.9), heart failure (n = 3, relative 8OHG = 5.9), and unknown (n = 1, relative 8OHG = 4.9), as we showed in other series of controls (Nunomura et al., 1999).

When we investigated relationship between % area of Aβ 42 or Aβ 40 burden and relative 8OHG levels in FAD, we found a significant inverse correlation between % area of Aβ42 burden and relative 8OHG levels, but no significant correlation between % area of Aβ40 burden and relative 8OHG levels (Figs 3B, C), as we observed in Down syndrome (Nunomura et al., 2000). In controls, only 7 cases showed Aβ42 burden and only 3 cases showed Aβ40 burden. No apparent relationship between %area of Aβ42 or Aβ40 and the levels of neuronal 8OHG was detected in control subjects (data not shown).

Discussion

Recently, an increasing number of *in vitro* and *in vivo* studies have suggested that oxidative stress is involved in the pathogenesis of AD and has an involvement in FAD with AβPP, PS-1, or PS-2 gene mutation. Indeed, increased oxidative stress, elevated vulnerability to oxidative stress-induced cell death and /or reduced antioxidant defenses have been demonstrated in: (i) cell lines expressing mutant human AβPP, PS-1, or PS-2 (Guo et al., 1997; Eckert et al., 2001; Hashimoto et al., 2002; Marcues et al., 2003); (ii) transgenic mice expressing mutant human AβPP and/or PS-1 as well as knock in mice expressing mutant human PS-1 (Smith et al., 1998; Guo et al., 1999; Leutner, et al., 2000; Takahashi et al., 2000; Matsuoka, et al., 2001; Praticò, et al., 2001; LaFontaine et al., 2002); (iii) fibroblasts and lymphoblasts from FAD patients with AβPP or PS-1 gene mutation (Cecchi et al., 2002); and (iv) cerebral cortex of autopsied brain samples from patients with AβPP gene mutation (Bogdanovic et al., 2001). The findings presented here represent the first evidence of increased oxidative damage to RNA in the cerebral cortex neurons of FAD, a finding previously made for the cerebral cortex neurons in sporadic AD and DLB (Nunomura et al., 1999; 2001; 2002) as well as for the substantia nigra neurons of Parkinson disease (Zhang et al., 1999). Therefore, RNA oxidation is a common phenomenon in vulnerable neurons of sporadic and familial types of AD as well as some disorders classified in the category of synucleinopathy. A recent biochemical study has revealed that some mRNA species are selectively oxidized in the cerebral cortex of AD, and as a biological consequence, abnormal processing of proteins occurred to the oxidized mRNAs when they are expressed in cell lines (Shan et al., 2003). These findings suggest that RNA oxidation itself is directly associated with neuronal deterioration instead of harmless epiphenomenon during the process of neurodegeneration.

Interestingly, a presymptomatic case carrying a PS-1 mutation, whose autopsied cerebral cortex exhibited substantial amount of Aβ42 deposition but no Aβ40 deposition (Lippa et al., 1998), showed a considerable level of neuronal RNA oxidation. This observation clearly suggests an early involvement of oxidative stress in the pathological cascade of FAD, which corresponds with our previous finding in Down syndrome cases where neuronal RNA oxidation precedes Aβ deposition (Nunomura et al., 2000). The early involvement of oxidative stress in FAD is supported also by experiments examining transgenic mice expressing human AβPP with FAD mutation and showing increased lipid peroxidation prior to Aβ plaque formation (Praticò, et al., 2001).

Furthermore, we found a significant inverse correlation of Aβ42 burden, but not Aβ40 burden, with neuronal RNA oxidation. Again, this observation is completely coincident with the results of our previous study on Down syndrome (Nunomura et al., 2000). Because Aβ 42 deposition is an upstream event in the pathological cascade of FAD (Iwatsubo et al., 1994; Kalaria et al., 1996; Lippa et al., 1998), early involvement of oxidative stress is suggested by the association of Aβ42 burden with the levels of neuronal RNA oxidation. We may be able to explain the *inverse* correlation between Aβ42 burden and neuronal RNA oxidation when we consider roles of transition metals such as copper and iron, efficient catalysts of oxidation, and zinc, a redox-inert antioxidant that are significantly elevated in the neocortex and especially enriched in Aβ plaques of individuals with AD (Lovell et al., 1998). Indeed, Aβ1-42 possesses high affinity for these transition metals and the binding promotes assembly of Aβ (Atwood et al., 1999). The *inverse* correlation may reflect a possible antioxidant property of Aβ peptide that chelates copper and iron to keep these transition metals in a redox-inactive form (Kontush, 2001;

Zou et al., 2002). Another possible explanation is that the *inverse* correlation may reflect zinc elevation as a homeostatic antioxidant response to oxidative stress with subsequent abundant Aβ plaques formation (Cuajungco et al., 2000). Because recent studies have suggested that pre-fibrillar Aβ, but not the Aβ fibril, shows toxicity (Lambert et al., 1998; Walsh et al., 1999), Aβ plaques themselves may represent a fraction of total Aβ in the brain that has been condensed and neutralized and no longer contributes to neurotoxicity. Further investigations on the relationship between intraneuronally accumulated Aβ (Gouras et al., 2000) and oxidative stress markers are necessary to elucidate whether intraneuronal Aβ peptide has pro- or antioxidant property.

Conclusion

We observed prominent nucleic acid oxidation marked by 8OHG immunoreactivity in FAD patients with PS-1 or AβPP gene mutation. The 8OHG was mainly restricted to cytoplasmic RNA of vulnerable neurons in FAD as we observed in sporadic AD. Early involvement of RNA oxidation in the pathological cascade of FAD was suggested by a presymptomatic case who carried a PS-1 mutation and showed a considerable level of neuronal RNA oxidation. An inverse correlation of Aβ42 burden with neuronal RNA oxidation in FAD, which was demonstrated also in Down syndrome, might suggest a link between the process of Aβ plaques formation and an effective tissue protective response to oxidative stress.

Acknowledgments

Work in the authors' laboratories is supported by funding from the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research (c) 14570902), Alzheimer Association, and National Institute of Health.

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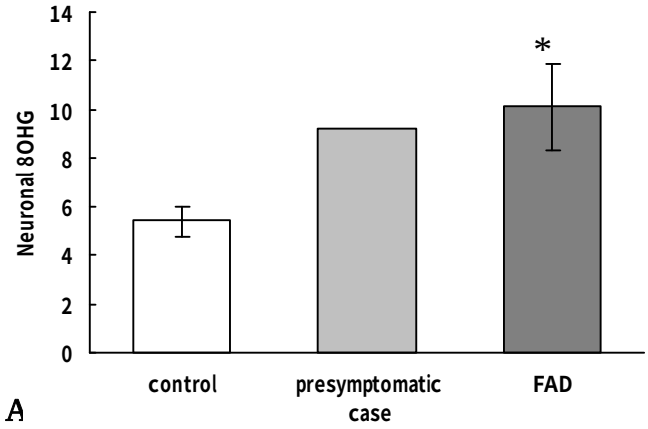
Figure legends

Fig. 1. Oxidized nucleoside, 8OHG, is abundant in vulnerable neurons in FAD. Neuronal 8OHG immunoreactivity showing cytoplasmic predominace is prominent in the frontal cortex from a case of FAD with a PS-1 gene mutation (C410Y, 55 years old) (A, B). In a case of FAD with an AβPP gene mutation (A692G, 57 years old) (C) and in a presymptomatic case with a PS-1 gene mutation (A264E, 51 years old) (D), moderately positive 8OHG immunoreactivity is observed in neurons of the frontal cortex. Whereas, in a control case (64 years old), the neuronal 8OHG immunoreactivity is faint in the frontal cortex (E). Scale bars, A = 100 μm, B-E = 50 μm.

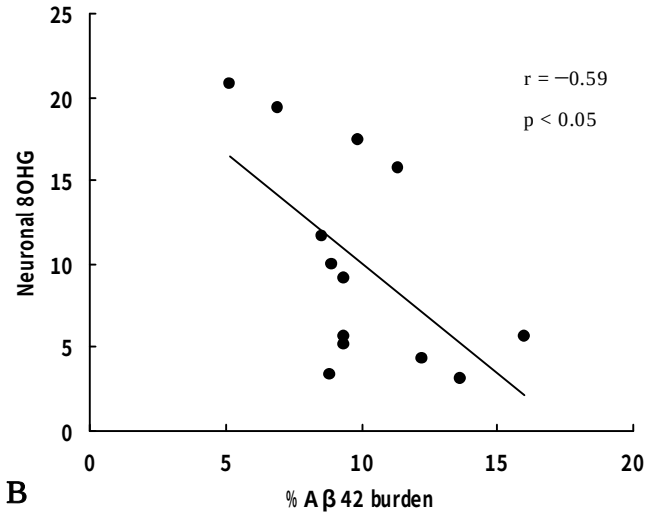
Fig. 2. RNA is a major site of nucleic acid oxidation in FAD. The immunoreaction with 1F7 antibody in FAD (A, C) is diminished greatly by the treatment with DNase-free RNase (B) but only slightly by the treatment with RNase-free DNase (D). A and B are adjacent serial sections, so are C and D. *indicates landmark blood vessel. The frontal cortex from a case of FAD with a PS-1 gene mutation (C410Y, 47 years old). Scale bar, 100 μm.

Fig. 3. Increased levels of neuronal 8OHG immunointensity and an inverse relationship between % area of Aβ42 burden and neuronal 8OHG immunointensity in the neocortex of FAD. Relative scale measurements reveal that the levels of 8OHG immunoreactivity are significantly increased in FAD cases (n = 13, average age 59 years) compared with controls (n = 15, average age 66 years) (*$p < 0.05$ by Mann-Whitney U-test). A presymptomatic case with a PS-1 gene mutation (51 years old) shows a similar level of neuronal 8OHG immunointensity to the average of the FAD group. Values shown are the averages with SE (A). In FAD cases, there is a significant inverse correlation of % area of Aβ42 burden (B), but not % area of Aβ40 burden (C),

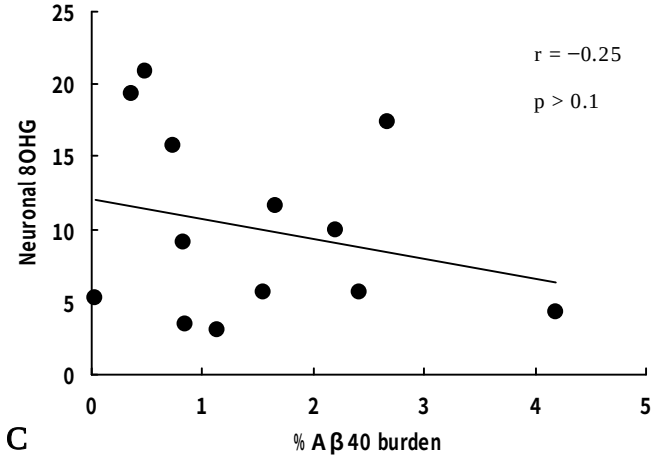
with the levels of 8OHG immunoreactivity by linear regression analysis.



A



B



C

Figure 3

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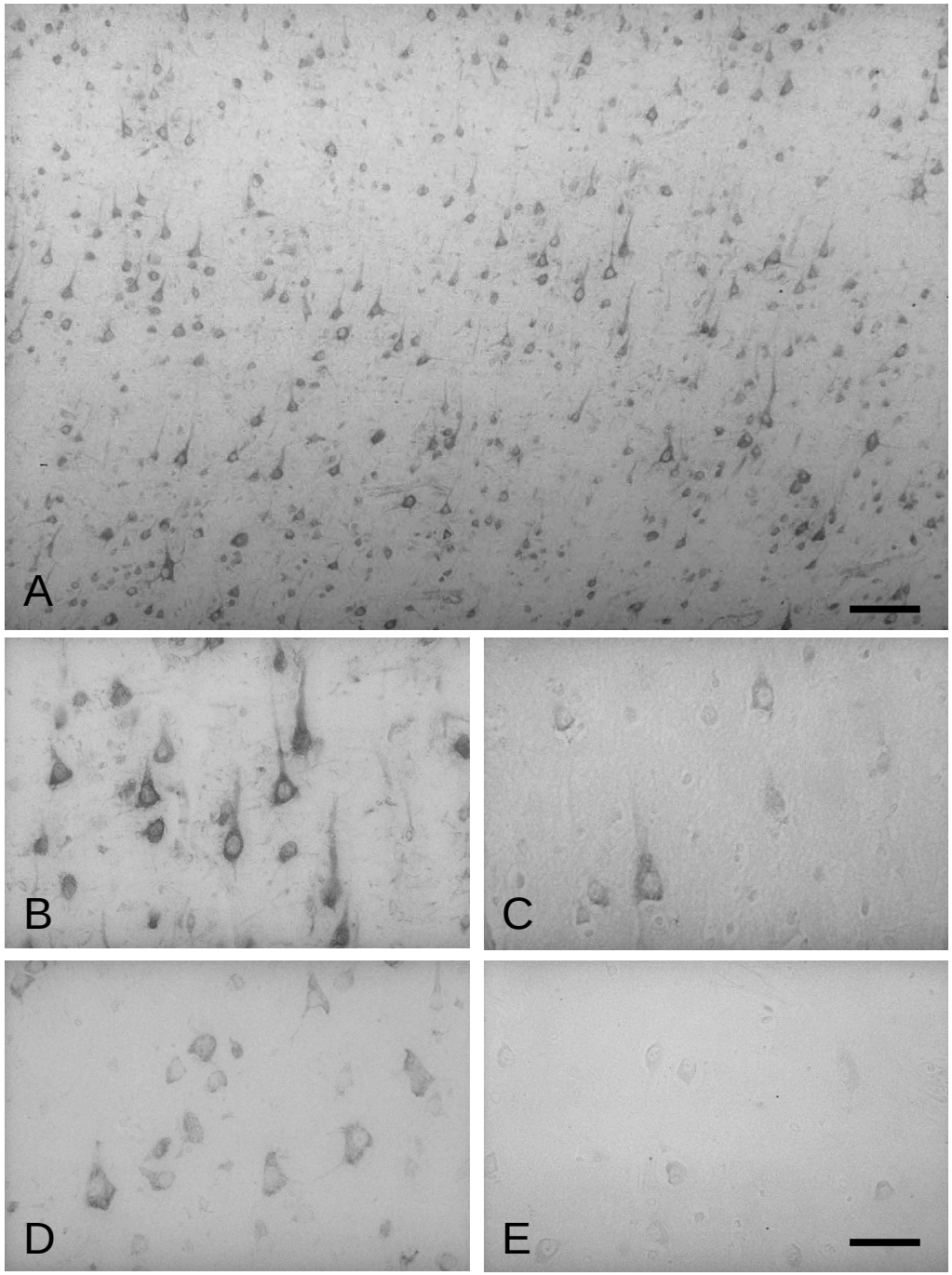

Figure 1
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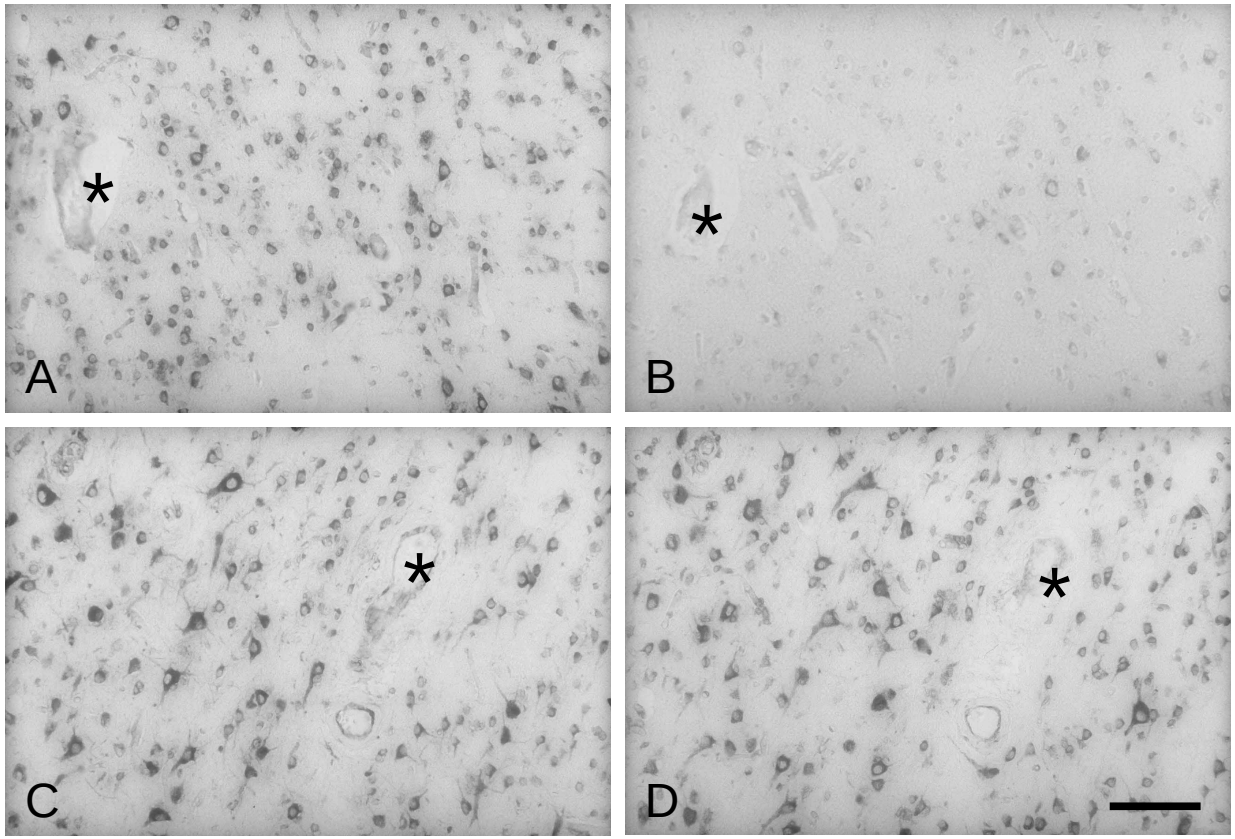

Figure 2
Nunomura et al.