# Immunocytochemical distribution of the AMPA receptor subunits GluR2/3 and GluR4 in the cristae ampullares of the hatched chicken

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## Summary

A preembedding immunoperoxidase method was used to study the distribution of the GluR2/3 and GluR4 AMPA subunits of the ionotropic glutamate receptors in the peripheral vestibular epithelium of the hatched chicken. At the light microscopic level, a strong immunoreactivity for both subunits was observed around type I hair cells in the *cristae ampullares*. At ultrastructural level, immunoreaction product was accumulated in calyx-type profiles of vestibular ganglion afferent fibres closely attached to type I hair cells. Occasionally, bouton-type endings supposedly on type II hair cells were immunostained for GluR4.

These observations, together with the electrophysiological evidence, suggest that GluR2/3 and GluR4 subunits play a role in the transmission of vestibular sensory information during early development of the chicken.

**Key words:** Vestibular organ – Glutamate receptors – Immunocytochemistry – Light microscopy – Electron microscopy – Avian

## Introduction

The vestibular information sensed in the *cristae ampullares* of the semicircular canals of the inner ear is transmitted from the periphery to the central nervous system through the vestibular ganglion neurons. The peripheral endings of these cells receive synaptic contacts from types I and II sensory hair cells. While type I is more abundant in the medial part of the *crista*, type II is more distributed in the periphery. Furthermore, type I hair cells receive calyceal nerve fibres and type II are associated with bouton-like ending fibres (Yamashita and Ohmori, 1990).

Both hair cell types exert an excitatory effect on the vestibular ganglion neurons, probably due to the release of the neurotransmitter glutamate (Raymond et al., 1988; Demêmes et al., 1990; Usami and Ottersen, 1995) acting through ionotropic glutamate receptors (Annoni et al., 1984; Soto and Vega, 1988; Prigioni et al., 1990; Dechesne et al., 1991; Soto et al., 1994a, 1994b; Puyal et al., 2002). However, these synaptic responses mediated by types I and II hair cells are already different as from early stages of development in chicken (Yamashita and Ohmori, 1990). Here we studied the distribution of AMPA receptor subunits in the vestibular end organ of the hatched chicken, which could underlie the different excitatory synaptic responses of types I and II hair cells at hatching.

## Materials and Methods

The protocols for animal care and use were according to the European Communities Council

Submitted: May 2, 2003
Accepted: July 10, 2003

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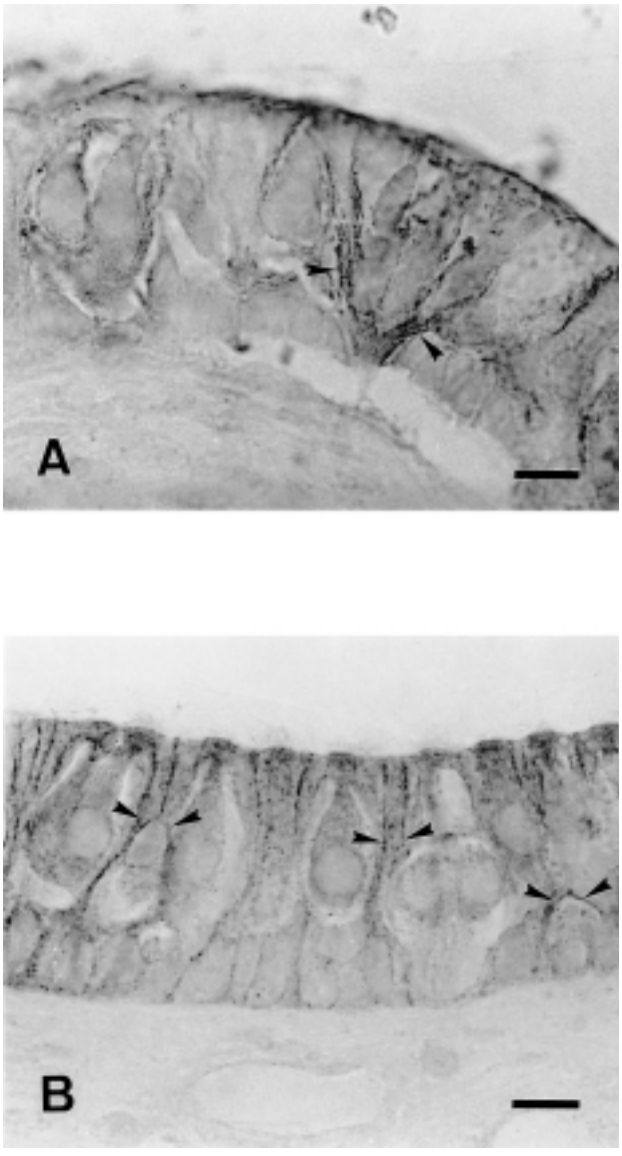


**Figure 1.-** Light microscopic distribution of the AMPA receptor subunits GluR2/3 (**A**) and GluR4 (**B**) in the *cristae ampullares* of 3-day old chicken semicircular canals. Preembedding immunoperoxidase method. GluR2/3 immunoreactivity is observed in fibres (arrowheads) within the vestibular epithelium running towards type I and type II hair cells. GluR4 immunoreactivity (B) is also observed in thin fibres between hair cells (arrowheads). Scale bars: 25 µm.

Directive (85/609/EEC). Eighteen chickens between 1 and 4 days posthatching were anaesthetised with sodium pentobarbital (60 mg/kg body weight, intraperitoneal injection) and transcardially perfused with phosphate-buffered saline (PBS; pH 7.4, at room temperature) for twenty seconds followed by 1 litre of a fixative containing 4% formaldehyde and 0.2% picric acid in ice-cold 0.1 M phosphate buffer (PB; pH 7.4) for 10-15 minutes. The semicircular canals with the ampullae were removed from the skull and postfixed in the same fixative used for perfusion for 15 minutes. After several washes in PB, ampullae were embedded in agarose and cut at 50 µm on a vibratome and preincubated with 20% normal serum for 1 hour at room temperature. Tissue sections were then incubated with a monoclonal antibody recognizing the GluR2/3 subunits (1F1, 0.02 mg/ml, Ottiger et al., 1995) and with commercially available polyclonal antibodies for the GluR4 subunit (0.2 mg/ml, Chemicon, Temécula, CA, USA) of the AMPA receptor for 48 hours at 4ºC. Then, they were processed for a conventional avidin-biotin horseradish peroxidase complex method (ABC Elite; Vector Laboratories, Burlingame, CA, USA). Briefly, sections were incubated sequentially with a biotinylated secondary antibody (1:200 anti-rabbit for GluR4; 1:400 anti-mouse for GluR2/3) and with an avidin-biotin complex, each for 1 hour at room temperature. Next, sections were preincubated with 0.05% 3,3'-diaminobenzidine for 5 minutes and then incubated for 5 minutes by adding 0.01% hydrogen peroxide to the same solution. Tissue sections were osmicated in 1% PB (0.1 M, pH 7.4) osmium tetroxide for 20 min on a shaker at room temperature, dehydrated in graded alcohols, transferred to propylene oxide and embedded flat in Epon 812 (Fluka Chemie AG, Switzerland). To improve the visualization of the immunostaining, ampullae were cut at 4 µm thickness obtained from labelled 50-µm-thick sections. The semithin sections were counterstained with Richardson´s solution, dried, dehydrated and coverslipped with DPX (Fluka Chemie AG, Switzerland). In addition, ultrathin sections from immunostained vibratome ampullar slices were collected on mesh nickel grids, stained with uranyl acetate and lead citrate, and examined using a JEOL X-100 electron microscope.

## RESULTS

At light microscopic level, a strong immunoreaction for GluR2/3 and GluR4 was mainly observed in the medial zone of the *crista ampullaris*. The stained elements for the AMPA subunits in the vestibular epithelium were distributed between and around types I and II hair cells (Fig. 1 A, B). In addition, some processes were tightly associated with vestibular hair cells (Fig. 1 A). The ampullar cells showing stained rings were immunonegative either for GluR2/3 or GluR4.

At ultrastructural level, we confirmed the presence of a diffuse immunoreaction product for GluR2/3 (Fig. 2 A, B, C) and GluR4 (Fig. 2 D-G) in profiles intimately appended to vestibular globular neurons with the characteristics of type I hair cells. The immunoreactivity was spread over the whole profile, as seen in portions of a full GluR4-immunoreactive calyceal fibre surrounding a globular cell (Fig. 2 F, G). Sometimes, afferent fibres labelled for GluR2/3 were seen to

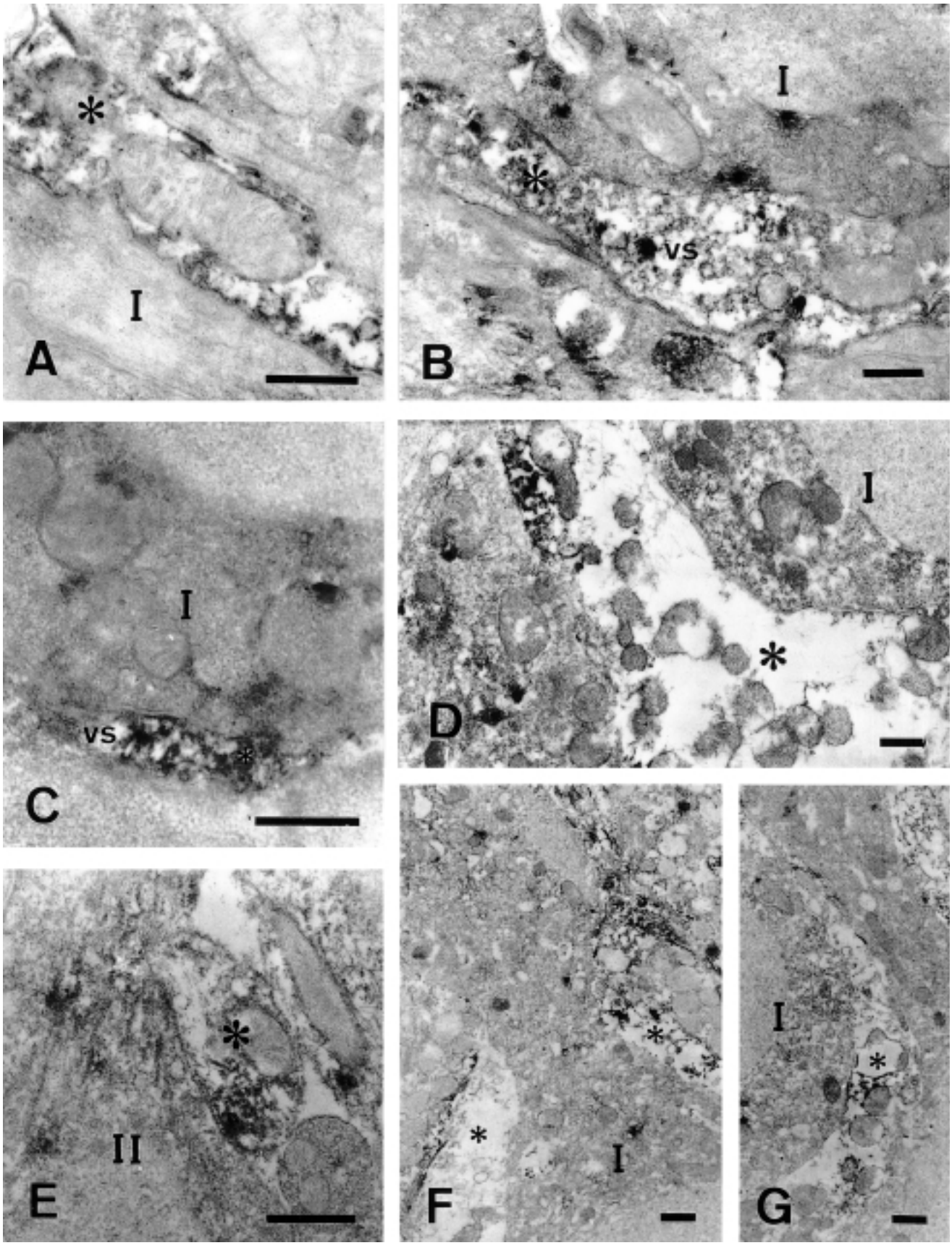


**Figure 2.-** Ultrastructural immunolocalization of GluR2/3 (**A-C**) and GluR4 (**D-G**) subunits of the AMPA receptor in chicken vestibular ampullae 3 days after hatching. Preembedding immunoperoxidase method. Note the presence of a diffused immunoreaction product for GluR2/3 within calyceal profiles (asterisks) on type I hair cells (I, **A-C**). In B, C, immunoreactivity is seen in vesicle-containing profiles (vs). GluR4 immunoreactivity is also in calyceal afferent fibres (asterisks) surrounding type I hair cells (I) (**D,F,G**) and in small bouton-like endings (asterisk in **E**) probably around a type II hair cell (II). Scale bars: 0.5 μm.

contain round and clear synaptic vesicles (Fig. 2 B), suggesting the localization of a presynaptic terminal zone to the hair cell. In turn, the sensory cell accumulated similar vesicles at the presynaptic membrane facing to the postsynaptic vestibular ganglion fibre. GluR4-immunoreactivity was also observed in some bouton-type endings (Fig. 2 E).

## Discussion

We have studied the immunocytochemical localization of AMPA receptor subunits in the vestibular end organ of the hatched chicken. Our results demonstrated that GluR2/3 and GluR4 subunits are differentially expressed in calyx-type afferent fibres receiving synapses from type I vestibular hair cells. Several lines of evidence have indicated that glutamate is the main excitatory neurotransmitter in these synapses (Raymond et al., 1988; Demêmes et al., 1990; Ottersen et al., 1998). However, until some years ago it was not clear which receptors are involved in the vestibular responses mediated by glutamate. Thus, in mammals GluR2/3 and GluR4 were seen in peripheral afferent fibres receiving excitatory synapses from type I hair cells (Demêmes et al., 1995; Matsubara et al., 1999), in agreement with the findings obtained in vestibular ganglion neurons by RT-PCR (Niedzielski and Wenthold, 1995). However, occasional synaptic terminals from peripheral fibres immunoreactive for GluR4 were in contact with sensory cells. On the other hand, vestibular hair cell bodies receiving these synaptic contacts, and those receiving GluR2/3 immunoreactive calyceal fibres, were not immunoreactive for the AMPA receptor subunits studied. A previous study has shown the distribution of GluR2/3 and GluR4 subunits on both type I and type II hair cells in the rat, using a postembedding immunogold method (Matsubara et al., 1999). Another immunocytochemical study also pointed out the possibility of the localization of GluR2/3 in sensory cells of the rat and guinea pig (Demêmes et al., 1995). Finally, there are some indications suggesting the presence of non-NMDA (N-methyl-D-aspartate) type of glutamate receptors in sensory hair cells of the frog (Prigioni et al., 1990; Guth et al., 1991). We cannot rule out the possibility of the presence of AMPA subunits in vestibular hair cells, since a good preservation of the tissue is always a determining factor in the intensity of immunoreaction. This was often not the case in our material due to the difficulty of an effective fixation of the hatched chickens.

Taken together, our observations of GluR2/3 and GluR4 distribution in calyceal fibres around type I vestibular hair cells fit in well with the distribution of these subunits in other species and this may be responsible for the distinctive electrophysiological responses detected in the isolated chicken ampullae (Yamashita and Ohmori, 1990).

## Acknowledgements

Supported by Ministerio de Ciencia y Tecnología grant BFI2002-01474, Fondo de Investigación Sanitaria grant FIS00/198, Gobierno Vasco grant PI-1999-129, and Universidad del País Vasco grants UPV212.327-G24/99, 9/UPV 212.327-14442/2002. I.E. and J.D. were in receipt of fellowships from the Universidad del País Vasco (1998 and UPV212.327-G24/99, respectively) and D.H. from the Ministerio de Ciencia y Tecnología (grant BES-2003-2722). J.J.A. was supported by the "Programa Ramón y Cajal" and N.P. by the Fondo de Investigación Sanitaria, grant FIS00/0198.

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