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Neuronal cell fate decisions

O_2 and CO_2 sensing neurons require *egl-13*/Sox5

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We recently conducted a study that aimed to describe the differentiation mechanisms used to generate O_2 and CO_2 sensing neurons in *C. elegans*. We identified *egl-13*/Sox5 to be required for the differentiation of both O_2 and CO_2 sensing neurons. We found that *egl-13* functions cell autonomously to drive O_2 and CO_2 sensing neuron fate and is therefore essential for O_2 and CO_2 sensing-induced behaviors. Through systematic dissection of the *egl-13* promoter we identified upstream regulators of *egl-13* and proposed a model of how differentiation of O_2 and CO_2 sensing neurons is regulated. In this commentary we discuss our findings and open questions we wish to address in future studies.

Keywords: neuronal specification, neuronal diversity, EGL-13, Sox5, oxygen and carbon dioxide sensing, BAG neurons, URX neurons

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Introduction

How the human brain, which contains ~86 billion neurons, generates the correct complement of diverse neuron types is a long-standing question.[1-3] In order for a sensory neuron to correctly differentiate to its terminal neuronal fate, and thus, be able to respond to specific sensory cues, it needs to obtain a specific morphological, spatial, and synaptic repertoire. Such differentiation events are tightly regulated during development by both intrinsic and extrinsic signals.[3] It has been proposed that terminal differentiation of specific neurons utilizes unique combinations of transcription factors to achieve neuronal diversity.[4-7] One example is the function of the conserved transcription factors AST-1 (ETS factor) and CEH-43 (homeobox factor), which act in a combinatorial manner to specify dopaminergic neurons in *C. elegans*.[8,9] The mechanism controlling dopaminergic specification seems to be conserved, at least for AST-1, since knockout mice for the AST-1 ortholog Etv1 fail to differentiate dopaminergic neurons in the olfactory bulb.[9] Another example showing mechanistic conservation across species is the specification of cholinergic neurons. This event requires the COE-type transcription factor UNC-3 in *C. elegans*.[10] The role of *unc-3* was further investigated in the chordate *C. intestinalis* where the ortholog of UNC-3, COE, also regulates its own expression in cholinergic motor neurons.[10] These studies are good examples of how neuronal differentiation and diversity utilizes conserved mechanisms across the phylogeny and how studies in model organisms can provide crucial information on questions that are difficult to address in higher organisms.

In their terminal differentiated state, oxygen (O_2) and carbon dioxide (CO_2) sensing neurons express specific gene batteries that enable them to sense and initiate a behavioral response to changes in these respiratory gases. In humans, O_2 and CO_2 are sensed by the carotid body.[11,12] CO_2 is also sensed in its hydrated form through changes in pH by neurons in the brainstem.[11-15] The carotid body comprises two major cell types, glomus cells (type I) and sustentacular cells (type II). Glomus cells are neurosecretory cells that release neurotransmitters in a Ca^{2+}-dependent manner, when the pressure of O_2 or CO_2 (pO_2/pCO_2) changes in the blood.[16] The signal is projected to the medulla oblongata

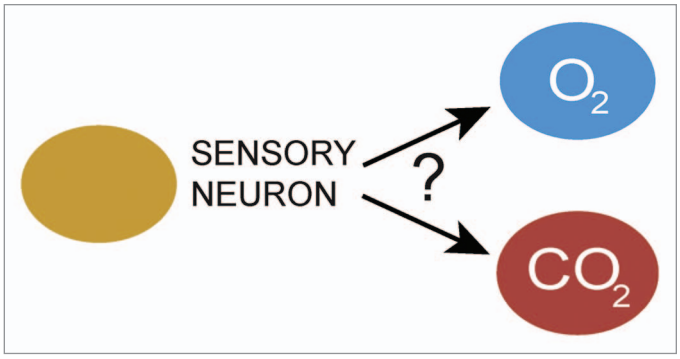

Figure 1. Specification of distinct sensory neuron fates. As in other organisms, *C. elegans* has specialized neurons that are able to detect changes in O_2 and CO_2. However, the molecular mechanisms that drive these specializations are poorly understood.

before it is transmitted to motor neurons in the diaphragm that responds with increased or decreased contractions.[17] In *C. elegans,* behavioral responses to changes in the environmental concentrations of O_2 and CO_2 are controlled by six neurons: BAGL/R, URXL/R, AQR, and PQR. The BAG neurons respond to downsteps in O_2 and upsteps in CO_2, whereas the URX, AQR, and PQR neurons respond to upsteps in O_2.[18-21] O_2 sensing is mediated by soluble guanylate cyclases (sGC), GCY-31/33 in BAG, and GCY-35/36 in URX, which bind O_2 through a heme group.[20,22] The binding of O_2 to a sGC induces the conversion of GTP to cGMP that in turn allows the cation channels TAX-2/4 to open and depolarize the neurons.[22,23] A response to upsteps in CO_2 is initiated by binding of CO_2 or a CO_2 metabolite to the receptor guanylate cyclase GCY-9 expressed by the BAG neurons.[24-26] The CO_2 response, like the O_2 response, utilizes cGMP and TAX-2/4 to transmit the signal.[23] Since the molecular basis of O_2 and CO_2 sensing in humans are not well understood, studies in model organisms may provide important insights. Humans do not have a direct ortholog of the guanylate cyclase GC-D used by rodents or GCY-9 used by *C. elegans* to detect CO_2. However, it is proposed that the human CO_2 response is activated, like GC-D in rodents, by a change in pH.[13,14] The conserved link is further strengthened by the direct regulation of *gcy-9* expression by ETS-5 in the BAG neurons of *C. elegans*[25,26] because the mouse ortholog Pet1b is expressed and required for differentiation of CO_2 responsive neurons.[27] Defining direct targets of Pet1b might therefore identify CO_2 responding receptor candidates.

Many diseases are characterized by an inability to sense O_2 and CO_2 or progress due to altered pO_2. These diseases include solid tumors where altered gene expression within hypoxic regions lead to resistance to radio- and chemotherapy[28] resulting in bad prognosis for patients.[29] In addition, the inability to sense increases in CO_2 concentration by infants is believed to be the major cause of sudden infant death syndrome (SIDS).[30] There is also an interest in understanding the CO_2-sensing mechanisms used by mosquitoes carrying malaria and how parasites infecting farm animals locate their hosts.[31,32] We believe that obtaining a deeper insight into the mechanisms used to sense O_2 and CO_2 can yield a better understanding of such pathophysiological and ethological situations. In our study, we aimed to describe how neurons required to sense environmental changes in O_2 and CO_2 are specified (**Fig. 1**).

egl-13 is Required for Differentiation of O_2- and CO_2-Sensing Neurons

To identify factors required for the differentiation of O_2- and CO_2-sensing neurons, we conducted a forward genetic screen using a fluorescent marker for the gas-sensing neurons. From this screen, we isolated four independent mutant alleles of *egl-13 (rp13, rp22, rp23*, and *rp26)*[33] that all phenocopied the previously published null allele *egl-13(ku194)*[34] (**Fig. 2A**). We found that *egl-13* is required for the correct expression of the terminal gene battery in both the BAG and URX neurons. Previous studies in vertebrates have described roles of the *egl-13* ortholog Sox5 in chondrogenesis and cell cycle progression of neuronal progenitors in the spinal cord in mice and chickens, respectively.[35-37] Data presented below indicate that, in the nervous system of *C. elegans*, *egl-13* specifically acts to determine O_2 and CO_2 sensing neuron specification (**Fig. 2B and C**). When we analyzed the expression of a transcriptional reporter of *egl-13*, we found that expression is confined to the O_2- and CO_2-sensing neurons, muscle and vulval cells. Further, we observed that *egl-13* expression is initiated during embryogenesis where the BAG and URX neurons are generated.[38] In addition, we found that *egl-13* is not required for expression of terminal differentiation markers of the sister cells of BAG and URX (SMDV L/R and CEPD L/R). These data indicate that in the nervous system EGL-13 functions specifically to regulate terminal differentiation of BAG and URX neurons.

In our rescue analysis we asked three questions that further characterized the role of EGL-13 during differentiation of the BAG and URX neurons: (1) Does EGL-13 act autonomously? (2) Is EGL-13 needed to initiate and maintain a correct cellular fate? (3) Since *egl-13* has four different splice variants (A to D) distinguished by the length of the N-terminal tail, we decided to test if one of the EGL-13 isoforms has a predominant role in specifying BAG and URX neuronal fate?

To answer the first question, we used BAG and URX cell-specific promoters that we developed during the study. Using these promoters to drive EGL-13, we rescued the expression of the terminal differentiation markers *flp-19::GFP* and *gcy-33::GFP* in BAG and URX neurons, respectively. These data showed that EGL-13 functions cell autonomously in BAG and URX neurons.

To answer the second question, we used a heat shock (HS) inducible promoter to drive the expression of *egl-13*. Using this technology to express *egl-13* at the L3 stage (URX and BAG neurons generated during embryogenesis), we were able to answer the fundamental

question of whether the BAG and URX neurons are generated at all or remain in an undifferentiated state in *egl-13* mutant animals. We concluded the latter was the case since we were able to induce expression of a URX terminal fate marker post-developmentally. Further, we showed that the URX neurons lost expression of the marker, and thus, their O_2-sensing fate, when the HS promoter was returned to an inactive state by lowering the incubation temperature. Together, these data demonstrated that EGL-13 is sufficient to drive terminal differentiation of both BAG and URX neurons, and EGL-13 is continuously needed to maintain the O_2-sensing fate in URX neurons.

The third question was addressed by evaluating the rescuing potency of the EGL-13 long isoform A and short isoform D. Previous studies have shown that Sox proteins often work in complexes with other proteins, via their long N-terminal tails,[39] and we speculated this could also be the case for EGL-13 during differentiation of O_2- and CO_2-sensing neurons. However, we found that the short isoform D of EGL-13 without a long N-terminal tail rescued equally well as the long isoform A, indicating no requirement of the N-terminal tail in this context. Although we did not further investigate EGL-13 protein interactions in our manuscript, we have not discarded the possibility of interaction domains in the remainder of the protein. To identify proteins that interact with EGL-13 to drive the differentiation of the BAG and URX neurons, we will express affinity-tagged EGL-13 specifically in BAG and URX neurons and use this to pull down potential interaction partners, which will be identified by mass spectrometry analysis. These experiments would add an additional layer of mechanistic insight on how transcription in BAG and URX is regulated at the protein level but also show how Sox proteins act in transcriptional complexes.

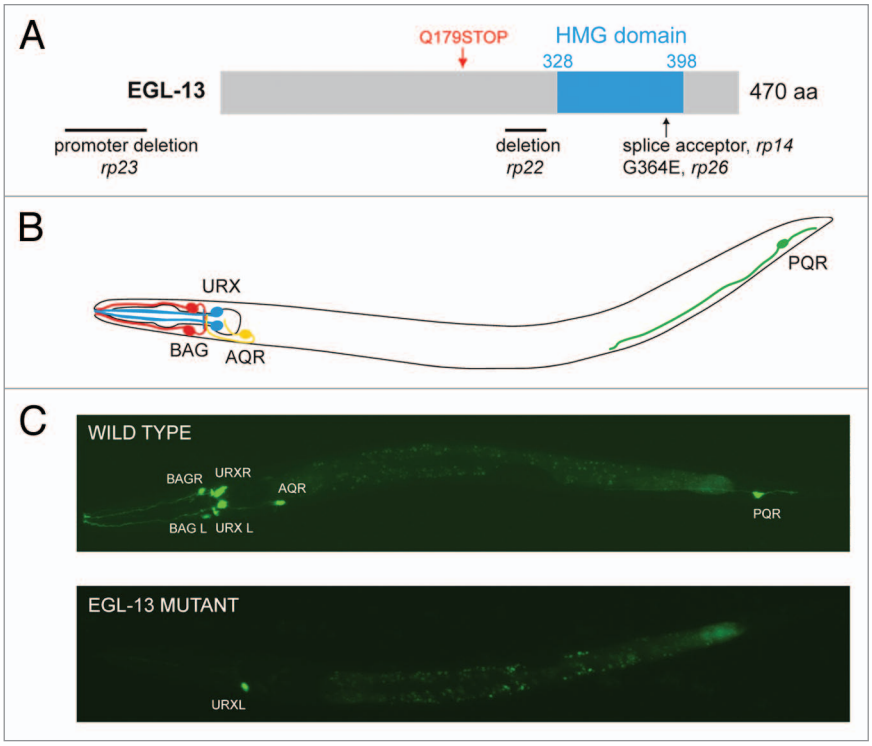

Figure 2. Identification of EGL-13, a factor that specifies both O_2- and CO_2-sensing neurons. (**A**) Schematic presentation of *egl-13* genetic lesions showing the resultant effects on the EGL-13 protein. (**B**) In *C. elegans*, the O_2/CO_2-sensing neural system comprises of six neurons called BAGL/R, URXL/R, AQR, and PQR (only single BAG and URX cells are shown). This neural system senses and transmits information regarding O_2/CO_2 status in the external environment and in the body fluid to the nerve ring. (**C**) Wild-type animals have six functional O_2/CO_2-sensing (top picture). *egl-13* mutant animals (bottom picture) fail to correctly express an O_2/CO_2-sensing cell fate marker and are unable to sense O_2 and CO_2.

Ectopic Expression of egl-13 Induces O_2 and CO_2 like Fates

We tested whether *egl-13* was sufficient to induce O_2- or CO_2-sensing neuronal fate in other neurons by ectopically expressing *egl-13* under the control of the *unc-86* promoter that is widely expressed in the nervous system.[40] Using this promoter to drive *egl-13* expression, we were able to induce expression of multiple O_2 and CO_2 neuronal markers in neurons that do not normally express these markers. Since the neurons we observed to adopt an O_2/CO_2 sensing-like fate were not consistent from animal to animal we were unable to experimentally show if they responded to changes in O_2 or CO_2, indicating a total change of fate, or alternatively, if their fate was pushed toward an O_2- or CO_2-sensing fate while still having features of their original fate. That ectopic *egl-13* expression can induce O_2 or CO_2 neuronal fate in other neurons further underlines the importance of EGL-13 in BAG and URX differentiation and resembles findings from other studies.[9,10] One such example is the adoption of dopaminergic neuronal fate by a selection of non-dopaminergic neurons when AST-1 is ectopically expressed.[9] Another study described how UNC-3 is able to induce cholinergic fate in a subset of non-cholinergic neurons.[10] The general assumption on cell identity and fate maintenance is that both positive and negative regulators tightly control it.[41,42] Negative control that prevent cells from changing fates has been shown to be directed by chromatin modifications that prevent expression of genes from other cell fate programs.[43,44] Recently, it was shown that removing LIN-53, a member of the PRC2 complex, thereby altering the chromatin state, made it possible to induce germ cell conversion into specific neurons, depending on the transcription factor used to drive the fate.[43,44]

egl-13 Mutants Are Unable to Respond to Changes in O_2 and CO_2

To test the functionality of the BAG and URX neurons in *egl-13* mutant animals, we conducted behavioral assays

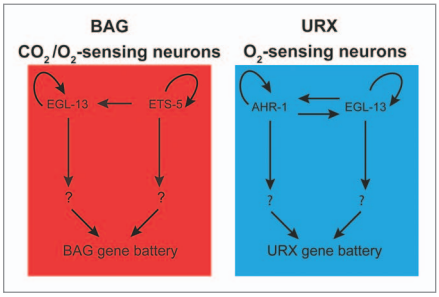

Figure 3. Working model for O_2/CO_2-sensing neuron specification. So far we have placed three factors (EGL-13, ETS-5, and AHR-1) into the genetic network of O_2/CO_2-sensing neuron specification. Ongoing work will help us understand how other factors work together to precisely control this important developmental decision.

where the locomotory response to changes in either O_2 or CO_2 was tested. Populations of wild-type animals decrease their average speed in response to changes in either O_2 or CO_2.[20,45,46] This behavior was absent in *egl-13* mutants but transgenic expression of *egl-13* under its own promoter rescued the sensory defects. These data clearly showed that *egl-13* was needed not only for differentiation but also for correct functionality of the BAG and URX neurons.

egl-13 Regulation in BAG and URX Neurons

Since *egl-13* is required for terminal differentiation of both BAG and URX neurons, we speculated whether the upstream regulator(s) of *egl-13* expression was the same for both neurons. To address that question, we drove expression of fluorescent protein with truncated fragments of the *egl-13* promoter and analyzed the expression patterns in BAG and URX neurons. It became apparent that *egl-13* expression is regulated through different motifs in the BAG and URX neurons. In the BAG neurons, we found that *egl-13* expression is regulated through two ETS-5 binding sites, whereas in URX, *egl-13* is regulated through AHR-1 and EGL-13 binding sites. We were able to confirm this regulation by crossing the mutants of *ahr-1, egl-13*, and *ets-5* into the full-length *egl-13* reporter, and by crossing *egl-13* mutants with reporters of *ets-5* and *ahr-1*. These experiments showed that the regulation is complex and the expression of the factors involved, in both BAG and URX, are dependent on each other for correct expression (**Fig. 3**). We speculate that *egl-13* is involved in the general specification of both O_2- and CO_2-sensing neurons, but in order to distinguish between the two different fate programs, giving rise to BAG and URX individually, there is a requirement for co-factors ETS-5 and AHR-1, respectively.

The question marks in our model illustrate that we are still questioning the directness of regulation of the terminal gene battery in the BAG and URX neurons (**Fig. 3**). However, ETS-5 was shown to bind directly to the promoter of *gcy-9* in the BAG neurons[26] and our preliminary data show that both AHR-1 and EGL-13 bind the promoter of the URX terminal differentiation gene *flp-8*. To complete the picture we are currently analyzing the direct regulation with the rest of the BAG and URX promoters.

Concluding Remarks

Collective data from other neuronal cell fate studies have been used to create a paradigm on how neuronal diversity is obtained by the selective expression of one or few key transcription factors referred to as terminal selectors.[8,10,47] In the definition of terminal selectors, four characteristics are listed: (1) Essential for terminal neuronal differentiation but not for general neuronal features. (2) Directly regulates the expression of the terminal gene battery through a specific motif. (3) Regulates its own expression to maintain differentiated features of the neuron. (4) One neuron might have more that one terminal selector and the correct fate is only obtained when co-expressed in the same cellular context.[4,5,48]

Reviewing the data we obtained during our studies, and in addition to our preliminary data showing that *ahr-1* and *egl-13* bind directly to the *flp-8* promoter in URX neurons, strongly suggests that EGL-13 is a terminal selector. However, since it has a dual role in both BAG and URX neurons, the need of co-factors is obvious. We believe that ETS-5 and AHR-1 are strong EGL-13 interaction candidates in the BAG and URX neurons respectively and might prove to work with EGL-13 in a terminal selector complex. However, we do not exclude the possibility that other factors are needed for the correct differentiation of the O_2- and CO_2-sensing neurons and additional genetic screens would enable the identification of such genes.

The information we have provided on the differentiation mechanism of O_2- and CO_2-sensing neurons deepens and solidifies the understanding of how specific neurons differentiate and neuronal diversity is obtained. Further work pursuing the role(s) of *egl-13*/Sox5 in neuronal differentiation in higher organisms may potentially provide a better understanding of aspects of complex diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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