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EFFECTS OF AGE ON AXON TERMINALS FORMING AXOSOMATIC AND AXODENDRITIC INHIBITORY SYNAPSES IN PREFRONTAL CORTEX

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Abstract—Much of the cognitive decline shown by aging primates can be attributed to dysfunction of prefrontal cortex and, as shown previously, about 30% of asymmetric (excitatory) and symmetric (inhibitory) axodendritic synapses are lost from the neuropil of layer 2/3 in prefrontal area 46 with age [Peters A, Sethares C, Luebke JI (2008) Neuroscience 152:970–981]. Whether there is a similar loss of inhibitory axosomatic synapses from this cortex has not been determined, but a study in primate motor cortex suggests that axosomatic synapses are not lost with age [Tigges J, Herndon JG, Peters A (1992) Anat Rec 232:305–315]. The present study is focused upon whether the remaining axon terminals forming inhibitory synapses in old monkeys hypertrophy to compensate for any age-related loss. Analysis of electron micrographs show that in layer 2/3 of area 46 in both young and old monkeys, axon terminals forming axosomatic synapses are significantly larger and contain more mitochondria than those forming axodendritic synapses and both axodendritic and axosomatic terminals become larger with age. However, while mitochondria in axodendritic terminals do not change in either size or amount with age, the mitochondria in axosomatic terminals become larger. Similarly, in terminals forming axodendritic synapses, the mean numbers of synaptic vesicle profiles is the same in young and old monkeys, whereas in terminals forming axosomatic synapses there is an increase in the numbers of synaptic vesicles with age. We also show that among these age-related changes, only the numbers of synaptic vesicles in axosomatic synapses are significantly correlated with the cognitive impairment indices displayed by the same monkeys. In summary, the data provide original evidence that axosomatic axon terminals increase in size and in their content of mitochondria and synaptic vesicles. Furthermore, based on our and previously published results, we speculate that these changes are linked to age-related cognitive decline.

Key words: aging, prefrontal cortex, synapses, inhibitory, axon terminals, ultrastructure.

Rhesus monkeys provide an excellent model in which to study normal aging, for although they live as long as 35 years (Tigges et al., 1988), they are not subject to Alzheimer's disease. But as they age, rhesus monkeys exhibit cognitive decline, which parallels that shown by humans (e.g. Gallagher and Rapp, 1997; Herndon et al., 1997; Moore et al., 2003; Moss et al., 2007). It is possible to assess the cognitive status of monkeys using behavioral tasks that are derived and adapted from those used for humans (e.g. Bachevalier et al., 1991; Albert and Moss, 1996; Herndon et al., 1997) and then to examine their brains to determine what changes in morphology parallel the cognitive decline (e.g. Peters, 2007, 2009). Much of the cognitive decline shown by primates has been attributed to dysfunction of prefrontal cortex, and age-related impairment in spatial and reversal learning tasks, as well as recognition memory tasks are considered to be a result of dysfunction of area 46 (e.g. Kojima and Goldman-Rakic, 1982; Lai et al., 1995; Fuster, 1997; Moore et al., 2003; Moss et al., 1997, 2007). Earlier it was assumed that the cognitive decline was due to loss of neurons, but more recent studies show there is not a significant loss of neocortical neurons with age (e.g. Peters et al., 1994, 1998a,b; Hof et al., 2000; Peters, 2002), and this is also true for area 46 (e.g. Peters et al., 1994; Smith et al., 2004). However, neurons are not spared because there is a regression or loss of some dendritic branches together with a reduction in the numbers of dendritic spines (e.g., Jacobs et al., 1997; Peters et al., 1998b; Duan et al., 2003; Kabaso et al., 2009).

Since dendrites and their spines are the main recipients of axon terminals forming synapses on cortical neurons, regression or loss of these structures should result in a loss of synapses with age. Indeed, with increasing age there is an overall loss of about 30% of synapses from layer 2/3 and both asymmetric (excitatory) and symmetric (inhibitory) synapses are lost at the same rate (Peters et al., 2008). When these data are correlated with the overall cognitive impairment shown by aging monkeys, a strong inverse correlation emerges between the numerical density of asymmetric synapses in layers 2/3 and cognitive impairment, but a somewhat less strong correlation between the numerical density of symmetric synapses and cognitive impairment (Peters et al., 2008). In layer 5 the situation is different. The overall loss of synapses is only 20% and this is almost entirely due to a loss of asymmetric synapses and there is no correlation between the numerical density of synapses in layer 5 and cognitive impairment (Peters et al., 2008). These results essentially agree with those of Bertoni-Freddari et al. (2006) who examined the frontal and temporal cortices of long-tailed macaques and found the ratio of synapses to neurons in the neuropil of frontal cortex to decrease by 21% with age, although there is little change in temporal cortex.

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Abbreviations: CII, cognitive impairment index; IPSCs, inhibitory postsynaptic currents.

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The functional significance of a decreased numerical density of inhibitory synapses with age is unclear. A recent patch-clamp study has found that normal aging results in increased frequency of spontaneous inhibitory post-synaptic currents (IPSCs), but not miniature IPSCs, in layer 2/3 pyramidal neurons in area 46 of rhesus monkeys (Luebke et al., 2004). Because spontaneous IPSCs reflect activity of inhibitory inputs, this result suggests that loss of inhibitory synapses may be compensated by an increased activity of remaining axon terminals. It is not known, however, if the remaining inhibitory synapses become altered to compensate for any loss and to make this determination, we have compared the morphological features of symmetric axodendritic and axosomatic synapses in layer 2/3 of area 46 of young and aged rhesus monkeys. The following parameters were examined: axon terminal and mitochondrial sizes, numbers of synaptic vesicles and junctional lengths.

EXPERIMENTAL PROCEDURES

Tissue specimens and processing

Tissue was taken from area 46 in the prefrontal cortex of 10 rhesus monkeys (*Macaca mulatta*), nine of which had been previously behaviorally tested to assess their cognitive status (Peters et al., 2008). The cognitive status is measured using a delayed nonmatching to sample task and a spatial delayed recognition memory span task. A cognitive impairment index (CII) for each monkey is derived from these measures (Peters et al., 2008). The identification number, age and CII of each monkey used in this study are shown in Table 1. Briefly, the monkeys were pre-anesthetized with ketamine (0.5 mg/kg), after which sodium barbitol was administered i.v. (15 mg/kg to effect) until the monkey was deeply anesthetized and a state of areflexia attained. Each monkey was then intubated and artificially respired with a mixture of 5% CO_2 and 95% O_2. The chest cavity was opened and the monkey perfused intracardially with a warm solution of 1% paraformaldehyde and 1.25% glutaraldehyde in either 0.1 M cacodylate or phosphate buffer at pH 7.4. The brain was then removed and one hemisphere stored in a cold solution of 2% paraformaldehyde and 2.5% glutaraldehyde in the same buffer used for the perfusion. The perfusions were carried out in full accordance with the approved Institutional Animal Care and Use Committee Regulations, and in accordance with the NIH Publication *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of animals used and their suffering.

Several pieces of cortex were removed from the lower bank inside the sulcus principalis of each monkey, at the level of the corpus callosum. This portion of the prefrontal cortex is area 46. The pieces of cortex were then osmicated, dehydrated, stained en bloc with uranyl acetate and embedded in araldite.

Table 1. Identification number, age and cognitive impairment index (CII) of monkeys used in this study

Monkey identification number	Age (y)	CII
AM16 (young)	5	No data
AM76 (young)	6	0.08
AM129 (young)	7	1.87
AM47 (young)	9	0.51
AM53 (young)	10	0.32
AM19 (old)	25	1.98
AM12 (old)	27	3.31
AM62 (old)	27	3.81
AM26 (old)	29	1.05
AM41 (old)	32	4.51

Preparation of sections and photography

The araldite embedded tissue blocks from area 46 were sectioned in a plane at right angles to the pial surface, so that the apical dendrites of pyramidal cells were sectioned along their lengths, as evidenced by examining 1 μm thick sections that had been stained with Toluidine Blue for light microscopic examination. Thin sections were then taken, mounted on copper grids, and stained with Lead Citrate. The location of layer 2/3 was determined and electron micrographs were taken of random samples of neuropil and of axon terminals forming symmetric synapses with the perikarya of pyramidal cells in layer 2/3. Axoaxonic synapses were excluded from this study because they are too sparse to sample readily. All micrographs were taken at an initial magnification of $\times$6000. Some of the micrographs of the neuropil used in this study were the same ones that had been used in our previous study of the effects of age on the numerical density of synapses in layer 2/3 (Peters et al., 2008). These micrographs had been printed photographically at a final magnification of $\times$12,500. Other micrographs of neuropil and the ones of axon terminals forming symmetric axosomatic synapses on the cell bodies of layer 2/3 pyramidal cells were scanned on an Epson Perfection V700 photo scanner using Epson scan plug-in software for Photoshop and saved as 150 dpi tif files. Prints from these scanned images were made at a magnification of $\times$12,500 using a Kodak Professional 9810 Digital Photo Printer.

Measurements

To measure the sizes of axon terminals forming symmetric synapses in the neuropil of layer 2/3 in area 46, the outlines of 426 such axon terminals profiles from the five young monkeys and 323 profiles from the five old monkeys were traced from the $\times$12,500 photographic prints onto acetate sheets. The outlines of the mitochondria within these terminals were also drawn, as well as the lengths of their synaptic junctions. Axon terminals forming symmetric axosomatic synapses with the cell bodies of pyramidal cells in layer 2/3 were similarly examined. Some 30 axon terminals from each of the monkeys were traced, so that a total of 159 terminals forming axosomatic synapses in the young monkeys and 150 terminals from the old monkeys were traced. The numbers of sampled axon terminals forming axosomatic synapses were lower than those forming axodendritic synapses because axosomatic synapses are less frequent than axodendritic synapses. In order to test the presumption that the numbers of sampled axon terminals are sufficient for accurate and reliable statistical analysis, we carried out a post-hoc analysis of achieved power using the G*Power3 software (Faul et al., 2009). Based on the group means, standard deviations and sample sizes, the numbers of sampled axon terminals forming axodendritic synapses or axosomatic synapses are sufficient to reach a power 1-β>0.8 (axodendritic: size effect=0.3845; 1-β=0.999 and axosomatic: size effect=0.3018; 1-β=0.8415).

The tracings of axon terminals and the mitochondria they contained were scanned on an Epson Perfection V700 photo scanner using the Epson scan plug-in for Photoshop CS3 and saved as 150dip tif files. These scans were imported into ImageJ 1.40g (Wayne Rasband, National Institutes of Health) and the areas of the profiles of the terminals and their mitochondria measured and expressed in square microns. These data were copied to, and organized in Excel X for Mac (Microsoft). Ultimately analyses and graphs were constructed using Prism 4 for Macintosh (version 4.0c, GraphPad Software, Inc).

Counts of the numbers of profiles of synaptic vesicles in axon terminals, the sizes (areas) of which had been previously deter-