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Increase of the ornithine decarboxylase/polyamine system and transglutaminase upregulation in the spinal cord of aged rats

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Abstract

We have investigated changes in ornithine decarboxylase (ODC) activity and in polyamine levels in the central nervous system of aged rats. We measured a significant increase of ODC catalytic activity in the spinal cord from 30 month-old rats (+105%) as compared to 4 month-old rats. No changes were noticed in the cerebellum, cortex and hippocampus from the same animals. A related putrescine increase was measured in the spinal cord of 30 month-old rats (+168%), together with a smaller increase of spermidine (+33%). A parallel increase (+78%) of the Ca^{2+}-dependent transglutaminase activity was detected in the spinal cord of 30 month-old rats, while no changes were apparent in the cortex and cerebellum. Our observations indicate a possible role of the ODC/polyamine system during the normal process of ageing in rats and point to the spinal cord as the most sensitive area for this kind of modification. A possible role of protein polyamination by tranglutaminase is discussed.

Keywords: Ageing; Rat; Spinal cord; Polyamines; Ornithine decarboxylase; Tissue transglutaminase

Polyamines are polycationic compounds largely distributed both in eukariotic and prokariotic cells, where they are involved in cell proliferation and differentiation. The biosynthesis of natural polyamines is under the tight control of ornithine decarboxylase (ODC), the rate-limiting enzyme that converts ornithine to putrescine. This diamino compound is then enzymatically converted into the two higher-order polyamines, spermidine and spermine [18]. In the central nervous system (CNS), a role for polyamines during the development is supported by the fact that ODC activity and, in parallel, putrescine levels peak during pre- and/or post-natal phases of neurogenesis and nerve cell differentiation, declining then towards the very low levels of the adulthood [15]. However, the ODC/polyamine system of the mature brain remains very sensitive to a variety of harmful stimuli and is transitorily induced in pathological states [2,11]. It is still debated whether this upregulation of the ODC/polyamine system is neuroprotective or whether polyamines may contribute to neuropathology [3,4,11]. The recent demonstration that polyamine accumulation induces apoptosis [13] while polyamine depletion delays apoptosis [14], adds more controversial results to the above issue. Poor evidence exists concerning the possible consequences of normal brain ageing on polyamine metabolic alterations or on their regional distribution and concentration in humans [9,10]. Experiments with transgenic mice overexpressing ODC did not show enhanced neurodegeneration with ageing [1]. Other animal data are lacking.

In addition to the rapidly inducible ODC/polyamine system, tissue transglutaminase (tTG) has been demonstrated to be another enzymatic activity induced in the brain under pathological conditions [7]. Tissue transglutaminase is a Ca^{2+}-dependent enzyme that catalyzes protein cross-linking through a reaction between a glutamine residue and a lysine residue, present in adjoining substrate proteins or in different parts of the polypeptide chain of the same protein [5]. Cross-linking of structural proteins has been deemed to be important in promoting the formation of insoluble protein complexes that are characteristic of neurodegenerative apoptotic processes [7]. For instance, tTG is overexpressed and is enzymatically active on specifically-altered proteins in brain samples from cases of Alzheimer's and Huntington's diseases [6,7]. It has been also shown that tTG catalyzes the incorporation of polyamines into various proteins by linking them to polypeptide-bound glutamine residues [7]. It is, therefore, very likely

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that increased availability of free polyamines in the brain tissue may result in increased rate of polyamination of several protein substrates of tTG, with possible induction of neuropathological processes. While the above-mentioned reports have provided interesting data on alterations of tTG expression and activity in human neuropathologies, no report has addressed so far the issue of possible modifications occurring with brain ageing either in humans or in laboratory animals.

The lack of information concerning alterations of both ODC/polyamine system and tTG in animal models of ageing, has prompted us to investigate these neurochemical markers in various regions of the CNS of young-adult and aged rats. We show that both systems are upregulated in the spinal cord of aged rats, while no apparent differences are present in brain regions such as the cortex, hippocampus and cerebellum.

Male Wistar rats aged 4 or 30 months and raised under microbiological barrier, were purchased from Harlan Italy and used within 1 or 2 weeks after reception. During this period, the animals were housed in conditions of controlled temperature and lighting with free access to standard food and water. Some experiments on ODC assay were also replicated on samples from groups of Wistar rats, respectively of 4 and 22 months of age, obtained from the colony bred in the facility of the National Institute for Aging Care and Research (INRCA, Ancona, Italy). Sacrifice of the animals was done according to the guidelines established by the Italian law on the use of animals for research and under veterinary control. Rats were killed by decapitation; portions of the cervico-thoracic spinal cord, cerebellum, cortex and hippocampus were rapidly dissected, frozen in solid CO_2 and stored at -80°C until use. For ODC assay, tissue from the various CNS areas was homogenized in ice-cold 50 mM Tris–HCl (pH 7.5) containing 0.1 mM EDTA, 5 mM dithiothreitol and 0.04 mM pyridoxal-5-phosphate. The homogenate was centrifuged at $20{,}000 \times g$ for 20 min and aliquots of the supernatant were assayed by measuring the $[^{14}C]CO_2$ released from [^{14}C]ornithine (NEN, specific activity 40–60 mCi/mmol, final concentration 0.05 mM) and trapped by hyamine hydroxide [16].

For polyamine determination, samples were sonicated in five to ten volumes of 0.3 N $HClO_4$ and centrifuged at $11{,}000 \times g$ for 20 min. The pellet was dissolved in 0.5 N NaOH for protein content determination, while aliquots of the supernatant were collected for HPLC separation and quantification after dansyl chloride derivatization [17]. Briefly, 300 μl samples were added to 20 μl of 100 μM 1,8-Diaminooctane, as an internal standard, 300 μl of 0.3 N $HClO_4$, 20 μl of 3 N KOH and 180 μl of 1.5 M Na_2CO_3. After adding 0.8 ml of dansyl chloride (10 mg dissolved in 1 ml acetone), the mixture was left in the dark overnight. At the end of the dansylation, 200 μl of 0.3 N KOH were added and the dansylated polyamines were extracted with 1 ml of diethyl ether, dried and resuspended in 100–500 μl of methanol. 20 μl of this sample were injected in an HPLC equipped with a reverse phase C_{18} column and with a fluorimetric detector (excitation: 340 nm; emission: 510 nm). Polyamines were eluted at 1 ml/min with a step gradient ranging from 60% A (50% water, 30% acetonitrile and 20% methanol) to 80% B (60% acetonitrile and 40% methanol) in 16 min.

As a measure of tTG catalytic activity, we evaluated the Ca^{2+}-dependent incorporation of [^{14} C] putrescine into casein as an exogenous protein substrate, as described [12] with some modifications. In all biochemical experiments, protein content was evaluated through the method of Lowry et al. [8]. For transglutaminase immunohistochemistry, 4 and 30 month-old male rats (two animals each) were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.3. Cervical spinal cords were immediately excised, postfixed in the same fixative for 3 h and cryoprotected in 30% sucrose for 3 days. The spinal cords were sectioned serially into 30 μm thick transverse sections with a freezing microtome. Two of every six sections were processed for tissue transglutaminase immunohistochemistry using an anti-mouse monoclonal antibody against tissue transglutaminase (NeoMarkers, CA) diluted 1:200 in 0.1 M PBS containing 0.1% NaN_3 and 0.5% Triton X-100. The primary antiserum was detected by the biotin-streptavidin technique according to the procedure suggested by the supplier (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), using 3-3′-diaminobenzidine tetrahydrochloride as chromogen. As a specificity control, some sections were incubated with normal mouse serum instead of the primary antibody. No staining was observed in these sections.

ODC activity exhibited the low levels usually found in adult rats in both the cerebellum and cortex, not showing any significant alteration in the same regions of aged (30 month-old) rats (Fig. 1). In the spinal cord, however, the level of enzymatic activity was remarkably higher (by a factor of approximately threefold as compared to the cerebellum and cortex) in adult rats and the activity was almost doubled in aged rats (Fig. 1). A similar result was obtained

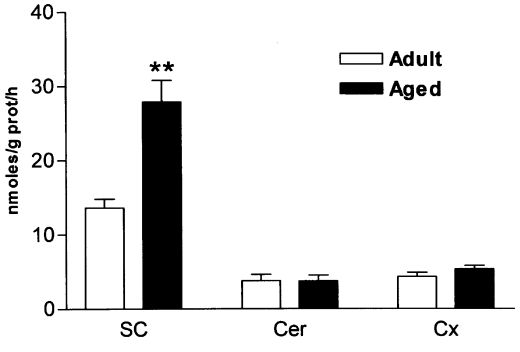

Fig. 1. Activity of ornithine decarboxylase (ODC) in various regions of the CNS in adult and 30 month-aged rats (SC, spinal cord; Cer, cerebellum; Cx, cortex). Bars represent the mean ± SEM of 11–14 experiments; $^{**}P < 0.01$, Student's *t*-test.

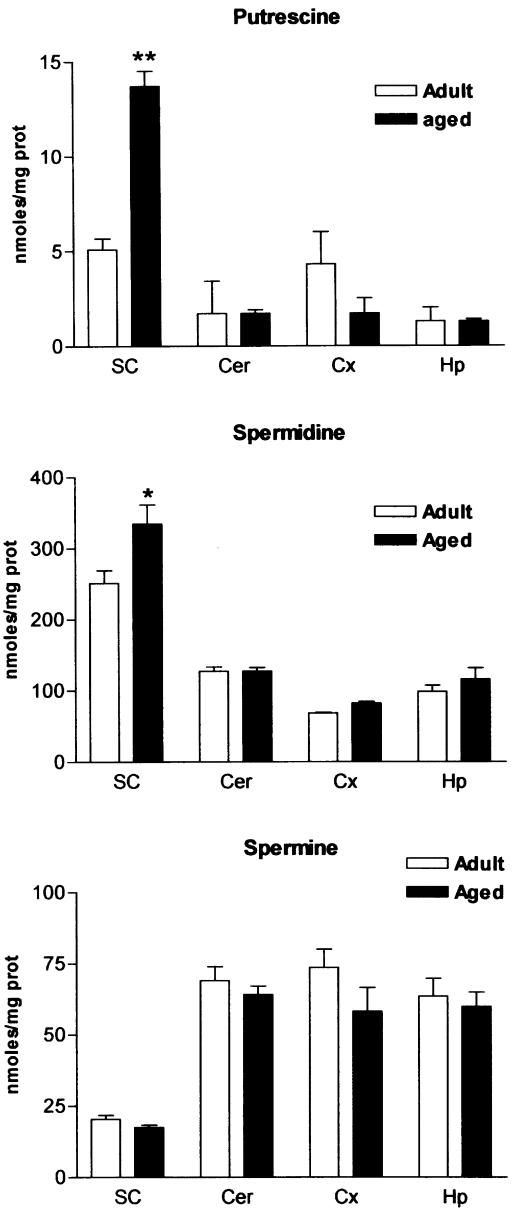

Fig. 2. Polyamine content of various regions of CNS in adult and 30 month-aged rats (SC, spinal cord; Cer, cerebellum; Cx, cortex; Hp, hippocampus). Bars are the mean ± SEM of 6-11 experiments; $*P < 0.05$; $**P < 0.01$, Student's *t*-test.

in the spinal cord of adult and intermediately-aged rats from a different breeding (4 month-old rats: 16.3 ± 1.5 nmol/g prot/h, 22 month-old rats: 31.2 ± 2.7 nmol/g prot/h; $n = 11$, $P < 0.01$ Student's *t*-test).

The putrescine level in the spinal cord of aged rats was found dramatically increased in comparison to adult animals (+168%), while no significant differences were apparent in the cerebellum, cortex and hippocampus. In the spinal cord of aged rats, also spermidine level was significantly increased (+33%) in comparison to adult rats (Fig. 2). Spermine levels were unchanged between adult and aged animals in all the CNS areas examined (Fig. 2).

Ageing had a clear effect also on the catalytic activity of tTG in the spinal cord, the level of this enzymatic activity in aged rats being increased by about 80% with respect to the one measured in adult controls (Fig. 3). The catalytic activity resulted, instead, unchanged in the cortex and cerebellum of aged rats (data not shown). After immunoreaction for tTG, the grey matter of the cervical spinal cord showed diffuse staining in both adult and aged rats (Fig. 4a,b). However, in the ventral horns of aged rats more motoneurons were intensely stained than in adults (Fig. 4a–c). No stained cells were present in the dorsal horns of both animal groups (not shown). The white matter, showed increased staining in fibrous and perivascular structures of aged rats as compared to the adult ones (Fig. 4a,b). Furthermore, positive glial cells intermingled with the fiber bundles of the white matter, were clearly observed in aged rats (Fig. 4d), while being apparently absent in adult rats.

The main finding of this study is that the spinal cord of aged rats shows a selective derangement of the ODC/polyamine system which brings to increased activity of the key enzyme for polyamine synthesis and to consequent accumulation of putrescine, and spermidine to a lesser extent. In parallel, also the activity and expression of tTG, of which polyamine are a natural substrate, are substantially increased in the same region of the aged rats, thus suggesting an increased level of protein polyamination and cross-linking in the spinal cord of aged rats.

In agreement with previous data in aged humans [9,10], we found no age-dependent differences in ODC activity and polyamine content in the forebrain and cerebellum. However, by extending our investigation to the spinal cord, we were able to demonstrate a large increase in ODC activity in this region of aged rats and to observe a related, dramatic rise in putrescine and, to a lesser extent, in spermidine concentrations. This is, at least to our knowledge, the first time that the ODC/polyamine system is reported to be activated not in response to acute stressful conditions, but in relation to a slowly-evolving process like senescence, and in the absence of any overt neuropathology. The fact that we measured a similar trend of increase of ODC activity also in a group of intermediately-aged rats from a different

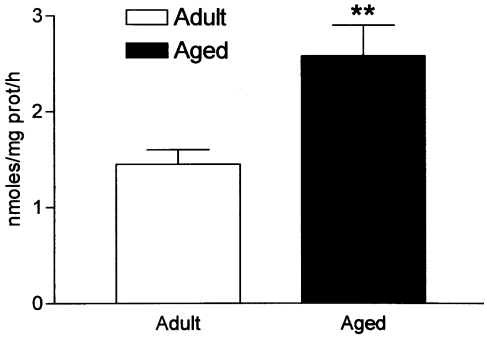

Fig. 3. Tissue transglutaminase (tTG) activity in the spinal cord of adult and 30 month-aged rats. Bars are the mean ± SEM of 18 experiments; $**P < 0.01$, Student's *t*-test.

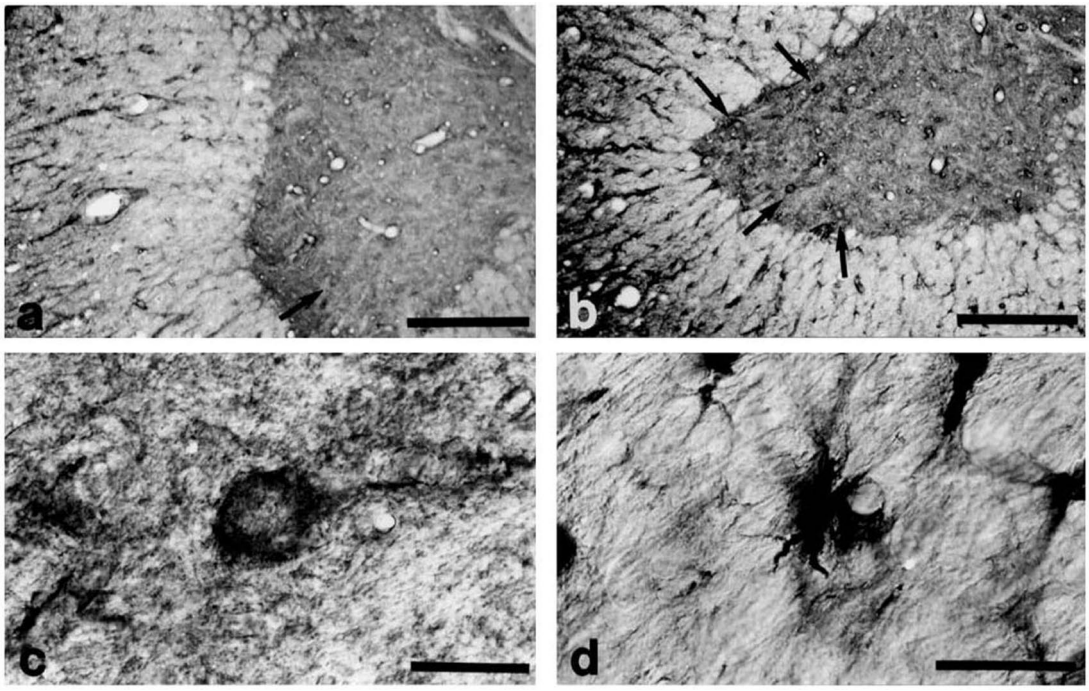

Fig. 4. Ventral horns of the cervical spinal cord after immunoreaction for tissue transglutaminase. (a) Four month-old rat. The gray matter is diffusely stained. The large motoneurons are unstained or only slightly stained (arrow). In the white matter, nerve fibers are weakly stained. Scale bar: 300 μm. (b) Thirty month-old rat. Note that several large motoneurons are strongly stained (arrows). Also in the white matter nerve fibers are apparently more intensely stained than in young-adult rats. Scale bar: 300 μm. (c) Higher magnification of a motoneuron in an aged rat. Scale bar: 30 μm. (d) Higher magnification of the white matter in an aged rat showing a strongly labeled glial cell. Scale bar: 30 μm.

breeding, suggests that this perturbation of the ODC/polyamine system, specifically localised in the spinal cord, starts at relatively early stages in the course of the ageing process. It is tempting to speculate whether the increased ODC activity and rise in putrescine content detected in the aged spinal cord, mirrors a senescence-related, subtle pathological condition preferentially occurring in the spinal cord as compared to other CNS regions. A previous study on the neurochemistry of ageing has, indeed, demonstrated that the spinal cord of aged rats shows a widespread decrease in the activity of several neurochemical markers [19]. These alterations may be related to the impairment of motor performances commonly occurring in aged animals.

In order to investigate in the spinal cord of aged rats alterations of other biochemical systems that could be functionally linked to the upregulated polyamine system, we studied the catalytic activity and the expression of tTG, that has been reported to be involved in neurodegenerative damage and apoptosis through its action of polyamination and cross linking of proteins [7]. Our finding of a large increase of tTG catalytic activity and protein expression in the spinal cord of aged rats suggests that this alteration, combined with the increased polyamine availability, gives rise to a condition potentially dangerous for the biochemical function and the structural integrity of spinal cord neurons. Our data do not clarify whether the observed rise of tTG is dependent on ODC activation and putrescine elevation or whether ODC and tTG increased activities are both consequences of other neurodegenerative processes. A direct control of tTG activity by polyamines cannot be excluded in the light of previous studies demonstrating that polyamine depletion reduces tTG activity and delays apoptosis in intestinal epithelial cells [14,20].

In conclusion, we have reported here for the first time evidence for an increased activity of the ODC/polyamine system and tTG activity and expression in the spinal cord during normal ageing in rats. These concomitant neurochemical alterations may constitute a marker for the ageing process specific for this region of the CNS, as similar modifications were not observed in the various brain areas examined. The fact that these neurochemical systems are known to be involved in various neurodegenerative conditions makes it interesting to further investigate their physiopathological role in the ageing process of the spinal cord.

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