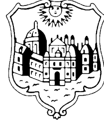

# "Cumulative Single-cell Laser Ablations of Functionally or Genetically Defined Respiratory Neurons Interrogate Network Properties of Mammalian Breathing-related Neural Circuits in vitro"


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Abstract


A key feature of many neurodegenerative diseases is the pathological loss of neurons that participate in generating behavior. To mimic the neuronal degeneration procedure of a functioning neural circuit, we designed a computer-automated system that algorithmically detects and sequentially laser-ablates constituent neurons from a neural network with single-cell precision while monitoring the progressive change of the network function in real time. We applied this cell-specific cumulative lesion technique to an advantageous experimental model, the preBötzinger Complex (preBötC), the mammalian respiratory central pattern generator (CPG) that can be retained in thin slice preparations and spontaneously generates breathing-related motor activity *in vitro*. As a consequence, we sought to investigate the issue: how many neurons are necessary for generating respiratory behavior *in vitro*? This question pertains to whether and how progressive cell destruction will impair, and possibly preclude, behaviorally relevant network function. Our ablation system identifies rhythm-generating interneurons in the preBötC based on genetically encoded fluorescent protein markers or imaged $Ca^{2+}$ activity patterns, stores their physical locations in memory, and then randomly laser-ablates the neuron targets one at a time in sequence, while continuously measuring changes to respiratory motor output via hypoglossal (XII) nerve electrophysiologically *in vitro*. A critical feature of the system is custom software package dubbed Ablator (in Python code) that detects cell targets, controls stage translation, focuses the laser, and implements the spot-lesion protocol automatically. Experiments are typically carried out in three steps: 1) define the domain of lesion and initialize the system, 2) perform image acquisition and target detection algorithms and maps populations of respiratory neurons in the bilateral volumes of the slice, 3) determine the order of lesions and then spot-lesion target neurons sequentially until all the targets are exhausted. Here we show that selectively and cumulatively deleting rhythmically active inspiratory neurons that are detected via $Ca^{2+}$ imaging in the preBötC, progressively decreases respiratory frequency and the amplitude of motor output. On average, the deletion of 120±45 neurons stopped spontaneous respiratory rhythm, and our data suggest ~82% of the rhythm generating neurons remain un-lesioned. Similarly, destruction of 85±45 homeodomain transcription factor *Dbx1*-derived ($Dbx1^{+}$) neurons, which were hypothesized to comprise the rhythmogenic core of the respiratory CPG in the preBötC, precludes the respiratory motor behavior *in vitro* as well. The fact that these two estimates of the size of the critical rhythmogenic core in the preBötC are different can be reconciled considering that the $Ca^{2+}$ imaging method identifies ~50% inhibitory neurons, which are found in the preBötC but are not rhythmogenic. $Dbx1^{+}$, on the other hand, identifies only excitatory rhythmogenic neurons. Serial ablations in other medullary respiratory regions did not affect frequency, but diminished the amplitude of motor output to a lesser degree. These data support the hypothesis that cumulative single-cell ablations caused a critical threshold to be crossed during lesioning, after which rhythm generation in the respiratory network was unsustainable. Furthermore, this study provides a novel measurement that can help quantify network properties of the preBötC and gauge its susceptibility to failure. Our results in turn may help explain respiratory failure in patients with neurodegenerative diseases that cause progressive cell death in the brainstem respiratory networks.