**Tonic signaling from O₂ sensors sets neural circuit activity and behavioral state**

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Tonic receptors convey stimulus duration and intensity and are implicated in homeostatic control. However, how tonic homeostatic signals are generated and how they reconfigure neural circuits and modify animal behavior is poorly understood. Here we show that *Caenorhabditis elegans* O₂-sensing neurons are tonic receptors that continuously signal ambient [O₂] to set the animal’s behavioral state. Sustained signaling relied on a Ca²⁺ relay involving L-type voltage-gated Ca²⁺ channels, the ryanodine and the inositol-1,4,5-trisphosphate receptors. Tonic activity evoked continuous neuropeptide release, which helps elicit the enduring behavioral state associated with high [O₂]. Sustained O₂ receptor signaling was propagated to downstream neural circuits, including the hub interneuron RMG. O₂ receptors evoked similar locomotory states at particular O₂ concentrations, regardless of previous d[O₂]/d.t. However, a phasic component of the URX receptors’ response to high d[O₂]/d.t, as well as tonic-to-phasic transformations in downstream interneurons, enabled transient reorientation movements shaped by d[O₂]/d.t.

Our results highlight how tonic homeostatic signals can generate both transient and enduring behavioral change.

Sensory neurons can exhibit phasic, tonic or phasic-tonic signaling. Phasic receptors adapt rapidly, signal changes in stimulus intensity, and typically function over broad dynamic ranges. They allow animals to monitor changing environmental features. Most sensory receptors that have been genetically dissected are phasic¹⁻³. In contrast, tonic receptors adapt slowly and communicate stimulus duration and intensity. Phasic-tonic receptors combine phasic and tonic properties⁴. For many sensory modalities, including vision, sound and smell, nervous systems convey both dynamic and static information⁵,⁶. For example, retinal photoreceptors and cochlear hair cells transmit a range of stimulus intensities for long periods by changing the tonic rate of transmitter release at ribbon synapses⁷. Tonic receptors are functionally diverse⁸ and include proprioceptors⁹, nociceptors¹⁰, chemoreceptors⁶,¹¹ and mechanoreceptors⁵. Typically, these receptor neurons have been characterized electrophysiologically, but the molecular mechanisms underlying their sustained signaling are unclear. Also poorly understood is how their tonic output influences neural circuit properties and behavior.

Many mammalian homeostatic responses involve tonically signaling circuits. These include circuits controlling body temperature¹², arterial pressure¹³, and CO₂ and O₂ levels¹⁴. Although these circuits are not well understood, in both vertebrates and invertebrates homeostatic imbalance often alters behavior. In *C. elegans*, homeostatic responses allow worms to escape high (21%) and low (<5%) ambient [O₂]¹⁵. Avoidance of high [O₂] involves the AQR, PQR and URX neurons¹⁵⁻¹⁹, neurons first characterized because they promote *C. elegans* aggregation²⁰,²¹. Rising [O₂] is thought to stimulate their activity by activating atypical soluble guanylate cyclases¹⁵,²¹, thereby opening cyclic GMP–gated cation channels. The BAG ciliated head neurons are activated by decreases in [O₂] and also help worms locate preferred oxygen levels¹⁹. Avoidance of low [O₂] is less well understood.

Here we show that AQR, PQR and URX are tonic receptors that cause long-lasting changes in neural circuit activity and persistently set *C. elegans* behavior according to [O₂]. We identify a Ca²⁺ relay that sustains this tonic signaling. By combining different stimulus dynamics, halorhodopsin and channelrhodopsin activation, and Ca²⁺ imaging, we elucidate how these neurons can evoke transient and enduring behavioral change.

**RESULTS**

**Tonic O₂ sensors couple behavioral state to O₂ levels**

To ask whether *C. elegans* behavioral state is persistently reset by [O₂], we recorded worms in different O₂ environments (Supplementary Fig. 1). We focused on mutants defective in the *npr-1* neuropeptide receptor, as the reference *C. elegans* strain, N2 (Bristol), has acquired a gain-of-function mutation in this receptor during laboratory cultivation that inhibits some O₂-evoked responses²²,²³. For comparison, we studied the Hawaiian wild isolate CB4856, which retains the natural *npr-1* allele²²,²³ (for a complete list of strains and mutants, see the Supplementary Strain List). Altering [O₂] evoked persistent behavioral change in *npr-1* and CB4856 worms. At 21% O₂ these worms moved rapidly on food, even after 2 h (Fig. 1a,b). By contrast, in 7% O₂ the worms moved slowly and dwelled locally, even after 2 h (Fig. 1c). These enduring behavioral states remained sensitive to [O₂]: changing [O₂] switched behavior in seconds (Fig. 1d–e). Thus, *C. elegans* persistently alters behavior according to [O₂].

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Two mechanisms could explain the impact of [O\textsubscript{2}] on behavior. First, O\textsubscript{2} receptors could be tonically activated, continuously signaling [O\textsubscript{2}] to downstream circuits. Second, O\textsubscript{2} sensors could respond transiently to changes in [O\textsubscript{2}] but persistently alter downstream circuits. *C. elegans* O\textsubscript{2} sensors include the head neurons BAG, URX and AQR, and the tail neuron PQR\textsuperscript{15,18,19}. AQR, PQR and URX neurons are activated by rising [O\textsubscript{2}]\textsuperscript{18,19} and regulate turning behavior and locomotory activity in response to transient changes in [O\textsubscript{2}]\textsuperscript{16,17}. To examine whether these neurons tonically signaled [O\textsubscript{2}], we visualized their responses to long-lasting stimuli in immobilized worms using the calcium sensor cameleon YC3.60 (ref. 24). In parallel, we imaged O\textsubscript{2}-evoked Ca\textsuperscript{2+} responses of URX and PQR neurons in fast-moving animals. Together, our data suggest that tonic signaling from AQR, PQR and URX neurons signal tonically in high [O\textsubscript{2}].

The O\textsubscript{2} responses of AQR, PQR and URX require the GCY-35 subunit\textsuperscript{15,18–21}. Disrupting these channels abolishes O\textsubscript{2}-evoked Ca\textsuperscript{2+} responses in these neurons\textsuperscript{18,19}. Disrupting EGL-19, the sole *C. elegans* L-type voltage-gated Ca\textsuperscript{2+} channel (L-VGCC) \alpha\textsubscript{1} subunit, yielded similar phenotypes (Supplementary Fig. 4 and ref. 25), suggesting that L-VGCCs amplify the sensory potential, as reported in other *C. elegans* neurons\textsuperscript{26,27}.

We hypothesized that sustained O\textsubscript{2}-evoked Ca\textsuperscript{2+} signaling might involve Ca\textsuperscript{2+} release from the endoplasmic reticulum. To test this, we imaged mutants defective in the inositol-1,4,5-trisphosphate (IP\textsubscript{3}) receptor, encoded in *C. elegans* by the itr-1 gene, and in the ryanodine receptor, encoded by unc-68. In worms bearing a reduction-of-function mutation of the IP\textsubscript{3} receptor, itr-1(sa73), URX and PQR often did not respond to a rise in [O\textsubscript{2}], although in some worms the neurons responded normally (Fig. 2d and Supplementary Fig. 5f). Cell-specific knockdown of itr-1 in AQR, PQR and URX by RNA interference gave a stronger phenotype: a switch from 7% to 21% O\textsubscript{2} evoked an initial Ca\textsuperscript{2+} peak, but the sustained Ca\textsuperscript{2+} plateau at 21% O\textsubscript{2} was strongly reduced (Fig. 2d and Supplementary Fig. 5e). Mutations in the ryanodine receptor also strongly reduced the sustained Ca\textsuperscript{2+} responses evoked in URX and in PQR by high [O\textsubscript{2}] (Fig. 2f and Supplementary Fig. 5b,c). These data suggest that activation of L-VGCCs by the sensory potential is potentiated by Ca\textsuperscript{2+} release through the ryanodine and IP\textsubscript{3} receptors during sustained signaling.

**HaloRhodopsin and Channelrhodopsin control of O\textsubscript{2} sensors**

To investigate whether tonic signaling from O\textsubscript{2}-sensing neurons was required for continuous fast movement at 21% O\textsubscript{2}, we acutely inhibited these neurons using the light-driven chloride pump halorhodopsin (NpHR)\textsuperscript{28}. Upon exposure to green light, *npr-1 lite-1* worms grown on the rhodopsin cofactor retinal and expressing NpHR tagged with the red fluorescent protein mCherry in AQR, PQR and URX sharply reduced their movement on food. Control worms grown without retinal showed no behavioral change (Fig. 3a,b and Supplementary Video 1). These data suggest that tonic signaling from AQR, PQR and URX sustains rapid movement in high O\textsubscript{2}.
Upon exposure to blue light, worms expressing functional ChR2 in AQR, PQR and URX substantially increased movement during a 30 s period before the downstep compared with 30 s afterwards). As [O2] is 21%, ***

Fast movement in nature neur OSCI enCe

To distinguish from that of the same worms kept at 21% O2 without light activation. To extend our analysis, we gave npr-1 lite-1 worms expressing pgy-c-32::ChR2-mCitrine light stimuli of different durations. We first illuminated worms kept at 11% O2 for 15 s with blue light, then, after an interval, exposed them to blue light for 15 min (Fig. 3e,f). When blue light came on briefly, worms only transiently sped up; by contrast, sustained blue light elicited sustained fast movement. In both cases, when light was switched off, the worms returned to the slow movement typical of npr-1 worms in 11% O2. These results suggest that tonic activation of AQR, PQR or URX can induce sustained fast movement in npr-1 worms kept at low O2.

To ask whether each of the O2-sensing neurons could activate locomotion, we exploited the variable expression of transgene arrays, and selected worms in which ChR2-mCitrine was visible in a subset of the neurons. In worms with ChR2-mCitrine visible only in URX, blue light stimulated movement, but to a lesser extent than in worms expressing ChR2 in AQR, PQR and URX (Fig. 3c,d). Worms expressing ChR2 in AQR or PQR alone also increased locomotory activity in blue light in comparison to control worms, but expression in both neurons gave stronger responses (Fig. 3g,h). In all cases, the increased speed persisted in blue light and returned to pre-stimulation levels when light was switched off. Our results suggest that tonic activation of any one of AQR, PQR and URX can stimulate sustained fast movement.

Neuroexocytosis from O2 sensors promotes rapid movement

To examine whether release of synaptic vesicles or dense core vesicles from O2 sensors helps sustain rapid movement in 21% O2, we created transgenic worms expressing a pgy-c-32::tetanus toxin::gfp polycistronic construct. By cleaving synaptobrevin, tetanus toxin disrupts neuroexocytosis29. We then monitored the behavior of worms expressing GFP, and therefore tetanus toxin, in AQR, PQR and URX, or only in URX (Fig. 4a). Worms with transgene expression visible only in URX showed a weak reduction in locomotory activity in 21% O2. By contrast, worms expressing the transgene in all three neurons moved much more slowly than non-transgenic controls at 21% O2, but they retained the ability to slow down appropriately in 7% O2. These data suggest that neuroexocytosis from one or more O2-sensor sustains rapid movement in high [O2].
O₂ sensors tonically release neuropeptides in high O₂

As AQR, PQR and URX are peptidergic neurons, we examined whether they differentially release neuropeptides at high and low O₂. In C. elegans, secreted GFP-tagged proteins are removed from body fluid by coelomocytes, which consequently become fluorescent. This allows release of fluorescently tagged neuropeptides to be followed by monitoring coelomocyte fluorescence. To exploit this, we expressed red fluorescent protein (RFP)-tagged insulin-like peptide-1 (INS-1) specifically in AQR, PQR and URX, using the gcy-32 promoter. We confirmed that INS-1-RFP was correctly targeted by colocalizing it with the dense core vesicle marker IDA-1-GFP (Fig. 4b). We then tracked coelomocyte fluorescence intensity when worms were exposed to 7%, 21% or 25% O₂ for different lengths of time. During 4 h of exposure to 7% O₂, we observed no change in coelomocyte fluorescence (Fig. 4c). In contrast, after a switch to 21% O₂, coelomocyte fluorescence increased for 1 h and then plateaued. Worms switched to 25% O₂ showed larger increases in coelomocyte fluorescence, and it continued to rise throughout the 4-h exposure to this O₂ level.

To confirm that accumulation of tagged INS-1 in coelomocytes reflects evoked release, we repeated the experiment in unc-64 syntaxin mutants, which have defects in dense core vesicle release. unc-64 mutants showed increased fluorescence in the cell body of PQR but very little fluorescence in coelomocytes, and the coelomocyte fluorescence was unaltered by O₂ experience (Fig. 4c). These results suggest that high O₂ evokes tonic release of neuropeptides from one or more O₂ sensors.
Rapid movement requires carboxypeptidase E in O$_2$ sensors

To investigate to what extent neuropeptide release from O$_2$ sensory neurons modified behavior, we first asked whether disrupting egl-21, the worm ortholog of carboxypeptidase E (CPE), alters O$_2$ responses. EGL-21 removes basic C-terminal amino acids; without it, most prepropeptides cannot be processed into functional neuropeptides. Disrupting egl-21 switched the sign of the O$_2$ response: egl-21; npr-1 worms sped up when [O$_2$] fell and slowed down when [O$_2$] rose (Supplementary Fig. 6). Thus, peptide signaling is required for wild-type responses to changes in [O$_2$].

egl-21 is expressed widely in the nervous system. To examine whether it is required in O$_2$ sensors for appropriate O$_2$ responses, we downregulated it specifically in these neurons, using targeted RNAi. npr-1 worms expressing egl-21 sense and antisense RNA in AQR, PQR and URX moved appropriately slowly in 7% O$_2$, but they sped up much less than non-transgenic siblings when switched to 21% O$_2$ (Fig. 4d). This phenotype could be rescued by expressing an RNAi-insensitive egl-21 transgene in AQR, PQR and URX, confirming the effect was specific (Fig. 4d). These data suggest that neuropeptide release from one or more O$_2$ sensors when [O$_2$] is high promotes rapid *C. elegans* movement.

O$_2$ sensors tonically control downstream neural circuits

We next examined how the activity of O$_2$ sensors modulated downstream circuits in npr-1 worms, using YC3.60 as a Ca$^{2+}$ reporter. URX has gap junctions with the AUA and RMG interneurons and makes chemical synapses onto AUA37.

A switch from 7% to 21% O$_2$ elicited increased [Ca$^{2+}$] in the RMG and AUA cell bodies that persisted while [O$_2$] was at 21% (Fig. 5a,b). Upon returning worms to 7% O$_2$, [Ca$^{2+}$] quickly returned to levels previously observed at this [O$_2$]. The O$_2$-evoked Ca$^{2+}$ responses in RMG were abolished in *gcy-35; npr-1* mutants, suggesting they reflect input from URX and/or AQR and PQR (Fig. 5c). The O$_2$-evoked Ca$^{2+}$ responses in AUA interneurons were attenuated but not abolished in *gcy-35; npr-1* mutants (Fig. 5d). The residual O$_2$ sensitivity could reflect the activity of other atypical soluble guanylate cyclases expressed in AQR, PQR and URX, namely GCY-32, GGY-34 and GGY-37; these have previously been shown to modify behavioral responses to O$_2$ (ref. 16). Thus, AUA and RMG interneurons, like the O$_2$ sensors themselves, are tonically regulated by [O$_2$].

Ablating RMG and AUA interneurons alters O$_2$ responses

To investigate how O$_2$ sensors modulate AUA, we imaged its O$_2$-evoked Ca$^{2+}$ responses in mutants defective in synaptic transmission or neuropeptide synthesis. To control for the possibility that altered AUA activity reflects defects in the O$_2$ sensors, we also imaged URX responses in the mutants. Loss of *unc-13*, which disrupts synaptic vesicle release but does not prevent vesicle communication, did not reduce O$_2$-evoked Ca$^{2+}$ responses in AUA or URX but potentiated the initial responses of both neurons (Fig. 5e,f). By contrast, loss of egl-21 substantially reduced O$_2$-evoked responses in AUA without altering URX responses (Fig. 5e,g). These data suggest that O$_2$-evoked Ca$^{2+}$ responses in AUA partly reflect tonic peptidergic input.

To examine the functions of RMG and AUA in O$_2$-evoked behavioral responses, we ablated them using a laser microbeam. Removing RMG abolished [O$_2$]-dependent changes in speed: worms with ablated neurons moved at speeds intermediate between those of npr-1 worms kept at 21% and 7% O$_2$ (Fig. 5h,i). Ablating AUA had modest effects: naive AUA(-) worms were only slightly slower than mock-ablated controls at 21% O$_2$ and slowed down strongly when [O$_2$] fell to 7% (Fig. 5j). These data suggest that sustained O$_2$-evoked changes in [Ca$^{2+}$] in RMG contribute to the high and low locomotory states of *C. elegans* at high and low [O$_2$].

Head and tail O$_2$ sensors promote opposite escape responses

Besides evoking persistent changes in speed according to [O$_2$], O$_2$ sensors elicit transient reorientation movements in response to changing O$_2$ concentration, d[O$_2$/dt]. A rise in [O$_2$] from 7% to 21% induces a bout of reversals and turns lasting 1–2 min that usually leads to a change in direction of travel. These transient responses are disrupted in...
gy-35; npr-1 mutants but can be restored by transgenic expression of gcy-35 in AQR, PQR and URX, or in URX alone (ref. 17; see below).

To show explicitly that activating URX head neurons evokes reversal, we selectively activated them using ChR2-EYFP. To keep O₂ sensor activity low when blue light was absent, we maintained worms at 7% O₂ using a microfluidic arena. We then shone a circle of blue light that illuminated only the head of npr-1 worms expressing ChR2 in URX and PQR for 1.5–2 s. To restrict blue light to a small area we used a prototype programmable array microscope (PAM), a fluorescence microscope in which a spatial light modulator is placed in the primary image plane (Fig. 6a). Simultaneously, we observed behavioral changes using transillumination with yellow light. As predicted, activating URX elicited reversal behavior (Fig. 6d,e and Supplementary Video 3) in worms grown with the ChR2 cofactor retinal.

We next examined how selectively activating PQR alters C. elegans’ movements. Because O₂ diffuses slowly in water, sharp gradients can be generated even across the 1 mm separating the sensory endings of URX in the head and PQR in the tail of an adult (Fig. 6c) [17]. We speculated that, unlike activating URX neurons, activating PQR neurons would promote acceleration without first provoking reorientation, allowing worms to escape efficiently if the tail but not the head detects a high [O₂].

To test this, we used the PAM to selectively illuminate the tails of worms expressing pgcy-32::ChR2-EYFP while keeping them at 7% O₂. As predicted, activating PQR elicited acceleration without reversal (Fig. 6d,i and Supplementary Video 4). In both URX and PQR light activation experiments, we rarely observed light-evoked behavioral responses in worms grown without retinal, and we obtained similar results in npr-1 lite-1 mutants, confirming that responses do not reflect innate light avoidance.

To test directly whether elevated [O₂] at the head and tail elicited different behavioral responses, we directed a 2-s puff of 21% O₂ at either end of worms expressing ChR2-EYFP, while keeping them at 7% O₂. As predicted, activating PQR elicited acceleration without reversal (Fig. 6d,i and Supplementary Video 4).

ARX, AUA and URX are required for persistent behavioral change. Behavioral changes using transillumination are strongly attenuated in gcy-35 mutants. **P < 0.01, ***P < 0.001 (two-tailed t-test). NS, not significant.

**Figure 5** Tonic activation of O₂ sensors is propagated to downstream interneurons, which are required for persistent behavioral change. (a) A switch from 7% to 21% O₂ elicits a Ca²⁺ spike in RMG interneurons, followed by a Ca²⁺ plateau that persists while [O₂] is high. The plot shows relative ratio change (∆R/R) compared to the baseline (mean ratio during 1 min in the first period at 7% O₂, immediately before the shift to 21% O₂). (b) A switch from 7% to 21% O₂ elicits a rise in Ca²⁺ in AUA interneurons that persists while [O₂] is high. (c) O₂-evoked responses in RMG are disrupted in gcy-35 mutants. **P < 0.01, ***P < 0.001 (two-tailed t-test). NS, not significant. (d) O₂-evoked responses in AUA are strongly attenuated in gcy-35 mutants. (e) Mutations in gcy-35, but not egl-21, disrupt O₂-evoked Ca²⁺ responses in URX neurons. Loss of unc-13 transiently enhances O₂-evoked URX Ca²⁺ responses. (f) An unc-13 mutation enhances O₂-evoked Ca²⁺ responses in AUA transiently (f), whereas an egl-21 mutation strongly attenuates them (g). (h,i) Ablating RMG abolishes O₂-evoked changes in the locomotory activity of npr-1 worms. RMG(–) npr-1 worms move at speeds intermediate between those displayed by mock-ablated worms at 7% and 21% O₂. The O₂ stimulus is plotted in gray in i,j. Horizontal red bars indicate statistical comparison of the ablated worms with the mock controls during the given period. n > 40 worms. (j) Ablating AUA neurons only has a small effect on control of locomotory activity by [O₂]. n > 40 worms.
of npr-1 and gcy-35; npr-1 worms kept at 7% O₂ (Fig. 6b,c). To control for behaviors elicited by mechanical stimulation, we also puffed 7% O₂. Puffing 21% O₂ at the head of npr-1 worms robustly evoked reversal (Fig. 6g and Supplementary Video 5), whereas directing 21% O₂ at the tail elicited acceleration (Fig. 6h and Supplementary Video 6). Puffing 7% O₂ at the heads or the tails of npr-1 worms kept at 7% O₂ had minimal effects on behavior (Fig. 6g,h). Both head and tail responses to 21% O₂ were reduced to control (7% O₂) levels in gcy-35; npr-1 mutants. These data indicate that C. elegans head and tail O₂ sensors can elicit escape from high O₂ in opposite directions.

Neural coding of behavioral responses coupled to d[O₂]/dt
We explored how O₂ receptors can encode both persistent changes in speed and transient reorientation responses. Two models, not exclusive, could explain short-lived responses. First, a phasic component of the O₂-evoked Ca²⁺ responses in these neurons could drive the reorientations. Second, tonic output from the sensors could be transformed into phasic responses in downstream interneurons.

To investigate these models, we varied d[O₂]/dt 60-fold by switching worms between 7% and 21% O₂ at different rates, and analyzed Ca²⁺ traces and behavioral responses. The d[O₂]/dt stimuli we used were 2% s⁻¹, 0.2% s⁻¹ and 0.03% s⁻¹ (we used the last only for behavioral studies); each was experimentally validated (Supplementary Fig. 1a). For d[O₂]/dt = 0.2% s⁻¹, [Ca²⁺] in URX rose gradually as [O₂] increased from 7% to 21%, and it then plateaued (Fig. 7a). By contrast, at d[O₂]/dt = 2% s⁻¹ URX generated an overshoot—a transient Ca²⁺ peak that decayed to the plateau level observed for the slower d[O₂]/dt stimulus (Fig. 7a). Thus, URX displayed both phasic and tonic signaling properties, but the phasic Ca²⁺ responses were evident only at high d[O₂]/dt under our imaging conditions. AQR and PQR showed small Ca²⁺ peaks at both fast and slow d[O₂]/dt (Supplementary Fig. 7a,b).

We next measured the behavioral responses of worms exposed to the d[O₂]/dt used in our imaging experiments. The final speed of worms after [O₂] had stabilized was similar, regardless of d[O₂]/dt, as expected if speed were coupled to the [Ca²⁺] plateau in the O₂ sensors (Fig. 7b and Supplementary Fig. 7c). By contrast, and as expected for a phasic response, varying d[O₂]/dt altered the pattern of reversals and omega turns (within worms turn deeply and touch their own tails). Worms reversed and turned as long as [O₂] changed, even when d[O₂]/dt was very small (Fig. 7c,d and Supplementary Fig. 7d,e). Altering d[O₂]/dt also altered the probability of reversals and turns. Transgenic rescue of gcy-35 mutants confirmed that URX could account for most of these responses (Fig. 7c–f and Supplementary Fig. 8b,c), although this does not exclude redundant roles for other neurons. Together, our data suggest that URX encodes reversals and turns in two ways. The phasic component of its Ca²⁺ response at high d[O₂]/dt correlates with high probability of a reversal immediately after a fast O₂ switch.

Figure 6 Anterior O₂ sensors direct reversals, whereas posterior sensors direct forward movement. (a) Simplified working principle of the programmable array microscope. A liquid crystal on silicon (LCOS) chip acts as a spatial light modulator, wherein every pixel functions as an aperture that can be ‘opened’ or ‘closed’ independently. ChR2-EYFP was excited with blue light from a Nd:YAG laser. (b) Device for targeting a puff of O₂ to different body regions. The O₂ mixture is pumped from the narrow glass capillary (see g,h). (c) Worms expressing channelrhodopsin-EYFP in the URX and PQR neurons were selectively illuminated with a circle of blue light in the head or tail region only (see d–f). (d) Responses of npr-1 lite-1 worms expressing ChR2-EYFP in URX and PQR neurons when blue light is restricted to head (top) or tail (bottom) regions. The illuminated region is indicated by a circle. Stimulation of the head induces a reversal; stimulation of the tail induces forward movement. (e,f) Quantification of behavioral responses of npr-1 gcy-32::ChR2-EYFP and npr-1 lite-1 gcy-32::ChR2-EYFP worms elicited by light-activation of URX neurons (e) or PQR tail neurons (f). ATR, all-trans retinal. ***P < 0.001 (two-tailed Fisher’s exact test). (g,h) Behavioral responses of npr-1 worms kept at 7% O₂ to a puff of 21% or 7% O₂ directed at the head (g) or tail (h). The responses evoked by 21% O₂ are abolished in gcy-35;npr-1 mutants. ***P < 0.001 (two-tailed Fisher’s exact test). NS, not significant. Error bars, s.e.m.
Conversely, the absence of a phasic calcium response in URX at low d[O2]/dt suggests a tonic-to-phasic transformation of input from O2 sensors in downstream circuits, enabling reversals and turns to cease when dO2/dt and d[Ca2+]/dt = 0 even if [Ca2+] in URX remains persistently elevated.

Neural circuits mediating transient responses to O2

We next examined how manipulations that disrupt tonic control of speed modify phasic reorientation responses. Expressing tetanus toxin in AQR, PQR and URX attenuated both reversal and turning when [O2] rose (Fig. 7g and Supplementary Fig. 8d,e), implicating

![Figure 7 Behavioral and neuronal responses to different d(O2)/dt. (a) Ca2+ imaging of URX neurons in immobilized worms exposed to slow (0.2% s−1) and fast (2% s−1) switches between 7% and 21% O2. Measured [O2] is plotted in gray. ***P < 0.001 (two-tailed t-test). (b–f) Behavioural responses of worms on food to the stimulus regime in b. Horizontal bars indicate comparison of the genotype of the same color with npr-1 (c,d,g,j) or gcy-35; npr-1 (e,f) or mock ablated (k,l) worms during the given period. (b) Locomotory speed follows [O2] regardless of d[O2]/dt in npr-1 worms. Control gcy-35; npr-1 worms show little O2 response. (c,d) Proportion of npr-1 and gcy-35; npr-1 worms reversing (e) or turning (d) during slow and rapid rises in [O2]. (e,f) Expressing gcy-35; gfp in AQR, PQR and URX neurons or i in URX alone in gcy-35; npr-1 mutants restores reversal (e) and turning (f) responses to both slow and fast O2 changes. ***P < 0.001 (two-tailed chi-squared test). n > 80 worms for each genotype. (g) O2-evoked reversal and turning (bottom) behaviors of worms expressing tetanus toxin in AQR, PQR and URX or only in URX. Data from same movies as in Figure 4a. n > 70 worms. (h) RNAi knockdown of egl-21 carboxypeptidase E in AQR, PQR and URX reduces O2-evoked reversals and turning. Data from same movies as in Figure 4d. n > 80 worms. (i) The RMG interneuron contributes to O2-evoked reversal and turning. Data from same movies as in Figure 5i. n > 40 worms.]
AVA interneurons display phasic responses to changes in O2. (a) AVA neural activity visualized in individual immobilized npr-1(ad609) worms expressing pmnr-1::YCD3 cameleon. E. coli food was spread around the head of the worms glued onto the agarose pad. Traces show inhibition of AVA activity in response to a drop in [O2] and activation of AVA when [O2] rises. Worms kept in 21% O2 show prolonged bouts of high AVA activity, alternating with periods of low activity that are not observed in freely moving worms (see b). Gray shading indicates 21% O2. (b) AVA neural activity visualized in individual freely moving npr-1(ad609) worms expressing pmnr-1::YCD3 cameleon. AVA activity and reversals increase when [O2] rises from 7 to 21%. Blue, red and green sections of the traces indicate periods of forward movement, reversals or no movement, respectively. Missing sections of individual traces are due to worms moving out of the field of view. In both a and b, AVA displays not tonic (sustained) activation but phasic responses (peaks and troughs).

AVA interneurons respond phasically to changes in O2

C. elegans reversal behavior is controlled by a set of interconnected pre-motor interneurons called AVA, AVD and AVE37,38. Ca2+ imaging of freely moving worms suggests that reversals occur whenever AVA [Ca2+] rises39. AQR, PQR, URX and RMG and AVA each synapse onto one or more of AVA, AVD and AVE. We therefore examined whether tonic activation of AQR, PQR, RMG and AVA following a rise in [O2] elicited persistent or transient changes in AVA [Ca2+].

We first imaged O2-evoked Ca2+ responses in AVA in npr-1 worms tethered by glue with food present (Fig. 8a). In these worms, AVA [Ca2+] decreased when [O2] dropped from 21% to 7%. Returning [O2] to 21% led to a rapid rise in AVA Ca2+, consistent with the increased reversal rates we observed in behavioral assays. High [Ca2+] in AVA was not, however, tonically maintained at 21% O2, but fluctuated between high and low levels as expected if the worms were sporadically attempting to reverse. Thus, AVA neurons respond to the ‘onset’ and ‘offset’ of an O2 stimulus but do not display sustained changes in [Ca2+], suggesting a tonic-to-phasic transformation between O2 sensors and AVA interneurons.

In the imaging experiments above using tethered npr-1 worms, AVA was active more than half the time when the worms were at 21% O2. However in our behavioral experiments, much fewer than 50% of npr-1 worms were reversing at any one time in 21% O2 (Fig. 7c and Supplementary Fig. 8d, f, h). Moreover, reversals were much briefer than the bouts of high AVA activity we imaged in immobilized worms (Fig. 8a and Supplementary Fig. 9a, b). We speculated that this discrepancy might reflect either attempt by immobilized worms to escape by long reversals or a need for feedback from locomotor activity to terminate AVA responses. To investigate this, we imaged AVA responses to O2 stimuli in freely moving worms while simultaneously monitoring locomotion (Fig. 8b). Under these conditions, AVA exhibited much shorter bouts of high [Ca2+] for a much smaller proportion of the time (compare Fig. 8a, b). As in immobilized worms, a rise in [O2] elicited a rise in AVA [Ca2+], and this coincided with reversal behavior. The short periods when [Ca2+] was rising correlated with reversals, whereas long periods when [Ca2+] was falling or low correlated with forward locomotion or rest. Unlike in immobilized worms, we did not observe sharp drops in AVA [Ca2+] when we switched freely moving worms from 21% to 7% O2, probably because AVA [Ca2+] at the end of the 3-min 21% O2 period was already close to baseline. Our data suggest that tonic signaling from O2 sensors evokes phasic responses in AVA neurons, which correlate with reversals.

DISCUSSION

Tonically signaling neurons and neural circuits are widespread but poorly understood. Here we show that the C. elegans O2-sensing neurons AQR, PQR and URX are tonic receptors whose sustained signaling sets the behavioral state according to ambient [O2]. High [O2] evokes tonic high [Ca2+] in these neurons, leading to tonic peptide release, long-lasting changes in downstream neural circuit activity, and sustained rapid movement in feeding worms (Supplementary Fig. 10a, b). Low [O2] results in persistently low [Ca2+] and sustained slow movement.

To maintain elevated [Ca2+] for minutes and hours, the O2 sensors use a relay of cGMP-gated ion channels, L-type voltage-gated Ca2+ channels, and IP3 and ryanodine receptor Ca2+ channels (Supplementary Fig. 10a). IP3 receptors are generally thought to require IP3 for activation, suggesting that O2-sensing neurons have an unknown source of this molecule40. In other systems ryanodine receptors are activated either through physical interaction with L-type VGCCs, as in skeletal muscle, or by Ca2+-induced Ca2+ release (CICR), as in cardiac muscle41. Varying d[O2]/dt affected neither the persistent Ca2+ plateau nor the consequences for locomotory activity once [O2] has stabilized, suggesting that the signaling system sets up and maintains a steady state of elevated Ca2+ according to [O2]. CICR might be expected to operate in ‘all-or-none’ fashion, but is often finely tuned to the magnitude and duration of the trigger, typically Ca2+ entry via L-VGCCs, suggesting unknown counteracting...
control mechanisms. Both behavior and steady state Ca\textsuperscript{2+} can change in seconds when [O\textsubscript{2}] varies, indicating that the activity of IP\textsubscript{3} and ryanodine receptors remains coupled to the signal transduction mechanism measuring [O\textsubscript{2}]. A simple model is that different O\textsubscript{2} concentrations elicit different persistent levels of cGMP, leading to graded changes in sensory potential by activating cGMP-gated channels. The membrane potential change is amplified by L-VGCCs. The cGMP channels, or a proportion of L-VGCCs that inactivate very slowly but whose activity remains linked to [cGMP], could provide the Ca\textsuperscript{2+} influx to sustain ryanodine and IP\textsubscript{3} receptor signaling. Such a tonically acting mechanism requires a balanced return of Ca\textsuperscript{2+} to the endoplasmic reticulum, to enable continued signaling for many minutes and probably hours at high [O\textsubscript{2}].

Part of the sustained response to changes in [O\textsubscript{2}] is mediated by neuropeptides. Targeted RNAi knockdown of neuropeptide biogenesis in AQR, PQR and URX neurons strongly attenuated their ability to promote rapid movement in 21% O\textsubscript{2}, without interfering with the ability to inhibit movement at 7% O\textsubscript{2}. The downstream targets are unknown, but neuropeptide signaling by volume transmission can allow communication with cells not in direct synaptic communication. It will be interesting to examine whether sustained neuropeptide release is a leitmotiv of tonically signaling neurons.

The two post-synaptic targets of URX we imaged, AUA and RMG, both exhibited persistent changes in [Ca\textsuperscript{2+}] following changes in [O\textsubscript{2}] (Supplementary Fig. 10b). In the case of AUA, this partly depended on peptidergic signaling. In the case of RMG, on the basis of the anatomy, communication is likely to be via gap junctions. The weak behavioral effect of tetanus toxin expression in URX alone would be consistent with this scenario. We have not explored the ion channels that allow persistent elevated Ca\textsuperscript{2+} in either AUA or RMG. However, RMG is functionally required for the O\textsubscript{2}-evoked persistent changes in locomotory activity. It will be interesting to examine how far tonic signaling from O\textsubscript{2} sensors propagates through the C. elegans nervous system.

Besides persistently coupling rate of movement to [O\textsubscript{2}], URX can transiently elicit reversals and turns when [O\textsubscript{2}] rises\textsuperscript{16}. At least two features enabled tonically signaling URX sensors to elicit reversals with appropriate timing across a 60-fold range of d[O\textsubscript{2}]/d\textit{t}. First, rapid but not slow rises in [O\textsubscript{2}] elicited a Ca\textsuperscript{2+} spike that correlated with a high probability of reversal. Second, Ca\textsuperscript{2+} imaging suggested that the AVA interneurons, which elicit reversals, respond phasically to tonic input from O\textsubscript{2} sensors. This enables a slow rise in [O\textsubscript{2}] to elicit a sequence of reversals, and ensures that reversals and turns are suppressed when [O\textsubscript{2}] stabilizes, despite continued signaling from the sensors.

The locations of URX in the head and PQR in the tail, together with the circuitry properties associated with these neurons, allow C. elegans to avoid high O\textsubscript{2} in two ways: by reversing, turning and accelerating away without changing direction of travel when the tail (and PQR) but not the head encounters high [O\textsubscript{2}]. The opposite signs of these behavioral responses would facilitate migration down spatial gradients of O\textsubscript{2} away when the head (and URX) encounters high [O\textsubscript{2}]; and by accelerating away without changing direction of travel when the tail (and PQR) but not the head encounters high [O\textsubscript{2}]. The opposite signs of these behavioral responses would facilitate migration down spatial gradients of O\textsubscript{2} away from high [O\textsubscript{2}]. This arrangement is reminiscent of gentle touch avoidance in C. elegans, where anterior and posterior touch sensors evoke opposite escape responses\textsuperscript{38}. A potential difference is that O\textsubscript{2} sensors could allow head-to-tail comparisons if PQR tonically sustains forward movement in high [O\textsubscript{2}] unless overruled by increased phasic URX/AQR–AVA activity. We have not explored this here. Sustained activation of locomotion provides a way to escape aversive environments, particularly when directional avoidance cues are absent, whereas sustained inhibition of movement facilitates accumulation in favorable ones.
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ONLINE METHODS

Molecular cloning and worm maintenance. Molecular cloning and worm maintenance followed standard protocols. Gases were supplied by BOC and humidified before use.

Microfluidics. Soft lithography was used to create microfluidic devices45. Devices were designed in AutoCAD (Autodesk) and printed on a photomask at 128,000 dpi. To make master moulds, a silicon wafer was spin coated with a 200-µm layer of SU-8 2150 photoresist (MicroChem) at 2,000 r.p.m. for 30 s and patterned by photolithography. To create devices, 3 mm of polydimethylsiloxane (PDMS) prepolymer mixture (Sylgard 184, Dow Corning) was poured over the mould and cured for >1 d at room temperature (Supplementary Fig. 1a). Inlets and outlets were connected to gas supplies with PE50 polyethylene tubing (Intramedic).

Temporal gradient assays. 21–25 adult C. elegans were picked and allowed to settle for 1 h on 5 cm NGM plates seeded 1 d earlier with 40 µL of E. coli OP50. The microfluidic behavioral arena (Supplementary Fig. 1a) was then lowered over the worms. After 1 min a syringe pump (PHD 2000, Harvard Apparatus) started pumping gas at a flow rate of 3 ml/min. Video recording using a Grasshopper 2048M-C CCD camera (Point Grey Research) began after a further minute. Teflon valves (AutoMate Scientific) allowed rapid switching between two gas mixtures delivered from the same pump, by shunting flow either to the chamber or to an air exhaust. Valves were controlled by TTL pulses from the camera, using a custom-built frame counter that enabled switching at precise time points.

In the gyy-35 rescue experiments, transgenic worms were preselected by picking worms expressing GCY-35::GFP.

Spatial assays. Experiments were performed in a hypoxia chamber at 7% O2 to maintain low activity in the O2-sensing neurons. A drawn-out microcapillary was connected to 21% (test) or 7% (control) O2 pump at 800 µl min−1. The tip of the microcapillary was brought close to the nose or tail of individual worms and their behavioral responses scored visually. Responses were categorized as (1) reversal, (2) no change of behavior, (3) acceleration (from resting or backward movement) or (4) stalling (slowing after prior forward or backward movement). Responses were only counted if they occurred within ≤2 s of stimulation. Significance was assessed using Fisher’s exact test for each response category.

Assays with slow temporal gradients of O2. Two syringe pumps were programmed to deliver a constant gas flow into the microfluidic arena, but with varying contribution from each pump (Supplementary Fig. 1b). For a 2-min shift from 21% to 7% O2, pump A (supplying 21% O2) changed its speed from 2 ml/min to 0 ml/min over 2 min, while pump B (supplying 7% O2) was ramped up from 0 to 2 ml/min at the same time. Pump speeds were adjusted every 0.5 s. To obtain smooth piston movement we used glass syringes (Summit). [O2] in behavioral or imaging microfluidic chambers was measured using a spot optode (PreSens).

Behavioral analysis. Speed, reversals and omega turns were quantified as described previously46. We excluded objects representing anything other than single animals from analysis by monitoring the size of tracked objects and sudden increases in their perimeter (indicating two worms touching).

Reversals and omega turns were identified from changes in the path direction of each tracked object’s centroid, with characteristic shape properties used to distinguish the two. Parameters for reversal detection were a minimum turn angle of 150° and a minimum reversal distance of 0.3 mm. The analysis, data from multiple replicates of each assay were pooled. Statistics are based on the total number of valid observations in all video frames in the corresponding time period. Proportions are an instantaneous measure of a behavioral state in the population. Behavioral results are shown in 8-s bins for speed or 10-s bins for reversal and omega turns.

Optogenetic control of neural activity. ChR2 (ref. 47) codon-optimized for C. elegans and C-terminally tagged with mCherry, or halorhodospin48 tagged with mCherry, were expressed from the gcy-32 promoter. Worms were grown on plates supplemented with 30 µl of 5 mM all-trans retinal (Sigma) dissolved in ethanol. Control worms were grown on plates supplemented with 30 µl of ethanol. Worms were preselected for fluorescence in one or more of the AQR, PQR, URX neurons. For each assay, 10–13 adult hermaphrodites were transferred to low peptone NGM plates seeded 2 d earlier with 30 µl of E. coli OP50, and left for 1 h, 10 min before the recording, the plate was transferred to a Perspex chamber and kept at 7, 11 or 21°C. Films were recorded on a Leica M165FC dissecting microscope with a Grasshopper camera at 2 frames s−1. Worms were exposed to 18 mW/cm2 green light (halorhodopsin) or 13 mW/cm2 blue light (channelrhodopsin) for 3 min. Worms were also observed 3 min before and after stimulation using dim white light. Speed was determined as described above and statistically significant differences in mean speeds assessed (excluding the first 10 s after stimulus change) using one-way ANOVA with Bonferroni’s multiple comparison test.

In the mosaic experiments, we excluded worms with detectable fluorescence in non-selected neurons visible under a dissecting microscope. To test whether ChR2 expression below this detection limit could affect behavior, we stimulated worms from a line expressing ChR2 in AQR, PQR and URX stably but so weakly that it was visible under a compound microscope with a 40x objective, but not under the dissecting microscope. Blue light illumination did not significantly increase speed of these worms (data not shown).

Spatially selective light activation of ChR2 in O2-sensing neurons with a programmable array microscope. A detailed description of the programmable array microscope (PAM) prototype used for selective illumination of head or tail regions can be found in ref. 48. The PAM is an epifluorescence microscope that includes in the primary image plane an array of small liquid crystal apertures that can be independently activated at high speed. This array serves as the spatial light modulator (SLM) and consists of a 1,280 × 1,024 pixel liquid crystal on silicon (LCOS) chip. By dynamically changing the pattern of apertures on the SLM, any illumination scheme can be implemented. Both excitation and emission light paths pass through the SLM. Blue excitation light (473 nm) was generated with a Nd:YAG laser (intensity ~0.5 mW/cm2 at the specimen plane). The transmission image was not synchronized with and was unaltered by the state of the LCOS, which has a much higher refresh rate than the frame rate of the CCD camera used for acquiring images (1.44 kHz versus 30 fps). Therefore, worm behavior could be observed using transillumination while using the PAM’s selective illumination capabilities. Pixels at the border between ‘on’ and ‘off’ regions on the SLM have slightly different intensity. By adjusting illumination intensity to emphasize these border regions, it was possible to accurately locate the excitation spot in the final camera images.

Transgenic worms expressing ChR2-EYFP in URX and/or PQR neurons were picked onto seeded NGM plates >30 min before the experiment. The worms were placed under a custom-built microfluidic arena connected to a syringe pump delivering 7% O2 at 1.6 ml min−1 starting 3 min before light stimulation. The stimulation could only be performed with still animals. Worms exhibiting frequent spontaneous reversals or moving at high speed were excluded. Individual worms were stimulated up to 10 times with a 1-min delay in between; responses generally did not decrease across repetitions. Responses were classified and statistically assessed in the same way as for the spatial stimulation with O2 puffs.

Calcium imaging. Ca2+ imaging of immobilized worms was done as described previously49. Recordings were carried out at 2 frames s−1 on an inverted compound microscope (Axiovert, Zeiss), using a 40x C-Achromat lens, a Cascade II 512 EMCCD camera and MetaMorph software (Molecular Devices). Worms were glued to agarose pads (2% in M9 buffer) using Nexaband S/C or Dermabond tissue adhesive (Closure Medical). Glued worms were placed under the microfluidic device and remained alive in the chamber for >30 min. Photobleaching was limited using a 2.0 neutral density filter. Exposure time was 100 ms except for AVE (250 ms). The ratio of the background-subtracted fluorescence in the CFP and YFP channels was calculated with Jmalyze48. Mean fluorescence ratio (YPF/CFP) plots and heat maps of individual responses were made in Matlab (MathWorks). For statistical comparisons, all data points from individual traces during selected periods of the recording were collated. Statistical tests were made with Prism (GraphPad). Generally, worms that showed no responses to O2 stimuli were excluded from the mean ratio traces, except where the proportion of responding worms in the compared genotypes differed: then, all traces were included in the calculation. Transgenes used for imaging include: ppyg-32 :: YC3.60 (AQR, PQR, URX); pfp-8 :: YC3.60 (URX, AUA); pnmr-1 :: YC3.60 (AVA); pnr-1 :: YC3.60 (RMG) (Supplementary Strain List).
Calcium imaging in freely moving worms. Worms were imaged on 5-cm plates filled with low-peptone NGM. To reduce surface inhomogeneities, plates were incubated at 60°C for 1 h 16–20 h before imaging, dried at room temperature for another hour, then seeded with 5 μL of *E. coli* OP50. Imaging was performed using a Zeiss Axio Observer D1 inverted epifluorescence microscope with a ProScan II motorized stage system (Prior). A diffuser in the aperture stop helped homogenize and reduce the intensity of the excitation light, which was then filtered for CFP excitation and reflected by a dichroic mirror onto the worms. Light was collected through the same 10×. 0.3 NA Zeiss Achromat objective used to deliver excitation light. Emitted light was passed through an Optosplit II beam splitter (Cairn Research) to selectively collect YFP and CFP emission wavelengths. The two channels were recorded side-by-side on a Cascade II 1024 EMCCD camera (Photometrics). Worms were kept in the field of view by moving the stage manually using a joystick, with the acceleration rate set to its lowest value to disturb the worm as little as possible. To reconstruct the speed and trajectory of the worm, stage position was continuously queried and logged using a Prior Terminal macro.

To deliver gas stimuli, we placed a 500-μm-deep rectangular PDMS chamber over the worm, with inlets connected to a PHD 2000 syringe pump (Harvard Apparatus) at a flow rate of 120 μL s⁻¹. Valves placed between the syringes and the PDMS chamber allowed rapid switching between different gas mixtures at prespecified frames of the recording. We analyzed the resulting movies using a custom-written Matlab program. By selecting worms where the neuron of interest strongly expressed the fluorescent sensor, we could track the centroid of the neuron using an algorithm identifying the brightest pixel in both YFP and CFP channels independently. We then calculated the mean of the 20 brightest pixels within a 7-pixel radius of the centroid for both channels independently, and subtracted from this the median pixel intensity of the respective channel as the background. We obtained the YFP/CFP ratio from these intensity values and subtracted a crosstalk correction value of 0.6.

Cell-specific RNA interference. Nucleotides 565–1302 of gcy-32 cDNA and its reverse sequence were separately cloned into pExpression vectors downstream of the gcy-32 promoter (0.6kb). *unc-119* worms were co-injected with an *unc-119* rescue construct and either ccGFP (gcy-32 sense) or ccRFP (gcy-32 antisense) injection marker. Lines established from these injections were crossed with *npr-1(ad609)* worms and then each other to create *npr-1(ad609) ccGFP ccRFP gcy-32::egl-21(sense) gcy-32::egl-21(antisense)* strains. Several such lines obtained from different injection events showed a reduction in aggregation. To rescue the *egl-21* knockdown, an RNAi-resistant and codon-optimized50 gcy-32 cDNA construct under control of the gcy-32 promoter was co-injected with *ehl-2::GFP* into the knockdown strain. RNAi of *itr-1* employed the same procedure; the *pgcy-32::itr-1* sense and antisense transgenes spanned IV:768922–7690120 of the *itr-1* sequence.

**Coelomocyte assay.** For coelomocyte imaging, we grew an integrated *pgcy-32::ins-1::EYFP* transgenic line on food at 21% O₂ until adulthood. Worms were separated and incubated for different lengths of time at 7%, 21% and 25% O₂. The posterior coelomocyte was imaged in young adults oriented laterally. Coelomocyte average fluorescence was determined using MetaMorph software. For colocalization studies, the maximum intensity fluorescence along the PQR ventral neurite was evaluated using the linescan function.

**Cell ablations.** Laser ablations were conducted following standard procedure51. RMG/AUA were identified by GFP fluorescence and ablated in L1 or L2 stage. Loss of the ablated cells was confirmed by observing loss of fluorescence in the adult.

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