A network of growth and transcription factors controls neuronal differentiation and survival in the developing ear

HORTENSIA SÁNCHEZ-CALDERÓN¹, MARTA MILO², YOLANDA LEÓN¹,³ and ISABEL VARELA-NIETO*,¹

¹Instituto de Investigaciones Biomédicas “Alberto Sols”, CSIC-UAM, Madrid, Spain, ²Department of Biomedical Science, University of Sheffield, U.K. and ³Departamento de Biología, Unidad de Fisiología Animal, Universidad Autónoma de Madrid, Spain

ABSTRACT Inner ear neurons develop from the otic placode and connect hair cells with central neurons in auditory brain stem nuclei. Otic neurogenesis is a developmental process which can be separated into different cellular states that are characterized by a distinct combination of molecular markers. Neurogenesis is highly regulated by a network of extrinsic and intrinsic factors, whose participation in auditory neurogenesis is discussed. Trophic factors include the fibroblast growth factor, neurotrophins and insulin-like peptide families. The expression domains of transcription factor families and their roles in the regulation of intracellular signaling pathways associated with neurogenesis are also discussed. Understanding and defining the key factors and gene networks in the development and function of the inner ear represents an important step towards defeating deafness.

KEY WORDS: otic neurogenesis, IGF-I, FGF, inner ear, auditory ganglion, cochlear microarray

Otic neurogenesis

The inner ear develops from the otic placode, an ectodermal patch located close to the neural tube that invaginates to form a transitory structure, the otic cup. This cup subsequently closes and pinches off from the ectoderm to generate the otic vesicle (Figure 1). The otic vesicle is an autonomous structure that contains the information required to generate most of the cell types and structures of the adult inner ear (Bissonnette and Fekete, 1996; Bever et al., 2003; reviewed in Varela-Nieto et al., 2004). The mammalian inner ear is composed of six distinct sensory organs: the three cristae of the semicircular canal, the two maculae of the saccule and utricle and the organ of Corti in the cochlea. The cristae and the maculae are vestibular organs, whereas the organ of Corti is the organ of hearing (reviewed in Moller, 2006). The inner ear cochlear ganglion is formed by bipolar neurons that connect the peripheral sensory receptors or hair cells with central neurons in auditory brain stem nuclei. The correct organogenesis of the inner ear involves a dynamic balance of cell proliferation, differentiation, survival and death, processes that are tightly regulated by a network of extrinsic and intrinsic factors (Frago et al., 2000; Varela-Nieto et al., 2003 and 2004; Leon et al., 2004).

The process of otic neurogenesis can be separated into different cellular states, each characterized by a distinct combination of molecular markers (summarized in Figure 1A). The first visible output of otic neurogenesis is the delamination of neural cells (otic neuroblasts) from the otic cup (Fig. 1B). These neuroblasts are committed to generate otic neurons and they populate the cochleo-vestibular ganglion (CVG). This ganglion is generated from a region of the otic vesicle epithelium denominated the prospective neural-sensorial domain and that is defined by the domain in which Ngn1 and Delta1 are co-expressed at early stages (Adam et al., 1998; Abu-Elmagd et al., 2001). It is believed that both neurons and sensory cells derive from a common multipotent progenitor cell (MPe, in Figure 1A) (Brigande et al., 2000; Lang and Fekete, 2001). Indeed, there is a loss of ganglion neurons and a decrease in the number of hair cells in the mouse Ngn1 null-mutant (Ma et al., 2000). Interestingly, Notch and its ligands Delta1, Serrate1 and Serrate2 (Ser in the chicken, Jag in the mouse) are expressed in the otic placode (Adam et al., 1998; Edsson et al., 2000; Cole et al., 2000). While Ser1 is expressed at the anterior and posterior poles of the otic cup, Ser2 overlaps with the neural sensory domain defined by Delta1 (Lang and Fekete, 2001). The Delta-Notch pathway is again essential at later stages when the cell fate decision of becoming a hair cell or a supporting cell is taken (Lanford et al., 1999; Lang and Fekete, 2001).

Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; bHLH, basic helix-loop-helix; CVG, cochleo-vestibular ganglion; FGF, fibroblast growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; NT, neurotrophin.
Another set of proneural genes, NeuroD and NeuroM, are expressed both in the otic cup epithelium and in the delaminating neuroblasts (Liu et al., 2000; Abu-Elmagd et al., 2001), suggesting that they act after the selection and specification of neural precursors. The null mouse for NeuroD exhibits deficient neuroblast delamination but it does not display alterations in the generation of neuronal precursors (Liu et al., 2000; Kim et al., 2001; reviewed in Chae et al., 2004). Therefore, the expression of NeuroD defines a second cell state in the neural lineage after neural fate specification, the epithelial neuroblast (NBe, Figure 1A).

Delamination of otic neuronal precursors has been studied extensively in the chicken embryo (D’Amico-Martel and Noden, 1983; Alvarez et al., 1989; Hemond and Morest, 1991). This delamination starts at the otic cup stage and their migration peaks when the otic cup closes to form the early otic vesicle. The delaminating cells are a population committed to a neural fate able to proliferate that constitutes a transit-amplifying cell population (Adam et al., 1998; Begbie et al., 2002). We can identify this third cellular state as the ganglionar neuroblast (NBg, Figure 1A).

The LIM-homeodomain gene family of transcription factors is crucial to specify the identity of neurons derived from the neural tube (Hobert and Westphal, 2000). Islet-1 is one member of this family that is expressed in a few cells within the epithelium, but it is intensely expressed in cells located at the sites of delamination and in those that have delaminated (Figure 1B, and Chae et al., 2004). Therefore, the expression of NeuroD defines a second cell state in the neural lineage after neural fate specification, the epithelial neuroblast (NBe, Figure 1A).

The LIM-homeodomain gene family of transcription factors is crucial to specify the identity of neurons derived from the neural tube (Hobert and Westphal, 2000). Islet-1 and G4 expression in ganglion neuroblasts (E,F) and Islet-1 and IGF1R expression in the otic vesicle (G). Abbreviations: CVG, cochlear-vestibular ganglion; HC, hair cell; INP, immature neural precursor; MPe, multipotent progenitor; NBe epithelial neuroblast; NBg, ganglionar neuroblast; NP, neuronal precursor; OC, otic cup; OCE: otic cell epithelial; ON, otic neuron; OV, otic vesicle; SC, stem cell; ZN, neurogenic zone. Orientation: A, anterior; D, dorsal; M, medial. Scale bars: A, 50 μm; B-D, 100 μm.
brain derived neurotrophic growth factor (BDNF) and neurotrophin 3 (NT-3) (Friedman et al., 2005). Since Tbx1 is expressed in the otocyst epithelium but is excluded from the prospective endolymphatic duct and proneural regions, it has been postulated that this gene could inhibit neural fate by suppressing Ngn1 and Delta1 expression (Raft et al., 2004; Xu et al., 2007).

After the last cycle of cell division, neuroblasts are post-mitotic and they become immature neurons, which then differentiate and extend projections towards their peripheral and central targets (Whitehead and Morest, 1985). These cells are immature neuronal precursors (INP, Figure 1A), a fourth cell state characterised by being post-mitotic but not yet differentiated. Accordingly, these precursors down-regulate the expression of most of the early neural genes and start to express another set of markers related to neurite extension and survival. These markers include fasciculin G4 (Fig. 1B; Camarero et al., 2003), the neurofilament associated antigen 3A10 (Adam et al., 1998; Camarero et al., 2003) and the TrkB and TrkC neurotrophin receptors (Brumwell et al., 2000; Kim et al., 2001, Sanchez-Calderon, unpublished observations). The final cellular state is that of a mature otic neuron (ON, Figure 1A), which generates action potentials and expresses synaptic receptors and neurotransmitters (Raphael and Altschuler, 2003).

Adult inner ear cells retain certain capacity to proliferate. Natural regeneration of the sensory hearing epithelia is displayed by teleosts, amphibians and birds. In contrast, there is no evidence of regeneration in the cochlear sensory epithelium or the cochlear ganglion neurons in mammals and only vestibular hair cells retain some capacity for regeneration. This lack of regeneration in the adult mammalian inner ear has made cell replacement therapy by transplanting extrinsic stem cells into the inner ear, or by activating intrinsic stem cells residing in the inner ear, an interesting proposition to counteract the degeneration and loss of sensory and neuronal cells (Hu and Ulfendahl, 2006). Several growth factors, including epidermal growth factor (EGF), IGF-I and basic fibroblast growth factor (bFGF) promote the proliferation of embryonic stem cells to produce inner ear precursors (Li et al., 2003a and 2003b; Hu et al., 2005), in accordance with observations implicating these factors in the proliferation, differentiation and survival of developing inner ear cells (Leon et al., 1995; Zheng et al., 1997; Hossain and Morest, 2000; Ladher et al., 2000; Varela-Nieto et al., 2004). In particular, IGF-I has been shown to promote neural stem cell proliferation and survival in different contexts and to induce differentiation in combination with neurotrophins (Arsenijevic and Weiss, 1998; Arsenijevic et al., 2001) (Figure 1A).

It is clear that hair cells can be generated from embryonic and adult mammalian stem cells and that in some cases they can integrate into the developing chicken sensory field (Malgrange et al., 2002; Tateya et al., 2003; Li et al., 2003a, 2003b and 2004a). In contrast, there is only limited evidence for the presence of neuronal stem cells in the mammalian ear (Rask-Andersen et al., 2005). Therefore, an attractive alternative for the replacement of neurons lost from the cochlear ganglion is the transplantation of neural stem cells (Okano et al., 2005). These stem cells play a major role in the development of the embryonic central nervous system and their expansion depends on signals from the microenvironment in which they are found, including a number of growth factors such as IGF-I and bFGF (reviewed in Merkle and Alvarez-Buylla, 2006; Doetsch, 2003). Adult mouse neural stem cells differentiate into a neuronal fate but only few survive in the inner ear. Nevertheless, it is promising that the survivors migrate to the spiral ganglion, the auditory nerve tract and the organ of Corti, where they differentiate and extend neurites into the auditory system of adult mammals (Hu et al., 2005; Hu and Ulfendahl, 2006).

Otic neurogenesis is regulated by trophic factors

The Fibroblast Growth Factor family

The FGF family is comprised of 25 members that have been implicated in different cell functions, including cell differentiation, proliferation, motility and survival (Wright and Mansour, 2003). Two of them, FGF24 and FGF25, have been identified in zebrafish (Fischer et al., 2001; Fritzsch et al., 2004). FGF’s act through the specific FGF receptors produced from four genes in mammals, which generate many different protein isoforms through alternative splicing (Wright and Mansour, 2003).

FGF2, FGF3, FGF8, FGF10 and FGF19 participate in otic neurogenesis (Table 1), and FGF16 is present in the otic vesicle epithelium in areas not associated with neurogenesis but rather with sensory organ specification (Chapman et al., 2006). Fgf2 (bFGF) is expressed in mouse and chicken otic placode and otic vesicle (Vendrell et al., 2000). This FGF augments the migration and differentiation of CVG neurons (Hossain et al., 1996; Zheng et al., 1997; Adamska et al., 2001) and it has been implicated in

| Table 1: DIFFUSIBLE FACTORS INVOLVED IN INNER EAR NEUROGENESIS |
|---------------------------------|----------------|--------|--------|--------------|----------------|
| **Factor** | **Migration** | **Proliferation** | **Survival** | **Apoptosis** | **Early Differentiation** |
| Insulin | - | + | - | nd | - |
| IGF-I | - | + | - | nd | - |
| IGF-II | - | + | - | nd | - |
| BDNF/TrkB | - | + | - | + | - |
| NT-3/TrkC | - | + | - | + | - |
| NGFp75^{TR} | - | + | - | + | nd |
| FGF2 | - | + | - | + | - |
| FGF3 | - | + | - | + | - |
| FGF8 | - | - | nd | nd | - |
| FGF10 | - | - | nd | nd | - |
| FGF19 | - | - | nd | nd | - |

Data compiled from transgenic mouse and from in vitro experiments performed in mouse and chicken otic cultures. The list is not intended to be comprehensive. -, positive modulation; - negative modulation; nd, not determined.
the differentiation of sensory cells in the chicken embryo. However, the inner ear forms normally in mice lacking Fgf2 and no abnormalities are observed in adults (Carnicero et al., 2004; Slawińska-Kowalska et al., 2005). Interestingly, FGF2 promotes BDNF activity by up-regulating its high affinity TrkB receptor in cultured mouse auditory neurons (Brumwell et al., 2000). Significantly, the sequential interaction between FGF2, BDNF, NT-3 and their receptors define critical periods of the development of auditory neurons (Hossain et al., 2002). Moreover, co-administration of FGF2 and glial cell line-derived neurotrophic factor (GDNF) supports the survival of adult mouse cochlear neurons in vitro, whilst FGF2 alone promoted neurite generation (Wei et al., 2006). Indeed, either FGF2 or FGF1 induced bipolar neurite formation in cultures of US/VOT-N33 (N33) cells, which are derived from mouse cochlear neuroblasts, a characteristic of mature cochlear neurons (Nicholl et al., 2005).

In mice and Xenopus, Fgf3 (Int2)mRNA is confined to the otic vesicle sensory epithelium and to the CVG (Wilkinson et al., 1989; Tannahill et al., 1992), but not in the chicken embryo (Mahmood et al., 1995). The CVG ganglion is diminished in mice carrying a null-mutation for Fgf3, on a background of morphogenetic defects (Mansour et al., 1993). Indeed, mouse embryos lacking both Fgf3 and Fgf10 fail to form otic vesicles and therefore, these two genes are clearly implicated in otic induction (Wright and Mansour, 2003; Alvarez et al., 2003). It was recently shown that a human syndrome characterized by type I microtia, microdontia and profound congenital deafness due to the complete absence of inner ear structures is caused by any of three different homozygous mutation in FGF3 (Tekin et al., 2007).

FGF8 (AIGF) is expressed in the chicken otic vesicle and later in the CVG (Colvin et al., 1999) and it is implicated in zebrafish, chicken and mouse otic induction (Leger and Brand, 2002; Ladher et al., 2005; Hans et al., 2007). Both loss of function of Fgf8 in zebrafish and the knock down of Fgf3with antisense morpholinos cause profound alterations in the induction and formation of the otic vesicle (Phillips et al., 2001; Hans et al., 2007). However, little is known about the alterations of sensory neurons or sensory receptors under these circumstances. The formation of the otic placode in the chicken and mouse requires at least two signals, one from the mesoderm and one from the ectoderm. In the chicken, the mesodermal signal is FGF19 while in the mouse it is FGF10. Moreover, a signal from the endoderm is necessary for Fgf8 to induce mouse and chicken otic development (Ladher et al., 2005). Previous studies in the chicken showed that the ectopic
application of FGF2 and FGF8 enhances the transcription of several otic patterning genes and enlarges the CVG (Adamska et al., 2001), supporting a role for FGF8 in the generation of the CVG (Hossain et al., 1996). Moreover, delaminating neuroblasts are observed at the boundary between the domains of chicken Fgf8 and Otx2 expression (Hidalgo-Sanchez et al., 2000; Sanchez-Calderon et al., 2004).

Fgf10 mRNA is expressed in the chicken and mouse otic placode and otic vesicle, within the proneural-sensory epithelium. Indeed, Fgf10 in the mouse is also expressed in CVG neurons (Pirvola et al., 2000; Alsina et al., 2004; Lillevall et al, 2006) and overexpression of chicken Fgf10 increases the number of cells expressing NeuroD and NeuroM, but not Delta1 (Alsina et al., 2004). The number of otic neurons is reduced in mouse null mutant for Fgf10 and defects are detected in the sensory epithelium (Pauley et al., 2003). Based on binding studies, FGFRIIib is thought to be the target of FGF10, although this factor also binds to FGFR-1 (Powers et al., 2000). Fgfr-2is expressed dorsally in the prospective non-sensory epithelium of the otocyst, whereas Fgf10 and Fgfgare co-expressed with neurotrophins in the ventral domain, suggesting that FGfs and FGFRs may act in a paracrine manner (Pirvola et al., 2000). FGFRIIib null mutants fail to form the CVG at early stages and although the detailed mechanism remains unknown neural precursors are probably affected. Disruption of Fgfr1, the other potential receptor for FGF10, causes defects in the organ of Corti associated with the reduced proliferation of the precursor pool that generates the auditory sensory epithelium (Pirvola et al., 2002). Moreover, FGF10 signalling was proposed to be regulated by GATA3, a zinc finger transcription factor (Ohuchi et al., 2005; Lillevall et al., 2006).

Finally, mouse FGF15 is the homologue of chicken and human FGF19 (Wright et al., 2004). Mesodermal chicken Fgf19 has been implicated in otic induction (Ladher et al., 2000; Wright et al., 2004) and it is expressed both in the ganglionar neuroblasts delaminating from the otic epithelium and in the CVG (Figure 1B). FGF19 transcripts remain in the CVG until E8, suggesting a relationship between this factor and neural differentiation (Figure 1B) (Sanchez-Calderon et al., 2007). Fgf19 and Fgf8 mRNA expression determine two subdomains within the Fgf10-positive proneural-sensory territory (Sanchez-Calderon et al., 2007). Human FGF19 appears to bind only to FGFR4 (Xie et al., 1999), which is expressed in the chicken and mouse neuroectoderm and in the chicken mesoderm (Wright et al., 2004). In the mouse, Fgf15 is not expressed in the CVG and accordingly, Fgf15 null mutants do not display clear otic abnormalities (Wright et al., 2004). Thus, FGF signalling clearly plays an important role in every aspect of the development and innervation of the ear. Hence, it will be interesting to explore of FGF-cooperation or looping and the interactions of these factors with other growth factors during otic neurogenesis.

Nerve growth factor and neurotrophins

The neurotrophin (NT) family is made up of the small, basic, secreted proteins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). These proteins are essential for neuronal survival and differentiation (reviewed by Bibel and Barde, 2000; Huang and Reichardt, 2001; Pirvola and Ylikoski, 2003) and they mediate their effects through two types of receptors: the low affinity p75 neurotrophin receptor (p75NTR), a modulator of cell survival and cell death; and the Trk tyrosine kinase receptors that interact specifically with NGF (TrkA), BDNF (TrkB) and NT3 (TrkC) (Casaccia-Bonnefil et al., 1999; Barrett, 2000; Lee et al., 2001; Chao and Bothwell, 2002). Both NT-3 and BDNF, but not NGF or NT-4/5, are expressed in the inner ear sensory epithelia and in delaminating neuroblasts suggesting an early source of neurotrophic support for newborn CVG cells (Farinas et al., 2001; Friedman et al., 2005). The high affinity receptors TrkB (BDNF) and TrkC (NT3) receptors are expressed in otic sensory neurons and, while cochlear neurons depend on NT-3, vestibular neurons depend on BDNF for survival and differentiation in chicken, mouse and humans (Table 1) (reviewed in Fritzsch et al., 2004). Indeed, the majority of cochlear neurons are lost during inner ear development between E13.5 and E15.5 in N73-/- mutant mice. The double BDNF and TrkB mutant mainly lose vestibular neurons and in BDNF/NT-3 or TrkB/TrkC double homozygous mutants all cochlear and vestibular neurons are lost (Bianchi et al., 1996; Fritzsch et al., 1998 and 2004; and reviewed in Pirvola and Ylikoski, 2003). Specific binding of NGF to the TrkA receptor is involved in growth, survival and differentiation of neurons in the central nervous system (Williams et al., 2006). NGF is not expressed in the inner ear, but TrkA is localised in the early-developing CVG (Pirvola et al., 1994; Schecterson and Bothwell, 1994; Farinas et al., 2001) and in the adult mouse and rat organ of Corti. Hence, NGF may exert a specific effect on the peripheral auditory system (Dai et al., 2004). The development and survival of spiral ganglion neurons depends on neurotrophic factors such as NT-3 and GDNF (reviewed in Roehm and Hansen, 2005). In stem cells obtained from adult human and guinea pig spiral ganglia, neural differentiation and neurite outgrowth is induced by BDNF, GDNF and NT-3 (Rask-Andersen et al., 2005). Neurons differentiated from human progenitors co-express receptors for neurotrophic factors such as TrkB and TrkC (Rask-Andersen et al., 2005), consistent with the co-expression of these receptors in auditory neurons (Pirvola et al., 1992; Farinas et al., 2001). On this basis, neurotrophic factors have been proposed as candidates for pharmacological treatment to prevent secondary auditory nerve degeneration and to promote neurite re-growth following hearing loss (reviewed in Atar and Avraham, 2005; Holley, 2005).

All neurotrophins bind to p75NTR, which also interacts with Trk receptors to modulate ligand binding specificity, affinity, and function in certain cell types (Benedetti et al., 1993; Bibel et al., 1999). Mature neurotrophins preferentially bind to Trk receptors whereas the immature “proforms” of these proteins have higher affinity for p75NTR, which fulfils a critical role in controlling the balance between cell survival/death. Sortilin acts as a co-receptor for p75NTR essential for pro-neurotrophin-induced cell death (Nykjaer et al., 2004). The p75NTR receptor is encoded by two proteins, the full-length (FL-p75NTR) and short (S-p75NTR) isoforms of p75 (von Schack et al., 2001). Indeed, NGF induces apoptotic cell death in organotypic cultures of otic vesicles and in the CVG through binding to p75NTR in the chicken (Table 1) (Frago et al., 1998; Sanz et al., 1999a). Moreover, NGF-induced cell death only occurs in specific areas of the otic vesicle and CVG. The presence of p75NTR has been reported at different stages of inner ear development in several animal species, and p75NTR expression has been associated with the early specification of vestibular
sensory patches in the chicken embryo (von Bartheld et al., 1991; Schecterson and Bothwell, 1994; Wu and Oh, 1996; Frago et al., 2003 and 2006). The precise signalling pathway(s) used by p75NTR to activate cell death remain unclear. They may involve the generation of ceramide (Frango et al., 1998 and 2006; Hirata et al., 2001) and the activation of Jun-N-terminal kinase (Sanz et al., 1999a; Harrington et al., 2002), the caspase cascade (Gu et al., 1999; Troy et al., 2002; Frago et al., 2003) and cyclin-dependent kinases (Frade, 2000). The pro-apoptotic activity of NGF/p75NTR during early chicken neurogenesis is strictly controlled by survival factors such as IGF-I (Frango et al., 2003). In mice carrying a null mutation for the FL-p75NTR but that still expressing S-p75NTR, there is progressive hearing loss four months after birth, which is associated with the degeneration of cochlear neurons and hair cell loss at the basal turn of the cochlea. It was proposed that p75NTR may play a significant role in the maintenance of cochlear function and that mice carrying a mutation in the p75 gene could represent a new animal model of early onset progressive hearing loss (Sato et al., 2006). These data contrast with that suggesting that the activation of p75NTR is associated with cell death in the adult cochlea. In a normal cochlea, the hair and supporting cells of the organ of Corti produce mature BDNF that activates TrkB receptors on cochlear neurons, triggering their survival. After amingolysin administration, sensory cells of the organ of Corti progressively degenerate, reducing the availability of mature BDNF and producing a decrease in TrkB expression. In parallel, p75NTR expression and c-Jun phosphorylation augments in cochlear neurons, suggesting an activation of apoptotic pathways when pro-survival neurotrophic support is diminished (Tan and Shepherd, 2006).

New insights into the activity of profactors and the transcriptional regulation of the genes encoding neurotrophins and their receptors have re-kindled interest in these elements. Thus, by controlling cell death and survival decisions the NGF family of neurotrophins contributes to inner ear development and the maintenance of its activity. Therefore, these elements may be of interest for hearing protection and regeneration.

The insulin-like growth factor system in inner ear neurogenesis

The insulin gene family is made up of insulin, the insulin-like growth factors (IGF) I, II, relaxin and several insulin-like peptides in different species (reviewed in Russo et al., 2005). Indeed, the gene encoding IGF-I is highly conserved in mammals, birds and amphibians (reviewed in Russo et al., 2005). Insulin-like peptides bind to specific receptors in the plasma membrane of target cells which can be grouped into two categories, the tyrosine kinase receptors (insulin receptor and IGF1R) and the mannos-6-phosphate IGF2R. In addition, the activity of IGF is modulated by a family of plasma-transporters referred to as the IGF binding proteins (IGFBP).

The actions of IGF-I are mediated by IGF1R, whose gene has also been highly conserved during evolution (Fig. 2A). IGF1R is a heterotetrameric transmembrane protein containing two α (extracellular ligand-binding) and two β (intracellular) subunits with protein tyrosine kinase activity (LeRoith et al., 1995). Proinsulin and insulin are also able to bind to this receptor with high affinity and both are abundant in serum, typically masking the activity of IGF-I. The IGF-I protein displays an amino acid identity above 91% in the tyrosine kinase encoding domain in animals as divergent as Xenopus, fish, chicken, rat and human (Russo et al., 2005). In the zebrafish embryo, Igf1r is expressed abundantly, particularly in the tail, eye, ear primordia and brain (Ayaso et al., 2002). The inhibition of IGF1R signalling during zebrafish embryogenesis causes significant defects in the inner ear that are accompanied by reduced embryonic growth, arrested development, increased lethality and induced neuronal apoptosis (Schlueter et al., 2007).

During embryogenesis, Igf-1 and Igf1r mRNA are expressed in brain regions where there is active nerve sprouting in spinal and sensory ganglia and in cranial and spinal nerves (reviewed in Varela-Nieto et al., 2003). However, the transcription of the IGF-I and IGF1R genes declines significantly postnatally and reaches very low levels in the adult, a decrease that is correlated with the degree of cell maturation (reviewed in Varela-Nieto et al., 2003). Nonetheless, both proteins are still present in neurons and Schwann cells of the autonomic and peripheral nervous systems, as well as in structures of the central nervous system that undergo cell renewal in adulthood, such as the olfactory bulb, choroid plexus and the dentate gyrus of the hippocampus (reviewed in Varela-Nieto et al., 2003). Both IGF-I and IGF-II appear to be able to increase the number of neural brain cells and prevent neuronal apoptosis (reviewed in Varela-Nieto et al., 2003; Russo et al., 2005; Ye and D’Ercole, 2006). IGF induces growth and DNA synthesis in a variety of neuronal cell types in vitro, including the neurons of the CVG (Leon et al., 1995). IGF-I shortens the length of the cell cycle in neuronal progenitors during embryonic life and influences the growth of all neural cell types (reviewed in Varela-Nieto et al., 2003; Ye and D’Ercole, 2006). IGF-I may also act as a competence factor, a factor required for other growth factors to act. For example, in the absence of IGF-I neither EGF nor FGF-2 were able to stimulate proliferation of cultured neural stem cells from E14 mouse embryos (Arsenijevic et al., 2001). However, the role of IGF-I in cell lineage specification remains controversial, possibly due to species specific activity.

IGF-I is also required for the early differentiation and survival of neuroblasts in the chicken otic vesicle (Table 1; Fig. 2B) (Camargo et al., 2003). IGF-I and IGF1R are expressed in the developing chicken otic epithelium and CVG (Camargo et al., 2003) and in the postnatal cochlear and vestibular ganglia (Camargo et al., 2001 and 2002; reviewed in Varela-Nieto et al., 2003 and 2004). In organotypic cultures of chicken otic vesicles, the addition of exogenous IGF-I causes an increase in cell number in the otic vesicle and its associated CVG, mimicking the normal pattern of in vivo morphogenesis (Leon et al., 1995). Addition of exogenous IGF-I to the isolated chicken CVG increases cell proliferation, causes neurite outgrowth and elevates the expression of the neuronal differentiation marker G4 (Camargo et al., 2003) (Fig. 2C). Blockage of endogenous IGF-I activity inhibits CVG formation in growth factor-free medium and increases cell death, revealing that endogenous IGF-I activity is essential for ganglion generation and survival (Camargo et al., 2003). These studies suggest that proliferation, differentiation and survival of neural progenitors and neurons of the inner ear are dependent on IGF-I.

Even though much progress has been made in recent years, the intracellular signaling pathways that mediate organ-specific IGF-I activity remain to be clarified. The response of target cells to IGF-I is mediated by its high affinity receptor, IGF1R, a classic transmembrane tyrosine kinase receptor. IGF1R is expressed in multiple neural tissues in the embryo and its activation plays an important role in both proliferation and neuronal differentiation during embryonic development and also in regeneration (reviewed
in Varela-Nieto et al., 2003). Binding of IGF-I to IGF1R activates distinct signalling cascades, which in turn mediate the trophic effects of IGF. Tyrosine kinase activity results in the autophosphorylation of IGF1R and triggers the activation of two main intracellular signalling pathways: the phosphatidylinositol-3 kinase/Akt (PI-3K/Akt) survival pathway; and the Raf/mitogen-activated protein kinase (Raf/MAPK) that activates the proliferation of neuronal cells during development (reviewed in Varela-Nieto et al., 2003; Russo et al., 2005). Recently, IGF-I treatment has been shown to promote a neuronal phenotype in mouse olfactory bulb stem cells by regulating the PI-3K/Akt pathway (Otaegi et al., 2006; Kalluri et al., 2007) and significantly, IGF-I can activate these pathways during early development of the otic vesicle (Sanz et al., 1999b; Frago et al., 2003). The activation of the Raf/MAPK cascade is involved in the proliferation of the epithelial inner ear cells, whereas the activation of PI3-kinase/Akt pathway controls otic cell survival (Sanz et al., 1999b; Frago et al., 2003; Leon et al., 2004). In addition to canonic intracellular pathways, ceramide kinase has also been proposed as a key target in IGF-I protective pathways (Sugiura et al., 2002; Frago et al., 2003 and 2006).

Insight into the role of IGFs in inner ear cochlear ganglion neurogenesis has come from the study of genetically modified mice. While there is an increase in cell size and less apoptosis in a transgenic mouse over-expressing IGF-I (reviewed in Varela-Nieto et al., 2003 and 2004), conversely there is growth retardation, reduced brain size, loss of selective neuronal populations, hypomyelination and reduced peripheral conduction velocities in mice lacking IGF-I (igf1−/−) (Figure 3) (reviewed in Varela-Nieto et al., 2003 and 2004). In addition, postnatal cochlear development is severely impaired in mice igf1−/−, which develop smaller cochlea and cochlear ganglia, an immature tectorial membrane and they display a significant decrease in the number and size of auditory neurons (Camarero et al., 2001 and 2002). The marked reduction in neural cell number seems to be due to multiple processes, including increased cell death (Camarero et al., 2001). These results demonstrated that IGF-I contributes to the maturation and maintenance of cochlear neurons. In addition, the myelin sheath that envelops the bipolar neurons of the postnatal vertebrate cochlear nerve is severely affected (Camarero et al., 2002). In summary, lack of IGF-I in mice affects the postnatal survival, differentiation and maturation of cochlear ganglion cells and causes abnormal innervation of the sensory cells in the organ of Corti. Interestingly, mutations in the gene encoding human IGF-I cause syndromic sensorineural hearing loss (Woods et al., 1996 and 1997; Walenkamp et al., 2005; Walenkamp and Wit, 2006). Furthermore, IGF-I deficit in the mouse causes all-frequency bilateral sensorineural hearing loss and a delayed response to acoustic stimuli (Cediel et al., 2006) (Figure 3N). In the diseased or injured nervous system, IGF-I infusion enhances nerve regeneration and in vitro, stimulates regeneration of adult sensory neurons (reviewed in Varela-Nieto et al., 2003). Hence, IGF-I may be used as a treatment cofactor to combat hearing loss.

### Transcription factors and gene networks

During the development of the inner ear there are several families of transcription factors that are implicated in defining boundaries and polarity and in the regulation of intracellular signalling (Table 2). It follows a brief overview of the transcription factors that are expressed in the inner ear during the stages of early neurogenesis.

### Zinc finger transcription factors

Two of the six members of the GATA family of zinc finger transcription factors are expressed in the inner ear, GATA3 and GATA2 (Lillevali et al., 2004). Their expression overlaps in the mouse otic vesicle at E9.5-10.5 but during subsequent development, it diverges considerably. GATA3 is confined to the sensory

### Table 2

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Description</th>
<th>Family</th>
<th>Process</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gata3</td>
<td>Gata3 zinc finger</td>
<td>Zinc fingers</td>
<td>Expressed only in auditory neurons; Selective loss of cochlear ganglion neurons in the null mouse.</td>
<td>Lillevali et al., 2004; Lawoko-Kerali et al., 2002 and 2004; Karis et al., 2004</td>
</tr>
<tr>
<td>Gata2</td>
<td>Gata2 zinc finger</td>
<td>Zinc fingers</td>
<td>nd</td>
<td>Lillevali et al., 2004; Holley et al., 2007</td>
</tr>
<tr>
<td>Gli1</td>
<td>Growth Factor Independence 1</td>
<td>Zinc fingers</td>
<td>Hair cells differentiation and survival</td>
<td>Wallis et al., 2003</td>
</tr>
<tr>
<td>Bm3a</td>
<td>POU domain, class 4, transcription factor 1</td>
<td>POU domain</td>
<td>Growth and migration of sensory neurons; innervation and axon guidance</td>
<td>Huang et al., 2001a</td>
</tr>
<tr>
<td>Bm3c</td>
<td>POU domain, class 4, transcription factor 3</td>
<td>POU domain</td>
<td>Hair cell differentiation</td>
<td>Xiang et al., 1997; Zheng et al., 2000</td>
</tr>
<tr>
<td>Ngn1</td>
<td>Neurogenin 1</td>
<td>Basic helix-loop-helix (bHLH)</td>
<td>Differentiation of all inner ear sensory neurons</td>
<td>Ma et al., 1998</td>
</tr>
<tr>
<td>NeuroD</td>
<td>Neurogenic differentiation 1</td>
<td>bHLH</td>
<td>Survival of inner ear sensory neurons. The NeuroD null mouse presents a general loss of otic neurons, being more affected the cochlear ones.</td>
<td>Liu et al., 2000†; Kim et al., 2001</td>
</tr>
<tr>
<td>Hes1</td>
<td>Hairy and enhancer of split 1</td>
<td>bHLH</td>
<td>Ear histogenesis; effectors for the Notch pathway</td>
<td>Zine et al., 2001; Kageyama et al., 2005</td>
</tr>
<tr>
<td>Hes2</td>
<td>Hairy and enhancer of split 3</td>
<td>bHLH</td>
<td>Effectors for the Notch pathway</td>
<td>Kageyama et al., 2005</td>
</tr>
<tr>
<td>Hes5</td>
<td>Hairy and enhancer of split 5</td>
<td>bHLH</td>
<td>Ear histogenesis; effectors for the Notch pathway</td>
<td>Zine et al., 2001; Kageyama et al., 2005</td>
</tr>
<tr>
<td>Atoh1</td>
<td>Atonal homolog 1</td>
<td>bHLH</td>
<td>Hair cell differentiation</td>
<td>Berghammer et al., 1999</td>
</tr>
<tr>
<td>Isl1</td>
<td>Islet 1</td>
<td>LIM homeodomain (LIM – HD)</td>
<td>Development of both sensory and neuronal lineages of inner ear</td>
<td>Radde-Gallwitz et al., 2004; Li et al., 2004b</td>
</tr>
</tbody>
</table>

Data compiled from null mouse studies. The list does not pretend to be comprehensive. nd, not determined.
domains, epithelial cells, auditory sensory neurons (spiral ganglion neurons) and periotic mesenchyme (Lawoko-Kerali et al., 2002) and by E18.5 it becomes restricted to the cochlea. Without GATA3 a small otic vesicle forms while the cochlear duct, the semicircular canals and auditory ganglion fail to develop (Karlis et al., 2001). Its expression is particularly high in spiral ganglion neurons during migration and differentiation (Lawoko-Kerali et al., 2004) and it is specifically down-regulated in hair cells when they start to differentiate within the sensory fields (Rivolta and Holley, 1998). There is evidence that GATA3 regulates the expression of the transcription factor NeuroD (Lawoko-Kerali, 2004), as well as that of Fgf10 in otic epithelium (Lillevali et al., 2006).

The role of GATA2 in the inner ear is not yet clear and no abnormalities in early otic development have been observed in Gata2 deficient embryos (Lillevali et al., 2004). However, a recent microarray study of GATA2 functionally related genes based on the similarity of expression patterns in inner ear cell lines have identified interesting co-regulated genes such as the signal transducer and activator of transcription, Stat3 (Holley et al., 2007).

Another zinc finger transcription factor that is expressed in the inner ear is the growth factor independence 1 (Gfi1), which is essential for hair cell differentiation and survival (Wallis et al., 2003; Hertzano et al., 2004).

**POU domain transcription factors**

There are two members expressed in the inner ear: Brn3a and Brn3c. Brn3a is required for proper growth and migration in the inner ear and gustatory sensory neurons and it is critically involved in target innervation and axon guidance by spiral and vestibular ganglion neurons. Brn3a controls survival and differentiation of sensory neurons by regulating different downstream genes. Loss of Brn3a results in severe retardation in the development of axon projections to the cochlea and the posterior vertical canal as early as E13.5. In addition, efferent axons that use the afferent fibers as a scaffold during pathfinding also show severe misrouting (Huang et al., 2001).

Brn3c is specifically expressed by hair cells within the adult mice inner ear; moreover, Brn3cnull mutant mice contain immature hair cells but have a normal development of cochlear innervation (Xiang et al., 1997 and 2003). Ectopic overexpression of Brn3c does not lead to the production of hair cells, indicating that it is only required for the later aspects of hair cells differentiation (Zheng and Gao, 2000).

**bHLH transcription factors**

During development of the otocyst the auditory and vestibular neuroblasts are amongst the first cell types to be specified (Hemond and Morest, 1991; Hossain and Morest, 2000; Fekete and Wu, 2002). They migrate from the prospective neural-sensory domain epithelium at E9.5–10.5, simultaneously down-regulating epithelial cytokeratin. Their differentiation depends on the two basic helix-loop-helix (bHLH) transcription factors, neurogenin 1 (Ngn1) and NeuroD. In null mutants for Ngn1, both auditory and vestibular neurons are absent, showing that this gene is essential for the differentiation of all inner ear sensory neurons (Ma et al., 1998). Indeed, it was suggested that NeuroD and JIL-1/tubulin are part of the same regulatory cascade downstream of Ngn1 (Ma et al., 1998). In null mutants for NeuroD, both auditory and vestibular neuroblast migration is compromised, although there is greater depletion of auditory ganglion neurons (Liu et al., 2000; Kim et al., 2001). Other bHLH genes are necessary to realize the full proliferative capacity of progenitor neurons: Hes1, Hes3, Hes5 (Kageyama et al., 2005). Hes5 and Hes1 are relevant for ear histogenesis (Zine et al., 2001) since they appear to interact with Notch signalling in supporting cells and with Atoh1 in hair cells (Zheng et al., 2000; Lanford et al., 2000). Atoh1 is a bHLH transcription factor and it is ‘necessary and sufficient’ for hair cell differentiation (Bermingham et al., 1999).

**LIM-HD transcription factors**

Islet-1 (Isl-1) is a transcription factor belonging to the LIM homeodomain (LIM-HD) family. In the nervous system, the cooperation between the bHLH and LIM-HD transcription factors is responsible for generating cell diversity. As described earlier, Islet-1 is expressed in the otic neurogenic zone (Li et al., 2004b). Its expression is later maintained in otic neurons but is lost in the sensory lineages (Radde-Gallwitz et al., 2004).

**Microarrays and gene networks**

One very unique aspect of the ear is the complex combination of forms and functions that enable the reception and the transduction of specific physical sensations. As an additional level of complexity, the precise geometry of the inner ear permits the interpretation of only certain mechanical stimuli. Very little is known about the molecular interactions and the genetic hierarchies that guide the morphogenesis of such a specialized epithelium. In the effort to unravel the regulatory gene networks that characterize the development and the maturation of the inner ear organs, new experimental techniques such as microarray analyses have been employed.

Microarrays provide a practical means to measure the expression of thousands of genes simultaneously (Schena et al., 1995; Lockhart et al., 1996). They have been successfully applied to the study of gene expression patterns in inner ear cell differentiation in vitro (Rivolta et al., 2002) and more recently, in the studies of gene expression changes in the cochlea following exposure to noise and ageing (Kirkegaard et al., 2006; Gong et al., 2006). Very early efforts have also been made to analyse changes in gene expression during embryonic stem cell differentiation along the inner ear cells pathways (De Silva et al., 2006). However, this technology is associated with many significant sources of experimental uncertainty that makes the data analysis very challenging. In studies of complex biological systems like the inner ear, the gene expression signal of fundamental processes may often be in the same order of magnitude as the background noise and therefore, very difficult to detect. For this reason the uncertainty generated by the experimental and biological noise must be carefully analyzed and quantified in order to obtain meaningful information from the data. An estimate of uncertainty is not fully achieved using repeat experiments because outliers are often due to flaws in the microarray technique, or to problems in the hybridization of the biological material. The most commonly used microarrays are high-density oligonucleotide arrays, such as Affymetrix GeneChip®. They are characterized by multiple probes associated with each target paired as perfect match (PM) probes and mismatch (MM) probes. The probe-set is used to measure levels of target gene expression and this measurement is then
Growth factors in otic neurogenesis

utilized to detect genes differentially expressed between different conditions, to visualize clustering or to define gene networks. To estimate gene expression levels a summary of these probe-level values is required. The level of uncertainty associated to low gene expression signals is very high and a summary that is less sensitive to the noise is needed. The most popular methods to analyse probe-levels at present only provide a single point estimate of the gene expression levels but most of them are unable to provide credibility intervals for each measurement. (Affymetrix, Microarray Suite User Guide version 5.0., 2001, Affymetrix Inc.) (Irizarry et al., 2003). Probabilistic models have been introduced more recently that describe the probe data set in terms of probabilistic functions. The Bayesian Gene Expression (BGX, http://www.bgx.org.uk; Hein et al., 2005) has developed a Hierarchical Bayesian model for probe-level analysis but it is computationally very expensive and therefore not feasible to run on very large datasets. An affordable alternative are the models developed by PUMA group (Propagating Uncertainty in Microarray Analysis, http://www.bioinf.man.ac.uk/resources/puma/) and the gMOS models (gamma model of oligonucleotide signal) show improvements both in accuracy and computational efficiency over the BGX model. They estimate gene expression levels with credibility intervals that quantify the measurement variance associated with the estimates of target concentration within a sample. This within sample variance is a significant source of uncertainty in oligonucleotide arrays, especially for weakly expressed genes. These models also run efficiently and perform accurately on different types of data including inner ear data (Milo et al., 2003;
Liu et al., 2005; Holley et al., 2007). A still ongoing microarray study on the Ift172 null mutant carried out by Sanchez-Calderon et al., has shown that using a sensitive probe-level analysis, such as gMOS and related models, very low expression of signals can be detected in highly variable biological samples. Such types of gene expression analysis were not successful when performed with models that do not quantify variance at the probe-level. The high number of false positives made the detection of true positives almost impossible. However, the results obtained from gene expression analysis are now being validated in vivo.

Although microarray technology is becoming more affordable, there is still an increasing demand for robust bioinformatics tools to analyse large scale gene expression data. The high cost of arrays and the limitations in performing biological experiments, due to the lack of biological material and/or the complexity of the system makes it even more important to have robust predictions in silico before undertaking in vitro or in vivo experiments. Developing mathematical models that integrate the experimental data with a statistical inference is advantageous to identify and describe the dynamic changes in a network of genes of interest and ultimately to infer changes in key signalling pathways. In an organ like the mammalian inner ear where there are several different biological processes regulated in a very precise manner, it is more informative to look at these processes as dynamic networks of genes of which key components are transcription factors and their gene expression patterns.

Neurosensoric hearing loss is the most common disabling disease worldwide, a large proportion of which involves the loss of hair cells and their associated neurons. Understanding and defining the key gene networks in the development and function of the mammalian inner ear would represent an important step towards defeating deafness. Such advances will potentially lead to new signalling pathways to assist hypothesis generation in regenerative studies and discovery of drug targets.

Acknowledgments

This work has been supported in part by grants from the Spanish Ministries of Health and Education (PI0-5168 and BFU-200500084), from the Community of Madrid (CAM-PRICIT0530), the Royal Society, DIGNA (Ministries of Health and Education (PI0-5168 and BFU-200500084), from the Fondo Social Europeo contract. We thank Drs. Sánchez, Gervasio Martín-Partido, Dawn Davies and Matthew Holley for their help and useful comments on the manuscript.

References


Growth factors in otic neurogenesis


Related, previously published *Int. J. Dev. Biol.* articles

See our Special Issue *Ear Development* edited by Fernando Giraldez and Bernd Fritzsch at:

**Equivalent genetic regulatory networks in different contexts recover contrasting spatial cell patterns that resemble those in Arabidopsis root and leaf epidermis: a dynamic model**
Mariana Benitez, Carlos Espinosa-Soto, Pablo Padilla-Longoria, José Díaz and Elena R. Alvarez-Buylla
*Int. J. Dev. Biol.* (2007) 51: 139-155

**Expression and regulation of Xenopus CRMP-4 in the developing nervous system**
Jacob Souopgui, Tiemo J. Klisch, Tomas Pieler and Kristine A. Henningfeld
*Int. J. Dev. Biol.* (2007) 51: 339-343

**XSu(H)2 is an essential factor for gene expression and morphogenesis of the Xenopus gastrula embryo**
Motoaki Ito, Tomohisa Katada, Seiji Miyatani and Tsutomu Kinoshita
*Int. J. Dev. Biol.* (2007) 51: 27-36

**Gene network analysis in plant development by genomic technologies**
Frank Wellmer and José Luis Riechmann
*Int. J. Dev. Biol.* (2005) 49: 745-759

**Single-cell transcriptional profiles and spatial patterning of the mammalian olfactory epithelium**
Ian Tietjen, Jason Rihel and Catherine G. Dulac
*Int. J. Dev. Biol.* (2005) 49: 201-207

**The chemokine network in cancer - much more than directing cell movement**
Hagen Kulbe, Neil R. Levinson, Fran Balkwill and Julia L. Wilson
*Int. J. Dev. Biol.* (2004) 48: 489-496

**Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo**
Edward Eivers, Karena Mccarthy, Catherine Glynn, Catherine M. Nolan and Lucy Byrnes

**Developmental gene network analysis.**
Roger Revilla-i-Domingo and Eric H Davidson
*Int. J. Dev. Biol.* (2003) 47: 695-703

**Adaptive walks in a gene network model of morphogenesis: insights into the Cambrian explosion.**
Ricard V Solé, Pau Fernández and Stuart A Kauffman
*Int. J. Dev. Biol.* (2003) 47: 685-693

**XMam1, the Xenopus homologue of mastermind, is essential to primary neurogenesis in Xenopus laevis embryos.**
Tomohisa Katada and Tsutomu Kinoshita
*Int. J. Dev. Biol.* (2003) 47: 397-404

**Conserved genetic mechanisms for embryonic brain patterning.**
Heinrich Reichert
*Int. J. Dev. Biol.* (2002) 46: 81-87