Rat hippocampal GABAergic molecular markers are differentially affected by ageing

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Abstract

We previously reported that the pharmacological properties of the hippocampal GABAA receptor and the expression of several subunits are modified during normal ageing. However, correlation between these post-synaptic modifications and pre-synaptic deficits were not determined. To address this issue, we have analysed the mRNA levels of several GABA-ergic molecular markers in young and old rat hippocampus, including glutamic acid decarboxylase enzymes, parvalbumin, calretinin, somatostatin, neuropeptide Y and vasoactive intestinal peptide (VIP). There was a differential age-related decrease in these interneuronal mRNAs that was inversely correlated with up-regulation of the a1 GABA receptor subunit. Somatostatin and neuropeptide Y mRNAs were most frequently affected (75% of the animals), then calretinin and VIP mRNAs (50% of the animals), and parvalbumin mRNA (25% of the animals) in the aged hippocampus. This selective vulnerability was well correlated at the protein/cellular level as analysed by immunocytochemistry. Somatostatin interneurons, which mostly innervate principal cell distal dendrites, were more vulnerable than calretinin interneurons, which target other interneurons. Parvalbumin interneurons, which mostly innervate perisomatic domains of principal cells, were preserved. This age-dependent differential reduction of specific hippocampal interneuronal subpopulations might produce functional alterations in the GABAergic tone which might be compensated, at the post-synaptic level, by up-regulation of the expression of the a1 GABAA receptor subunit.

Keywords: calcium-binding proteins, GABAA receptor, interneurons, neuropeptides, reverse transcriptase competitive polymerase chain reaction.


Inhibitory interneurones constitute a heterogeneous cell population that have a powerful inhibitory effect on excitatory cells and other interneurones, contributing to the generation of oscillatory activity which seems to be important for the integration of synaptic inputs and memory formation (Buzsaki and Chrobak 1995; Paulsen and Moser 1998). In rat hippocampus, interneurones have been classified according to different criteria (morphology, location, innervation, specific protein expression) (Buzsaki and Chrobak 1995). The existence of such cell diversity raises questions about their function. Indeed, there is a relationship between the selective synaptic targeting of different domains of post-synaptic cells and the expression of specific molecular markers (see Freund and Buzsaki 1996 for review). Most perisomatic inhibitory cells express either parvalbumin (PV) or cholecystokinin and vasoactive intestinal polypeptide (VIP); mid-proximal dendritic inhibitory cells express calbindin D28k; distal dendritic inhibitory cells express somatostatin (SOM) and 60–70% of this population also express neuropeptide Y (NPY); and interneurones that specifically innervate other interneurones express CR and, frequently, VIP (Nunzi et al. 1985; Feucht et al. 1999; Maccaferri et al. 2000).

At the post-synaptic level, GABA_A receptors are formed by particular heteropentameric combination of α, β, γ, δ, ε, π and θ subunits, which confers specific kinetic and pharmacological properties (Sieghart 1995; Sigel et al. 1990). Moreover, single-cell RT–multiplex PCR has revealed that a single neurone can express several isoforms of the different subunit families (Ruano et al. 1997), supporting

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Abbreviations used: CaBP, calcium-binding protein; CR, calretinin; GAD, glutamic acid decarboxylase; HIPP, hilar perforant path-associated; NPY, neuropeptide Y; O-LM, oriens/lacunosum molecular; PV, parvalbumin; SOM, somatostatin; VIP, vasoactive intestinal polypeptide.
the hypothesis that GABA\textsubscript{A} receptors of different molecular composition might mediate GABAergic transmission by different interneuron subunits (Nusser et al. 1995; Tietz et al. 1999; Maccaferri et al. 2000). However, the precise location of the GABA\textsubscript{A} receptors in relation to synaptic inputs is poorly understood (Nyiri et al. 2001).

We have recently demonstrated the existence of an age-dependent up-regulation in the expression of several GABA\textsubscript{A} receptor subunits (Ruano et al. 2000). These modifications were interpreted as a post-synaptic adaptive response, occurring during normal ageing, as a consequence of deficits in the GABAergic inputs. In the present study we have addressed this question directly by quantifying, by RT-competitive PCR and immunocytochemistry, the expression of several pre-synaptic markers of GABAergic neurones. These markers included glutamic acid decarboxylase (GAD) 65 and GAD67, as markers of the whole GABAergic population, and the calcium-binding proteins (CaBPs) and neuropeptides PV, CR, SOM, NPY and VIP, as markers of specific subpopulations of GABAergic neurones. Moreover, we have extended our previous work in order to clarify relationship between the pre- and post-synaptic modifications. Use of RT–competitive PCR and immunocytochemistry has allowed us to obtain a global view of the cellular and molecular modifications occurring within the GABAergic system during normal ageing.

Materials and methods

Isolation of hippocampus

Adult (3 months) and aged (24 months) Wistar rats were killed by decapitation and both hippocampi (150–160 mg wet-weight) were dissected, frozen in liquid N\textsubscript{2} and stored at -80°C until use. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the committee of animal use for research at Sevilla and Malaga Universities. All efforts were made to minimize the number of animals used and their suffering.

RNA extraction

Total RNA was extracted using the TriPure\textsuperscript{TM} Isolation Reagent kit (Roche Molecular Biochemicals, Germany), according to the instructions of the manufacturer. The contaminating DNA was removed by incubation with Dnase (Sigma, St Louis, MO, USA) and confirmed by PCR analysis of total RNA samples before RT. Integrity of the RNA samples was assessed by agarose gel electrophoresis. The yield of total RNA was determined by measuring the absorbance at 260/280 nm. As a control, samples from adult and aged hippocampi were reverse transcribed as above but in the presence of 2 \( \mu \)M digoxigenin–dUTP, dotted on nylon membranes and developed. The mean ± SD aged/adult ratio of 1.08 ± 0.25 (\( n = 5 \)) demonstrated a similar RT efficiency for both ages (Ruano et al. 2000). As an additional control, β-actin was amplified in adult and aged rats, for which the aged/adult ratio was 0.99 ± 0.11 (\( n = 15 \)).

Competitive PCR was performed essentially as described previously (Ruano et al. 2000). Briefly, 100 ng cDNA was mixed with an increasing amount of each internal standard. Each internal standard was synthesized as described by Vela et al. (2001) and was different in size with respect to the specific PCR product. The range of internal standards used was established previously in control experiments using adult hippocampus.

The PCR reaction was performed using 1 \( \mu \)M each of 5’ and 3’ primers from the following set (from 5’ to 3’): GAD65 5’: TCTT-TTCTCCGTGGTGTTGCC, GAD65/67 3’: CCCCCAGCACAT- CACAT (one mismatch with the sequence of GAD67, indicated by the underlined base; Bochet et al. 1994) (position 713 and 1085); GAD67 5’: TACGGGGTTCGCACAGGTC (position 1159); SOM 5’: ATCGTCCCTGGCCTTGGGG, SOM 3’: GCCCTACATCTGCTT- GCTCA (position 43 and 231); NPY 5’: GCCCAGAGCAC- CACC, NPY 3’: CAAGTTTTACATTCCCATACCA (position 45 and 292); VIP 5’: GCCGTAGCAGGACAATGACA, VIP 3’: CCTCACGTCTCCTCTCCCA (position 167 and 434); PV 5’: AAGAGTCCGGATGATGTGGAAGA, PV 3’: ATTTGTTCCAC- CATTTCACC (position 115 and 480); CR 5’: CTTGAGAGGG- TACTGGAAATGACA, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085); β-actin 5’: CTGGAGAAAGG- CAAGAAAGGT, CR 3’: AGTGTGCCTAAGGACGHTG (position 142 and 429); β-actin 5’: CGGAACCCTCATTGCC, β-actin 3’: ACCCACACTGTCCTGGGTA (position 713 and 1085).

RT-competitive PCR

RT was performed in a final volume of 20 \( \mu \)L using 1 \( \mu \)g total RNA as template. After RT, samples were treated with Rnase and cDNA was purified using Microcon PCR cartridges (Millipore, Corporation, Bedford, MA, USA) and quantified by measuring the absorbance at
and aged animals were incubated overnight at room temperature (22°C) with one of the following primary antibodies: CR goat polyclonal antiserum (Swant Bellinzona, Switzerland) diluted 1:4000, PV monoclonal antibody (Sigma) diluted 1:10 000, or SOM rabbit antiserum (Rodriguez et al. 1987) diluted 1:1000. Sections were rinsed and incubated for 1 h at room temperature in a 1:500 dilution of the corresponding biotinylated secondary antibody: anti-goat IgG (Vector Laboratories), anti-mouse IgG (Dako, Carpinteria, CA, USA); anti-rabbit IgG (Vector Laboratories), for CR, PV and SOM primary antibodies respectively. Finally sections were incubated with extra-avidin–peroxidase complex (1:500) (Sigma) for 2 h at room temperature. Immunoreactive products were visualized by staining with 0.05% 3,3′-diaminobenzidine, 0.01% hydrogen peroxide and 0.03% nickel ammonium sulphate in phosphate-buffered saline. Sections were mounted on gelatin-coated slides, air dried, dehydrated in alcohol, cleared in xylene, coverslipped with DPX resin and studied in a Nikon Microphot FXA microscope (Nikon, Japan) with bright-field illumination. Omission of primary antibody or use of pre-immune serum served as negative controls for all immunocytochemical studies.

Image analysis
The cellular density in the different hippocampal regions (CA1, CA2 and CA3) and dentate gyrus was determined with a computerized image analysis system (Visilog 5.2; Noesis S.A., France). Images of immunostained sections were taken with a Sony CCD video camera (Sony, Japan) connected to a Nikon Labophot microscope. The regional areas (CA1, CA2, CA3 and dentate gyrus) were delimited by (morphological criteria) and only immunopositive cells showing a stained unit area sufficient to ensure that the majority of their volume was contained within the selected area (discriminated by eye) were scored. Cell counting was done in a minimum of six slices per antibody and animal, separated by a minimum of four consecutive slices. Cell density values were obtained by dividing the number of immunoreactive neurones of each type by the total area of the corresponding hippocampal region studied. The density of each type of immunolabelled neurones from different hippocampal regions was averaged and expressed as the mean number of positive cells per mm². It was not our intention to determine the total immunostained cell number in the whole rat hippocampus. We are aware that, if this was the aim, Cavalieri point-counting in combination with three-dimensional optical disector methods would be more appropriate. Our principal interest was to corroborate the molecular data at the morphological and protein levels. However, in order to avoid the potential artifacts related to age-related morphological differences, such as an increase in hippocampal volume, some control measurements were performed. We determined the hippocampal volume, the average area of each hippocampal region and the average area of the scored cells. We observed only a small increase, mean ± SD 8.9 ± 2.2%, in hippocampal volume in aged rats, but this did not account for the decrease in average cell density observed with age (41 ± 19%; see Fig. 4).

Statistical analysis
One-way or multifactor ANOVA followed by Bonferroni post-hoc multiple comparison tests were used. Comparison of the expression levels of all molecular markers in both age groups was done using a discriminant analysis (Mardia et al. 1989).

Results
Age-dependent modifications in the expression of the GABAergic markers
GAD65 and GAD67
The expression of both isoenzymes was significantly reduced in the aged group (ANOVA $F_{1,18} = 15.66$, $p < 0.01$ and $F_{1,17} = 18.10$, $p < 0.01$ for GAD65 and GAD67 respectively). The inter-individual variability in the expression of both isoforms was notably higher in old than in adult rats (Fig. 1a), suggesting a heterogeneous effect of ageing. For all animals tested (Fig. 1b), the age-dependent modification in both GAD65 and GAD67 mRNAs (expressed as percentage variation) was similar. Six aged rats displayed a decrease in the expression of both GAD65 and GAD67 isoenzymes, whereas the other two aged rats showed no differences (numbers 10 and 26). To rule out the possible differences in the amount of starting cDNA, we determined the expression of β-actin in parallel experiments. No differences were observed (Fig. 1b). These results demonstrated the existence of a severe age-dependent reduction in the expression of the specific enzymes for the synthesis of GABA in rat hippocampus. However, this effect was heterogeneous in our aged rat population.

The GABAergic interneurones in rat hippocampus constitute a highly heterogeneous cell population characterized by the expression of different CaBPs and/or neuropeptides (Freund and Buzsáki 1996). We therefore analysed whether the diminution in GAD expression observed in aged hippocampus was reflected in the expression of several interneuronal markers.

Neuropeptides
SOM, NPY and VIP mRNAs were quantified in the same adult and aged rat populations. The interindividual variability in the expression of all three neuropeptides was higher in old than in adult rats (Fig. 1c). The expression of SOM and NPY decreased significantly during ageing in most of the animals examined (ANOVA $F_{1,12} = 15.19$, $p < 0.01$ and $F_{1,12} = 21.26$, $p < 0.01$ for SOM and NPY respectively). For the whole aged population, the decrease in the expression of VIP was not statistically significant. The expression profile of SOM and NPY mRNAs in the aged population was different from that observed for VIP mRNA (Fig. 1c). Expression of SOM and NPY mRNAs declined over a broad range of aged hippocampi, whereas aged rats could be clearly segregated into two groups according to the levels of VIP mRNA: aged animals numbers 1, 12, 18 and 24 showed a remarkable decrease (32.2 ± 7.0 fg per 0.1 μg cDNA $(n = 5)$ vs. 6.1 ± 4.8 fg per 0.1 μg cDNA $(n = 4)$ for adult and aged rats respectively), whereas the other four aged rats, numbers 7, 10, 16 and 26, displayed no change compared with...
adult values (32.2 ± 7.0 fg per 0.1 μg cDNA (n = 5) vs. 35.1 ± 2.6 fg per 0.1 μg cDNA (n = 4) for adult and aged rats respectively). The expression of VIP in the first group of aged rats was significantly different both from that in adults and that in the other aged group (ANOVA $F_{2,13} = 35.53$, $p < 0.01$; Bonferroni $p < 0.05$; Fig. 1c).

### CaBPs

The expression of PV and CR was quantified in the same adult and aged samples. The coefficients of variation for these markers were also higher in aged than in adult rats. The expression of PV mRNA was not significantly modified in the aged population (ANOVA $F_{1,13} = 13.40$, $p < 0.01$). As with VIP, the aged rat population could be separated clearly into two groups according to the expression of CR mRNA (compare Figs 1c and 1d). Again, aged rats numbers 1, 12, 18 and 24 had a significantly lower level of CR expression than rats 10, 26, 16 and 7, and than the adult population (1.5 ± 1.2 fg per 0.1 μg cDNA (n = 4) vs. 9.8 ± 1.0 fg per 0.1 μg cDNA (n = 4) and 13.1 ± 4.8 fg per 0.1 μg cDNA (n = 5) respectively; ANOVA $F_{2,13} = 114.38$, $p = 0.001$; Bonferroni $p < 0.01$). These results suggest the existence of a clear relationship between the expression of VIP and CR in the aged population.

### Relationships between the expression of GAD, neuropeptide and CaBP mRNAs in aged rats

Different molecular markers are frequently co-expressed in the same GABAergic cells (Freund and Buzsaki 1996). We therefore analysed whether the expression of molecular markers that are usually co-localized in the same cell population (CR/VIP and SOM/NPY) were similarly affected in aged rats. We first compared the aged/adult ratio of CR mRNA expression in each aged rat. As shown in Fig. 2a, animals with little or no diminution in VIP mRNA expression also showed little or no diminution CR mRNA expression (numbers 7, 10, 16 and 26). Similarly, animals that had a substantially lower level of VIP mRNA expression showed a similar low level CR mRNA expression (numbers 1, 12, 18 and 24). We also compared the aged/adult ratios for SOM and NPY mRNA expression (Fig. 2b). Apart from rats 1...
and 26, all other aged animals showed a diminution compared with adult values, in the level of expression of both SOM and NPY. However, as also shown in Fig. 2(b), a different degree of expression of these two markers could also be observed within this aged population (i.e. rats 7 and 24). Finally, the level of mRNA for PV, which is frequently expressed alone, decreased exclusively in rats 12 and 24 (Fig. 2c).

The age-dependent variations in the expression of the different markers showed ‘animal-dependent’ specificity. Taking advantage of the fact that the expression of all GABAergic markers in each individual adult and aged rat can be analysed by means of RT–competitive PCR, it is possible to compare individually the age-dependent variation in the expression of the different markers with expression in the total adult population. For this purpose, we applied a discriminant statistical technique to the values for the different molecular markers in individual adult and aged rats. The aged animals were classified according to the existence of significant variations \((p < 0.05)\) compared with the adult values. The degree of alteration was heterogeneous (Fig. 2d). First, in aged rats 10 and 26, the level of GAD65 mRNA was no different from adult values \((2082 \pm 517 \text{ fg} \ (n = 6) \text{ and } 2107 \pm 209 \text{ fg} \ (n = 2))\), for adult and aged rats respectively. Only the expression of SOM showed a decrease in rat 26. The aged rat 10 displayed no modification in the expression of any molecular marker.

Second, aged rats 1, 7 and 16 showed an intermediate decrease in the expression of the enzyme GAD65 \((853 \pm 243 \text{ fg} \ (n = 3))\); 59% decrease with respect to that in adults). In addition, rats 7 and 16 also showed a decrease in the expression of SOM and NPY, and rat 1 in the expression of NPY, CR and VIP.

Third, aged rats 12, 18 and 24 showed a substantial decrease in the expression of GAD65 \((367 \pm 214 \text{ fg} \ (n = 3); 82\% \text{ decrease compared with that in adults})\) and were highly affected, particularly rats 12 and 24, attending to the expression of all molecular markers (SOM, NPY, CR, VIP and PV). The amounts of GAD65 mRNA expressed were significantly different between these three groups of aged rats (ANOVA \(F_{2,13} = 110.40, p < 0.01; \text{Bonferroni } p < 0.01\)).

Importantly, global analysis of mRNA expression in the aged rats revealed that the expression of SOM and NPY mRNAs was more frequently affected by ageing (six of eight aged rats) than the expression of CR and VIP (four of eight) or that of PV (two of eight). Overall, these results demonstrate the existence of a differential vulnerability within the GABAergic system from aged hippocampus.

**Pre- and post-synaptic relationship in the GABAergic system during normal ageing**

During normal ageing expression of the \(\zeta_1\) and \( \gamma_2 \) GABA\(_A\) receptor subunits is up-regulated (Ruano *et al.* 2000). Moreover, in aged rat hippocampus, the \(\zeta_1\)-containing GABA\(_A\) receptors are more sensitive to GABA and/or benzodiazepines (Ruano *et al.* 1995; Griffith and Murchison 1995). These post-synaptic modifications might be a consequence of a pre-synaptic deficit in GABAergic tone. As the same aged animals were used in the previous work (Ruano *et al.* 2000) and in the present study, it was possible to determine directly the relationship between the age-dependent decrease in pre-synaptic molecular markers and the age-dependent up-regulation of the GABA\(_A\) receptor subunit mRNAs. We first quantified the expression of the \(\zeta_1\) GABA\(_A\) receptor subunit mRNAs in aged rats numbers 16,
Age modifications of the GABAergic system

In summary, these results demonstrate that the age-related decreases in the expression of molecular markers observed at the mRNA level are reflected at the protein level and, importantly, a similar differential age-related susceptibility was observed.

Discussion

We have extended and completed our previous studies of the age-related changes of the GABAergic system in rat hippocampus (Ruano et al. 1995, 2000; Gutierrez et al. 1996). The most relevant findings may be summarized as follows: (i) the expression of several molecular markers of different subpopulations of hippocampal interneurons decreased during normal ageing; (ii) the expression of SOM and NPY was more frequently affected than that of CR and VIP, whereas the expression of PV was rarely affected; and (iii) the GABAergic system might be adapted postsynaptically in response to pre-synaptic deficits during normal ageing in rat hippocampus.

Our results demonstrated a progressive decrease in the expression of different molecular markers of the GABAergic system. Indeed, the expression of SOM and NPY was more...
frequently reduced than that of CR and VIP, whereas PV was well preserved in aged rats. However, we also found two aged rats in which the expression of PV was decreased. Importantly, in these two animals, the decrease in PV expression occurred in combination with a decrease in all other GABAergic markers. We are aware that the expression of a particular molecular marker is not restricted to a single interneuronal population. However, it is also true that each marker is mostly expressed by a particular subset of interneuronal populations in hippocampus. Taken that we have analysed expression by RT–competitive PCR, using total RNA extracted from whole hippocampi, the observed age-related modifications in expression of the different molecular markers should reflect modifications in the cell types that more frequently express these markers. Bearing in mind this limitation, our results suggest that the interneurones innervating distal dendrites of the principal cells (mostly SOM- and NPY-positive cells) and interneurones targeting other interneurones (mostly CR- and VIP-positive cells) are firstly affected, whereas the interneurones innervating the perisomatic region of principal cells (PV-positive cells) are less affected, or affected later during the normal ageing process. Therefore, the PV interneuronal subpopulation displays a higher degree of resistance to age-dependent neurodegenerative processes than the SOM and CR interneuronal subpopulations. A higher level of susceptibility of SOM-positive neurones has also been observed recently in experimental temporal lobe epilepsy (Cossart et al. 2001) and in rats subjected to transient cerebral ischaemia (Bering...
et al. 1997). However, the reason for this increased susceptibility remains unknown.

An important question is whether this age-dependent decrease in the expression of different molecular markers reflects a cell degeneration process or, simply, a loss of their phenotypic properties (hypofunctionality). There is a large body of evidence to indicate that the number of principal cells remains unaltered (or slightly decreases) in normal aged hippocampus, even in aged rats that displayed spatial learning deficits (Gallagher et al. 1996; Rapp et al. 1996; Merrill et al. 2001). However, as far as we know, no such information (i.e. stereological counting) is available for non-principal cells. From the present data, we cannot determine whether the decreased expression of molecular markers, and diminution in immunoreactive cell density, reflects selective age-related GABAergic neuronal death or loss of the GABAergic phenotype (i.e. molecular markers). Several lines of evidence strongly support the existence of GABAergic cell loss during ageing: (i) two different molecular markers that frequently co-localize in the same cell type (such as SOM and NPY or CR and VIP) were similarly affected in most individual aged rats; (ii) expression of both GAD65 and the molecular marker mRNAs decreased in parallel in individual aged rats; and (iii) the age-related decrease in the molecular markers was detected at both mRNA and protein levels. However, in some aged rats (numbers 1 and 26) a parallel decrease in the expression of SOM and NPY was not observed, which may argue against a cellular death process. Therefore, we cannot completely exclude the existence of both processes, hypofunctionality and cellular death, of the hippocampal interneurones during ageing (see Feucht et al. 1999). Indeed, the two processes might be sequential and, in both situations, the GABAergic tone should be compromised in the aged hippocampus.

The molecular mechanisms that underlie either GABAergic cell loss and/or hypofunctionality are currently unknown. However, for some neurodegenerative disorders, such as Alzheimer’s disease (in which ageing is the principal risk factor), it has been proposed that unfolded proteins accumulate which, ultimately, might cause endoplasmic reticulum stress and cellular death (Mattson et al. 2000; Imaizumi et al. 2001). In our aged hippocampal samples, we have recently demonstrated the up-regulation and accumulation of non-functional (unassembled) γ2 subunit of the GABA_A receptor (Ruano et al. 2000). This suggests that accumulation of non-functional protein during normal hippocampal ageing may be responsible for the neuronal degeneration.

Expression of either GAD65 or SOM/NPY decreased concomitantly with an increase in the expression of the α1 GABA_A receptor subunit mRNA. This response seems to be specific for the hippocampal formation as a decrease in both GAD65 and α1 subunit expression has been described in other brain areas, such as inferior colliculus (Gutierrez et al. 1994b; Caspary et al. 1999). An increase in the efficacy of the GABA_A receptors, independent of age-dependent molecular modifications, has also been reported in other brain areas (Griffith and Murchison 1995; Caspary et al. 1999).

According to immunocytochemical data, the SOM-positive cells correspond to either the oriens/lacunosum molecular neurons (O-LM cells) in the CA regions and the hilar perforant path-associated cells in the dentate gyrus (HIPP cells) or to the oriens-bistratified cells (Maccarelli et al. 2000). Both the O-LM and HIPP cell types are involved in feedback circuits (Katona et al. 1999). The age-related mRNA up-regulation in the α1 GABA_A receptor subunit was restricted to the stratum pyramidale and stratum granulare of the hippocampal formation (Gutierrez et al. 1996). The α1 increase (mRNA and protein) was directly reflected in the pharmacological properties of the GABA_A receptor (i.e. increase in density and sensitivity of receptors with high affinity for zolpidem) (Ruano et al. 1995, 2000). Interestingly, the increase in the density of high-affinity zolpidem-binding sites was mostly restricted to the stratum lacunosum moleculare of CA1 and CA2 fields and to the outer molecular layer of the dentate gyrus (Ruano et al. 1995). It is noteworthy that the O-LM cells in the CA1 and CA2 regions, and the HIPP cells in the hilus, project their axons from the stratum oriens to the stratum lacunosum moleculare and from the hilus to the outer molecular layer of the dentate gyrus respectively (Freund and Buzsaki 1996). Their post-synaptic elements are mainly dendritic shafts and spines of GABA-negative principal cells (Katona et al. 1999; Maccarelli et al. 2000). Thus, we propose that the pharmacological modifications and up-regulation of the expression of the α1 GABA_A receptor subunit constitute an adaptive process of the principal cells, as a consequence of the possible loss of GABAergic input, or decreased GABA release, from both the O-LM and HIPP cells, to maintain their network properties (i.e. feedback inhibition).

Considering that hippocampal neuronal networks are composed of inhibitory neurons that control the excitability and synchronization of the principal cells (Cobb et al. 1995; Paulsen and Moser 1998; Feucht et al. 1999), a specific and differential loss of inhibitory inputs might be reflected in some of the properties of the network (integrative properties, firing rate, general excitability, etc.). The O-LM neurons seem to be activated by repetitive action potentials, discharged by different pyramidal cells during theta frequency (Sik et al. 1995; Feucht et al. 1999). Moreover, it has been proposed that O-LM cells ( SOM positive), along with axoaxonic and basket cells (both PV positive), may cooperate with theta frequency inputs to CA1 pyramidal cells in dendrites and perisomatic terminals respectively (Katona et al. 1999; Maccarelli et al. 2000; Van Hooff et al. 2000). The differential vulnerability of these interneuronal populations to the ageing process ( SOM-positive cells more vulnerable than PV-positive cells) might produce an imbalance between dendritic and perisomatic inhibition in
the principal cells of the aged rat hippocampus. This differential vulnerability of GABAergic cells to the normal ageing process might be implicated in the lower mean discharge rate of hippocampal theta cells located in the stratum oriens (probably corresponding to O-LM cells) with respect to those located on stratum pyramidale, observed in aged hippocampus (Mizumori et al. 1992).

The reasons for this differential susceptibility of hippocampal GABAergic neurones to the ageing process are unknown. Differences in afferent input, targeting the GABAergic neurones, might represent a potential source of the differential vulnerability. Indeed, the density of inputs as well as the total number of excitatory plus inhibitory synapses was several times higher on PV cells than on calbindin or CR cells (Gulyas et al. 1999). On the other hand, a specific decrease in the number of SOM-immunopositive cells in rat hippocampus has been observed by cholinergic deafferentation of subcortical pathways to the hippocampus, but not by noradrenergic or serotoninergic deafferentation (Jolkonen et al. 1997). The preferential susceptibility of the SOM- and/or NPY-positive cells might reflect a lower degree of innervation as well as a decrease in the cholinergic inputs in the aged hippocampus.

In conclusion, our data provide the first evidence for a progressive loss of GABAergic input to the hippocampal GABAergic neurones during normal ageing that seems to be compensated at the post-synaptic level. Moreover, our results suggest a cellular and molecular basis for the higher basal neuronal excitability observed in the aged hippocampus.

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