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Cellular localization and developmental changes of the different isoforms of divalent metal transporter 1 (DMT1) in the inner ear of rats

Dalian Ding · Richard Salvi · Jerome A. Roth

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Abstract Divalent metal transporter 1 (DMT1) is generally considered to be the major transmembrane protein responsible for the uptake of a variety of divalent cations. Four isoforms of DMT1 have been identified in mammalian cells encoded by a single gene that differ both in their N- and C-terminal sequences with two mRNA isoforms possessing an iron response element (IRE) motif downstream from the stop codon on the message. Two distinct promoter sites regulate production of the 1A or 1B isoforms (translation starts at exon 2) for both the +IRE or –IRE species of the transporter resulting in the generation of four distinct configurations of this protein. Prior studies from our laboratory using cochlear organotypic cultures isolated from postnatal day three rats (P3) have demonstrated that Mn causes significant and selective damage to sensory hair cells and auditory nerve fibers and spiral ganglion neurons in a time and concentration dependent manner. Since DMT1 plays a critical role in controlling the uptake of a variety of essential and toxic metals into the cochlea, we compared the distribution and developmental changes of the 1A, +IRE and −IRE isoforms in rat inner ear. Results reveal that all three isoforms of DMT1 are selectively expressed in different cell populations within the cochlea and, additionally, demonstrate their cellular and subcellular distribution changes with development.

Keywords Divalent metal transporter 1 (DMT1) · Cochlea · Inner ear · Hair cells · Iron · Manganese

D. Ding · R. Salvi
Center for Hearing and Deafness, University at Buffalo, 137 Cary Hall, Buffalo, NY 14214, USA
e-mail: dding@acsu.buffalo.edu

R. Salvi
e-mail: salvi@buffalo.edu

D. Ding
Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China

J. A. Roth (✉)
Department of Pharmacology and Toxicology, University at Buffalo, 11 Cary Hall, Buffalo, NY 14214, USA
e-mail: jaroth@buffalo.edu

Introduction

Divalent metal transporter 1 (DMT1) is generally considered to be the major transmembrane protein responsible for the uptake of a variety of essential, but potential toxic divalent metals including Fe^{2+}, Mn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, and Pb^{2+} (Gunshin et al. 1997). Four isoforms of DMT1 have been identified in mammalian cells encoded by a single gene (Fleming et al. 1997; Gunshin et al. 1997; Hubert and Hentze 2002) and all are 12 putative membrane-spanning

domain proteins (Gruenheid et al. 1995; Fleming et al. 1997; Gunshin et al. 1997; Lee et al. 1998; Su et al. 1998) that differ both in their N- and C-terminal sequences with two mRNA isoforms possessing an iron response element (IRE) motif downstream from the stop codon on the message. Two distinct promoter sites regulate production of the 1A or 1B isoforms (translation starts at exon 2) for both the +IRE or −IRE species of the transporter resulting in the generation of four distinct configurations of this protein. All four isoforms starting at exon 2 share 543 residues in common but differ structurally in the last 18 (+IRE) or 25 (−IRE) carboxy terminal residues. The operative necessity and differences in the physiological functions of these four isoforms of DMT1 have not been adequately established although all are equally capable of transporting transition metals. Thus, the action of the four species does not appear to be related to their absolute or native binding capacity but most likely, due to factors that regulate their synthesis, degradation and subcellular distribution in any given cell. This is clearly evidenced by prior studies demonstrating that expression of each isoform of DMT1 is selectively regulated at the levels of transcription, translation and protein degradation (Mims and Prchal 2005; Paradkar and Roth 2006; Roth 2009). Since DMT1 is generally regarded as the major transport protein for uptake of iron, the independent mechanisms governing formation of the four species of DMT1 allow for adaptive adjustments in intracellular levels of iron under a variety of normal and abnormal conditions. The overall objective preserves homeostatic levels of iron within the cell which need to be acutely regulated in order to prevent both iron deficiency anemia and iron toxicity.

Our interest in evaluating the distribution of the DMT1 isoforms in the inner ear stems, in part, from the fact that iron deficiency and overload can result in hearing loss and cochlea pathology (Khalkova and Kostadinova 1986; Sun et al. 1990, 1991; Josephs et al. 2005; Kurekci et al. 2006; Bouchard et al. 2008; Ding et al. 2011b; Jougleux et al. 2011). This is predicated on the studies of Ma et al. (2008) demonstrating that DMT1 is the major divalent transport protein in the inner ear and is likely the transporter responsible for uptake of Mn and other divalent metals as well. Prior studies have, in fact, suggested that DMT1 may be the rate limiting step controlling the severe and debilitating neurotoxic disorder associated with excess accumulation of Mn in the CNS (Krieger et al. 1995; Pomier-Layrargues et al. 1995; Roth et al. 2013). Thus, it is reasonable to hypothesize that DMT may play a critical role in controlling the uptake and subsequent accumulation of a variety of essential and non-essential metals into the cochlea including their selective distribution within the different cells of the inner ear.

Recent studies from our laboratory using cochlear organotypic cultures isolated from P3 rats have demonstrated that Mn causes significant damage to sensory hair cells, peripheral auditory nerve fibers (ANFs) including the ANF and spiral ganglion neurons (SGN) in a time and concentration dependent manner (Ding et al. 2011b). The toxic effects of Mn are highly selective as the peripheral ANFs were particularly more vulnerable to Mn toxicity than the sensory hair cells. Mn also induced an atypical pattern of sensory cell damage with a greater and relatively uniform degeneration inner hair cells (IHCs) along the length of the cochlea compared to outer hair cells (OHCs). Since DMT1 is the major divalent cation transporter in the inner ear, the question is raised as to whether the relative abundance and distribution of the various isoforms of this protein are at all responsible for the selective neurotoxic actions of Mn observed. The role of DMT1 in controlling Mn-induced ototoxicity is not known, however, other DMT1 substrates, specifically Co, Pb and Cd have also been reported to cause hearing impairment and cochlear pathologies (Ozcaglar et al. 2001; Chuang et al. 2007; Prasher 2009; Choi et al. 2012; Apostoli et al. 2013). To address the issue as to the involvement of DMT1 in metal-induced ototoxicity, we investigated the distribution and intensity of DMT1 immunolabeling in the cochlea using specific antibodies that recognize three different isoforms of the DMT1 protein.

Experimental procedures

Materials

Preparation of rabbit polyclonal antibody used to identify −IRE, +IRE and 1A isoforms of DMT1 in the cochlear tissue has been described previously (Roth et al. 2000; Lis et al. 2004). Alexa Fluor 488 conjugated goat anti-rabbit IgG, the secondary antibody for DMT1 immunolabeling, TO-PRO-3 used to

identify the cell nucleus and Alexa Fluor 555 donkey anti-mouse IgG secondary antibodies were purchased from Life Technologies. Mouse monoclonal antibody against neurofilament 200 used to identify SGN and ANF was obtained from Sigma.

Animals

P3 and adult Sprague–Dawley rats were used for this study (Charles River Laboratories, Wilmington, MA). Animals had free access to water and were fed ad libitum normal rat chow. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University at Buffalo, and conform to the guidelines issued by the National Institutes of Health.

Immunocytochemistry

Cochlear tissue was carefully removed from the rats and initially fixed with 10 % formalin in PBS overnight at 4 °C. After rinsing with PBS, the cochlear basilar membrane, spiral ligament containing the stria vascularis, and SGN in Rosenthal's canal were carefully dissected out. Specimens were subsequently incubated with 1 % Triton X100 and 5 % goat serum in 0.1 M PBS plus primary antibody (rabbit polyclonal antibody against −IRE, +IRE or 1A) at 4 °C for 24 h. Specimens were rinsed 3 times in PBS for 15 min each and then incubated with Alexa 488 conjugated goat anti-rabbit IgG for 60 min at room temperature. After rinsing with PBS, ANF and SGN were double labeled with neurofilament antibody as described in our recent publications (Ding et al. 2011a, b, 2012). Briefly, specimens were incubated in 1 % Triton X100 and 5 % donkey serum in 0.1 M PBS plus mouse primary antibody against neurofilament 200 kDa at 4 °C for 24 h. On the second day, specimens were rinsed and then incubated in Alexa 555 donkey anti-mouse IgG secondary antibody for an additional 60 min. All specimens were routinely stained with TO-PRO-3 to identify the nucleus. Specimens were mounted on glass slides in glycerin and coverslipped.

Confocal microscopy

Specimens were examined under a confocal microscope (Zeiss LSM-510 Meta) using appropriate filters to detect green fluorescence of Alexa 488 labeled product to the different species of DMT1 (excitation 488 nm, emission 520 nm). The red fluorescent signal of Alexa 555 in SGN and ANFs was monitored at excitation of 550 nm and emission at 570 nm and purple fluorescence of TO-PRO-3 at excitation of 642 nm and emission at 661 nm. Confocal images were processed using Confocal LSM Image Examiner and Adobe Photoshop 5.5 software as described previously (Ding et al. 2011a). Using the Ortho function feature in the Zeiss LSM Image Examiner software, a single horizontal x–y image plane can be displayed, multiple horizontal images can be merged into a single plane or a z-plane image plane can be reconstructed from a series of horizontal x–y image plane.

Results

DMT1 expression in the organ of Corti

We examined the immunolabeling patterns of the three isoforms of DMT1 in the organ of Corti of P3 rats and adult rats. The 1A, −IRE, and +IRE isoforms of DMT1 (green fluorescence) were largely undetectable in the organ of Corti of P3 rats (Fig. 1a, b, c respectively) with the exception of a thin strip of +IRE immunolabeling in supporting cells in the lateral region of the outer sulcus (Fig. 1c), a region occupied by Hensen's cells in the mature cochlea. In contrast, immunolabeling of both +IRE and –IRE forms of DMT1 was detected in the adult organ of Corti. A lightly stained band of DMT1 1A immunolabeling was observed in the supporting cell region lateral to the OHC (Fig. 1d). Many fascicles strongly immunolabeled with −IRE were detected in fibers projecting out from the SGN (green arrowhead Fig. 1e), the inner spiral bundle (ISB) (green or yellow from red/green overlap) of nerve fibers medial to the IHC and scattered nerve fibers projecting out radially across the tunnel of Corti to the OHC and ending as puncta or patches resembling synapses beneath the OHC. The +IRE immunolabeled fibers resemble cochlear efferent nerve fibers (Fig. 1e). Finally, strong +IRE immunolabeling was detected at the openings of habenula perforata (Fig. 1f, green arrowhead, yellow labeling from green/red overlap).

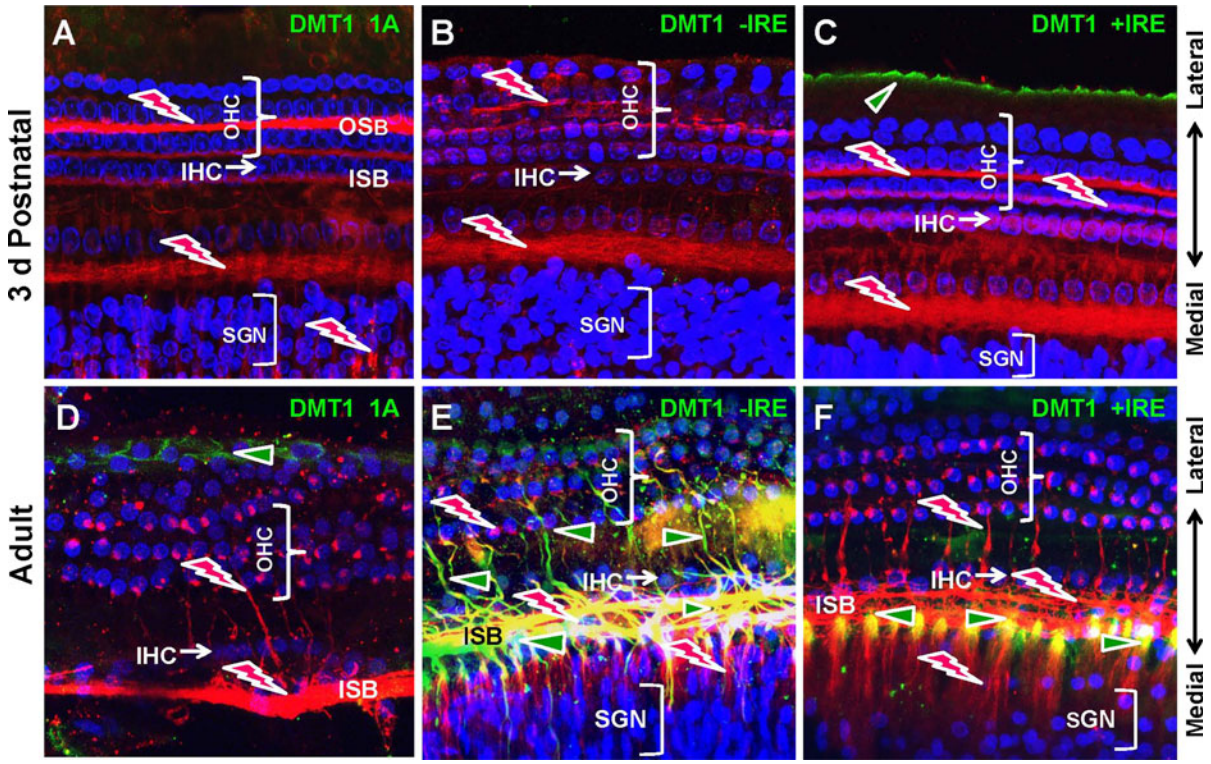

Fig. 1 Confocal images of surface preparations of the organ of Corti in the middle turn of the cochlea of P3 rats (*top row*) and adult rats (*bottom row*). Specimens immunolabeled with primary antibodies recognizing DMT1 1A (*left column*), DMT1 −IRE (*middle column*) and DMT1 +IRE (*right column*) and Alexa Fluor 488 conjugated secondary antibody (*green*). Nuclei labeled with TO-PRO-3 (*blue*) and nerve fibers immunolabeled with primary antibody recognizing neurofilament 200 kD and visualized with a Alexa Fluor 555 conjugated secondary antibody (*red*, *jagged triangle*). Green arrowheads point to patches of DMT1 labeling (*green* or *yellow*). *White pointed-brackets* delineate three rows of OHC, *white arrows* show single row of IHC, *plain-brackets* shows the location of SGN and *red*, *jagged triangles* point to neurofilament 200 labeled nerve fibers. Outer spiral bundle and ISB. SGN located medially and OHC located laterally. (Color figure online)

DMT1 expression in nerve fibers

The distribution of the three forms of DMT1 were further studied in more detail in the nerve fibers projecting out from the SGN cell bodies to the hair cells in the organ of Corti. In P3 rats, sparse patches of DMT1 1A immunolabeling were only detected in supporting cells, possibly immature Schwann cells, surrounding the ANF (Fig. 2a). Labeling of the –IRE DMT1 isoform was not observed either in supporting cells or nerve fibers at P3 (Fig. 2b). In contrast, the +IRE isoform was lightly stained and scattered in and around the nerves fibers at P3 (Fig. 2c). In the adult animals, DMT1 1A immunolabeling failed to be detected in either the ANFs or supporting cells (Fig. 2d). Interestingly, the −IRE species was clearly expressed only in a small number of nerve fibers resembling cochlear efferent nerve fibers (Fig. 2e) which is consistent with the findings in the organ of Corti as shown in Fig. 1e, whereas the −IRE species was not seen in the supporting cells. In contrast to the 1A and −IRE species, the +IRE form was positively expressed in some nerve fibers possibly representing cochlear efferent nerve fibers and supporting cells which may represent Schwann or glial cells (Fig. 2f).

DMT1 expression in SGN

The distribution of the three forms of DMT1 was also assessed in the cell bodies of SGN in P3 and adult rats. At postnatal day 3, staining of the 1A isoform of DMT1 was extremely limited and consisted of sparse immunolabeling of puncta confined to vesicles within the cytoplasm of SGN (Fig. 3a). In contrast, intense DMT1 −IRE immunolabeling (green nuclei, orange/yellow cytoplasm) was present within the nucleus and

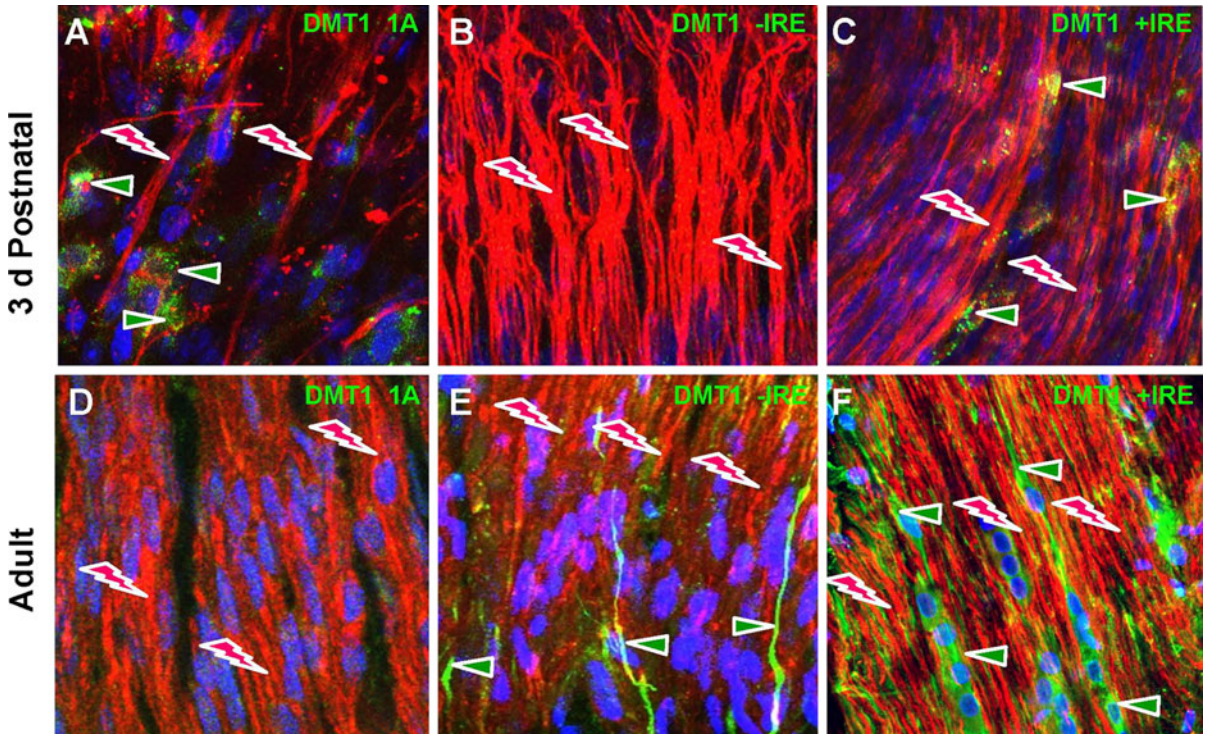

Fig. 2 Confocal images of the nerve fibers projecting out to the hair cells in the middle turn of the organ of Corti of P3 rats (*top row*) and adult rats (*bottom row*). Specimens immunolabeled with primary antibodies recognizing DMT1 1A (*left column*), DMT1 −IRE (*middle column*) and DMT1 +IRE (*right column*) and Alexa Fluor 488 conjugated secondary antibody (*green*). Nuclei labeled with TO-PRO-3 (*blue*) and nerve fibers immunolabeled with primary antibody recognizing neurofilament 200 kD and visualized with a Alexa Fluor 555 conjugated secondary antibody. *Red*, *jagged triangles* point to nerve fibers. *Green arrowheads* point to DMT1 labeled structures. (Color figure online)

cytoplasm of most SGN (Fig. 3b). Immunolabeling of the DMT1 +IRE isoform was very weak and detected in supporting cells surrounding SGN (Fig. 3c). In adult rats, DMT1 1A labeling was strongly expressed in the cytoplasm of SGN (Fig. 3d, orange/yellow) whereas immunolabeling of the DMT1 −IRE species was primarily observed in the cytoplasm of most SGN and occasionally the nucleus and cytoplasm. In addition, a few nerve fibers, possibly cochlear efferent fibers, were labeled (Fig. 3e). The DMT1 +IRE species was also expressed in the cytoplasm and nucleus of SGN (Fig. 3f).

DMT1 expression in stria vascularis

The distribution of the three isoforms of DMT1 was also evaluated in the stria vascularis in the lateral wall of the cochlea in P3 and adult rats. Z-axis imaging of the surface preparations of the stria vascularis was used to determine if DMT1 labeling was in the marginal cell, intermediate cell and/or basal cell layers of the stria. All three DMT1 isoforms exhibited moderate to strong immunolabeling in the stria vascularis of P3 rats (Fig. 4a–c). Z axis confocal images revealed that DMT1 1A, −IRE and +IRE immunolabeling was largely confined to the outer marginal cell layer of the stria vascularis that faces the endolymph; Z axis imaging showed little or no DMT1 labeling in the intermediate cell and basal cell layers of the stria. In adult rats, DMT1 1A immunolabeling was mainly expressed in the intermediate cell layer of stria vascularis (Fig. 4, upper panel). In addition, immunolabeling was also evident at the intercellular boundaries of the outer marginal cells that face the endolymphatic space in the cochlea (Fig. 4d, lower panel). The Z axis images show that the −IRE isoform was mainly detected near the surface of marginal cells and also in tubular projections between the marginal cell and intermediate cell layers of the stria vascularis (Fig. 4e). In contrast, the +IRE species was strongly

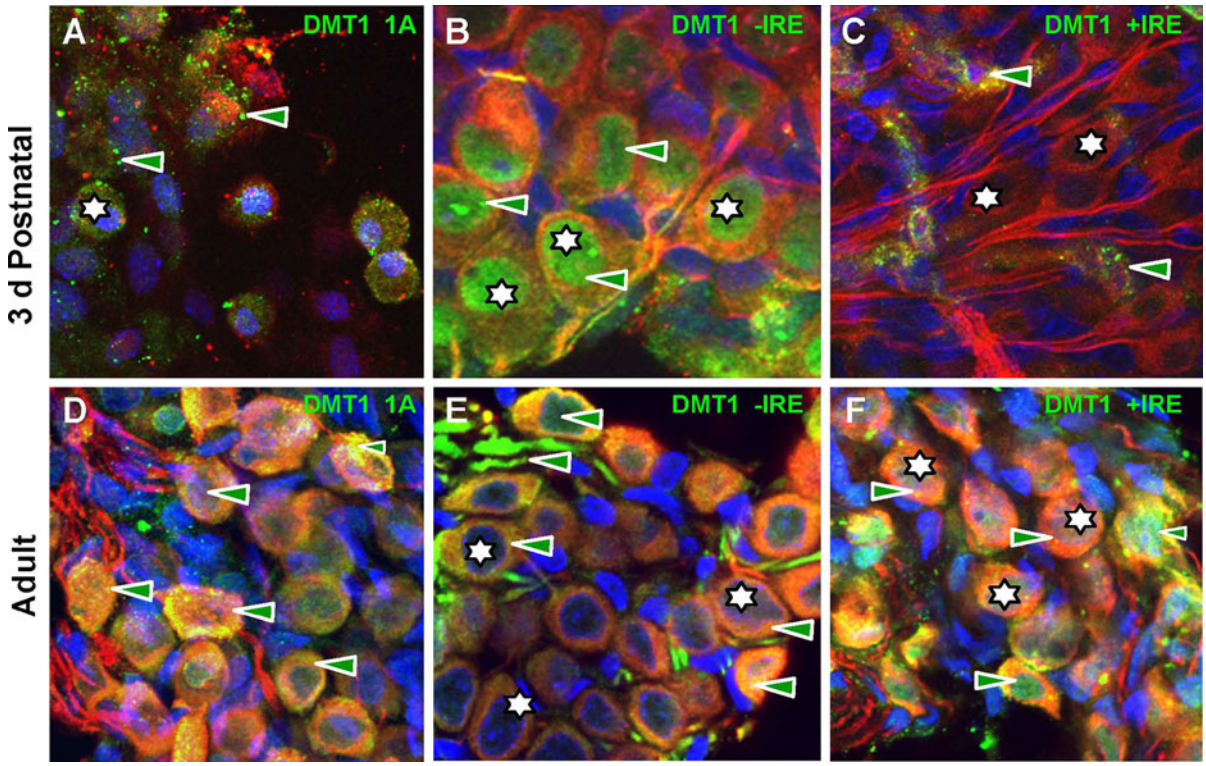

Fig. 3 Confocal images of SGN somas (*white stars*) in the middle turn of the organ of Corti of P3 rats (*top row*) and adult rats (*bottom row*). Specimens immunolabeled with primary antibodies recognizing DMT1 1A (*left column*), DMT1 −IRE (*middle column*) and DMT1 +IRE (*right column*) and Alexa Fluor 488 conjugated secondary antibody (*green*). Nuclei labeled with TO-PRO-3 (*blue*) and the soma and fibers of SGN immunolabeled with primary antibody recognizing neurofilament 200 kD and Alexa Fluor 555 conjugated secondary antibody. *Green arrowheads* point to DMT1 labeled structures (*green* or *orange/yellow*). (Color figure online)

expressed near the surface of the marginal cells facing the endolymphatic space (Fig. 4f).

Discussion

Divalent cations such as iron, Mn and Co, which are transported by DMT1, are essential cofactors for a number of enzyme systems (Garrick et al. 2003; Au et al. 2008; Apostoli et al. 2013; Roth et al. 2013). However, abnormally high or low concentrations of these divalent cations can be neurotoxic resulting in a diverse array of neurological symptoms (Roth 2006; Apostoli et al. 2013). These results imply that in vivo concentrations of these divalent cations must be tightly controlled to prevent potential toxicity. Although the neurotoxic mechanism for many of the divalent cations transported by DMT1 have been extensively investigated in the CNS, few studies have systematically examined the actions of these cations on the structure and function of the cochlea.

Although DMT1 can transport a variety of essential divalent metals, it is generally accepted that its major function is to preserve homeostatic levels of iron which is fundamental for cell survival (Garrick et al. 2003; Roth and Garrick 2003; Mims and Prchal 2005). The existence of four species of DMT1, capable of efficiently transporting iron and other essential transition metals with equivalent relative affinities, implies a functionality and specificity which is likely to be independent of their actual activity or uptake capacity. Differential regulation of the expression of the four transporters, thus, provides an effective and highly selective method to maintain and adjust iron content in any given cell during periods of injury, stress and other adverse physiological states. The role of the different isoforms of DMT1 in maintaining homeostatic levels of iron, therefore, is governed

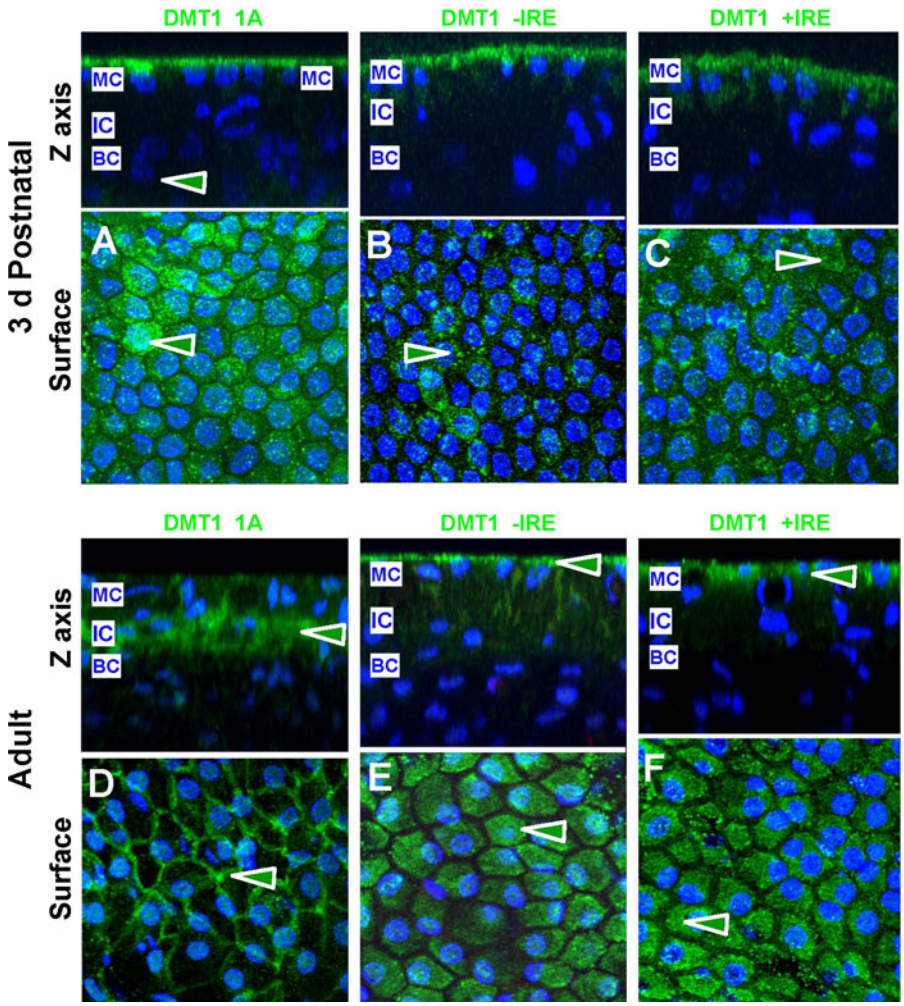

Fig. 4 Confocal images of the stria vascularis in the middle turn of the cochlea of P3 rats (*2 top rows*; Z axis image plane in *upper row* and surface view in *second row* from *top*) and adult rats (*2 bottom rows*, Z axis image plane in *third row* and surface view in *bottom row*). Specimens immunolabeled with primary antibodies recognizing DMT1 1A (*left column*), DMT1 −IRE (*middle column*) and DMT1 +IRE (*right column*) and Alexa Fluor 488 conjugated secondary antibody (*green*). Cell nuclei labeled with TO-PRO-3 (*blue*). Green arrowheads point to DMT1 labeled structures (*green*). (Color figure online)

chiefly by the degree to which each is differentially expressed within the various cells of the body. For example, the 1A forms predominate in the intestines and kidney whereas the +IRE form prevails in the liver and testis (Hubert and Hentze 2002). In contrast, the −IRE species are more evenly distributed throughout the different organs in the body whereas the 1B isoforms are regulated by the stress-related transcription factor, NF-κB (Paradkar and Roth 2006). These findings support the premise that the basic mechanisms governing the selective contribution and distribution of the four species is likely controlled by adaptive changes in cell signaling proteins and other regulatory factors.

Our results represent the first report of the distribution of all three isoforms of DMT1 in any given organ in both an adult and developing animal. The data clearly reveal that all three isoforms have the potential to play a selective role in accumulation of iron and other divalent metals in the inner ear. Our results show that the three species of DMT1 are expressed in distinctly different cell populations within the cochlea and that their distribution changes with development. While it is difficult to understand the biological significance behind the selective organization of the different species of DMT1 within the cells of the inner ear, several consistencies emerge. For example, supporting cells principally express the +IRE isoform of DMT1 suggesting a responsiveness related to intracellular iron; i.e. high iron concentration lead to decreased expression, while low iron increases expression (Gunshin et al. 2001). Staining of the +IRE species in these cells appears to be punctate possibly indicative of its localization in endosomal vesicles

(Roth et al. 2000; Lam-Yuk-Tseung and Gros 2006). Recent studies suggest that supporting cells in the inner ear can actually help repair damaged sensory hair cells (May et al. 2013). At this time, the role of iron in this repair process is not known but, clearly, iron is needed for normal functioning of mitochondria and thus, it is not totally surprising that levels of this metal have to adapt to maintain the supporting role in the repair of sensory hair cells.

In contrast to the +IRE species of DMT1, the −IRE isoform appears to predominate both in the cytoplasm and nucleus of neurons. Nuclear localization of the −IRE was especially evident in the 3 day old cultures of the spiral ganglion which is consistent with prior findings from our laboratory demonstrating its presence in the nucleus of a variety of neuronal like cells maintained in culture (Roth et al. 2000; Lis et al. 2004). The absence of the 1A form of DMT1 in the nucleus suggests that the 1B –IRE isoform is likely to be the predominant species.

The −IRE isoform of DMT1 as well as 1A and +IRE were also present in the cytoplasm of marginal cells which line the stria vascularis as was the 1A and +IRE in the 3 day old cultures. The distribution of the 1A species in these cells changed in adult rats as they redistributed mainly to the surface of these cells. The presence of the 1A species in the inner ear is interesting as it was previously observed only in the intestinal epithelium and kidney. Several studies have suggested that the kidney and stria vascularis of the inner ear are very similar given the fact that many drugs that are nephrotoxic are also ototoxic (Humes 1999). The cells in the kidney and lateral wall of the cochlea presumably play a major role in ion transport and help maintain the chemical balance of the fluids in both structures. In addition, both the ear and kidney develop around the 5th–8th week of pregnancy. Thus, it is not totally surprising that the 1A species of DMT1 is also present in the inner ear.

The presence of + and −IRE isoforms of DMT1 in cells lacking the 1A species suggests that the N-terminal 1B species must predominate. This is important since the promoter of this form of DMT1 contains an NF-κB element responsive to inflammatory cytokines (Paradkar and Roth 2006). Thus, in the presence a variety of proinflammatory agents or excess noise, expression of DMT1 will selectively increase in these cells leading to increases in the accumulation of iron, Mn and other divalent cations. This may help explain the increase hearing loss in occupations associated with excess noise and exposure to toxic metals such as cisplatin (Gratton et al. 1990).

Our results also demonstrate that the expression levels and distribution of the different isoforms of DMT1 change during development. For example, in the organ of Corti of P3 rats only the +IRE form of DMT1 is present whereas in the adult all three forms are expressed throughout the different cell types. In the nerve fiber region of P3 rats, the 1A species was only detected in supporting cells, whereas the −IRE and +IRE isoforms were present in and around the nerves fibers. In contrast, the 1A isoform was not observed in the nerve fibers of adult rats yet both the + and −IRE species were expressed in nerve fibers. Similar developmental changes were observed in the SGN and stria vascularis further implying that during development the various signaling mechanisms controlling the expression of these isoforms of DMT1 adapt to the homeostatic needs of the cells.

What was most remarkable in these studies was the almost total lack of staining of the hair cells by any of the three isoforms of DMT1 in both P3 and adult animals. Since our previous studies clearly demonstrated that Mn was toxic to both OHC and IHC in P3 rats (Ding et al. 2011b), this finding implies that other transport systems are likely involved in its uptake. The studies by Ma et al. (2008) previously indicated that the cochlea also contains the solute carrier-39 (SLC39) metal-transporter proteins, ZIP8 and ZIP14 (He et al. 2006; Girijashanker et al. 2008) both of which are reported to have a similar affinity for Mn as DMT1. Although the qPCR data suggested that DMT1 predominates in the inner ear, it is conceivable that either or both of these ZIP proteins are responsible for the transport of Mn and other divalent cations in hair cells (Fujishiro et al. 2011). Interestingly, both transporters have high affinity for Cd which has previously been associated with degeneration of hair cells and hearing loss (Girijashanker et al. 2008; Fujishiro et al. 2011; Jenkitkasemwong et al. 2012).

In summary, our results show for the first time the relative abundance and distribution of the 1A, +IRE and −IRE isoforms of DMT1in the cochlea of P3 and adult rats. These results reveal that all three isoforms of the transporter have the capacity to contribute to the accumulation of iron and other divalent metals in the inner ear. The three species of DMT1 are selectively expressed in different cell populations within the

cochlea and their distribution changes with development.

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Conflict of interest There is no conflict of interest which effects objectivity in regard to publishing this paper.

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