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Localization of the calcium channel subunits $Ca_v1.2$ (α1C) and $Ca_v2.3$ (α1E) in the mouse organ of Corti

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Summary. Voltage-activated Ca^{2+} channels play an important role in synaptic transmission, signal processing and development. The immunohistochemical localization of $Ca_v1.2$ (α1C) and $Ca_v2.3$ (α1E) Ca^{2+} channels was studied in the developing and adult mouse organ of Corti using subunit-specific antibodies and fluorescent secondary antibodies with cochlear cryosections. $Ca_v1.2$ immunoreactivity has been detected from postnatal day 14 (P14) onwards at the synapses between cholinergic medial efferents and outer hair cells as revealed by co-staining with anti-synaptophysin and anti-choline acetyltransferase. Most likely the $Ca_v1.2$ immunoreactivity was located presynaptically at the site of contact of the efferent bouton with the outer hair cell which suggests a role for class C L-type Ca^{2+} channels in synaptic transmission of the medial efferent system. The localization of the second Ca^{2+} channel tested, $Ca_v2.3$, showed a pronounced change during cochlear development. From P2 until P10, $Ca_v2.3$ immunoreactivity was found in the outer spiral bundle followed by the inner spiral bundle, efferent endings and by medial efferent fibers. Around P14, $Ca_v2.3$ immunoreactivity disappeared from these structures and from P19 onwards it was observed in the basal poles of the outer hair cell membranes.

Key words: Calcium channel, $Ca_v1.2$, $Ca_v2.3$, Cochlea, Development, Innervation

Introduction

Voltage-activated Ca^{2+} channels mediate influx of Ca^{2+} ions that serve as second messengers of electrical signalling. In neurons and sensory cells, they initiate intracellular processes such as synaptic transmission, secretion, Ca^{2+} oscillations, and gene expression, and they can modulate a variety of enzymes and ion channels. Voltage-activated Ca^{2+} channels are formed by at least four different subunits (α1, α2-δ, ß, sometimes also γ; for review see Hofmann et al., 1994; Catterall, 1998). The physiological and pharmacological diversity of voltage-gated Ca^{2+} currents that have been classified into L-, P-, Q-, N-, R-, and T-type arises primarily from the existence of multiple pore-forming a1 subunits (Hofmann et al., 1994; Catterall, 1998; Randall, 1998). Ten different α1 genes have been found so far that have been classified into three families and according to a new nomenclature are now termed $Ca_v1.1$ (α1S), $Ca_v1.2$ (α1C), $Ca_v1.3$ (α1D), and $Ca_v1.4$ (α1F) all giving rise to L-type Ca^{2+} currents; $Ca_v2.1$ (α1A), $Ca_v2.2$ (α1B) and $Ca_v2.3$ (α1E) giving rise to P/Q, N and R-type Ca^{2+} currents, respectively; and $Ca_v3.1$ (α1G), $Ca_v3.2$ (α1H), $Ca_v3.3$ (α1I) all giving rise to T-type Ca^{2+} currents (Ertel et al., 2000).

In the mouse cochlea, the occurrence of the α1 subunits $Ca_v1.2$ (formerly α1C), $Ca_v1.3$ (formerly α1D) and $Ca_v2.3$ (formerly α1E) subunits has been demonstrated using PCR analysis of total cochlear mRNA (Green et al., 1996). These subunits should give rise to L-type currents with high voltage of activation and intermediate kinetics of inactivation ($Ca_v1.2$), L-type currents with low voltage of activation and slow inactivation ($Ca_v1.3$, Koschak et al., 2001), and to R-type currents resistant to all known organic Ca^{2+} channel blockers, with an intermediate threshold of activation and fast inactivation ($Ca_v2.3$; Randall and Tsien, 1997; Randall, 1998). As we are interested in the role of Ca^{2+} channels in cochlear development and function we performed immunohistochemical and electrophysiological studies. Recently, we could show by recording from hair cells from wild-type mice and mice deficient for the Ca^{2+} channel $Ca_v1.3$ subunit that class D L-type Ca^{2+} channels ($Ca_v1.3$) are the dominating α1 subunits in both neonatal inner hair cells (IHC; Platzer et al., 2000) and outer hair cells (OHC; Michna et al.,

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2001). Staining of $Ca_v1.3$ subunits in hair cells was unsuccessful as yet due to the lack of suitable antibodies. Nothing is known so far about $Ca_v1.2$ and $Ca_v2.3$ channels and currents. Here we show the cellular and subcellular localization of $Ca_v1.2$ and $Ca_v2.3$ Ca^{2+} channel subunits in the neonatal and adult organ of Corti using immunohistochemistry.

Materials and methods

Animals

NMRI mice (Charles River Inc., Germany) aged P2-P30 (day of birth was defined as P0) were used. The care and use of the animals was approved by the University's special animal care unit and carried out according to the guidelines of the Declaration of Helsinki. Age-dependent findings reported in this study refer to the mean value of the staining results from several (4-6) animals from different litters. Within a series of cryosections from one cochlea, the staining procedure was repeated several (3-6) times. Litters were limited to a number of 10 pups at P0 to reduce the variability in maturation due to large differences in litter sizes.

Tissue preparation

Animals were decapitated (animals older than P9 were first subjected to anesthesia with CO_2), cochleae were rapidly removed and fixed by immersion in 2 % paraformaldehyde, 125 mM sucrose and 2 mM protease inhibitor (Pefabloc, Boehringer Mannheim) in 50 mM phosphate-buffered saline (PBS), pH 7.4 at 0 °C for 30 min. After washing in 25% sucrose in PBS cochleae were incubated overnight in 25 % sucrose and 2 mM Pefabloc in PBS at 4 °C. After embedding in Tissue-Tek (Sakura Finitek Europe B.V.) and storage at -70 °C cochleae were cryosectioned at 10 to 14 μm thickness, mounted on glass slides, dried for 30 min and stored at -20 °C before use. In some cases cochleae were decalcified with 1 % ascorbic acid in 0.8 % NaCl and 1 mM Pefabloc for 10 days before sectioning.

Immunofluorescence staining

Cochlear sections were thawed and dried for 30 min at room temperature, permeabilized with 0.1% Triton X-100 (Sigma) in 50 mM PBS, washed 3 times and blocked with 1% BSA in PBS. After overnight incubation with the primary antibody at 4 °C the sections were rinsed and incubated with the secondary antibody for 1 h. For double labeling, the two primary antibodies were incubated simultaneously as were the two secondary antibodies. For specification of the immunoreactivity, antibodies were incubated with the appropriate blocking peptide as described by the manufacturer. Sections were embedded with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and viewed using an Olympus AX70 microscope equipped with epifluorescence illumination.

Photomicrographs were taken on Kodak TMY-400 film (400 ASA) or images were taken using a CCD Color view camera and the acquisition imaging system Analysis (SIS, Münster, Germany). The following antibodies were used: rabbit anti-rat α1C Ca^{2+} channel subunit antibody (Alomone Labs, Israel, 1:50); rabbit anti-rat α1E Ca^{2+} channel subunit antibody (Alomone Labs, Israel, 1:50); sheep anti-human synaptophysin (The Binding Site, 1:500); sheep anti-human neurofilament (The Binding Site, 1:500); and goat anti-choline acetyltransferase (Chemicon, 1:100). Primary antibodies were detected using the secondary antibodies Cy3-conjugated goat anti-rabbit-IgG (Jackson Immuno Research, 1:1500), Alexa 488-conjugated goat anti-sheep IgG (Molecular Probes, 1:1500) or Alexa 488-conjugated donkey anti-goat IgG (Molecular Probes, 1:1500).

Results

To test the performance of $Ca_v1.2$ and $Ca_v2.3$ antibodies sagittal cerebellar sections from adult mice were stained (Fig. 1). $Ca_v1.2$ (1:100) immunoreactivity was found in Purkinje cell somata and especially in their main dendritic shafts located in the molecular layer (Fig. 1A) while immunofluorescence could not be detected in the presence of the antigenic control peptide (Fig. 1B). The staining pattern observed is consistent with the findings of Hell et al. (1993). Application of $Ca_v2.3$ antibodies (1:100) resulted in a pronounced staining of Purkinje cell somata. Staining was also observed on Purkinje cell dendrites, especially at dendritic branch points, and to a lesser extent in the granular and molecular layer (Fig. 1C). Incubation of anti-$Ca_v2.3$ antibodies with the antigenic control peptide abolished the immunofluorescence (Fig. 1D). A similar staining pattern of $Ca_v2.3$ in the cerebellum has been described before (Yokoyama et al., 1995).

After having established that both Cav1.2 and $Ca_v2.3$ antibodies worked well in brain sections we tested them in cochlear sections of different ages. $Ca_v1.2$ immunoreactivity was not observed in the organ of Corti before P14 (data not shown). Distinct staining was found in small structures at or below the base of the outer hair cells shown in Fig. 2 for a mouse cochlea at P17. They were identified by using neuronal markers such as anti-synaptophysin antibody, which stains efferent synaptic endings in the organ of Corti (Knipper et al., 1995), and anti-neurofilament, which stains mainly afferent nerve fibers (Hafidi et al., 1990). Anti-neurofilament antibodies did not cross-react with the $Ca_v1.2$-positive structures below the OHCs (data not shown). In contrast, synaptophysin stained the large axosomatic synapses onto OHCs (Fig. 2B, solid arrows below OHCs) and diffuse aggregates below the inner hair cell which most likely are efferent fibers projecting to IHC afferents (inner spiral bundle, Fig. 2B, arrowhead below IHC).

Double labeling with both $Ca_v1.2$ and synaptophysin suggested that $Ca_v1.2$ immunoreactivity below the OHCs was located at the outermost part of each efferent terminal that contacted the OHC (Fig. 2C, solid arrows).

To test if the $Ca_v1.2$ staining at the OHC efferent synapse was a transient phenomenon during development sections from mature cochleae were investigated. In cochlear sections obtained at P27, small structures below the OHCs were labeled (Fig. 3A,B; arrows) but no specific labeling could be observed in the presence of the $Ca_v1.2$ antigenic control peptide (Fig. 3C). Identification of the labeled structures was attempted by co-staining with antibodies directed either against the efferent marker synaptophysin or against choline acetyltransferase (ChAT), a marker for cholinergic efferents (Eybalin, 1993). Anti-synaptophysin stained the inner spiral bundle (arrowheads in Fig. 3D,F) and the efferent terminals onto OHCs (arrows in D,F). Anti-ChAT showed essentially the same staining pattern (Fig. 3E, arrowhead indicates the inner spiral bundle; arrows point to the efferent boutons onto OHCs). Using double-excitation the distinct $Ca_v1.2$ immunoreactivity below the outer hair cells could be localized to the synaptophysin-positive efferent endings and ChAT-positive efferent endings that contacted the OHCs as indicated by the yellow fluorescence (arrows) in Fig. 3G and Fig. 3H, respectively. Higher magnification images of the double-labeled synapses of medial efferents onto OHCs suggested that the $Ca_v1.2$ channels were located presynaptically and thus may be involved in release of acetylcholine from the medial efferents onto OHCs (Fig. 3J, K). As a control, $Ca_v1.2$ antibodies were incubated with the antigenic peptide which resulted in loss of the specific staining of efferent endings (Fig. 3C,I,L).

To localize R-type Ca^{2+} channels formed by $Ca_v2.3$ subunits in the organ of Corti, cochlear sections between P2 and P30 were analyzed. From P2 to P10 $Ca_v2.3$ immunoreactivity was observed diffusely below IHCs and, much more pronounced, in a pearl-chain-like pattern below the OHCs (Fig. 4A-C). At P10, efferent boutons onto OHCs also seemed to be immunoreactive for $Ca_v2.3$ (Fig. 4C, small arrowheads). The staining was abolished by adding the antigenic control peptide (Fig. 4A, insert A1). To identify the pearl-chain-like structures below the OHCs, co-staining with anti-neurofilament was employed. The neurofilament staining (Fig. 4D-F) also showed a pearl-chain-like staining pattern below the OHCs which corresponded to the cross-sectioned outer spiral bundle (Hafidi et al., 1990 and Discussion). In

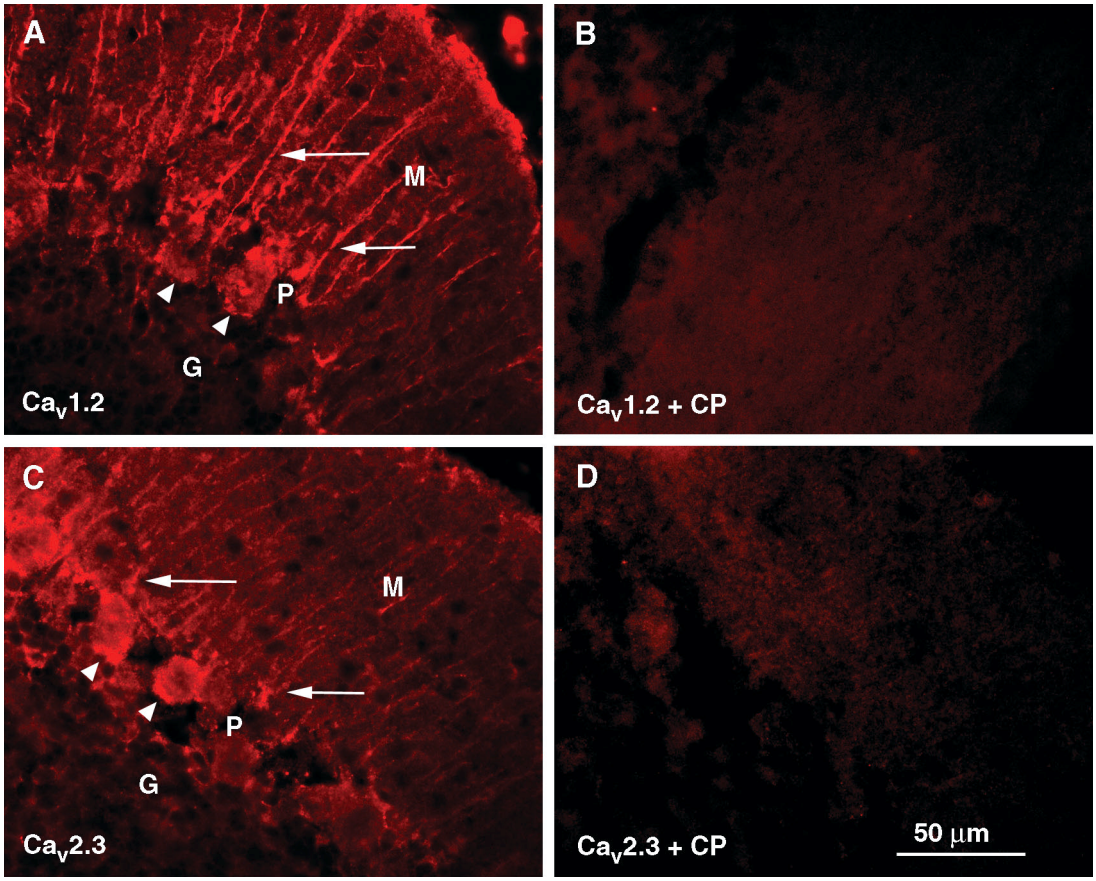
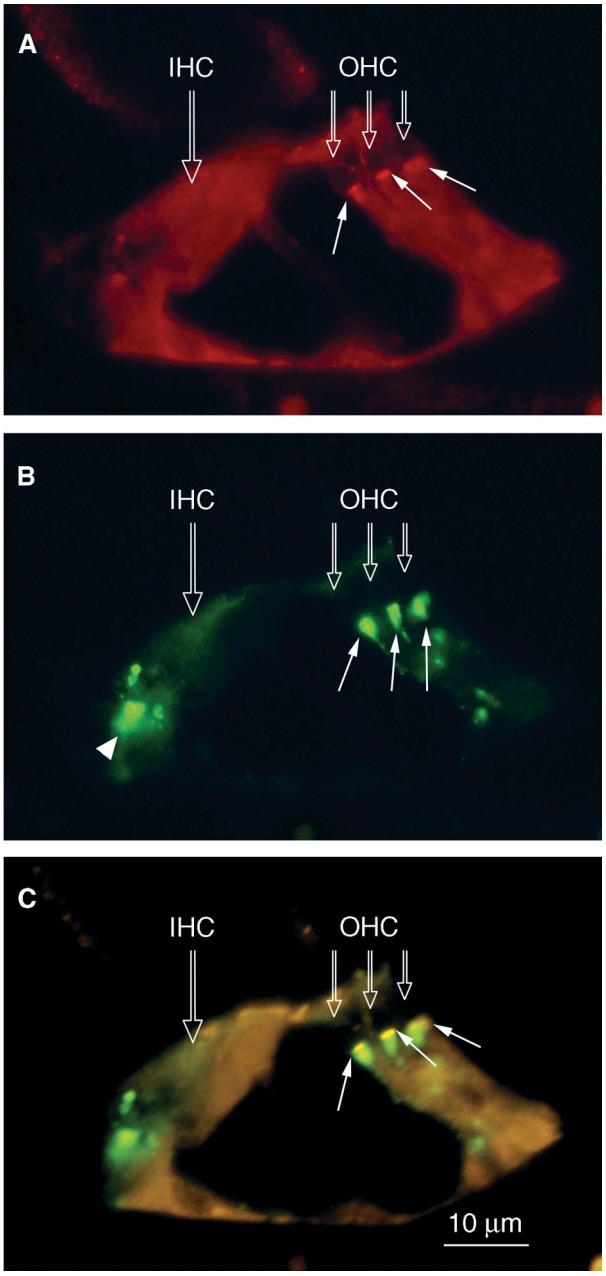


Fig.1. Immunostaining of $Ca_v1.2$ and $Ca_v2.3$ Ca^{2+} channel subunits in the cerebellum. Sagittal sections are labeled with $Ca_v1.2$ and class $Ca_v2.3$ channel antibodies using the immunofluorescence method described in Materials and Methods. **A.** Cerebellar section showing $Ca_v1.2$ immunoreactivity in the granular layer (G), Purkinje cell layer (P), and molecular layer (M). Immunoreactivity is prominent in somata of Purkinje cells (arrowheads) and at proximal and distal (arrow) dendrites. **B.** Class C Ca^{2+} channel immunoreactivity is lacking in the presence of the $Ca_v1.2$ control peptide (CP). **C.** $Ca_v2.3$ immunoreactivity is found in the somata (arrowheads) and especially at dendritic branch points (arrows) of Purkinje cells. **D.** $Ca_v2.3$ immunoreactivity is abolished in the presence of the $Ca_v2.3$ control peptide.



addition, anti-neurofilament stained a diffuse region below the IHCs (afferent fibers and inner spiral bundle) and presumptive efferent tunnel fibers at P5 and P10 (Fig. 4E, F). From P2 to P10, the neurofilament staining matched the $Ca_v2.3$ pearl-chain staining pattern below the OHCs as confirmed by double excitation (Fig. 4 G-I) indicating that the type II afferents comprising the outer spiral bundle contained $Ca_v2.3$ Ca^{2+} channel subunits.

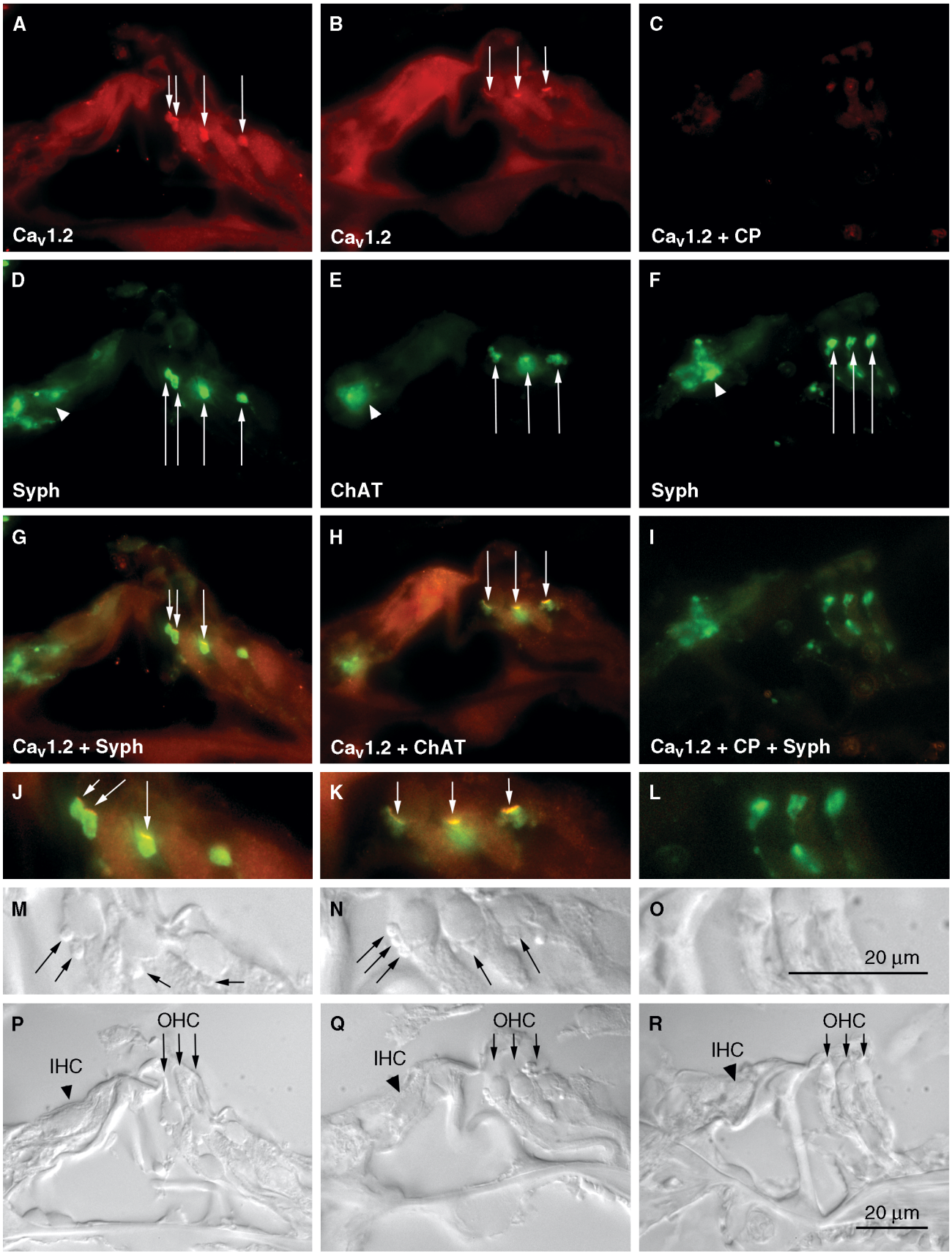
Upon further maturation of the organ of Corti, the $Ca_v2.3$ staining pattern changed completely: immunoreactivity vanished from the outer spiral bundle, the efferent endings and mostly also below the IHCs around P14. In contrast, a distinct $Ca_v2.3$ membrane staining in the basal parts of the outer hair cells was observed at P20 (Fig. 5) that could be blocked with the antigenic peptide (not shown). This finding suggests the presence of $Ca_v2.3$ channels in mature OHCs. The age-related switch in the staining pattern was found in both decalcified and non-decalcified specimens, ruling out that it was due to decalcification artifacts.

Discussion

The immunohistochemical analysis of the two Ca^{2+} channel $\alpha1$ subunits $Ca_v1.2$ and $Ca_v2.3$ revealed that the occurrence of both subunits changed upon maturation of the cochlea. R-type Ca^{2+} channel forming $Ca_v2.3$ subunits showed a remarkable switch during postnatal maturation of the mouse organ of Corti. The transient occurrence of $Ca_v2.3$ in the inner spiral bundle, medial efferent endings and, most prominently, in the outer spiral bundle from P2-P14 raises the question as to why $Ca_v2.3$ expression is restricted to this short temporal

Fig. 2. $Ca_v1.2$ Ca^{2+} channels colocalize with efferent synaptic boutons onto outer hair cells in the developing organ of Corti. Cochlear sections (P17) are double-stained with anti-$Ca_v1.2$ antibodies (red fluorescence) and anti-synaptophysin antibodies (green fluorescence) as described in Material and Methods. The position of IHCs and OHCs is indicated by the open arrows, respectively. **A.** $Ca_v1.2$ immunoreactivity is found below outer hair cells (solid arrows). **B.** Anti-synaptophysin antibody stains synapses onto IHC afferent fibers (arrowhead) and large efferent boutons below OHCs (solid arrows). **C.** Double-excitation reveals that $Ca_v1.2$ channels colocalize with the upper edge of the efferent synaptic boutons that contacts the base of the outer hair cells (yellow fluorescence, solid arrows).

Fig. 3. $Ca_v1.2$ channels colocalize with cholinergic efferent synaptic boutons onto outer hair cells in the mature organ of Corti. Cochlear sections (P27) are double-stained with anti-$Ca_v1.2$ antibodies (red fluorescence) and either anti-synaptophysin (green fluorescence, left and right row) or anti-choline acetyltransferase antibodies (ChAT; green fluorescence, middle row). The right row shows $Ca_v1.2$ staining including the $Ca_v1.2$ antigenic peptide (CP). **A, B:** $Ca_v1.2$ immunoreactivity is observed in structures just below the base of the outer hair cells (arrows). The specific staining is lacking in the presence of the $Ca_v1.2$ control peptide **(C). D, F:** Anti-synaptophysin stains the inner spiral bundle (arrowhead) and the large efferent boutons contacting the OHCs (arrows). A similar staining pattern is obtained using anti-ChAT antibody **(E)**. **G, H:** Double-excitation reveals that $Ca_v1.2$ channels are colocalized with the upper edges of the efferent synaptic boutons just at the contact site to the base of the OHCs (yellow fluorescence, arrows). Efferent endings onto the OHCs are shown at higher magnification under double-excitation **(J-L)** and as differential interference contrast (DIC) images **(M-O)**. Large synaptic boutons can be seen in the DIC image (black arrows in M,N). **P-R.** Corresponding DIC images of the complete cochlear sections. For the control staining (right row, $Ca_v1.2$ antibody plus control peptide) anti-synaptophysin is used as a marker for efferent endings (F). The characteristic $Ca_v1.2$ labeling of efferent endings is lacking in this case **(C,I,L)**.


A
$Ca_V1.2$
B
$Ca_V1.2$
C
$Ca_V1.2$ + CP
D
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E
ChAT
F
Syph
G
$Ca_V1.2$ + Syph
H
$Ca_V1.2$ + ChAT
I
$Ca_V1.2$ + CP + Syph
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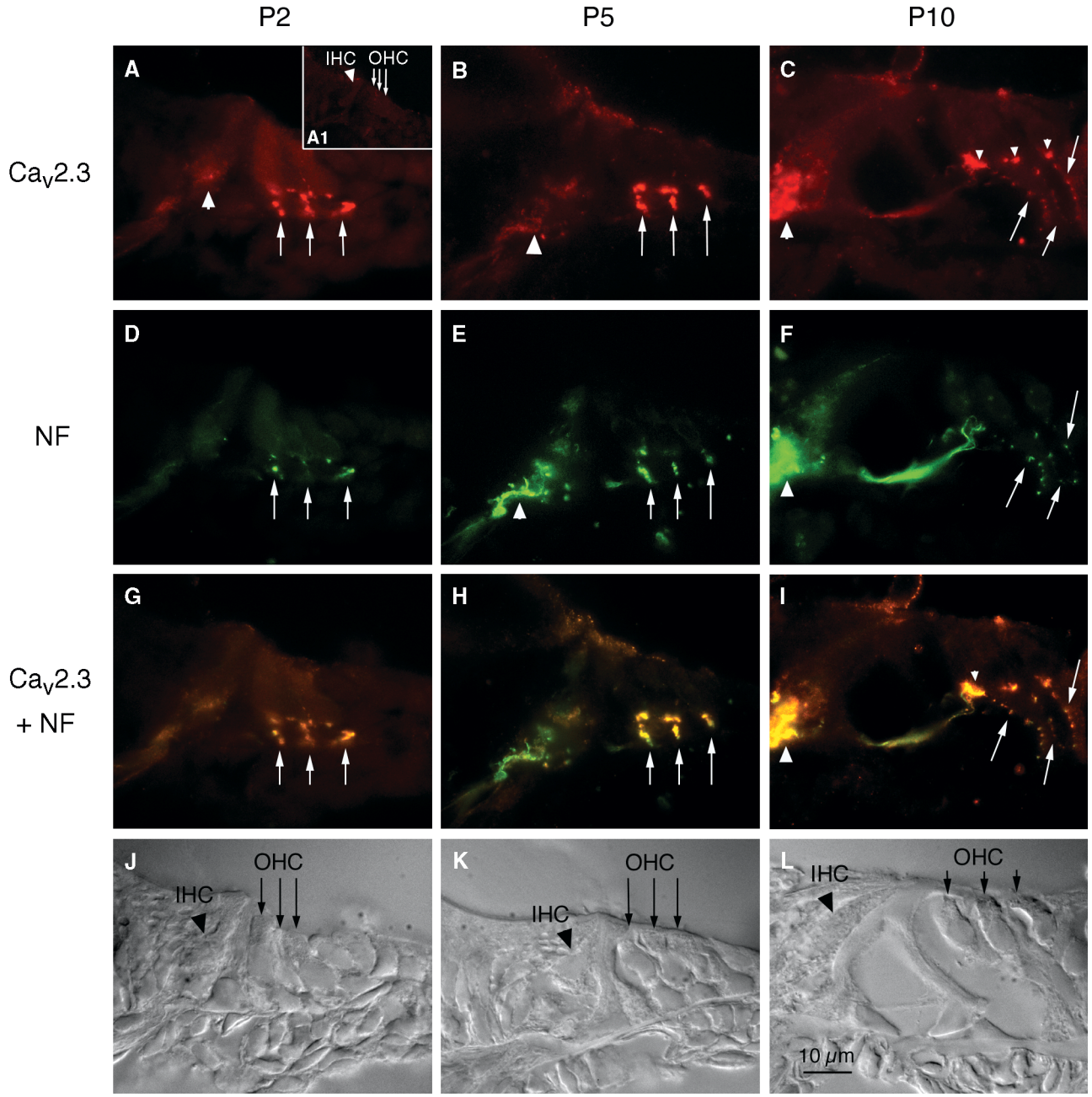

Fig. 4. Localization of $Ca_v2.3$ Ca^{2+} channels in the developing organ of Corti shown by double-labeling with anti-neurofilament. Left row: P2; middle row: P5; right row: P10. A-C show $Ca_v2.3$ immunoreactivity (red fluorescence). **A.** At P2, $Ca_v2.3$ immunoreactivity is prominent in a dot-like manner below the OHCs (arrows) and is sparsely found below IHCs (arrowhead, see corresponding DIC images at the bottom of each row for comparison). The insert A1 shows the lack of $Ca_v2.3$ immunostaining in the presence of the $Ca_v2.3$ antigenic control peptide. **B.** At P5, the $Ca_v2.3$ immunostaining pattern is essentially the same as at P2, but more intense below the OHCs (arrows). **C.** At P10, $Ca_v2.3$ immunoreactivity is found below the IHC (large arrowhead), in tunnel-crossing fibers, in circular to oval structures just below the OHCs (small arrowheads), and in a pearl-chain-like fashion extending from the OHCs towards the basilar membrane (arrows). **D-F.** Neurofilament immunoreactivity (green fluorescence) is found in a dot-like manner below the OHCs corresponding to the cross-sectioned outer spiral bundle (arrows). At P5 and P10, neurofilament immunoreactivity is also found below the IHCs representing afferent fibers and the inner spiral bundle (arrowheads, E, F) and at P10 in tunnel crossing fibers (F). **G-I.** Double excitation reveals the colocalization of $Ca_v2.3$ immunoreactivity and anti-neurofilament staining (yellow fluorescence) below the OHCs from P2 to P10 indicating that $Ca_v2.3$ channels are present in the outer spiral bundle at that age (G-I, arrows). At P10, $Ca_v2.3$ antibodies additionally label the inner spiral bundle (I, large arrowhead), tunnel-crossing fibers, and the efferent synaptic ending onto the innermost OHC (I, small arrowhead).

window. A maturational change in the composition of presynaptic Ca^{2+} currents including R-type currents has been found in several structures of rat brain; at the calyx of Held synapse, N-, P/Q-, and R-type currents were identified in the first postnatal days before they became predominantly P/Q-type around P13 (Iwasaki et al., 2000). In cerebellar granule cells, R-type currents were shown to be a prerequisite for Ca^{2+}-dependent action potentials present in the first neonatal weeks but which vanished around P21 (D'Angelo et al., 1997).

The transient occurrence of $Ca_v2.3$ immunoreactivity associated with nerve fibers falls in that period in which afferent and efferent innervation of IHCs and OHCs undergo final differentiation (for summary, see Pujol et al., 1997; Rubel and Fritzsch, 2002). At birth, OHCs are transiently innervated by collaterals of type I afferent fibers which retract from P6 onwards (Pujol et al., 1985; Gil-Loyzaga and Pujol, 1990). In parallel, OHCs are contacted by type II afferents that are not subject to damage in kainate-induced excitotoxicity (Pujol et al., 1985; Gil-Loyzaga and Pujol, 1990). On the other hand, dot-like structures have been stained with antibodies against the GluR4 subunit of AMPA receptors at the base of adult rat OHCs similar to the more numerous punctate staining at the base of IHCs suggesting postsynaptic AMPA receptors also in OHCs (Kuriyama et al., 1994). Taken together, the identity of both afferent transmitter and postsynaptic receptors in mature OHCs and their functional significance is still unclear (Rubel and Fritzsch, 2002).

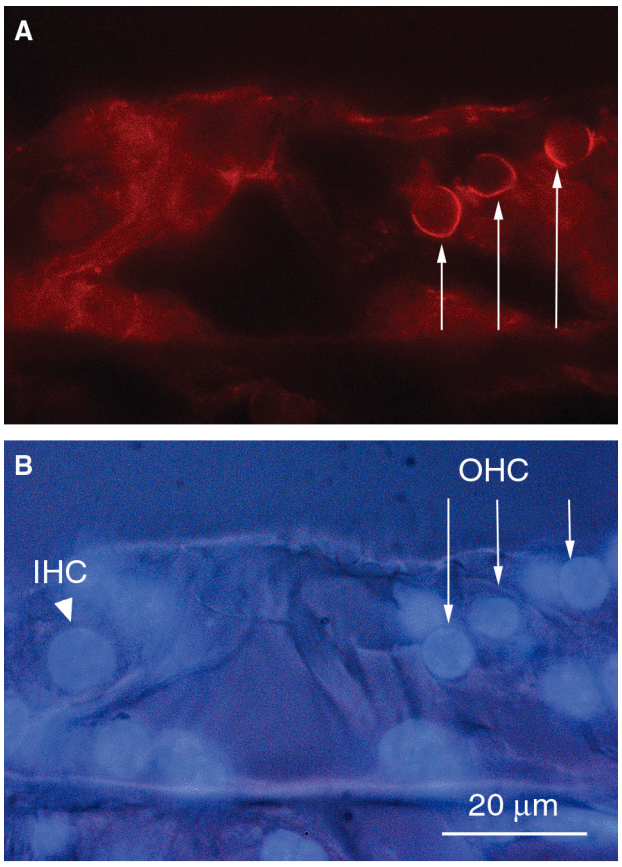

Fig. 5. Localization of $Ca_v2.3$ Ca^{2+} channels in the adult organ of Corti. **A.** $Ca_v2.3$ immunoreactivity is found at the basal poles of the OHCs (arrows, P20). **B.** Shows the corresponding DIC image combined with the nuclear stain DAPI to outline the position of the OHCs (arrows) and their nuclei.

From the neurofilament staining pattern (Fig. 4D) it follows that - irrespective of whether synapses are yet formed - type II afferent fibers comprising the outer spiral bundle are present below OHCs as early as P2. The increasing number of cross-sectioned type II afferents below each OHC (2-3 at P2, about 10 at P10) indicates that type II afferents substantially grow in a spiral fashion to make contact to an increasing number of OHCs in this period (Simmons et al., 1991; Echteler, 1992). The transient expression of $Ca_v2.3$ subunits in type II afferents could serve two functions: first, these channels could play a role in the pathfinding towards OHCs, a process that involves neurotrophin signalling (Knipper et al., 1997, 1999; Wiechers et al., 1999; Rubel and Fritzsch, 2002). Induction of the neurotrophin BDNF for example is controlled by the transcription factor cAMP-response element (CRE) and its binding protein CREB, both of which are activated by Ca^{2+} influx via voltage-activated Ca^{2+} channels or via ionotropic glutamate receptors (Tao et al., 1998; Tabuchi et al., 2000; West et al., 2001). If the CREB signalling pathway was involved in the pathfinding process of the growing type II afferent fibers, low- to mid-voltage-activated R-type Ca^{2+} channels (Randall, 1998) in type II afferents might provide the Ca^{2+} influx required for its activation. Secondly, neonatal OHCs possess $Ca_v1.3$-mediated Ca^{2+} currents (Michna et al., 2001) and are able to produce slow Ca^{2+}-dependent action potentials upon mild current injection in vitro (Marcotti and Kros, 1999). If afferent synaptic transmission worked between neonatal OHCs and type II afferents similar to that between neonatal IHCs and type I afferents (Beutner et al., 2001; Glowatzki and Fuchs, 2002) there would be two differences in signalling: First, the threefold smaller whole cell Ca^{2+} currents of OHCs (Michna et al., 2001) would result in lower transmitter release and smaller EPSPs in type II afferent fibers. Secondly, since many OHCs converge on the same type II fiber (Sobkowicz, 1992), a single EPSP would further decline when a particular small diameter collateral joins a larger diameter main fiber. Due to their relatively low threshold of activation, R-type Ca^{2+} channels could serve to enhance EPSPs in type II afferents. Enhancement by R-type channels may be obsolete after the onset of hearing (P12-P18) when OHCs converging on the same type II fiber are excited simultaneously by an acoustic stimulus, given that afferent signalling is still working in mature OHCs.

The complete lack of $Ca_v2.3$ immunoreactivity from type II afferent fibers after P14 supports the notion that it

plays an important role in the morphological and functional maturation of OHC innervation. In adult OHCs, a weak but consistent $Ca_v2.3$ staining was found along their basal poles, a membrane region that comprises ion channels and receptors but is devoid of the motor protein prestin (Weber et al., 2002). Type II afferent fibers contact OHCs at their basal pole (Pujol et al., 1985), so the relatively low voltage-activated $Ca_v2.3$ channels might mediate presynaptic Ca^{2+} influx in a presumptive afferent signalling of adult OHCs. Clarifying the presence and significance of $Ca_v2.3$ channels has to await the electrophysiological analysis of Ca^{2+} currents in adult OHCs.

The second type of voltage-activated Ca^{2+} channel that was subject of this study, $Ca_v1.2$ forming class C L-type Ca^{2+} channels, appeared relatively late in postnatal cochlear development. It was detected around P14 at the synapse between cholinergic medial efferents with OHCs at a time shortly after the onset of hearing when efferent signalling starts to work in the mouse (Pujol et al., 1997).

At present, we cannot rule out a postsynaptic localization of $Ca_v1.2$ channels on the side of the OHC without using immunostaining at the electron microscopic level or recording Ca^{2+} currents from mature OHCs. It seems, however, more likely that the $Ca_v1.2$ channels were located presynaptically: First, $Ca_v1.2$ immunoreactivity was never found in patches of OHC membranes not in contact with efferent boutons. Secondly, it is hard to conceive that high-voltage-activated Ca^{2+} channels should be involved in the ACh-mediated postsynaptic inhibition of OHCs that is thought to be mediated by Ca^{2+} influx through ionotropic ACh receptors and subsequent fast activation of Ca^{2+}-dependent SK2 potassium channels (Housley and Ashmore, 1991; Oliver et al., 2000).

A presynaptic localization of $Ca_v2.1$ channels would imply that class C L-type Ca^{2+} channels at least contribute to presynaptic Ca^{2+} influx which in central neurons is generally believed to be mediated by the non-L-type Ca^{2+} channel subunits $Ca_v2.1$ (α1A), $Ca_v2.2$ (α1B) and/or $Ca_v2.3$ (α1E) (Dunlap et al., 1995; Reuter, 1996; Catterall, 1998). However, a contribution of L-type Ca^{2+} channels to transmitter release has been identified for example in cortical and subthalamic axon terminals projecting on rat midbrain dopaminergic neurons (Bonci et al., 1998) and in axon terminals of bipolar cells (Satoh et al., 1998).

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