IMMUNOCYTOCHEMICAL DETECTION OF SYNAPTOHYSIN IN C57BL/6 MICE COCHLEA DURING AGING PROCESS

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Abbreviations

Age-related hearing loss gene (Ahl)

Electron transmission microscopy (ETM)

Inner hair cells (IHCs)

Outer hair cells (OHCs)

Phosphate buffer saline (PBS)

Synaptophysin (Syp)

Anti-Syp monoclonal antibody clone SY-38 (SY-38)

ABSTRACT

Aged mammals frequently exhibit a bilateral, progressive, and symmetric deafness related to the degeneration of auditory receptor. However, little is still known about aging effects on synapses in this receptor. Synaptophysin (Syp) is a 38 kDa Ca^{2+} binding glycoprotein widely found in presynaptic membrane and vesicles. The Syp has been found in presynaptic buttons of efferent auditory fibers, within the developing and adult auditory receptor. The detection of Syp in aged cochleae could provide relevant information about synaptic changes and receptor degeneration process observed in old animals. This paper focuses on aging linked changes related to the presence of Syp in cochleae of C57BL/6J mice (from 1 to 24 months old). Results shown that during the first months of age, no significant changes were observed in the Syp distribution under the basal pole of inner (IHCs) neither the outer (OHCs) hair cells. At six months of age, a significant decrease of Syp immunocytochemical detection appeared in fibers under the most external row of OHCs, but restricted to the cochlear basal coil. Only a very scarce reduction of was noted under the IHCs and the other OHCs rows, also at the basal coil. From mice 9 months old on, a progressive decreased of the presence of SYP was found under IHCs and all OHCs rows starting at the basal coil and reaching the apical coil in the oldest mice.

All these data could indicate that the cochlea aging process early affects to presynaptic membrane proteins of efferent endings fibers. This early alteration of cochleae efferent synapses could be involved in the whole degeneration of the Corti's organ.

Section: Sensory and motor system.

Theme: Auditory system: periphery.

Key words: Auditory efferent system, cochlea, aging, C57BL/6 mouse, sinaptophysin immunohistochemistry, morphometry.

1. Introduction

The mammalian auditory receptor exhibits a progressive, bilateral and symmetric hearing loss related to aging that, at least for in human clinics, is known as presbycusis (Schuknecht and Gacek, 1993). This hearing loss is characterized by an increase of hearing thresholds, which starts at high frequencies and progressively affects middle and low frequencies (Castillo et al., 2006; Johnson et al., 1997; Willot et al., 1996). This agrees with the progressive base-to-apex sensory-neuronal degeneration, associated to loss of both inner (IHCs) and outer (OHCs) hair cells (Bartolome et al., 2001, 2002; Bohne et al., 1990; Gil-Loyzaga 2002; Ingham et al., 1999; Keithley and Feldman 1982), and spiral ganglion neurons (Bartolome et al., 2001, 2002; Cohen et al., 1990; Gil-Loyzaga 2002; Keithley et al., 1989).

In the auditory receptor, synaptic alterations have been described in noise induced hearing loss (Liberman and Dodds 1984), but also in congenital (Ruygo et al., 1998) or aged-related deafness (Schimiedt et al., 1996; Stamataki et al., 2006). Synaptic alterations of efferent nerve endings (Stamataki et al., 2006) fit well with changes in efferent neurotransmitters turnover (e.g. dopamine) that were found in the aged cochleae (Vicente-Torres and Gil-Loyzaga 2002; Vicente-Torres et al., 2001, 2002).

The C57BL/6J mouse has two copies of the age-related hearing loss gene (Ahl), which renders these mice more susceptible to a rapid and progressive hearing loss. Anatomical and physiological data obtained from aged C57BL/6J mice (Bartolome et al., 1999, 2001, 2002; Castillo et al., 2006; Gil-Loyzaga 2002; Henry and McGinn, 1992; Johnson et al., 1997) exhibited high similarities

with human prebycusis, as it was previously described a presbycusis (Schuknecht and Gacek, 1993).

Synaptophysin (official symbol: Syp, Syn or p38) is a calcium-binding hexametric thyrosine-phosphorylated protein of 38 kDa (Südhof, 1995). The Syp is a specific component of presynaptic membrane and the small synaptic vesicles (SSVs) (Navone et al., 1986; Thomas et al., 1988). In the auditory receptor, Syp has been identified in efferent nerve endings under IHCs and OHCs (Counter et al., 1997; Gil-Loyzaga and Pujol, 1988; Khalifa et al., 2003; Knipper et al., 1995; Simmons et al., 1996). The Syp was clearly found in vesicles in all efferent terminals, in both medial and lateral efferent systems (Gil-Loyzaga and Pujol, 1988). The Syp immunolabelling has been considered as an optimal technique to quantify pathological alterations at a specific location into the cochlea (Canlon et al., 1999; Counter et al., 1997).

The present study focuses on changes in the presence of Syp in the auditory receptor during aging. Aging related modifications of efferent nerve fibers can be early detected by using Syp as a sensible marker of synaptic degeneration and, also, as an indicative of functional modifications. The Syp is involved in the synaptic-physiology, because it participates in the formation of transmembrane channels during synaptic vesicle exocytose (Johnston et al., 1989, Südohof and Jahn, 1991; Thomas et al., 1988).

Nevertheless, mutant synaptophysin-deficient mice exhibit functional neurotransmission process, indicating that Syp is important but it is not an absolute requirement for neurotransmitter release (Eshkind and Leube, 1995; McMahon et al., 1996). On the other hand, it was found that Syp binds to

other synaptic proteins such as synaptobrevins (Becher et al., 1999; Südhof, 1995) or dynamin (Daly et al., 2000). The Syp-synaptobrevins complex has been demonstrated as essential protein during synaptic plasticity process with high synaptic activity (Becher et al., 1999; Fernández-Chacón and Südhof, 1999) and Syp-dynamin was required for the efficient synaptic transmission (Daly et al., 2000). Thus, this first approach must be followed by further research in order to determine the sequence of possible alterations for other synaptic or complex proteins during aging of the auditory receptor.

2. Results

2.1. Immunocytochemical results

The cochleae of 1 month old mice showed a strong SY-38 immunolabelling, around IHC bodies (Figs. 1A). Also, SY-38 immunolabelled efferent fibers were observed under the three OHC rows (Fig. 1A). The most internal OHC (near or pillar cell) is denominated OHC1, the second cell is denominated OHC2, and the most external is OHC3. Big immunolabelled buttons were found in contact with the OHCs cell body, at the basal (Fig. 1B) or medial (Fig. 1C) coils. Also, a similar SY-38 immunolabelling was found in cochleae from 3 months old mice (Fig. 1D) around the IHC body (Figs. 1 D, 1E and 1F), and on the basal pole of OHCs (Fig. 1D). The SY-38-immunolabelled fibers clearly corresponded with efferent nerve labelling under IHCs (Fig. 1F), and OHCs (Figs. 1B, 1C).

The basal cochlear coil of C57BL/6 mice 6 month old showed a strong SY-38 immunolabelling, around IHC, and under OHC1 and OHC2. The qualitative SY-38 immunolabelling distribution in middle (Fig. 1G), and apical coils was similar to results previously described for mice of 1 and 3 months old.

The cochleae of C57BL/6 mice from 21 months old exhibited a total absence of Syp (negative SY-38 immunolabelling) at the basal and at the middle coils. The Syp was restricted to the apical coil under the basal pole of IHCs (Fig. 2A). Cochleae from 24 months old mice only exhibited a scarce SY-38 immunolabelling around the IHC cell bodies and the habenula perforata (Figs. 2B, 2C). At the same apical coil, OHCs were completely absent (Fig. 2D).

In addition, a total absence of SY-38 immunolabelling was observed in the negative controls obtained by incubation in the absence of primary antibody (SY-38).

2.2. Morphometric results

The quantitative results, carried out in 18 midmodiolar sections (6 cochleae and 3 sections) by cochlea by group of age (1, 3, 6, 9, 12, 15, 18, 21, 24). In all sections the whole SY-38 immunolabelled area was measured in each (basal, medial and apical) cochlear coil.

The SY-38 immunolabelling around the inner hair cells (IHCs) was separately quantified in each cochlear coil: the basal (BIHC), the medial (MIHC) and the apical (AIHC) coils of the mice cochleae.

The SY-38 immunolabelled area was statistically significant ($p< 0.01$) between at the basal, the medial and the apical coils (Fig. 3A). The basal coil (BIHC) showed the lesser SY-38 immunolabelled area than the other ones, being the biggest area at the apical coil (AIHC) (Fig. 3A). Also, the SY-38 immunolabelling significantly ($p<0.01$) changed during aging, being bigger in young adult mice (1-6 month old) with respect to older (from 9 month old on). A significant interaction effect was detected ($p<0.01$) between age and cochlear coil in the data corresponding to the mice 1 to 12 months old aged. This fact implies that the age-related decrease of the SY-38 immunolabelled area below the IHCs is significantly different for each of the three cochlear coils, so these changes in the SY-38 immunolabelled area depends on the cochlear coil considered and not only on the age of the animal. This interaction effect was not

detected in the comparisons of the data from mice 12 months old on, so the age-related variations in the SY-38 immunolabelled area in this period were not statistical different ($p = 0.087$) (between the three cochlear coils (Fig. 3A).

The quantitative analysis of SY-38 immunolabelled area under OHCs exhibited two relevant characteristics: a) the immunolabelling was lesser at the basal than at the upper coils, b) the immunolabelled area under each of three OHCs showed statistically significant differences ($p < 0.01$).

The quantitative results of SY-38 immunolabelled area under OHCs at the basal coil (BOHC1, BOHC2, and BOHC3) showed statistically significant differences ($p< 0.01$) between them (Fig. 3B). Early modifications in the SY-38 immunolabelling restricted to BOHC3 (the most external OHCs) were observed at the basal coil in 6 months old mice cochleae. All sections analyzed of basal coil of cochleae of mice 9 months old on appeared quite devoid of SY-38 immunolabelled. They exhibited no significant difference in SY-38 immunolabelling under OHC1 ($p= 0.999$) and OHC2 ($p= 0.116$) (Fig. 3B). A significant interaction effect ($p< 0.01$) was detected between age and OHCs at the basal coil (BOHC1, BOHC2, AND BOHC3). This effect demonstrated that the age did not affect the SY-38 immunolabelled area in the same way for each BOHC (Fig. 3B).

From middle cochlear coil, the SY-38 immunolabelled area under MOHC1, MOHC2, and MOHC3 showed significant differences between them ($p= 0.002$) (Fig. 3C). Significant differences were also observed between the different ages analyzed ($p< 0.01$). The SY-38 immunolabelled area at the middle coil of cochleae of mice 9 months old on, was not statistically significant between the

three MOCH1 (p = 0.102), MOHC2 (p = 0.704) and MOHC3 (p = 0.885) (Fig. 3C). An interaction effect was detected between age and MOHCS (p = 0.04). Like in the basal coil, a significant (p = 0.04) interaction effect was observed between age and MOHCs, so the age did not affect the SY-38 immunolabelled area in the same way for each OHC at this middle cochlear coil.

A very relevant finding was detected in the apical coil, within the AOHC1, AOHC2, and AOHC3 rows. The Sy-38 immunolabelling area was significantly different between the three AOHCs (p= 0.006) (Fig. 3D). Also the significant differences were detected between different groups of ages analyzed (p< 0.001). The SY-38 immunolabelled area under AOHC2 and AOHC3 was significantly different (p= 0.007) (Fig. 3D). In contrast none statistically significant differences (p= 0.895) were observed in the SY-38 immunolabelling between 21 and 24 months old mice (Fig. 3D). In this cochlear coil, no interaction effect was observed (p = 0.604) between age and the three rows of OHCs (AOHC1, AOHC2, and AOHC3) (Fig. 3D). These results demonstrated that the age-related was statistically similar for the three AOHCs rows.

3. Discussion

Present study has shown that the presence of Syp, a main synaptic protein found in cochlear efferent nerve endings, progressively changes during the aging of C57BL/6 mice. The SY-38 immunolabelling was restricted to the nerve fibers connecting the sensory cells (Gil-Loyzaga and Pujol, 1988). Also present MET findings serve to recognize the presence of Syp exclusively within efferent nerve endings, in both under IHCs (inner spiral bundle) and under OHCs. A total absence of SY-38 immunolabelling was observed in Deiters or Hensen's cells (Burgess et al., 1997; Gil-Loyzaga and Pujol, 1988).

The SY-38 immunolabelling around the IHCs showed significant differences between base, middle and apical coils. In all C57BL/6 mice cochleae analyzed, the IHCs SY-38 immunolabelled area was bigger at the apex than at the other cochlear coils. Different findings were obtained from the adult guinea pig cochlea were SY-38 immunolabelled fibers were more abundant in the basal and middle coil than in the apex (Counter et al., 1997). In addition, in the cat (Liberman et al. 1990) and human beings (Khalifa et al., 2003) a uniform base to apex cochlear distribution was found. All these findings could be related to interspecies differences without excluding some minor differences in morphometric methods used.

In C57BL/6 mice, very early modifications of the presence of Syp were observed in the basal cochlear coil in animals 6 month old. These alterations appeared to be restricted to the fibers under the most external row of OHCs (OHC3). Also at the basal coil, a relevant decrease of the presence of Syp was noted, under IHCs and under the OHCs, from 9 month old on. This Syp

absence was also evident in the three rows of OHCs of the middle coil in mice cochleae from 15 months old on. A progressive decrease of SY-38 immunolabelling was noted, under IHCs and OHCs, from the middle until the apical coil. Middle and apical coils from 24 months old mice cochleae only showed a very scarce SY-38 immunolabelling butons under the IHCs. These results suggested that the Syp of medial efferent nerve endings, projecting to OHCs completely disappeared during aging process. In contrast, some scarce nerve endings belonging to the lateral efferent system (projecting to the IHCs) still contained Syp in oldest animals (24 months old mice) studied. The progressive decrease of Syp within the cochleae during mice aging could be linked to a reduced ability of neurons to synthesize this protein (Cheng et al., 1998; Kazee and West 1999; Kazee et al., 1995).

The SY-38 immunolabelled area around the IHCs corresponds to the peripheral distribution of lateral efferent fibers. In contrast, the presence of SY-38 immunolabelling under the OHCs is due to the presence of Syp in medial efferent fibers. Taken together, all these findings indicate that the efferent nerve endings (immunolabelled by SY-38 antibody) degenerated before the IHCs and OHCs die or disappeared. These results fit well with previous morphological (Bartolome et al., 2001, 2002) electrophysiological (CAP) studies (Castillo et al., 2006). The Syp reduction during aging process might modify the efferent activity, including a reduction of the efferent modulation of afferent nerve fibers (under IHCs) and on OHCs physiology. The alteration of Syp, a main synaptic protein, during aging process, also could explain a fail in dopamine turn-over

previously reported (Vicente-Torres and Gil-Loyzaga 2002; Vicente-Torres et al., 2001; 2002).

In conclusion, the alteration of synaptic efferent physiology during aging could be an earlier and relevant event to justify the physiopathology of deafness linked to aging, including presbycusis pathology. Further studies will be devoted to determine firstly, if other synapse proteins, as synaptobrevins or dynamins are also affected by the aging process in the cochlea of C57BL6J mice. A decrease of several of these proteins could justify the rapid inner ear deafness usually observed in these mice. Secondly, it will be highly relevant to analyze the neurons at the brainstem olivary complex.

4. Materials and methods

4.1. Animals

In the present study 40 adult C57BL/6 mice aged of: 1, 3, 6, 9, 12, 15, 21 and 24 months old were used. The immunohistochemical procedure and posterior morphometric results were carried out with 24 mice. A total of 18 midmodiolar sections (6 cochleae and 3 sections by cochlea) were obtained each group of age. A second group of 16 mice (2 mice or 4 cochleae by group of age) were used for electron transmission microscopy (ETM) studies.

The care and use of all animals in this study were in strict accordance with the animal welfare guidelines of the Declaration of Helsinki. All animals were sacrificed under deep general anesthesia with 300mg/Kg body weight of chloral hydrate (8% in distilled water).

4.2. Immunohistochemical procedures in paraffin sections

All 24 mice were perfused with a fixative solution containing 2% acetic acid in 98% ethanol. This fixative solution was chosen because, for morphometric purposes, it warranties the maximum Syp preservation instead of formaldehyde or paraformaldehide solutions. However, morphological images were of a poor quality of other obtained after formaldehyde or paraformaldehide fixation (Khalifa et al., 2003). All cochleae were immediately removed and postfixed in the same solution for about 72h. Then, the cochleae were decalcified using a 2% ascorbic acid solution (Merchán-Pérez et al., 1999), dehydrated and embedded in paraffin. Serial sections of 7 µm thick parallel to the mid-modiolar plane were obtained.

Sections were rinsed three times, for 5min each, in phosphate buffer saline at pH= 7.4, 0.1M (PBS). Preincubation was carried out for 30min in a PBS solution containing 0.1% Triton X-100 (Sigma). Sections were then incubated overnight, at 4ºC, in a solution containing 1/250 of an anti-Syp monoclonal antibody (clone SY-38, Novocastra) (Bartolome et al., 1993; Gil-Loyzaga and Pujol 1988; Gil-Loyzaga et al., 1998). After three washes in PBS (5 min each), the sections were incubated for 1h in a 1/200 biotinylated horse anti-mouse IgG (Vectastin, Vector) in PBS solution. Antigen-antibody immunoreaction was revealed using the avidin-biotin-peroxidase method (Vectastin, Vector), as previously described. A series of negative controls was performed omitting the primary antibody and used the same procedure described above but. The slides were studied and photographed in a DMRB Leica photomicroscope.

4.3. Immunoelectron microscopy procedures

For TEM immunohistochemistry, all cochleae were perfused with a fixative solution containing 1% of paraformaldehyde and 1% of glutaraldehide in phosphate buffer at pH 7.4, 0.1 M (PB). The cochleae were immediately removed and postfixed in the same fixative solution for about 24h. Then, the cochleae were decalcified using 2% ascorbic acid (Merchán et al., 1999). All cochleae were processed by pre-embedding procedure using the same immunohistochemical method described above. Then, the cochleae were osmicated (1h with 2% osmic acid in PBS) and processed for electron microscopy (Gil-Loyzaga and Pujol, 1988). Ultrathin serial sections 0,25 µm thick were obtained parallel to the mid-modiolar plane. The sections were

obtained from the different coils of each cochlea. Ultrathin sections were then studied and photographed in a 902 Zeiss electron microscope

4.4. Morphometric analysis

A total of 18 midmodiolar sections (6 cochleae by age group, and 3 mid-modiolar sections by cochlea) were analyzed for each age group. In all sections, the three cochlear coils (basal, middle, and apical) were observed and individually analyzed. Data are expressed as mean and standard error deviations of n=18 sections by each group of age.

The morphometric analysis was carried out by measuring the SY-38 immunolabelled areas. For each section, and each coil, the SY-38 immunolabelling around the IHC and under the each OHC (OHC1, OHC2 and OHC3) rows were quantified separately. Previous morphometrical studies described that the mid-modiolar plane corresponds to those cochlear sections in which, the variations within width and height of the scala media were minimal (Gil-Loyzaga et al., 1987).

The cochlear sections were analyzed using a high resolution Sony CCD monochrome camera XC-75CE coupled to a DMRB Leica photomicroscope (40x lenses). The morphometric analysis was performed with a Leica Q500MC Image Analysis System, using 8 bits grey scale images (256 grey levels: 0 represents white and 255 black colors). The binary edition was used to remove artifacts that not corresponded with IHCs and OHCs SY-38 immunolabelled area. For all measurements, the same optical objective and lighting conditions were used. The calibration of the system was carried out with a stage

micrometer (Leizt), that allowed the computation of the object area (immunocytochemical detection of Syp under sensory cells) in μm^2 unities.

An ANOVA of repeated measures with one covariate (age) has been used for statistical purposes to establish significant differences between factors along the inner ear aging. Two within-subjects factors (cell type and cochlear coil) have been considered to make the analysis. *Post-hoc* multiple comparisons were carried out by using Bonferroni tests. Statistical analysis was performed using SPSS 15.0 and Statgraphics-Plus 5.1 statistical packages. Data were plotted in graphs in which each dot represents the mean, plus the standard error of the mean (SEM), of the measures carried out in 18 histological sections by each group of age.

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References

Bartolome, M.V., Ibáñez, M.A., López-Sánchez, J.G., Merchán-Pérez, A., Gil-Loyzaga P., 1993. Synaptophysin immunoreactivity in the cochlear nuclei of mammals: A comparative study. ORL and Its Related Specialities 55, 317-332.

Bartolome, M.V., Vago, P., Gil-Loyzaga, P., Humbert, G., Joubert-Caron, R., Pujol, R., Lenoir, M., 1999. Sequential changes in anti-GAL-1 labelling of the rat organ of Corti following amikacin exposure. Brain Res. 822. 43-51.

Bartolome, M.V., Maestre, L., Gil-Loyzaga. P., 2001. Galectine-1 expression in cochleae of C57BL/6 mice during aging. NeuroReport 12, 3107-3110.

Bartolome, M.V., Castillo, E., Maestre, L., Carricondo, F., Poch, J., Gil-Loyzaga, P., 2002. Effects of aging on C57 BL/6J mice. An electrophysiological and morphological study. From Rational pharmacotherapy of the inner ear. Advances in Oto-Rhino-Laryngology, Vol. 59, D. Felix, E, Oestreicher, eds. Basel Karger, Suiza, pp. 106-111.

Becher, A., Drenckhahn, A., Pahner, I., Margittai, M., Jahn, R., Ahnert-Hilger, G., 1999. The synaptophysin-synaptobrevin complex: a hallmark of synaptic vesicle maturation. J. Neurosci. 19, 1922-1931.

Bohne, B.A, Gruner, M.M., Harding, G,W., 1990. Morphological correlates of aging in the chinchilla cochlea. Hear. Res. 48, 79-92.

Burgess, B.J., Adams, J.C., Nadol, J,B., 1997. Morphologic evidence for innervation of Deiters' and Hensen's cells in the guinea pig. Hear. Res 108, 47-82.

Canlon, B., Fransson, A., Viberg, A., 1999. Medial olivocochlear efferent terminals are protected by sound conditioning. Brain Res. 850, 253-260.

Castillo, E., Carricondo, F., Bartolome, M.V., Vicente-Torres, M.A., Poch-Broto, J., Gil-Loyzaga, P., 2006. Presbycusis: neural degeneration and aging on the auditory receptor of C57/BL6J mice. Acta Otorrinolaringol. Esp. 57, 383-7

Cheng, Y.C., Chen, Q.S., Lei, J.L., Wang, S.L., 1998. Physiological training modifies the age-related decrease of GAP-43 and synaptophysin in the hippocampal formation in C57BL/6J mouse. Brain Res. 806, 238-245.

Cohen, G.M., Park, J.C., Grasso, J.S., 1990. Comparison of demyelination and neural degeneration in spiral and Scarpa's ganglia of C57BL/6 mice. J. Electron Micros. Tech. 15, 165-172.

Counter, S.A., Canlon, B., Borg, E., Aldskogius, H., 1997. Pattern of synaptophysin immunoreactivity in the efferent nerve terminals of the guinea pig cochlea. Neurosci. Letts. 222, 199-203.

Daly, C., Sugimory, M., Moreira, J.E., Ziff, E.B., Linás, R., 2000. Synaptophysin regulates clathrin-independent endocytosis of synaptic vesicles. Proc. Natl. Acad. Sci. USA 97, 6120-6125.

Eshkind, L.G., Leube, R.E., 1995. Mice lacking synaptophysin reproduce and form typical synaptic vesicles. Cell Tissue Res. 282, 423-433.

Fernández-Chacón, R., Südhof, T.C., 1999. Genetics of synaptic vesicle function: toward the complete functional anatomy on an organelle. Annu. Rev. Physiol. 61, 753-776.

Gil-Loyzaga, P., 2002. Deafness and aging: studies in experimental models. Ann. Real Acad. Nac. Med. (Madrid). 119, 273-84.

Gil-Loyzaga, P., Pujol, R., 1988. Synaptophysin in the developing cochlea. Int. J. Dev. Neurosci. 6, 155-160.

Gil-Loyzaga, P., Merchán Perez A., Martinez Alvarez C., Perez-Cao A., Merchan Fuentes J., 1987. Morphometrical study of the development of the cochlear duct. Acta Microscópica 10, 67-73.

Gil-Loyzaga, P., Bartolome, M.V., Ibáñez, A., 1998. Synaptophysin immunoreactivity in the cat cochlear nuclei. Histol. Histophatol. 13, 415-424.

Gil-Loyzaga, P., Poch-Broto, J., Vicente-Torres, M.A., Bartolome, M.V., Carricondo, F., San Roman, J., Jiménez, L., 1999. GABA, dopamine and serotonin in the cochlear innervation. Models of Protection against Neurotoxicity. From Cochlear Pharmacology and Noise Trauma. Vol. 3, D. Prasher, B. Canlon, eds. Noise Res. Network Publications. London, pp.23-42.

Henry, K.R., McGinn, M.D., 1992. The mouse as a model of human audition. Audiology 31, 181-189.

Ingham, N.J., Comis, S.D., and Withington, D.J., 1999. Hair cell loss in the aged guinea pig cochlea. Acta Otolaryngol. (Stockh) 119, 42-47.

Johnston, P.A., Jahn, R.J., Südhof, T.C., 1989. Transmembrane topography and evolutionary conservation of synaptophysin. J. Biol. Chem. 264, 1268-1273.

Johnson, K.R., Erway L.C., Cook S.A., Willot J.F., Zheng Q.Y., 1997. A major gene affecting age-related hearing loss in C57BL/6J mice. Hear. Res. 114, 83-92.

Kazee, A.M., West, N.R., 1999. Preservation of synapses on principal cells of the central nucleus of the inferior colliculus with aging in the CBA mouse. Hear. Res. 133, 98-106.

Kazee, A.M., Ying Han, L., Spongr, V.P., Walton, J.P., Salvi, R.J., Flood, D.G., 1995. Synaptic loss in the central nucleus of the inferior colliculus correlates with sensorineural hearing loss in the C57BL/6 mouse model of presbycusis. Hear. Res. 89, 109-120.

Keithley, E.M., Feldman, M.L., 1982. Hair cell counts in an age-graded series of rat cochleas. Hear. Res, 8, 249-262

Keithley, E.M., Ryan, A.F., Woolf, N.K., 1989. Spiral ganglion cell density in young and old gerbils. Hear. Res. 38, 125-134.

Khalifa, S.A.M., Friberg, U., Illing, R.B., Rask-Andeersen, H., 2003. Synapthophysin immunohistochemistry in the human cochlea. Hear. Res. 185, 35-42.

Knipper, M., Zimmermann, U., Rohbock, K., Kopschall, I., Zenner, H.P., 1995. Synapthoysin and Gap-43 proteins in efferent fibers of the inner ear during postnatal development. Dev. Brain Res. 89, 73-86.

Liberman, M.C., Dodds, L.W., 1984. Single-neuron labelling and chronic cochlear pathology. II. Stereocilia damage and alterations of spontaneous discharge rates. Hear. Res. 16, 43-53.

Liberman, M.C., Dodds, L.W., Pierce, S., 1990. Afferent and efferent innervation of the cat cochlea: quantitative analysis with light and electron microscopy. J. Com. Neurol. 301, 443-401.

McMahon, H.T., Bolshakov, V.Y., Janz, R., Hammer, R.E., Siegelbaum, S.A., Südhof, T.C., 1996. Synaptophysin, a mayor synaptic vesicle protein, is not essential for neurotransmitter release. Proc. Natl. Acad. Sci. USA 93, 4760-4764.

Merchán-Pérez, A., Gil-Loyzaga, P., Bartolome, M.V., Remezal, M., Fernández, P., Rodríguez, T., 1999. Decalcification by ascorbic acid for immuno- and affinohistochemical techniques on the inner ear. Histochem. Cell Biol. 112, 125-130.

Navone, F., Jahnm R., Di Gioia, G., Stukenbrok, H., Greengard, P., Di Camilli, P., 1986. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J, Cell Biol. 103, 2511-2527.

Ryugo, D.K., Rosembaum, B.T., Kim, P.J., Niparko, J.K., Saada, A.A., 1998. Single unit recordings in the auditory nerve of congenitally deaf white cats: morphological correlates in the cochlea and cochlear nucleus. J. Com. Neurol.. 397, 532-548.

Schmiedt, R.A., Mills, J.H., Boettcher, F.A., 1996. Age-related loss of activity of auditory-nerve fibers. J. Neurophysiol. 76, 2799-2803.

Schuknecht, H.F., Gacek, M.R., 1993. Cochlear pathology in presbycusis. Ann. Otol. Rhinol. Laryngol. 102, 1-14.

Simmons, D.D., Moulding, H.D., Zee, D., 1996. Olivocochlear innervation of inner and outer hair cells during postnatal maturation: an immunocytochemical study. Dev. Brain Res. 95, 213-226.

Stamataki, S., Francis, H.W., Lehar, M., May, B.J., Ryugo, D.K., 2006. Synaptic alterations at inner hair cells precede spiral ganglion cell loss in aging C57BL/6J mice. Hear. Res. 221, 104-118.

Südhof, T.C., 1995. The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375, 645-653.

Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W.W., Betz, H., 1988. Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. Science 242, 1050- 1053.

Vicente-Torres, M. A., Gil-Loyzaga, P., 2002. Age- and gender-related changes in the cochlear sympathetic system of the rat. Neurosci. Letters 319, 177-179.

Vicente-Torres, M.A., Muñoz, E., Dávila, D., Gil-Loyzaga, P., 2001. Changes in the cochlear dopaminergic system of the aged rat. Brain Res, 917, 112-117.

Vicente-Torres, M.A., Dávila, D., Muñoz, E., Gil-Loyzaga, P., 2002. Effects of aging on cochlear monoamine turnover. From Rational pharmacotherapy of the inner ear. Advances in Oto-Rhino-Laryngology, Vol. 59, D. Felix, E, Oestreicher, eds. Basel Karger, Suiza, pp.112-115.

Willot, J.F., Bross, LS., 1996. Morphological changes in the anteroventral cochlear nucleus that accompanies sensorineural hearing loss in DBA/2J and C57BL/6 mice. Dev. Brain Res. 91, 218-226.

Legends of figures

Figure 1. Fig. 1A. C57BL6J mouse cochlea of 1 month old. Paraffin midmodiolar section (light microscopy) of the basal coil exhibited the SY-38 immunolabelling in the organ of Corti. An intense immunoreaction can be observed around the inner hair cell (BIHC) (arrows) and under basal pole of outer hair cells (BOHCs) (arrows). The SY-38 immunolabelling was similarly strong under the three OHCs BOHC1, BOHC2, and BOHC3 rows. BOHC1 (1), BOHC2 (2), and BOHC3 (3). Tunnel of Corti (CT). Scale bar: 10 µm.

Figs. 1B and 1C. C57BL6J mouse cochlea of 3 month old. The transmission electron micrographs of two ultrathin sections showed a positive SY-38 immunolabelling. Efferent nerve endings (asterisks) showed a dense immunostaining under outer hair cell (OHCs) of the basal coil (BOHC) (Fig. 1B, asterisk), or medial coil (MOHC) (Fig. 1C, asterisk). Scale bar: 2 µm.

Fig. 1D. C57BL6J mouse cochlea of 3 month old. Paraffin midmodiolar section (light microscopy) of the medial coil showing an intense SY-38 immunolabelling. Positive immunoreaction can be recognized under the inner hair cell (MIHC) (arrows) and under basal pole of outer hair cells (MOHCs) (arrows). MOHC1 (1), MOHC2 (2), and MOHC3 (3). Tunnel of Corti (CT). Scale bar: 10 µm.

Figs. 1E and 1F. C57BL6J mouse cochlea of 3 month old. The transmission electron micrographs of the medial coil exhibited positive SY-38 immunolabelling (Fig. 1E arrows) within efferent nerve buttons (Fig. 1F asterisks). Inner hair cell of medial coil (MIHC). Fig. 1E: Scale bar: 5 µm. Fig. 1F: Scale bar: 1 µm.

Fig. 1G. C57BL6J mouse cochlea of 6 month old. Paraffin midmodiolar section (light microscopy) of the medial coil showing an intense SY-38 immunolabelling under inner hair cell (MIHC) (arrows) and under the basal pole of three outer hair cells (MOHCs) (arrows). The SY-38 immunolabelling was equally strong in efferent nerve endings of younger mice. MOHC1 (1), MOHC2 (2), and MOHC3 (3). Tunnel of Corti (CT). Scale bar: 10 µm.

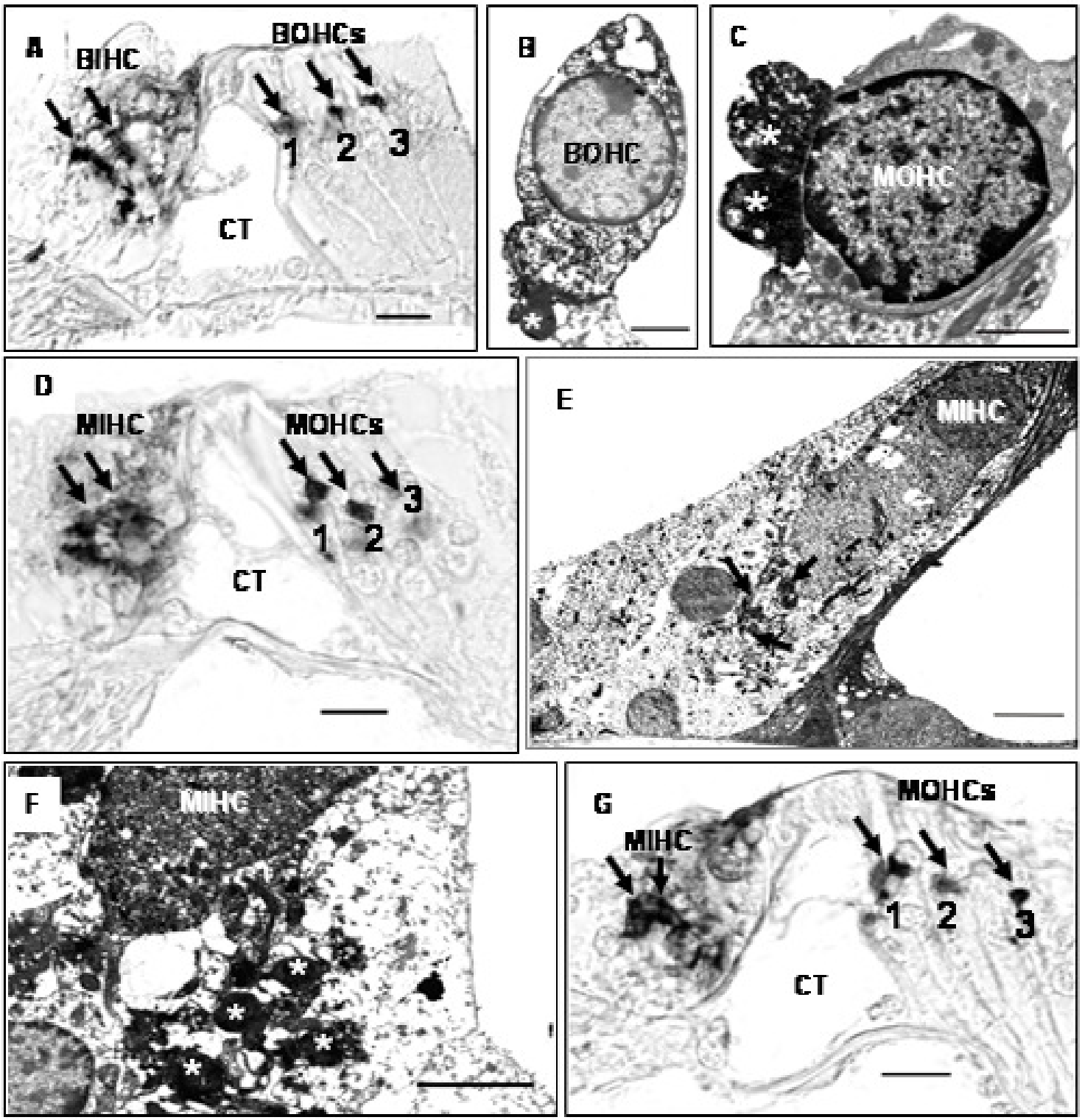
Figure 2. Fig. 2A. C57BL6J mouse cochlea of 21 months old. Paraffin midmodiolar section (light microscopy) at the apical coil. The SY-38 immunolabelling can be recognized only around the inner hair cell (AIHC) (arrows). The external part of the organ of Corti appeared devoid of OHCs. Tunnel of Corti (CT). Scale bar: 10 µm.

Fig. 2B. C57BL6J mouse cochlea of 24 months old. Paraffin midmodiolar section of the organ of Corti (light microscopy) at the apical coil. The pattern of SY-38 immunolabelling around the apical inner hair cell (AIHC) is little lower than in cochlea of mice 21 months old (arrows). Tunnel of Corti (CT). Scale bar: 10 µm.

Figs. 2C and 2D. C57BL6J mouse cochlea of 24 months old. The transmission electron micrograph allows the recognition of SY-immunolabelling in the habenula perforata (arrows) at the apical coil (Fig. 2C). The OHcs were absent (starts) at the apical coil due to the progressive degeneration linked to aging (Fig. 2D). Scale bar: 5 µm.

Figure 3. Plots of quantitative results (mean + SEM) of SY-38 immunolabelling distribution around the inner hair cells (IHCs) (Fig. 3A) and outer hair cells (OHCs) (Figs 3B, 3C and 3D). The SY-38 immunolabelling distribution around IHCs at the basal (BIHC), the medial (MIHC) and the apical (AIHC) coils (Fig 3A). The SY-38 immunolabelling distribution under OHCs in the basal (BOHCs) (Fig. 3B), the medial (MOHCs) (Fig. 3C) and apical (AOHCs) (Fig. 3D) coil. The most internal OHC (near or pillar cell) is denominated OHC1, the second cell is OHC2, and the most external is OHC3.

Figure 1


A
BIHC
BOHCs
1
2
3
CT
B
BOHC
C
MOHC
D
MIHC
MOHCs
1
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E
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F
MIHC
G
MIHC
MOHCs
1
2
3
CT

Figure 2

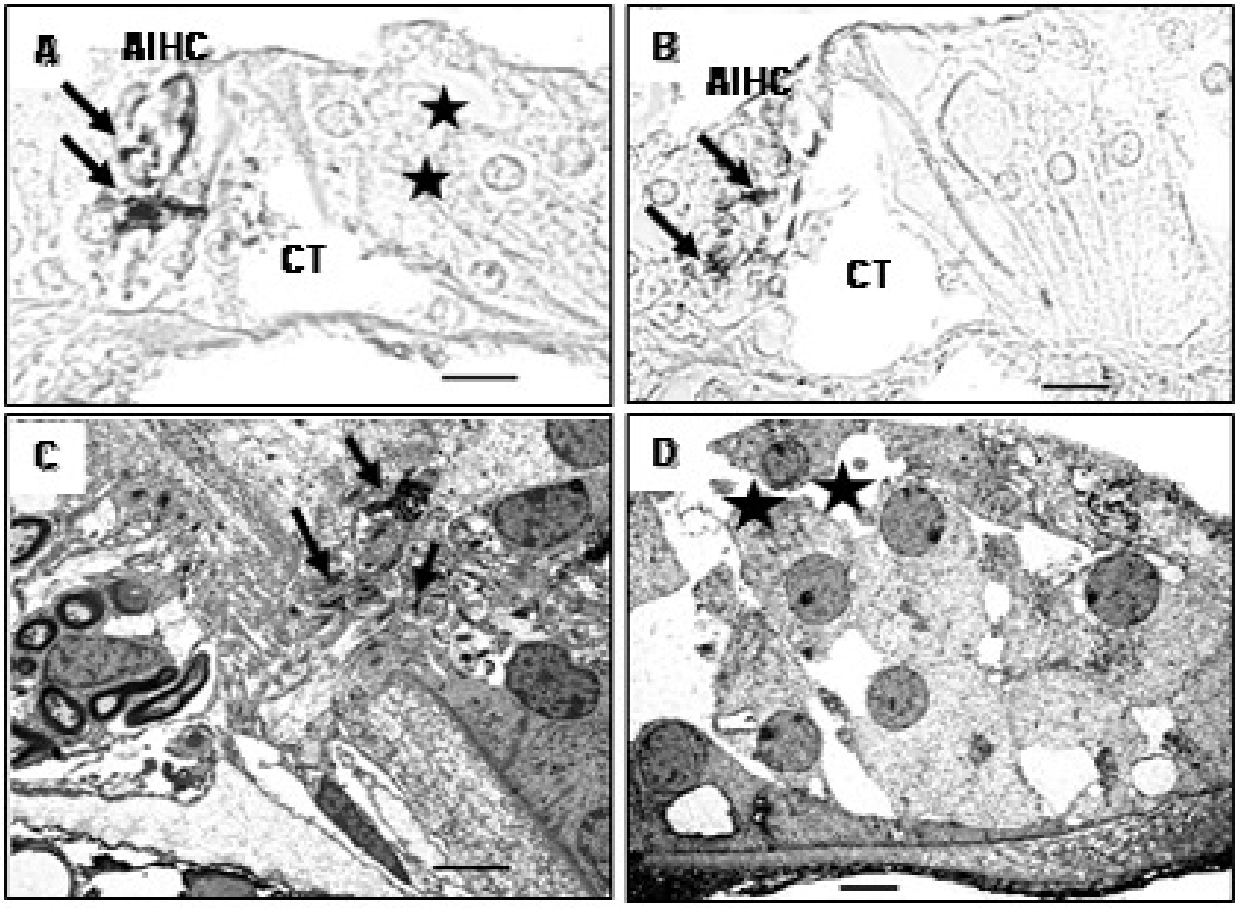
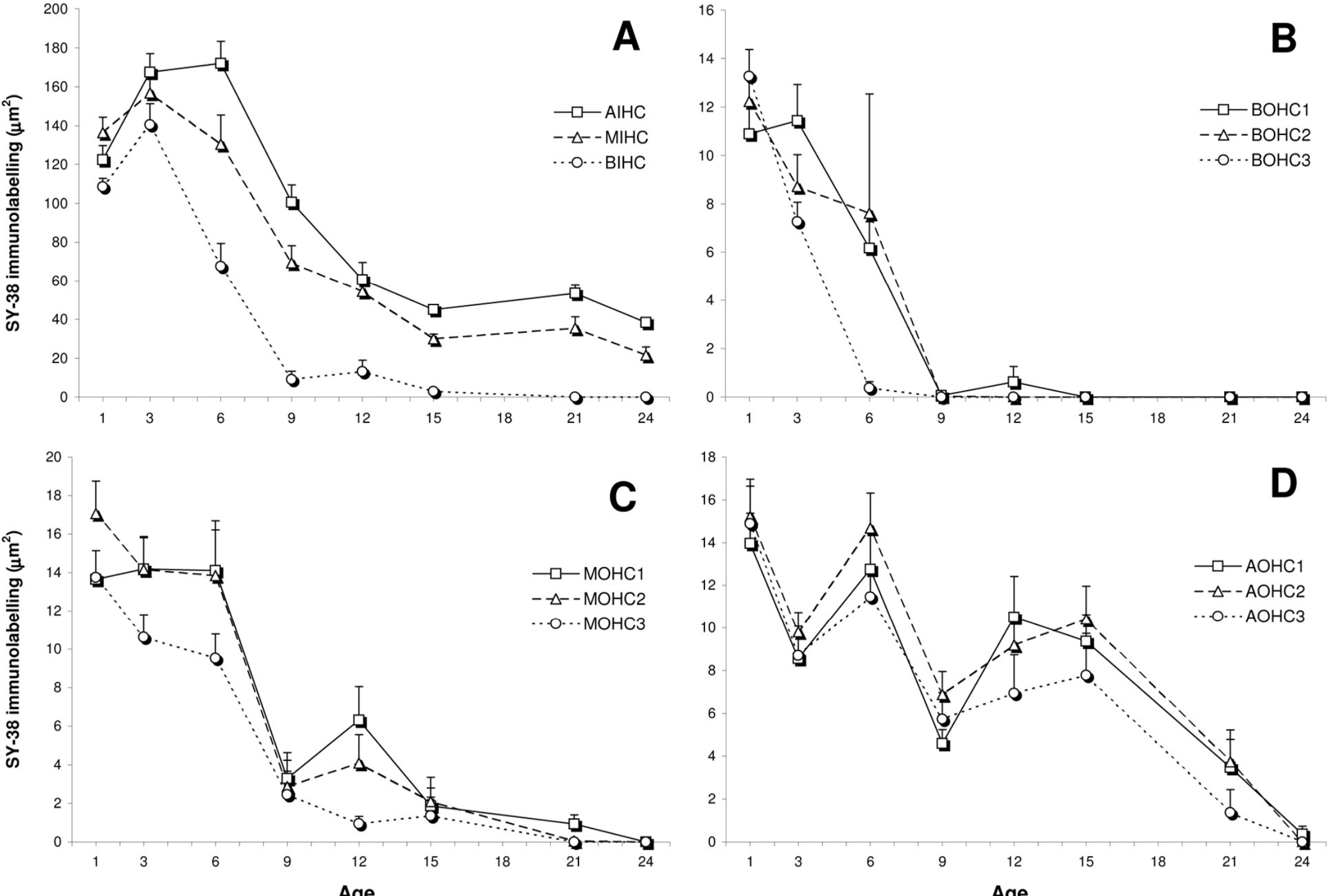


Figure 3


A
B
C
D
AIHC
MIHC
BIHC
BOHC1
BOHC2
BOHC3
MOHC1
MOHC2
MOHC3
AOHC1
AOHC2
AOHC3
SY-38 immunolabelling (µm²)
Age