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Gene Expression Patterns in Functionally Different Cochlear Compartments of the Newborn Rat

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Abstract

In an experimental model of organotypic cultures of the stria vascularis (SV), the organ of Corti (OC) and the modiolus (MOD), we compared the expression levels and injury/hypoxia induced response of 36 genes associated with the cells´ energy-producing and energy-consuming processes, using the microarray technique. A decrease of expression was observed for most of the voltage-dependent K^+- and Ca^{++}- channels as an effective mechanism to lower energetic demands. We identified two gene networks of transcripts that are differentially expressed across the three regions. One cluster is associated with the transcription factor hypoxia-inducing factor (*Hif-1a*) and the second one with the caspase and calpain cell death genes *Casp3, Capn1, Capn2* and *Capns1*. The *Hif-1a* gene subset consists of genes belonging to the glucose metabolism (glucose transporter *Slc2a1*, glycolytic enzymes *Gapdh*, *Hk1* and *Eno2*), the Na^+/K^+ homeostasis (ATPase *Atp1a1*) and the glutamate pathway (NMDA receptor associated protein 1 *Grina*, glutamate transporter *Slc1a1*, *Slc1a3*). The *Slc2a1*, *Gapdh*, *Hk1*, *Slc1a3*, *Grina* and *Atp1a1* transcripts are also members of the cell death subset indicating a role they have to play in the differential regional cell death rates. The newly identified genes *Grina* and calnexin (*Canx*) may play specific and yet unknown roles in regulating cell death induced by injury and hypoxia in the inner ear. We assume that the differential regional response occurs on the basis of endogenous gene regulatory mechanisms and may be important to maintaining the cochlea's function following damage from trauma and hypoxia.

Keywords: cell death, gene expression, hypoxia, injury, inner ear, microarray

1. Introduction

The cochlea consists of three main complex structures, each serving a specific function: the organ of Corti (OC), the modiolus (MOD) and the stria vascularis (SV). The OC with its inner and outer hair cells transforms the mechanical signal into an electrical one via hair cell depolarization and signal amplification. The specific function of the SV is to produce and maintain the ionic composition of the endolymph, a very specific fluid with high concentrations of potassium and low concentrations of sodium. The MOD, the conically shaped central axis in the cochlea, contains the spiral ganglion neurons (SGNs). These bipolar neuronal cells transmit the electrical signals from the hair cells to the cochlear nuclei in the brainstem.

Organotypic cultures of the SV, the OC and the MOD were used to experimentally study the differential gene expression of these regions to injury stress and hypoxia (Gross et al., 2007). In freshly prepared tissue, about 2-10 % of all nuclei were found to be stained by propidium iodide, indicating cell damage during preparation of the cochlear tissue. After 24h in culture, the number of necrotic cells in the MOD region increased from 8 % to 25-35 %), whereas the number of such cells remained unchanged in the OC and SV regions (Gross et al., 2008). Gene expression markers indicate that two basic pathogenetic mechanisms are involved in this experimental model: mechanically induced inflammation and hypoxia. The expression of genes involved in apoptosis and necrosis (*Casp3, Capn1, Capn2* and *Capns1*), reactive oxygen species metabolism (*Sod3, Nos2*), inflammation (*Ccl20*) as well as selected transcription factors (*Hif-1a*, *Jun,* and *Bmyc*) have to play a key role in the differential regional response (Mazurek et al., 2011; Gross, Olze, & Mazurek, 2014).

Other pathways implicated in injury and hypoxia include processes involved in energy production and regulation of ion homeostasis which could become critical for cell survival and regeneration (Michiels, 2004). The energy balance of a

cell under physiological and pathological conditions depends on its ATP production and consumption. The energy demand of the inner ear is high and can be compared to that of brain tissue. To meet the energy demand under conditions of inflammation and hypoxia, anaerobic ATP supply is triggered (Pasteur effect; Boutilier & St-Pierre, 2000). ATP production via glycolysis is associated with increased glucose transport and consumption (Frezza et al., 2011).

Maintaining ion homeostasis to allow de- and repolarization of the cells belongs to the processes that require high amounts of energy (Rolfe & Brown, 1997; Buttgereit & Brand, 1995). In the cochlea, the maintenance of ion homeostasis, K^+-cycling and its role in the endocochlear potential are coupled to Na,K-ATPase (Wangemann, 2002). K^+-cycling is particularly important, as in response to the stimulated stereocilia, endolymphatic K^+ flows into the sensory hair cells via the apical transduction channel and is released from the hair cells into the perilymph via basolateral K^+ channels. K^+ may be taken up by fibrocytes in the spiral ligament and transported from cell to cell via gap junctions into strial intermediate cells which secrete it to the endolymph.

Calcium entry and the maintenance of the multiple segregated transduction pathways is controlled by a combination of calcium channels, Ca^{++}-ATPases and buffering mechanisms. Elevated levels of intracellular calcium under hypoxia or injury stress are the result of a massive influx of extracellular calcium through activated channels or the release of calcium from intracellular stores like the endoplasmatic reticulum or the mitochondria (Brini & Carafoli, 2009; Lang, Vallon, Knipper, & Wangemann, 2007). Main routes for calcium influx are voltage-gated Ca^{++} channels, purinergic receptors and ionotropic glutamate receptors (Martinez-Sanchez et al., 2004). For active efflux of intracellular Ca^{++}, the main routes have found to be its export via the plasma membrane calcium ATPase (PMCA) and the transport via the Na^+/Ca^{++} exchanger. Another mechanism to decrease cytosolic Ca^{++} concentrations is uptake into intracellular stores via the sarco- and endo-plasmic reticulum calcium ATPase (SERCA). The calcium buffering mechanisms consist of several Ca^{++} binding proteins.

The aim of the present study is to analyze the basal and injury-induced expression of genes associated with the energy-producing and energy-consuming processes, i.e., of genes associated with the glucose metabolism, the regulation of Na^+-/K^+- and Ca^{++}-homeostasis, including the glutamate pathway. A microarray study was used as the guide that directed us to selecting a total of 36 genes associated with energy production and energy consumption, including the glutamate pathway (Mazurek et al., 2006). The use of the neurobiological array RN-U34 offers the possibility of identifying several transcripts in the inner ear that had not been described previously.

2. Materials and Methods

2.1 Explant Cultures

The cochleae from 3 to 5-day old Wistar rats were dissected into OC, MOD and SV (Sobkowicz, Loftus, & Slapnick, 1993). Details of the preparation of the fragments, the culture conditions and the testing of the viability of the explants were reported previously (Gross et al., 2007). Briefly, the fragments were incubated in four-well tissue culture dishes in Dulbecco`s Modified Eagle Medium/F12Nutrient (1:1) Mixtures (Gibco, Karlsruhe, Germany) supplemented with 10 % fetal bovine serum. Fragments of one ear were kept in culture under normoxic conditions, fragments of the second ear were exposed to moderate hypoxia (oxygen partial pressure inside the culture medium was 10-20 mm Hg) for 5 h, starting three hours after plating. The number of dead cells was determined in freshly prepared tissue (controls) and after 24 h in culture using the live/dead viability test by propidium iodide (PI) and calcein AM staining (Gross et al., 2008).

2.2 cDNA Microarray Analysis

The cDNA microarray analysis was carried out using the Affymetrix Rat Neurobiology U34 Array (RN-U34; Affymetrix, Santa Clara, USA). The complete data sets from this study have been deposited to the Gene Expression Omnibus (GEO) database according to the MIAME standard and can be accessed by ID GSE5446. Each of the total RNA samples of the MOD, OC and SV used in the microarray study originated from 6 animals. Altogether, 16 RNA preparations arising from three independent series were analyzed within one year: four samples from freshly prepared tissue (OC1, OC2, MOD, SV) and 12 experimental samples from cultures of OC, MOD and SV under normoxic (n = 2) and hypoxic conditions (n = 2). Further details of the RNA isolation and quantification and the cDNA microarray analysis were previously reported (Gross et al., 2007).

2.3 Statistical Analysis

Intensity of expression was classified on the basis of the histogram of normalized log2 signals and resulted in a normal distribution (data not shown). Values at or above the 75th percentile of the cumulative intensities are considered to be high level expression (> log2 = 12.55, = 6000 relative units; bold in Tables 1-3) and values below the 25th percentile to be low level expression (< log2 = 10.43, = 1380 relative units; italics in Tables 1-3); values between them are considered to be moderate (normal typeface in Tables 1-3). In this study, gene expression was

not found to differ significantly between normoxic and hypoxic environments, neither for the numbers of PI-stained nuclei nor for the expression of HIF-1a mRNA or for that of other genes, with the data having been combined to result in four samples per region. Obviously, the hypoxia conditions we used were too mild to induce specific expression changes. Overlapping gene expression patterns induced by hypoxia and mechanical injury may also contribute to this observation. Three features of the gene expression are presented: (i) The absolute expression levels, classified as low, moderate or high using the log2 data. (ii) The fold change of the expression level was calculated as the ratio between the expression intensity of the 24h cultures and the expression intensity of freshly prepared tissue. The mean coefficient of the variation was 11.8 ± 7.6 % (n = 36) for signal intensity and 17.6 ± 8.1 % (n = 108; Tables 1-3) for the expression change. To test the significance levels of the fold changes we used the paired t-test or the Wilcoxon paired test (Tables 1-3). (iii) We used the Pearson´s correlation analysis with the Bonferroni post hoc test to identify co-expression changes among selected transcripts across the three regions.

3. Results

3.1 Glucose Transporter and Glycolytic Enzymes

The chip comprises the transcripts of glucose transporters *Slc2a1* (Glut1) and *Slc2a3* (Glut3) and the glycolytic enzymes *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase)*, Hk1* (hexokinase 1) and *Eno2* (neuron-specific enolase; Table 1). As expected, the *Slc2a1* and the *Gapdh* transcripts show high expression levels in all regions, whereas *Hk1* and *Eno2* belong to the subset of genes with low or moderate expression levels. In culture, with the exception of *Eno2* in the MOD region, all transcripts increase significantly across the three regions.

Table 1. Expression of genes associated with glucose transport and glycolysis in the organotypic cultures of the modiolus, the organ of Corti and the stria vascularis

Gene	Expression			Fold change			Name/function
	MOD	OC	SV	MOD	OC	SV	
Slc2a1 (S68135)*[1]	**12109**	**8328**	**19903**	2.4	7.9[#1]	3.6	Glucose transp. 1 (Glut1)
Slc2a3 (D13962)*[2]	1764	1542	*1132*	1.3	2.1	3.4	Glucose transp. 3 (Glut3)
Gapdh (X02231.1)*[3]	**15383**	**24992**	**24879**	7.2	5.4	5.0	Gap-Dehydrogenase
Hk1 (J04526.1)*[4]	*1012*	1556	1866	2.2[#2]	1.6	1.3	Hexokinase 1
Eno2 (X07729)	5069	4818	1235	0.9	1.9[#3]	5.0[#4]	Enolase 2, gamma

Note. Expression intensity (relative units, RU) was categorized on the basis of the histogram of the normalized log2 signals (see materials and methods). Fold change (Wilcoxon paired test, n = 12): *[1]T(12) = 0.00, $p = 0.002$; *[2]T(12) = 0.00, $p = 0.002$; *[3]T(12) = 2.00, $p = 0.004$; *[4]T(12) = 2.00, $p = 0.004$. Glut - glucose transporter, Gap-glyceraldehyde-3-phosphate. [#]Significance of expression changes (paired t-test, n = 4): [#1]p<0.000 vs MOD, p<0.006 vs SV, [#2]p<0.02 vs SV, [#3]p<0.004 vs MOD, [#4]p<0.000 vs MOD, 0.006 vs OC.

3.2 Na^+/K^+-Homeostasis

Several hundred genes known to encode ion channel proteins are involved in the regulation of Na^+/K^+-homeostasis (Gabashvili, Sokolowski, Morton, & Giersch, 2007). The chip identified the transcript for one sodium and six potassium channels (Table 2), most of them previously not identified in the inner ear. These channels show low to moderate basal expression levels and do not change (*Scn3a, Kcnk1, Kcnq3* and *Kcns3*) or decrease (*Pias3, Alg10* and *Kcnh2*). The basal expression levels and the fold changes in culture are similar in all regions.

Sodium-potassium ATPase is of importance not only for the neuronal resting potential but also for re-establishing the ion homeostasis in the inner ear following injury and hypoxia (Johar, Priya, & Wong-Riley, 2012; Wangemann, 2002). The chip comprises two isoforms of the alpha subunit and two isoforms of the beta subunit (Table 2). The basal expression levels of the various isoforms are unevenly distributed in the three regions and, as expected, relatively high in the OC and SV regions. The most remarkable findings in the present work are the increase of the *Atp1a1* subunit and the decrease of the *Atp1a3, Atp1b2* and the *Atp1b3* subunits.

3.3 Calcium Homeostasis

Calcium entry and the maintenance of the multiple segregated transduction pathways is controlled by a combination of voltage-dependent calcium channels, purinergic and ionotropic glutamate receptors, the Ca^{++}-ATPases and several buffering mechanisms (Table 3).

3.3.1 Calcium Channels

The chip contains information for several voltage-dependent Ca^{++}-channels which respond to cell membrane potential changes and have a role to play in changes of the local intracellular Ca^{++} homeostasis and the formation of the nerve impulse (*Cacna1d* /D38101, *Cacna1g/*AF027984, *Cacna2d1/*M8662, *Cacnb3/*M88751). These transcripts show low to moderate basal expression levels and decrease in culture 0.4-0.7-fold (data not shown). The expression of the voltage-dependent calcium channel *Cacn1c* and of the purinergic channel *P2rx* remained unchanged in all regions.

Table 2. Expression of genes associated with Na^+/K^+ -transport in the organotypic cultures of the modiolus, the organ of Corti and the stria vascularis

Gene	Expression			Fold change			Name/function
	MOD	OC	SV	MOD	OC	SV	
Scn3a (Y00766)	*1206*	*1159*	*897*	0.8	0.8	0.7	VG Na^+channel, type III, alpha
Kcnk1 (AF022819)	*780*	3514	2542	1.2	1.2	1.2	K^+-channel, SF K, M1 (Twik)
Kcnq3 (AF091247)	*1280*	*1333*	*1084*	1.1	0.8	0.8	K^+-VGCh, SF KQT-like, M3
Kcns3 (Y17607)	*404*	2211	1433	1.0	0.7	1.0	K^+- VGCh, SF S, M3
Pias3 (AF032872)*[1]	4281	3549	3688	0.5	0.7	0.5	Binding protein to Kv-channels
Alg10 (U78090)*[2]	3299	3180	2446	0.6	0.8	0.5	Regulatory component
Kcnh2 (U75210)*[3]	4063	3846	2847	0.6	0.6	0.7	K^+-VGCh, SF H, M2 (ERG1)
Atp1a1 (M74494)*[4]	1498	**6001**	**15083**	**8.3**	**4.1**	**2.9**	ATPase, Na^+/K^+ transp., alpha1
Atp1a3 (M28648)*[5]	2475	5905	*1207*	0.2	0.4	0.3	ATPase, Na^+/K^+ transp., alpha3
Atp1b2 (J04629)*[6]	3914	**6888**	**2628**	0.5	0.4	0.3	ATPase, Na^+/K^+ transp., beta2
Atp1b3 (D84450)*[7]	**16726**	**8315**	**14474**	0.7	0.9	0.8	ATPase, Na^+/K^+ transp., beta3

Note. Expression see legend to Table 1. Abbr.: VGCh-Voltage gated channel; SF-subfamily; M-Member; V-voltage; Pias3 - Protein inhibitor of activated STAT, 3; Alg10 - Asparagine-linked glycosylation 10**,** regulatory component of non-inactivating K^+ channels, voltage-gated K^+ channel binding protein, involved in modulating the expression of Kv2 channels; transp.- transporting. Fold change (Wilcoxon paired test, n = 12): $^{*1\text{-}7}T(12) = 0.00$, $p = 0.002$.

3.3.2 Glutamate Pathway

The following transcripts involved in regulating the activity of the glutamate pathway were detected by the chip: three sequences associated with the ionotropic glutamate receptors (*Grina, Grin2B*, and *Grik5*), the glutamate receptor interacting protein 2 (*Grip2*), two glutamate transporters (*Slc1a1* and *Slc1a3*) and the glutamate-ammonia ligase (*Glul*). These transcripts have not been previously characterized in the inner ear. *Grina* encodes for a glutamate-binding subunit of an NMDA receptor-associated complex protein (NMDARA1), also called glutamate-binding protein (Kumar, Tilakaratne, Johnson, Allen, & Michaelis, 1991; Nielsen et al., 2011). It is characterized by a high basal expression level in all regions; in culture, its expression level remained unchanged in the OC and the SV and tended to increase in the MOD region. *Grin2b* encodes for the ionotropic NMDA2B receptor (NR2B), *Grik5* for the ionotropic kainate 5 receptor and *Grip2* for the glutamate interacting protein 2, which binds and affects AMPA receptors. These transcripts are moderately expressed and decrease in culture in all regions (Table 3).

Slc1a1 and *Slc1a3*, two glutamate transporters, are members of the excitatory amino acid transporter (EAAT) family of high-affinity sodium-dependent glutamate carriers encoded by the genes of the SLC1 family. *Slc1a1* encodes for the solute carrier family 1 (the neuronal/epithelial high affinity glutamate transporter, also known as EAAT3, EAAC1; Chen, Kujawa, & Sewell, 2010b). *Slc1a1* shows a low basal expression, and in culture, its expression tends to increase in the SV. *Slc1a3* encodes for the solute carrier family 1 member 3 (glial high affinity glutamate transporter, also known as EAAT1, GLAST). This transporter shows a high basal expression level; in culture, its expression decreases clearly in all regions. *Glul* encodes for the glutamate-ammonia ligase (also known as glutamine synthetase) which converts glutamate to glutamine; this transcript shows a high basal expression level, and in culture, its expression tends to increase in the OC.

Table 3. Expression of genes associated with Ca^{++} homeostasis in the modiolus, the organ of Corti and the stria vascularis

Gene	Expression			Fold change			Name/function
	MOD	OC	SV	MOD	OC	SV	
Cacna1c (M59786)	2396	*1292*	1845	0.9	1.1	0.8	VCa-ch, L-type,a-1C
P2rx2 (AF020756)	4585	**10920**	**9326**	0.7	1.3	1.1	Purinerg. rec. P2X, ch2
P2rx4 (U47031)	2416	1882	2194	0.9	0.9	1.1	Purinerg. rec. P2X, ch4
Grina (S61973)	**10282**	**21127**	**9227**	1.7[#1]	0.8	0.9	GR-NMDA-ass.prot.1
Grin2b (U11419)*[1]	2864	2414	2292	0.8	0.7	0.7	GR-NMDA2B
Grip2 (AF090113)*[2]	2794	2400	2052	0.7	0.7	0.7	GR-interact.prot.2
Grik5 (Z11581)*[3]	2567	3917	3010	0.6	0.6	0.8	GR-kainate5
Slc1a1 (D63772)	1658	*1060*	*827*	0.8	1.1	2.4[#2]	Eaat3
Slc1a3 (S59158)*[4]	**12059**	**23828**	**9227**	0.2[#3]	0.6	0.5	Eaat1
Glul (M91652)*[5]	**19899**	**17553**	**36544**	1.3	1.8[#4]	1.1	Glul
Atp2b1 (L04739)	1538	1486	*1120*	0.8	0.7	0.9	Pmca1b
Atp2b2 (J03754)*[6]	*785*	2190	*716*	0.1	0.5	0.3	Pmca2
Calm2 (M17069)*[7]	**37541**	**37770**	**33732**	0.5	0.7	0.7	Calmodulin 2
Calm3 (X14265)	**6740**	5556	5074	0.8	1.2	1.0	Calmodulin 3
Canx (L18889)*[8]	5700	**7439**	5467	2.2	2.2	2.6	Calnexin
Ppp3ca (D90035)*[9]	**6015**	**6021**	5400	0.9	0.8	0.7	Calcineurin

Note. Expression see legend to Table 1. Abbr.: VCa-ch – Voltage dependent calcium channel; Purinerg. rec.- purinergic receptor, ligand-ion channel; GR-NMDA-ass.prot.1 - glutamate receptor, N-methyl D-aspartate-associated protein 1; NMDA2B - glutamate receptor, ionotrop, N-methyl D-aspartate 2B; GR-interact.prot.2- GR-interacting protein 2; Eaat3 -neuronal/epithelial high affinity glutamate transporter, system Xag, member 1; Eaat1 - glial high affinity glutamate transporter, member 3; Glul - glutamate-ammonia ligase. Pmca – plasma membrane calcium ATPase. Fold change (Wilcoxon paired test, n = 12): $^{*1,2}T(12) = 0.00$, $p = 0.002$; $^{*3}T(12) = 1.00$, $p = 0.003$; $^{*4\text{-}9}T(12) = 0.00$, $p = 0.002$; $^{\#1}p<0.005$ vs OC and 0.001 vs SV; $^{\#2}p<0.022$ vs MOD; $^{\#3}p<0.001$vs OC; $^{\#4}p<0.02$ vs SV (n = 4).

3.3.3 Calcium ATPases and Calcium Binding Proteins

Ca^{++}-ATPases contribute largely to re-establishing Ca^{++}- ion homeostasis after unregulated Ca^{++}- influx, which is an energy demanding process (Buttgereit & Brand, 1995). The chip comprises two transcripts for plasma membrane calcium ATPase (PMCA; *Atp2b1*, *Atp2b2;* Table 3) and two for smooth endoplasmic reticulum calcium ATPase (SERCA; *Atp2a2*, *Atp2a3*). *Atp2b1* encodes for PMCA1, an enzyme with a housekeeping function. *Atp2b2* encodes for PMCA2, an enzyme with special functions in maintaining Ca^{2+} homeostasis in hair cells (Brini & Carafoli, 2009). Unlike PMCAs, SERCAs accumulate Ca^{++} into vesicles of the endoplasmic reticulum at the expense of ATP hydrolysis. The SERCA transcripts *Atp2a2* (J04739) and *Atp2a3* (M30581) show moderate expression levels and decrease significantly in all regions (0.5 - 0.6 fold, data not shown).

The present array data showed high to moderate expression levels for the Ca^{++}-binding proteins calmodulin (CaM) *Calm2 and Calm3,* for calnexin (*Canx*) and for calcineurin (*Ppp3ca*; Table 3). In culture, *Calm2* and *Ppp3ca* expression decreased in all regions, whereas *Canx* increased. *Calm3* remained unchanged. These features are in line with the finding that calcium-binding proteins constitute a high portion of the total cellular protein in all mammalian cells and are involved in protecting from calcium overload.

3.4 Co-Expression Analysis

Hypoxia inducible factor (HIF) is a key transcription factor regulating adaptation to hypoxia and tissue injury and it plays an important part in cell survival (Semenza, 2001). To characterize possible associations between the expression changes of *Hif-1a* and the transcripts analyzed in this study, we correlated the expression changes across the three regions. We observed that *Hif-1a* expression has correlations to nine transcripts associated with

the metabolism of glucose (*Slc2a1, Slc2a3, Gapdh, Hk1* and *Eno2)*, Na^+/K^+ homeostasis (*Atp1a1*) and the glutamate pathway (*Slc1a1, Slc1a3, Grina;* Figure 1A). A more detailed analysis of the data sets shows that the significance between two genes includes a different regional response (Figure 1B-E). The glucose metabolism associated genes belong to the classical target genes of HIF-1alpha (Greijer & van der Wall, 2004; Marin-Hernandez, Gallardo-Perez, Ralph, Rodriguez-Enriquez, & Moreno-Sanchez, 2009; Yu et al., 2012). Remarkably, the higher *Hif-1a* expression in the MOD region is associated with a relative decrease of expression levels of *Slc1a1/a3* and a relative increase of the *Grina* transcript.

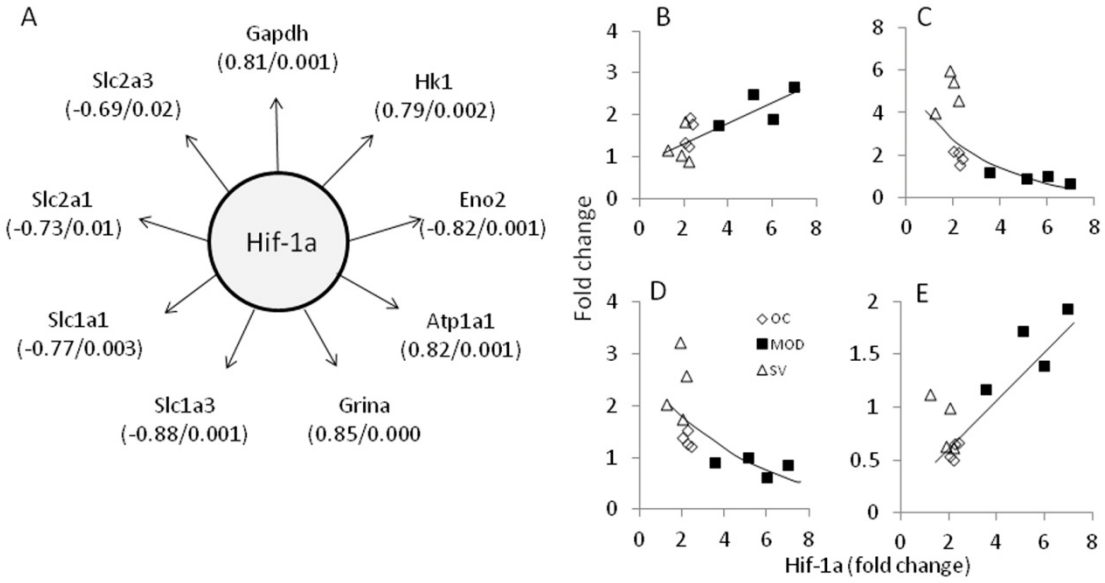

Figure 1. Relationship between *Hif-1a* expression and transcripts belonging to the glucose metabolism, the Na^+, K^+ homeostasis and the glutamate pathway

Note. (A) Diagram illustrating the cluster of transcripts associated with *Hif-1a* expression. Numbers indicate correlation coefficients *r* and significance levels *p*, n = 12. Marginal significance was observed for *Slc2a1* and *Slc2a3*. (B-E) Examples of scatter plots illustrating the correlations between *Hif-1a* and selected transcripts of Figure 1A. B - *Hk1*; C – *Eno2;* D - *Slc1a1;* E - *Grina*. The best fit curve to the *Eno2* and *Slc1a1* data is an exponential function.

To identify co-expression changes across the three regions in relation to cell death, we correlated the changes of these transcripts with molecules known to be mediators in apoptotic and necrotic cell death (Gross, Olze, & Mazurek, 2014). The present study shows that the *Slc2a1, Slc1a3, Grina, Atp1a1, Gapdh* and *Hk1* transcripts correlate closely with the cell death subunits (Figure 2A). These correlations are based on different responses in the MOD region compared to OC and SV (Figure 2B-E).

4. Discussion

4.1 Up-Regulated Transcripts

The up- and down-regulation of transcript levels appear plausible and efficient in terms of energy expenditure of the underlying processes. In the present study, we observe an up-regulation of transcripts involved in energy production and protective mechanisms. It is known that an increased mRNA synthesis which may contribute to an increased transcript levels is very energy-demanding (Simpson, Carruthers, & Vannucci, 2007). The parallel increase of the glucose transporter transcripts *Slc2a1* and *Slc2a3*, in particular in the OC and SV regions, and the up-regulation of *Gapdh* and *Hk1* may primarily contribute to increasing energy production (Edamatsu, Kondo, & Ando, 2011; Marin-Hernandez, Gallardo-Perez, Ralph, Rodriguez-Enriquez, & Moreno-Sanchez, 2009). The differential expression of *Hk1* and *Eno2* may have additional functions. The mitochondrial-bound isoform HK1 may interact with the membrane permeability transition (MPT) pore through the voltage-dependent anion channel (VDAC) which inhibits the cytochrome c release induced by the pro-apoptotic proteins Bax and Bid (Azoulay-Zohar, Israelson, Abu-Hamad, & Shoshan-Barmatz, 2004; Marin-Hernandez, Gallardo-Perez, Ralph,

Rodriguez-Enriquez, & Moreno-Sanchez, 2009). *Eno2* encodes for the neuron-specific enolase (NSE) which is the gamma-gamma enolase isoenzyme. The unchanged expression level in the MOD and the increase of *Eno2* in the OC and the SV may have a role to play in the cells' adaptation to stress (Yan et al., 2011). The expression increase of *Atp1a1* in all regions underlies the important functional role of Na,K-ATPase for cell survival, as studied using specific inhibitors (Johar, Priya, & Wong-Riley, 2012; Fu, Ding, Jiang, & Salvi, 2012). The calcium-binding protein *Canx* is important as it assumes the role of a chaperone in order to transport newly synthesized proteins from the endoplasmatic reticulum to the outer cellular membrane (Zuppini et al., 2002). Previous work showed that *Grina* is a member of the transmembrane BAX inhibitor motif (TMBIM3) known as an anti-apoptotic protein that controls apoptosis through the modulation of ER calcium homeostasis (Rojas-Rivera et al., 2012). *Grina's* high basal expression level and its increased expression in the MOD may well be involved in cell protection (Goswami et al., 2012). What is of interest here is the specific increase of *Slc1a1* (glutamate transporter) in the SV and of *Glul* (glutamate-ammonia ligase) in the OC. There is *in vitro* evidence of a polarized brain-to-blood transport of glutamate by endothelial cells co-cultured with astrocytes (Helms, Madelung, Waagepetersen, Nielsen, & Brodin, 2012). GLUL converts glutamate to glutamine and may contribute to the elimination of toxic glutamate (Takumi et al., 1997).

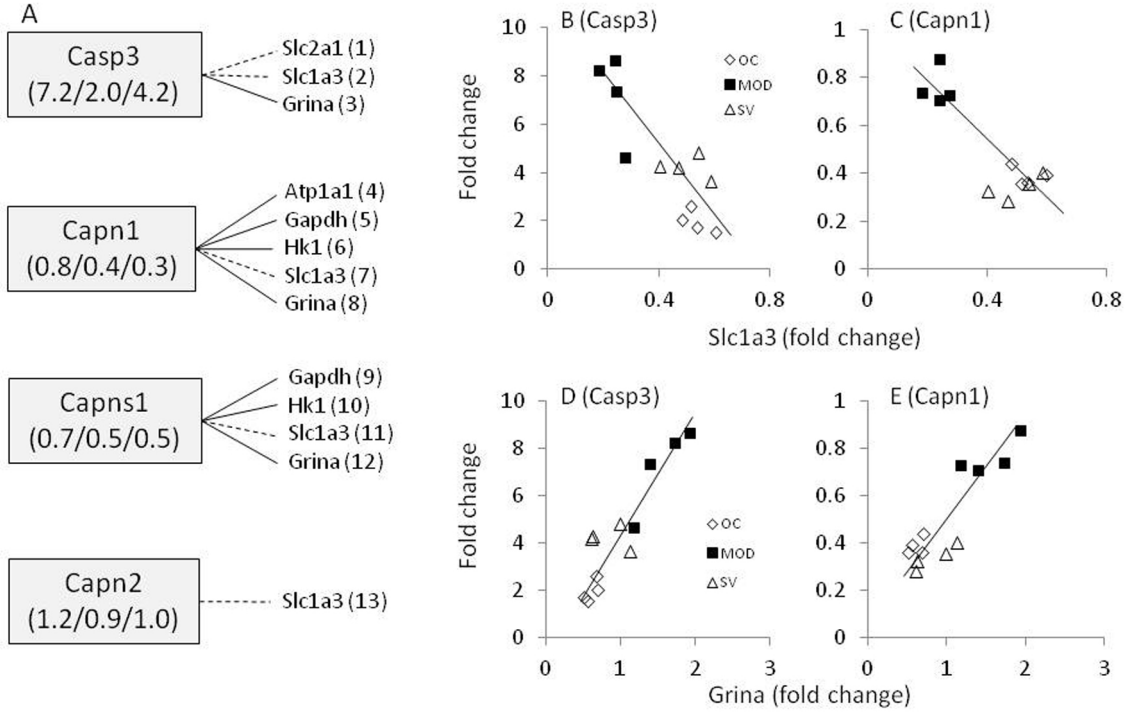

Figure 2. Members of the cell death cluster

Note. (A) Diagram illustrating the cluster of transcripts involved in apoptosis and necrosis. Lines 1-13 indicate significant correlations with correlation coefficients in the range $r = 0.78 – 0.92$ and significance levels in the range $p < 0.000 – 0.003$. Broken lines indicate negative correlations. Numbers within the rectangle indicate the fold change in MOD/OC/SV (Gross, Olze, & Mazurek, 2014). (B-E) Examples of scatter plots illustrating the correlations between transcripts of the glutamate system and cell death transcripts. Correlation coefficients and significance levels (*r*/*p*): B,- 0.87/0.000; C, -0.85/0.000; D, 0.92/0.000; E, 0.90/0.000.

4.2 Down-Regulated Transcripts

An important way of adapting to the energetic deficit following tissue injury and hypoxia is to decrease major ATP consuming functions. Several subsets of transcripts are characterized by a clear expression decrease, among them the K^+ and Ca^{++} - channels, the Atpase subunits *Atp1a3*, *Atp1b2*, the two SERCA transcripts, three glutamate receptors and the glutamate transporter subunit *Slc1a3*. Certain hypoxia-tolerant lower vertebrate species resort to decreasing ion channels as a mechanism to lower their energetic demands (Boutilier & St-Pierre, 2000). Because macromolecule turnover and ion-motive ATPases are major ATP consumers it comes as no surprise in the present

study that the mRNA levels of ion channels are found to be down-regulated. Suppression of ion channel densities probably associated with lower cell membrane permeability decreases the energetic costs of maintaining electrochemical gradients (so-called 'channel arrest'; Hochachka, Buck, Doll, & Land, 1996). Activities that are essential to the maintenance of life should be able to function at lower energy charge values (Atkinson, according to Buttgereit & Brand, 1995; Wieser & Krumschnabel, 2001). Whereas the changes of most of the transcripts may be advantageous for cell survival, the down-regulation of the glial high affinity glutamate transporter *Slc1a3* appears to exert a rather damaging effect, in particular in the MOD region, because this strong expression decrease of *Slc1a3* may contribute to elevated extracellular glutamate concentrations and cell damage in the MOD region (Bianchi, Bardelli, Chiu, & Bussolati, 2014; Gegelashvili & Schousboe, 1997).

4.3 Transcripts Without Significant Changes

Several K^+ channels, the purinergic ion channels, the voltage-dependent calcium channel *Cacn1c* and calmodulin *Calm3* belong to the subset of transcripts without significant changes. The unchanged expression of these channels may allow speculations to be made about the importance of these particular transcripts to cell survival (Rolfe & Brown, 1997). The *Cacn1c* subunit is part of the Cav1.2 channel that plays an important role in synaptic-activity-dependent gene expression and may be important for regenerative processes. With the exception of the *Cacnb3* data, no data are available for these channels in the inner ear (Kuhn et al., 2009). The high expression levels of the calcium-binding proteins *Calm3* and of the purinergic ion channel *P2rx2* in the OC and the SV made us assume that the corresponding proteins have a role to play in cell survival or cell repair. Calcineurin plays an important role in refilling Ca^{2+} stores of the endoplasmatic reticulum and maintaining optimum conditions for protein processing and folding (Bollo et al., 2010).

4.4 Hif-1a Associated Genes

Observations to the extent that the energy balance, glucose uptake by the cells, ion homeostasis and glutamatergic neurotransmission are interlinked, support the assumption of a membership of these genes in a functional gene network, with *HIF-1a* being an important regulator of efficient adaptation across the three regions (Greene & Greenamyre, 1996; Rodriguez-Rodriguez, Almeida, & Bolanos, 2013). For example, glutamate transport depends on the ATPase to remove Na^+ from the cytoplasm, and the activity of Na,K-ATPase is dependent on ATP production (Casey, Pakay, Guppy, & Arthur, 2002). Our data suggest that the differential expression changes of *Atp1a1*, the two glutamate transporters and of *Grina* optimize the adaption process of the glutamate system during repair and regeneration.

The co-expression changes between *Hif-1a* and several genes on the transcript levels could be explained by unique features observed for HIF-1 expression in recent years. Several authors showed a crucial role for Hif-1a mRNA turnover to exist in HIF-1 signaling, and a regulatory role of mRNA turnover as a modulator of HIF-1a function, independent of the oxygen tension (Fahling et al., 2012; Schodel, Mole, & Ratcliffe, 2013; Eltzschig & Carmeliet, 2011). Little is known about the factors that regulate expression changes of *Grina* and of the glutamate transporters. Endothelins, a family of peptides up-regulated in the injured brain, negatively regulate glial glutamate transporter expression (Rozyczka, Figiel, & Engele, 2004). Studies in astrocytes indicate that the suppression of *Slc1a3* is Ca^{++}-dependent (Liu, Yang, & Tzeng, 2008). By inhibiting the down-regulation of *Slc1a3* and buffering the glutamate homeostasis, taurine protects retinal cells *in vitro* under hypoxic conditions (Chen et al., 2010a).

Other than for these genes, we observed no significant correlations between *Hif-1a* and Ca^{++}-ATPases. The expression and activity of proteins involved in Ca^{2+} regulation are subject to the autoregulatory principle, which means that they are regulated by the Ca^{2+} signal itself (Brini & Carafoli, 2009). Other investigations have shown that the level of expression of proteins associated with Ca^{++} homeostasis is regulated by transcription (recently reviewed by Ritchie, Zhou, & Soboloff, 2011).

4.5 Cell Death Associated Genes

The present work suggests that *Slc2a1, Slc1a3, Grina, Atp1a1, Gapdh* and *Hk1* may be involved in the differential cell death rate. The type of cell death immediately after the preparation of the cultures can be categorized as accidental necrotic cell death (ANCD), whereas the cell death measured 24h after the damaging event corresponds most probably to secondary necrotic cell death (SNCD) or late apoptotic cell death (Krysko et al., 2011). The observed genetic responses may be important in terms of secondary cell-death prevention. Preventing the glutamate transporter *Slc1a3* from strongly decreasing in the MOD region or the increase of *Grina* can be assumed to be of special importance to the survival of the SGNs following injury and hypoxia. Up to now, it is unclear which factors show the highest regulatory strength to induce cell death or to maintain survival.

4.6 Conclusions

This study documents previously undescribed genetic features of the different compartments of the inner ear. Both gene clusters, the *Hif-1a* (Figure 1) and the cell death cluster (Figure 2), are based on the co-expression changes of genes across OC, MOD and SV and are the result of a differential, tissue-specific response to injury and hypoxia. The different response occurs on the basis of endogenous gene regulatory mechanisms developed in the course of evolution. The differential response appears important to maintain the cochlea's function following damage from environmental factors. For example, the decrease of ion channels in all regions or the differential response of *Hif-1a* and other transcription factors may be crucial for inner ear function.

The basal expression and the injury/hypoxia-induced patterns may contribute to the region-related difference in the cell death rates immediately after injury and after 24 h in culture. The newly identified genes *Grina* and *Canx* may play specific and yet unknown roles in regulating cell death induced by injury and hypoxia in the inner ear.

However, caution is indicated in the interpretation of these data for several reasons. First, the noise of microarray might influence some of the experimental results. However, the significant correlations between microarray data and quantitative RT-PCR values observed in several studies, justify our approach. Second, the data are not complete in the sense that only some members of pathways have been experimentally determined. Third, we are aware that the response of immature tissue is different from that of mature tissue. Nevertheless, responses are very similar to that in mature tissue (Kennedy, 2012). Fourth, the analysis quantified mRNA levels, but the data do not indicate whether subsequent proteins are generated and where they are located. Beyond the up and down-regulation of transcript expression, the question arises of whether such changes are functional. However, many similarities in the response of the corresponding proteins, even in *in vivo* studies, lead to the conclusion that these observations are far more important than only for the present model.

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