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Research paper

A subset of chicken statoacoustic ganglion neurites are repelled by Slit1 and Slit2

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ABSTRACT

Mechanosensory hair cells in the chicken inner ear are innervated by bipolar afferent neurons of the statoacoustic ganglion (SAG). During development, individual SAG neurons project their peripheral process to only one of eight distinct sensory organs. These neuronal subtypes may respond differently to guidance cues as they explore the periphery in search of their target. Previous gene expression data suggested that Slit repellants might channel SAG neurites into the sensory primordia, based on the presence of *robo* transcripts in the neurons and the confinement of *slit* transcripts to the flanks of the prosensory domains. This led to the prediction that excess Slit proteins would impede the outgrowth of SAG neurites. As predicted, axonal projections to the primordium of the anterior crista were reduced 2–3 days after electroporation of either *slit1* or *slit2* expression plasmids into the anterior pole of the otocyst on embryonic day 3 (E3). The posterior crista afferents, which normally grow through and adjacent to *slit* expression domains as they are navigating towards the posterior pole of the otocyst, did not show Slit responsiveness when similarly challenged by ectopic delivery of *slit* to their targets. The sensitivity to ectopic Slits shown by the anterior crista afferents was more the exception than the rule: responsiveness to Slits was not observed when the entire E4 SAG was challenged with Slits for 40 h *in vitro*. The corona of neurites emanating from SAG explants was unaffected by the presence of purified human Slit1 and Slit2 in the culture medium. Reduced axon outgrowth from E8 olfactory bulbs cultured under similar conditions for 24 h confirmed bioactivity of purified human Slits on chicken neurons. In summary, differential sensitivity to Slit repellents may influence the directional outgrowth of otic axons toward either the anterior or posterior otocyst.

1. Introduction

The vertebrate inner ear receives afferent innervation from bipolar neurons of the statoacoustic ganglion (SAG) that project a peripheral process to their sensory organ target and a central process into the hindbrain (Rubel and Fritzsch, 2002; Appler and Goodrich, 2011). These neurons originate from neuroblasts that delaminate from the floor of the otic cup and vesicle (Hemond and Morest, 1991). In the chicken embryo, delamination begins on embryonic day 2 (E2) and continues for several days, with the majority of neuroblasts generated by E4 (D'Amico-Martel, 1982; Hemond and Morest, 1991). The neuroblasts migrate into the mesenchyme, cluster into a cohesive group as the SAG and may continue to divide before differentiation commences. The earliest axons to emerge from the SAG project anteriorly and posteriorly towards the anlagen of the anterior and posterior cristae, respectively; these are the first organs to begin differentiation in the chicken (Wu and Oh, 1996). Over the next several days, as neuroblasts continue to delaminate, the tear-dropped-shaped otocyst develops into a complex membranous labyrinth that houses both auditory and vestibular organs.

Eventually, each peripheral axon will innervate only one of eight different sensory organs: anterior crista, lateral crista, posterior crista, utricular macula, saccular macula, macula neglecta, lagenar macula and the basilar papilla. Both attractive and repulsive cues

Abbreviations: CMV, cytomegalovirus; CNTF, Ciliary Neurotrophic Factor; df, degrees of freedom; E, embryonic day; EF-1α, elongation factor 1 alpha; GFP, green fluorescent protein; HBSS, Hank's Balanced Salt Solution; HEK, human embryonic kidney; HH, Hamburger and Hamilton stage; NF70, neurofilament 70; NT3, neurotrophin-3; PBS, phosphate-buffered saline; Robo, Roundabout; SAG, statoacoustic ganglion

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may be active to correctly match each neuron with its appropriate target. Several highly conserved families of axon guidance molecules and their receptors are present during this pathfinding phase, including Ephs/ephrins (Siddiqui and Cramer, 2005), Semaphorins (Chilton and Guthrie, 2003), and Slits/Robos (Holmes and Niswander, 2001; Battisti and Fekete, 2008; Wang et al., 2013). Molecules that can influence otic axon outgrowth include neurotrophins (Tessarollo et al., 2004; Fritzsch et al., 2005), inflammatory cytokines (Bianchi et al., 2005; Bank et al., 2012), ephrins (Bianchi and Gray, 2002; Brors et al., 2003; Zhou et al., 2011; Coate et al., 2012), Semaphorins (Gu et al., 2003; Fantetti et al., 2011) and members of the BMP, Shh, and FGF morphogen families (Hossain et al., 1996; Hossain and Morest, 2000; Hossain et al., 2008; Fantetti and Fekete, 2012). Peripheral otic axon pathfinding has been the subject of recent reviews (Pauley et al., 2005; Webber and Raz, 2006; Fekete and Campero, 2007; Appler and Goodrich, 2011; Coate and Kelly, 2013).

During development, Slit ligands function as long- and short-range chemorepellents by signaling through Roundabout (Robo) transmembrane receptors. Slit-Robo signaling is classically known for regulating commissural axon guidance at the central nervous system midline (reviewed by Dickson and Gilestro, 2006; Reeber and Kaprielian, 2009), but is now known to also regulate neuronal and non-neuronal cell migration, cell polarity, axon targeting, and axon guidance in several other neural systems (reviewed by Ypsilanti et al., 2010). Currently, two Robo homologs and three Slit homologs have been identified in the chick (Bashaw and Goodman, 1999; Li et al., 1999; Vargesson et al., 2001).

Expression data for the developing chicken inner ear suggest that Slits and Robos could be involved in several different aspects of otic patterning and axon guidance (Battisti and Fekete, 2008). *Slit -1*, *-2* and *-3* transcripts were detected within the otocyst adjacent to the forming SAG. Also, localized expression of *robo* transcripts in both the neurogenic domain of the otocyst and within the SAG led us to speculate that neuroblasts are probably Slit-responsive and that Slit-mediated repulsion might promote neuroblast delamination. However, neuroblast delamination is normal in Slit2 and Robo1/2 mutant mice, although at a later stage spiral ganglion cohesion and spatial positioning were disrupted in the cochlea (Wang et al., 2013). We also proposed that the earliest afferents projecting towards either the anterior or posterior crista might be repelled from entering territories where Slits are expressed, thereby channeling them toward their appropriate targets. Finally, we suggested that Slits might be involved in the establishment or maintenance of sensory/nonsensory boundaries because these adjacent territories express *Slits* (non-sensory) and *Robo1/2* (prosensory).

In this study, we used *in ovo* gain-of-function to ask whether Slit-Robo signaling might influence the formation of the SAG, channeling of neurites towards the anterior and posterior cristae, or the integrity of the prosensory patches. In addition, to test the hypothesis that SAG axons are repelled by Slits, isolated SAG explants were challenged with purified Slit proteins and then assayed for evidence that neurite outgrowth was inhibited. Our results show that most otic axons are not repelled by either Slit. One exception is the population of afferents projecting to the anterior crista; this group fails to enter its target if either *slit1* or *slit2* is ectopically expressed there. In contrast, posterior crista afferents are unimpeded when *slits* are introduced into their target. These data indicate that the myriad of sensory afferents projecting to different prosensory targets may have intrinsic differences in the guidance molecules that they use while pathfinding. Also, forced expression of *slits* does not alter SAG formation or the establishment of prosensory domains in the inner ear.

2. Materials and methods

2.1. Plasmids

A previous study inserted full-length coding sequences for human SLIT1 and SLIT2 into the pcDNA3.1/his-myc vector to encode bioactive proteins tagged with the myc epitope on their C-termini under the control of a cytomegalovirus (CMV) promoter (Patel et al., 2001). The myc-tagged proteins were purified from the supernatant of transfected cells by immunoprecipitation and Western blotted to show that ~200 kD full-length hSLIT1-myc and hSLIT2-myc were secreted, as well as a 55–60 kD cleavage fragment of hSLIT2 (Patel et al., 2001). For the experiments reported here, each gene was retained in its original plasmid backbone, but the CMV promoter was replaced with the EF1a promoter as follows. The pEF1-Slit1 and pEF1-Slit2 plasmids were constructed by replacing the CMV promoter in the original CMV-Slit:myc constructs with the EF-1α promoter derived from pEFX. The pEFX-GFP construct contains GFP under the control of an EF-1α (elongation factor 1 alpha) promoter (Agarwala et al., 2001). pEFX was generated by modifying pEF1/myc-His (version C, Invitrogen) such that a 2.2 kb fragment between the PvuII sites, containing neomycin and SV40 elements, was excised. The resulting pEF1-Slit1 and pEF1-Slit2 constructs are 10.7 kb and 10.1 kb, respectively. These two plasmids were used for transfection of HEK cells (ATCC) and for electroporations into the chicken otocyst. For some experiments, Slit expression plasmids were co-electroporated with pEFX-GFP (3:1 molar ratio of pSlit:-pEFX-GFP).

2.2. Electroporation into the otocyst

Eggs were windowed on E2 and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Chick Ringer's solution (7.2 g/L NaCl, 0.23 g/L CaCl, 0.37 g/L KCl, 0.115 g/L Na_2HPO_4, pH 7.4) was dripped onto the amnionic sac to facilitate opening it to expose the right otocyst. Plasmid DNA (4–8 μg/μl) was microinjected into the right otic cup/vesicle of HH15–18 embryos with pulled glass micropipettes (10–12 μm diameter) using a picospritzer. A pair of homemade platinum paddle-shaped electrodes was positioned adjacent to the anterior and posterior sides of the otocyst. Electrodes were constructed using insulated tubing (heat shrink 3/64″ BK 6″, SPC Technology) to shrink-wrap a small piece of platinum wire (0.01″, World Precision Instruments) to a longer piece of tungsten wire (0.02″, Alfa Aesar), such that only the platinum tip was exposed. Nail polish was used to coat the outside edges of the platinum paddles to direct the current between the two electrodes. Two or three 10-V square wave pulses, each 50 ms long and spaced 10 ms apart, were administered using a TSS20 Ovodyne electroporator connected to an EP21 Current Amplifier (Intracel, UK) following modified protocols (Momose et al., 1999; Krull, 2004). The cathode was connected to the electrode in front of the otocyst to target the anterior crista or behind the otocyst to target the posterior crista. Ringer's solution was dripped onto the electrodes before they were removed. The conducting surface of each electrode was cleaned with a damp Kimwipe after each embryo. Embryos were returned to the 37 °C incubator and sacrificed 24–72 h later at HH21–28 (anterior crista) and HH23–28 (posterior crista).

2.3. Histological analysis of electroporated tissue

Heads were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in 15% sucrose and frozen in Tissue Freezing Media (Triangle Biomedical Sciences). Transverse or horizontal sections of 15 μm thickness were collected onto