Hearing Research xxx (2016) 1–5

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Short review

Drug discovery for hearing loss: Phenotypic screening of chemical compounds on primary cultures of the spiral ganglion

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ARTICLE INFO

Article history:
Received 17 June 2016
Received in revised form
21 July 2016
Accepted 30 July 2016
Available online xxx

Keywords:
Phenotypic screening
Cochlea
Deafness
Neurite
Statins
In vitro

ABSTRACT

In the United States there are, at present, no drugs that are specifically FDA approved to treat hearing loss. Although several clinical trials are ongoing, including one testing D-methionine that is supported by the US Army, none of these trials directly address the effect of noise exposure on cochlear spiral ganglion neurons. We recently published the first report of a systematic chemical compound screen using primary, mammalian spiral ganglion cultures in which we were able to detect a compound and others in its class that increased neurite elongation, a critical step in restoring cochlear synapses after noise induced hearing loss. Here we discuss the issues, both pro and con, that influenced the development of our approach. These considerations may be useful for future compound screens that target the same or other attributes of cochlear spiral ganglion neurons.

The bipolar spiral ganglion neurons in the cochlea transfer sound information, such as intensity, timing and frequency, from the hair cells to the cochlear nucleus in the brain stem. If that link is broken, either by hair cell dysfunction or hair cell death, by synapse degeneration or by spiral ganglion neuronal dysfunction or death, no information can pass from surviving hair cells or from any future regenerated hair cells to the brain.

Physical (acoustic over-stimulation) or chemical (antibiotics, toxins) insults can initiate a variety of pathological changes leading to injury of spiral ganglion neurons within the cochlea, some of which occur even when the hair cells are not sufficiently damaged to undergo degeneration (Furman et al., 2013; Guthrie, 2008; Kujawa et al., 2009; Lin et al., 2011; Viana et al., 2015). Not all neurons die immediately after cochlear insult. The surviving neurons react by retracting their peripheral nerve fibers (neurites), the timing and extent of which depends upon the severity of the insult, the length of the recovery time as well as the species under study. After retraction, there is only limited spontaneous regeneration of neurites from spiral ganglion neurons. This, in turn, limits the ability of the neurites to reconnect to surviving hair cells to restore information flow. On the other hand, after cochlear insult, the centrally oriented fibers seem to degenerate along a slower time scale, and while they do survive, they maintain a generally organized frequency representation in the brain. Because the central connections are generally retained, even the damaged neurons can carry interpretable, tonotopic information from electric stimulation generated by cochlear implants.

For the military, the aim of focusing hearing loss drug discovery on spiral ganglion neurons is to design drugs that will promote one aspect of hearing regeneration after noise induced damage to cochlear neurons -that is, to reestablish a conduit to carry efficient auditory communication from the cochlea to the brain. This can take the form of developing interventions to protect or repair the neurons and to encourage the fibers to regrow toward surviving hair cells, cochlear implants, or, in the future, to regenerated hair cells. The exact biochemical pathways that prevent robust spontaneous neurite regeneration from spiral ganglion neurons are largely unknown, but can involve interference with any or all of the general mechanisms of neuronal survival, neurite initiation, neurite elongation, pathfinding and synaptogenesis. Since these mechanisms are likely to differ significantly from each other, a cocktail of different types of drugs, perhaps given along different timelines,

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http://dx.doi.org/10.1016/j.heares.2016.07.019
0378-5955/

may ultimately be necessary to successfully target the different stages of spiral ganglion neurite regeneration. Currently, there are no known drugs that are specifically approved by the FDA to promote neurite regeneration from spiral ganglion neurons. We became interested in uncovering molecular mechanisms that would promote the regrowth of retracted spiral ganglion neurites. To do this, we designed and validated a novel *in vitro*, small molecule screening approach to address the problem of biological regeneration of the length of spiral ganglion neurites.

Precise molecular mechanisms that control repair and regeneration in the auditory system are largely unknown. As a consequence, there is usually no specific protein to target for spiral ganglion neuron drug discovery. Thus the "target based" screening of chemical compounds, used in pharmaceutical companies to assay tens of thousands of compounds for binding, inhibition or activation of a particular molecular target (Sams-Dodd, 2005) are not yet useful at this stage of our knowledge of spiral ganglion neuron regeneration. Instead, without knowing any molecular target or mechanism or pathway, a "phenotypic screen" can be undertaken. An assay in a phenotypic screen uses as its endpoint a change in an observable physical trait, without initial regard for a molecular mechanism. Depending on the disease, phenotypic screening can be as good or better than target based screening for identifying "new molecular entities" for FDA approval (Swinney, 2013; Swinney et al., 2011).

Drug discovery is a challenging yet critically important business. Depending on the purpose and the assay thousands of compounds might be screened to acquire "hits", then more compounds might be made and screened to optimize the candidate molecule for Phase I clinical trials (Hughes et al., 2011). The rare compound that is promoted to a phase I clinical trial has only a 15% chance of success in acquiring full FDA approval. The biological reasons for this low success rate often have to do with the differences between in *vitro* and *in vivo* environments. Whole organism metabolism, toxicity, availability, clearance, and off target effects cannot adequately be modeled in a culture system. It is therefore especially important that the initial assay environment and measurements reflect, as closely as possible, the disease state under study, while balancing the need for speed and reproducibility.

The "rule of three" (Table 1). Designing a phenotypic screen for auditory drug discovery takes a great deal of up-front consideration as well as painstaking validation of methodology. As it turns out, our choices for design of our neurite elongation screen, as described below, were fully consistent with the recently formulated "rule of three" (listed below) for developing predictive phenotypic assays (Vincent et al., 2015).

1. **Develop a disease relevant assay system**. To mold the concept of a "disease relevant assay system" for the spiral ganglion, a clear focus on the purpose of the screen, an understanding of the available technology, and a healthy dose of pragmatism are all required. Time and resources preclude any screen on deaf animal models, which would require replicate animals for each tested compound, hearing testing, noise exposure, surgery for drug delivery, dissections, and histologic analyses for every cochlea. Drug evaluation in deaf animal models is best left to a secondary study of a limited number of promising compounds that are first highlighted in a more rapid, *in vitro*, screening procedure.

For *in vitro* studies of the auditory system, there are a variety of options that have different advantages depending on the question being addressed. Options include cell lines (Rivolta and Holley, 2002) genetically altered cells or cells from genetically altered animals (Teitz et al., 2016), stem cells (Kwan et al., 2015; Walters et al., 2015), explants (Mullen et al., 2012; Wang et al., 2011; Teitz et al., 2016) primary cells from the cochlea (Hegarty et al., 1997; Lie et al., 2010; Whitlon et al., 2006) or even more plentiful primary neurons from another region of the nervous system. Cell lines, genetically altered cells and stem or progenitor cells have the advantage of availability and ease of plating which allows the assaying of a large number of compounds, but they are fairly far removed from the *in vivo* environment. For a screen of compounds for effects on spiral ganglion neurons, it is worth considering that spiral ganglion neurons are embryonically derived from the otic vesicle (Rubel and Fritzch, 2002), a distinction they share only with vestibular neurons. Given their bipolar morphology (a minority in the nervous system) and their unique derivation, one must consider whether these neurons have mechanisms of neurite regulation that differ from those in cell lines or even from other neurons in the nervous system. Further, because neurites *in vivo* do not grow in isolation and are influenced by their microenvironments, purified cells may not adequately represent the growth of spiral ganglion neurites.

An additional consideration is whether to use dissociated spiral ganglion cells or spiral ganglion explants. The advantage to explants is that the neurons remain more or less adjacent to their normal non-neural counterparts – satellite cells, fibrocytes, etc. However, each explant is not identical to the next, not only due to the dissections, but also to the place along the cochlear spiral from base to apex from which the explant is extracted. Further, in explants, counting of neurons for survival assessment is problematic and the neurites themselves often cannot be traced back to the original neuron. Neurite length has to be measured from the edge of the explant, where it is difficult to determine how far away the parent neuron lies, whether branches of the same neurite are being measured, or whether two neurites from the same neuron are being evaluated. On the other hand, dissociated cells do not have the advantage of being connected to their normally adjacent, non-neural counterparts, and the microenvironment is not as close to that in the cochlea *in vivo* as an explant might be. Nonetheless, even in dissociated spiral ganglion cultures, Schwann cells and neurites grow along each other in a fashion similar to that in living tissue (Whitlon et al., 2009), suggesting that at least some of the normal

Table 1
"Rule of three" for developing predictive assays.

1. Develop a disease relevant assay system	**Spiral ganglia** vs cell lines, genetically altered cells, stem cells, more plentiful primary neurons **Dissociated** vs explant **Newborn** vs adult normal or damaged **Mixed cultures** vs purified neurons +**Serum** vs serum-free
2. Use a stimulus that has disease relevance	**Dissociated, denuded postnatal neurons** vs damaged adult neurons
3. Have an assay readout in proximity to the clinical endpoint	**Neurite length longest neurite** vs neurite marker density, number of neurites per neuron, number of neurons with neurites, total neurite length

On the left column of the table, the "Rule of Three" as presented by Vincent et al. (2015). On the right, possible options for carrying out a compound screen on spiral ganglion neurons. Choices made for the screen in Whitlon et al. (2015) are indicated in bold.

Please cite this article in press as: Whitlon, D.S., Drug discovery for hearing loss: Phenotypic screening of chemical compounds on primary cultures of the spiral ganglion, Hearing Research (2016), http://dx.doi.org/10.1016/j.heares.2016.07.019