Caspase-3 cleaves extracellular vesicle proteins during auditory brainstem development

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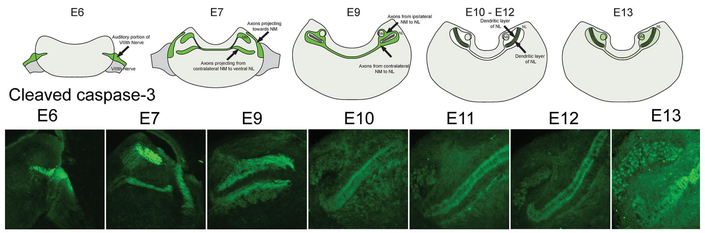
BACKGROUND:

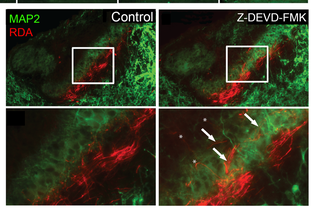
Information from both ears first converges in the auditory brainstem, which uses specialized circuitry to compute sound source location from interaural time differences. This circuitry is very precise, so its development must rely on precise molecular mechanisms, which our lab seeks to understand.

We previously showed that the **apoptotic protease caspase-3** is important for development of the projection from *n. magnocellularis* (NM) to *n. laminaris* (NL) in the chick auditory brainstem. This study had two major findings relevant to the current project:

- Cleaved (active) caspase-3 is **serially expressed** in ascending auditory brainstem structures throughout development.



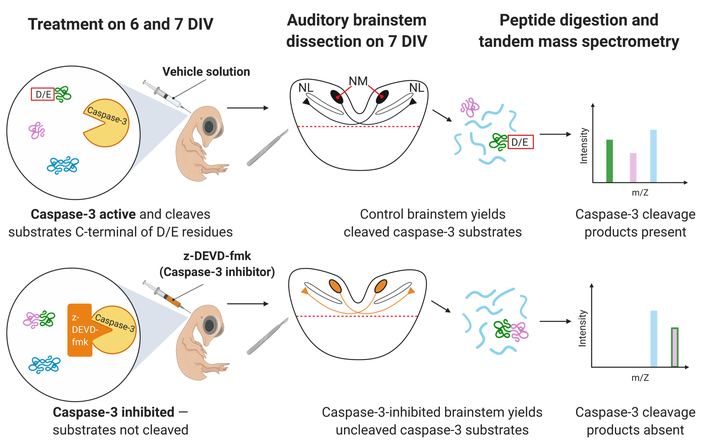
- Inhibition of caspase-3 activity causes **NM axon targeting errors.**



Both of these observations were made **prior to the period of cell death** in the auditory brainstem, suggesting a non-apoptotic function of caspase-3. We therefore asked: ***Which proteins does caspase-3 cleave in the auditory brainstem?***

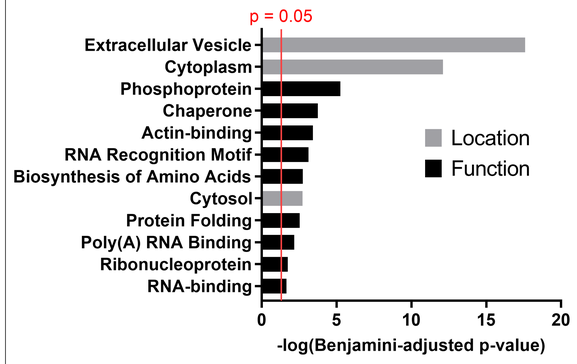
METHODS:

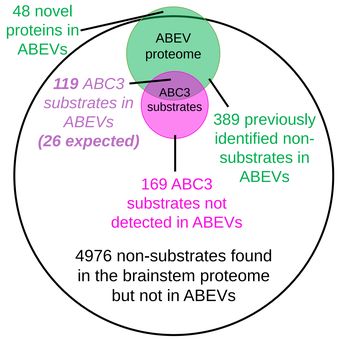
To find **caspase-3 substrates**: Inhibit caspase-3 activity, sequence the brainstem peptidome, and identify peptides that meet 2 criteria: **1. Cleavage after aspartate or glutamate** (suggesting proteolysis by a caspase *in vivo*), and **2. Present in control brainstems, absent in caspase-3-inhibited brainstems** (suggesting that they cannot exist without caspase-3 activity).



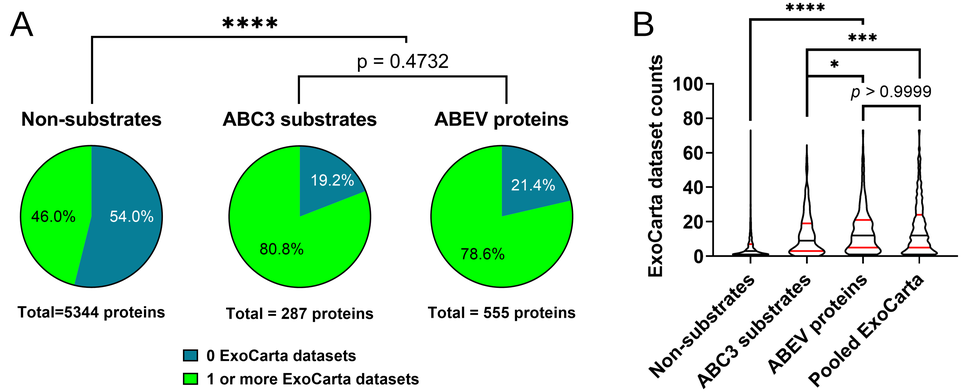
RESULTS

GO Term analysis of caspase-3 substrates shows enrichment for several protein categories, including EV proteins. 287 proteins had at least one peptide that met both criteria and were therefore considered **auditory brainstem caspase-3 (ABC3) substrates.** Functional annotation analysis of ABC3 substrates showed that they were significantly enriched in several categories, including **Extracellular vesicle (EV) proteins**, suggesting that caspase-3 may preferentially cleave EV proteins in the auditory brainstem.



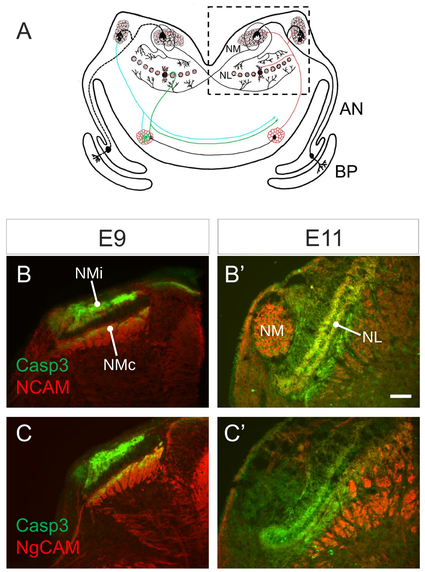


The auditory brainstem EV proteome contains many caspase-3 substrates. We isolated auditory brainstem EVs (ABEVs) and found that they contain nearly **5 times as many ABC3 substrates** as expected by chance ($p = 2.45 \times 10^{-53}$), strongly indicating that caspase-3 cleaves proteins actually found in ABEVs.

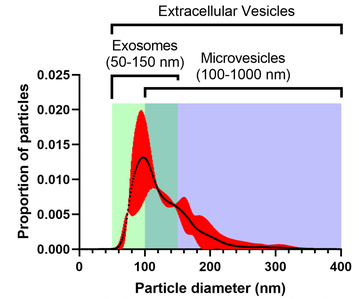


Caspase-3 substrates and auditory brainstem EV proteins resemble literature EV proteomes. We used the EV proteomics database ExoCarta to quantify how ABC3 substrates resemble published EV proteomes. More ABC3 substrates and ABEV proteins are found in at least one EV proteomic dataset on ExoCarta than non-substrates (**A**). Additionally, of the proteins on ExoCarta (green portion of **A**), ABC3 substrates and ABEV proteins are found in more ExoCarta datasets than non-substrates **(B),** suggesting that ABC3 substrates and ABEV proteins resemble EV proteomes more closely than the auditory brainstem proteome. ABEV proteins were found in the same number of ExoCarta datasets as an average EV proteome (Pooled ExoCarta), approximated by pooling all EV proteomic datasets on ExoCarta. Finally, ABC3 substrates are found in slightly fewer ExoCarta datasets than ABEV proteins and Pooled ExoCarta, consistent with ABC3 substrates being a heterogeneous mix of EV proteins and non-EV proteins.

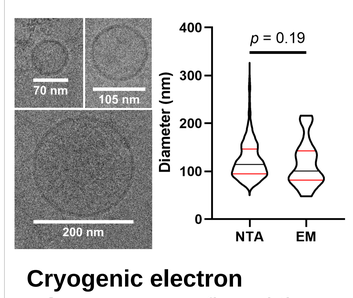
Cleaved caspase-3 colocalizes with its substrates in auditory brainstem circuitry. The ABC3 substrates Neural Cell Adhesion Molecule (NCAM) and Neuronal-Glial Cell Adhesion Molecule (NgCAM) are expressed alongside cleaved caspase-3 in **NM axons on E9 (B-C),** then in **NL dendrites on E11 (B'-C').** These caspase-3 substrates have well-documented functions in axon guidance, so this result suggests that **caspase-3 cleaves guidance molecules in multiple locations** to promote auditory brainstem development. NMi and NMc: NM ipsilateral and contralateral axons. Scale bar: 100 microns.



What are EVs, and how do we know we've isolated them?
Extracellular vesicles are membrane-bound nanoparticles that transport molecular cargo between cells. Common subtypes include exosomes (30-150 nm in diameter) and microvesicles (100 nm-1 µm in diameter). We used collagenase to digest brainstem tissue, then we separated EVs from free protein with size exclusion chromatography.



Nanoparticle tracking analysis (NTA) uses the Brownian motion of particles in a sample to determine their size. The brainstem particles are **the correct size for EVs.**



Cryogenic electron microscopy confirmed the presence of a **lipid bilayer** in our particles. It also validated the size of brainstem EVs measured by NTA.

CONCLUSIONS:

- We described the **first developmental degradome of caspase-3.**
- We identified several hundred caspase-3 substrates that are disproportionately **known non-apoptotic caspase-3 substrates** and **EV proteins** (both in the EV literature and in brainstem EVs).
- Caspase-3 **substrates** with roles in axon guidance **ascend the auditory brainstem** throughout development with caspase-3.

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