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Neurobiology of Aging 30 (2009) 973–986

NEUROBIOLOGY OF AGING

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Aging effects on the dopamine transporter expression and compensatory mechanisms

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Received 6 June 2007; received in revised form 31 August 2007; accepted 19 September 2007
Available online 31 October 2007

Abstract

Several studies report that the striatal dopamine (DA) uptake declines with age, but the underlying mechanisms are still unclear. The use of molecular, biochemical and morphological techniques, and antibodies which detect the glycosylated (80 kDa) and non-glycosylated (50 kDa) DA transporter (DAT) forms in the rat mesostriatal system, reveals that DAT is pre- and post-translationally damaged during aging. In middle age (18 months), the glycosylated DAT form decreases in the plasma membrane of striatal terminals, and the non-glycosylated form is accumulated in the endoplasmic reticulum–Golgi complex. Thereafter, in aged rats (24 months), DAT synthesis is also affected as the decrease in both DATmRNA and total DAT protein levels suggests. However, the evidence of a decrease in both DAT expression in the endosomal (vesicle-enriched) compartment and the phosphorylated DAT fraction from middle age, as well as its compartmental redistribution towards the terminal plasma membrane, with an increase in the membrane DAT/total DAT ratio in striatal synapotosomes, in aged rats, indicate that DA-cells activate compensatory mechanisms directed at maintaining DAT function during normal aging.

Keywords: Dopaminergic; Dopamine uptake; Subcellular fractionation; DAT regulation; Vulnerability; Elderly

1. Introduction

Data from different mammals including humans show that dopaminergic (DA)-mesostriatal neurotransmission undergoes a progressive decline during normal aging, and that this phenomenon affects the synthesis, release and reuptake of dopamine (DA) (Allard and Marcusson, 1989; Carfagna et al., 1985; Gerhardt et al., 2002; Haycock et al., 2003; Hebert and Gerhardt, 1999; Kish et al., 1992; Salvatore et al., 2003). Although age-related decrease of DA reuptake has been found in rats (Hebert and Gerhardt, 1999; Salvatore et al., 2003) and humans (Erixon-Lindroth et al., 2005; van Dyck et al., 2002), the basic mechanisms involved in this process are still unclear. DA is taken back into the terminals by the dopamine transporter (DAT), a 12 transmembrane domain protein which belongs to a family of Na^+/Cl^--dependent transporters (Giros and Caron, 1993; Horn, 1990; Shimada et al., 1991; Uhl, 2003), and is highly regulated at post-translational level (Li et al., 2004; Melikian and Buckley, 1999; Vaughan et al., 1997). Some studies propose that the DA reuptake decrease is due to a DAT redistribution away from the plasma membrane without changes in its steady-state levels (Salvatore et al., 2003). Others report a depletion in DATmRNA and protein levels (Bannon et al., 1992; Bannon and Whitty, 1997; Haycock et al., 2003; Himi et al., 1995; Ma et al., 1999) suggesting that pretranslational mechanisms are also involved. Interestingly, a comparative analysis between the decrease in DAT function and DATmRNA reveals an increase in the DAT binding/DATmRNA ratio with age (Bannon et al., 1995). This suggests that DA-cells activate post-translational compensatory mechanisms

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0197-4580/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.neurobiolaging.2007.09.009

directed at maintaining DA uptake, although these compensatory mechanisms have not been explored to date.

In order to gain a better understanding of the DA uptake regulation during normal aging, and particularly of the mechanisms directed at maintaining DA uptake, we have investigated the pre- and post-translational processing of DAT in the rat mesostriatal system during normal aging. In summary, our results show that DAT is affected at both levels. First (in middle age), it undergoes deglycosylation, expression decrease in the plasma membrane and accumulation in the endoplasmic reticulum–Golgi (ERG) complex. At a later stage (in aged rats), DAT synthesis is also affected, as revealed by the loss in both DAT messenger and total protein levels. However, the evidence of a decrease in its vesicular (endosomal) expression and phosphorylated form from middle age, and an increase in the membrane DAT/total DAT ratio in striatal synaptosomes of aged rats, indicate the activation of compensatory mechanisms directed at maintaining DA uptake.

2. Materials and methods

A total of 15 young (6 months), 15 middle-aged (18 months) and 15 aged (24 months) outbreed male Sprague–Dawley rats supplied by Charles River (Cedex, France) were used in this study. Five rats per group were used in qRT-PCR, Western-blot in midbrain and whole striatal protein extracts and catecholamine analysis; five in the morphological study (immunohistochemistry and in situ hybridisation), and five in experiments performed in synaptosomes (DA uptake, subcellular fractionation, plasma membrane biotynilation and immunoprecipitation). Samples from each animal were individually analysed ($n = 5$). Experimental protocols were approved by the Ethical Committee of the University of La Laguna, and are in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the care and use of animals for experimental procedures.

2.1. Quantitative RT-PCR and Western-blot analysis

The rats were sacrificed by decapitation and the brains were quickly removed. Ventral midbrains containing A9 and A10 DA-cell groups (between 3.00 mm and 4.00 mm rostral to the interaural axis) (Paxinos and Watson, 1998) corresponding to the substantia nigra (SN) and ventral tegmental area (VTA), respectively (Dahlström and Fuxe, 1964), and striata (between 10.00 mm and 8.60 mm rostral to the interaural axis) (Paxinos and Watson, 1998) were dissected in ice using a brain blocker. Samples from the left and right hemispheres were pooled, and RNA and proteins were extracted using the acid phenol method. Two micrograms of total RNA were reverse transcribed after heat denaturation (65 °C, 5 min) and annealing of oligo-dT primers for qRT-PCR. AMV-reverse transcriptase (Roche Diagnostics, Mannheim, Germany) was used for cDNA synthesis, under the conditions recommended by the manufacturer, in a final volume of 20 μl. The real-time assay was performed using a 96-well Opticon 2 cycler, the Opticon software (MJ Research, Waltham, MA), and the iQ™ SYBR® Green Supermix RT-PCR kit (BioRad, Hercules, CA). Twenty-five microlitre reactions were pipetted in each well, containing 1 μl of DNA template, 0.2 μM of each primer, 12.5 μl of the SYBR® Green reagent and 11 μl of sterile Milli-Q water. Amplification conditions were (1) incubation step at 95 °C (3 min); (2) DNA amplification for 35 cycles at 94 °C (1 min), 55.5 °C (45 s) and 72 °C (45 s). Primer sequences for DAT and reference genes (RPS18 and GAPD) are summarized in Table 1. The threshold cycles were calculated by plotting normalized fluorescence in relation to the cycle number, and the amplification efficiency of each primer pair was established using standard cDNA dilutions. The normalized expression of each gene was calculated using the *Q-Gene* software (Muller et al., 2002). All assays were performed in triplicate and with five animals per group.

For Western-blot, protein concentration was determined using the bicinchoninic acid method and bovine serum albumin as standard. Midbrain (70 μg) and striatal (50 μg) protein samples were diluted in Laemmli's loading buffer (62.5 mM Tris–HCl, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% β-mercaptoethanol and 0.05% bromophenol blue, pH 6.8), denatured (90 °C, 5 min.), separated by electrophoresis in 10% SDS-polyacrylamide gel, and transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Blots were blocked for 2 h at room temperature (RT) with 5% non-fat dry milk in TBST (250 mM NaCl, 50 mM Tris, pH 7.4, and 0.05% Tween20), and incubated overnight at 4 °C in blocking solution with one of the anti-DAT antibodies described below. After several rinses in TBST-5% milk, the membranes were incubated for 1 h in horseradish peroxidase conjugated anti-rabbit (1:10,000), or horseradish peroxidase conjugated anti-rat (1:10,000) IgG (Jackson-ImmunoResearch, West Grove, PA). Immunoreactive bands were visualized using enhanced chemiluminiscence (ECL; Amersham, Arlington, Heights, IL) and film exposure (Kodak Biomax, MR, Eastman Kodak, Rochester, NY). Different protein quantities, antibody dilutions and exposure times were tested to estab-

Table 1
Primers used in q-PCR

Genes	Gene reference	Forward (5′–3′)	Reverse (5′–3′)	cDNA product
DAT	NM_012694	AGC TAC CAT GCC CTA TGT GG	ATC CAC ACA GAT GCC TCA CA	129 bp
Rps 18	NM_213557	GTC ATC CCC GAG AAG TTT CA	TTG GTG AGG TCA ATG TCT GC	149 bp
GAPD	XM_579386	AAG GTC ATC CCA GAG CTG AA	CTG CTT CAC CAC CTT CTT GA	138 bp