C. elegans Notch Signaling Regulates Adult Chemosensory Response and Larval Molting Quiescence

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Article

C. elegans Notch Signaling Regulates Adult Chemosensory Response and Larval Molting Quiescence

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Summary

Background: The conserved DOS-motif proteins OSM-7 and OSM-11 function as coligands with canonical DSL (*D*elta, *S*errate, and *L*AG-2) ligands to activate *C. elegans* Notch receptors during development. We report here that Notch ligands, coligands, and the receptors LIN-12 and GLP-1 regulate two *C. elegans* behaviors: chemosensory avoidance of octanol and quiescence during molting lethargus.
Results: *C. elegans* lacking *osm-7* or *osm-11* are defective in their response to octanol. We find that OSM-11 is secreted from hypodermal seam cells into the pseudocoelomic body cavity and acts non-cell autonomously as a diffusible factor. OSM-11 acts with the DSL ligand LAG-2 to activate LIN-12 and GLP-1 Notch receptors in the neurons of adult animals, thereby regulating octanol avoidance response. In adult animals, overexpression of *osm-11* and consequent Notch receptor activation induces anachronistic sleep-like quiescence. Perturbation of Notch signaling alters basal activity in adults as well as arousal thresholds and quiescence during molting lethargus. Genetic epistasis studies reveal that Notch signaling regulates quiescence via previously identified circuits and genetic pathways including the *egl-4* cGMP-dependent kinase.
Conclusions: Our findings indicate that the conserved Notch pathway modulates behavior in adult *C. elegans* in response to environmental stress. Additionally, Notch signaling regulates sleep-like quiescence in *C. elegans*, suggesting that Notch may regulate sleep in other species.

Introduction

Notch signaling is a highly conserved signaling pathway with well-characterized roles in development and cell fate specification. In the canonical Notch pathway, transmembrane Notch receptors are activated by binding to transmembrane ligands containing a highly conserved DSL (*D*elta, *S*errate, and *L*AG-2) domain. Activated Notch receptors are cleaved by the gamma secretase/presenilin complex and the intracellular (IC) domain of the receptor translocates to the nucleus. Notch IC then acts in conjunction with a transcription factor called Suppressor of Hairless in *Drosophila* or LAG-1 in *C. elegans*, and transcription of target genes is activated [1]. The two *C. elegans* Notch receptors, LIN-12 and GLP-1, play well-characterized roles during *C. elegans* development. For example, LIN-12 plays critical roles at multiple steps of vulval cell fate specification and differentiation [2], whereas GLP-1 signaling negatively regulates mitotic exit and meiotic entry in the germline [3].

The *C. elegans* proteins encoded by *osm-7*, *osm-11*, *dos-1*, *dos-2*, and *dos-3* are predicted to encode secreted or transmembrane proteins containing an EGF repeat conforming to the DOS-motif consensus (*D*elta and *OS*M-11) [4]. DOS motifs are also found in canonical DSL ligands of other metazoans [4]. In *C. elegans*, OSM-11 acts with DSL ligands in vulval cell fate specification to help activate LIN-12 Notch [4]. Because *C. elegans* DSL-domain ligands lack the DOS motif, they may function as bipartite ligands with DOS-motif proteins to activate *C. elegans* Notch receptors. DOS-motif proteins also play poorly understood nondevelopmental roles. Loss of OSM-11 or OSM-7 causes defects in osmotic avoidance behavior, decreases defecation rates, increases internal osmolyte levels, increases sensitivity to anoxia [5], and alters expression of specific innate immunity proteins [6–8].

Several lines of evidence suggest that Notch signaling plays a nondevelopmental role in adult nervous systems across species. Notch receptors are expressed in the adult nervous systems of mammals [9], *Drosophila* [10], and *C. elegans* [11, 12] (this study). Altering Notch signaling changes neuronal activity [13, 14], and this affects various processes including spatial learning, memory, long-term potentiation, and neuromuscular junction synaptic plasticity [10, 15–18]. In *C. elegans*, LIN-12 Notch receptor function is required in the RIG interneurons of adult *C. elegans* to regulate the rate at which animals spontaneously initiate backward locomotion (i.e., reversals) during forward locomotion [11].

Here we describe roles for the Notch pathway in two *C. elegans* behaviors. First, Notch ligands function in adult animals to activate GLP-1 and LIN-12 Notch receptors expressed in neurons to modulate chemosensory avoidance of the odorant 1-octanol. Second, Notch ligands and receptors regulate quiescence during *C. elegans* molting lethargus, a sleep-like behavior. Decreased Notch signaling reduces arousal thresholds during the L4-to-adult molt, and ectopic expression of OSM-11 in adult animals is sufficient to induce anachronistic quiescence, which is dependent on the EGL-4 cGMP-dependent kinase (PKG) and on the LIN-3/LET-23 EGF pathway. Combined, these and other results presented

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herein reveal hitherto unsuspected roles for Notch signaling regulating *C. elegans* behavior.

Results

The Notch Coligand OSM-11 and Related DOS-Motif Proteins Are Required for Normal Octanol Response

Wild-type *C. elegans* avoid the noxious chemical odorant 1-octanol by rapidly initiating backward locomotion [19, 20]. We found that *osm-11(rt142)* complete loss-of-function (null) animals were defective in octanol response (Figure 1A). Another DOS gene was required for octanol response; *osm-7(tm2256)* null animals were as defective in octanol response as animals lacking *osm-11*. By contrast, *dos-1(ok2398)* null and *dos-2(tm4515)* loss-of-function animals were only modestly defective (Figure 1A). We confirmed these results by RNA interference (RNAi) knockdown (see Figure S1A available online). Octanol response defects were not further exacerbated in *osm-7;osm-11* double mutants (data not shown). Combined, these results suggest that *osm-11* and *osm-7* Notch coligand genes play essential and nonredundant roles in chemosensory avoidance of octanol; we focused here on the role of *osm-11*.

We found that *osm-11* acts in adult animals to modulate behavior. No morphological defects were observed in the octanol-sensing chemosensory amphid neurons in *osm-11(null)* animals (data not shown), consistent with a nondevelopmental role. To confirm that *osm-11* function in adult animals was sufficient to regulate octanol response, we manipulated *osm-11* levels in adult animals. First, wild-type animals were reared on *E. coli* expressing double-stranded RNA corresponding to *osm-11* [*osm-11(RNAi)*]; these animals were severely defective in octanol response. However, when moved as adults to standard, non-RNAi *E. coli*, octanol response was fully restored ("Recovered from *RNAi*," Figure 1B). In a reciprocal experiment, wild-type animals were transferred to plates containing *E. coli* expressing *osm-11(RNAi)* as L4 larvae; as adults, these animals were defective in octanol response (Figure 1B). Finally, an inducible heat-shock promoter was utilized to express *osm-11* cDNA (*hsp::osm-11*) after cell fate specification was complete. Induction of *osm-11* expression fully restored normal octanol response within hours (Figure 1B). Combined, these results indicate that the neural circuitry required for response to octanol is intact in animals lacking *osm-11* and that *osm-11* function is required only in adult animals for response to octanol.

Previous work has shown that *osm-11* animals have increased internal glycerol levels reminiscent of animals adapted to high external osmolarity [7]. To determine whether *osm-11* regulation of octanol response is dependent on glycerol accumulation, we examined the octanol response in *osm-11* animals lacking the glycerol biosynthesis genes *gpdh-1* and *gpdh-2*. The loss of both *gpdh-1* and *gpdh-2* did not suppress the octanol response defect of *osm-11* animals (Figure S1B), suggesting that this behavioral defect may be independent of glycerol accumulation.

osm-11 Acts Non-Cell Autonomously to Regulate Octanol Avoidance in Adults

osm-11 is expressed in the hypodermal seam cells and spermathecae of adult animals but is not expressed in neurons [4]. The seam cells lie along the lateral sides of the body contacting both the cuticle and the body cavity (pseudocoelom); they help secrete cuticle at each molt, but their roles in adult

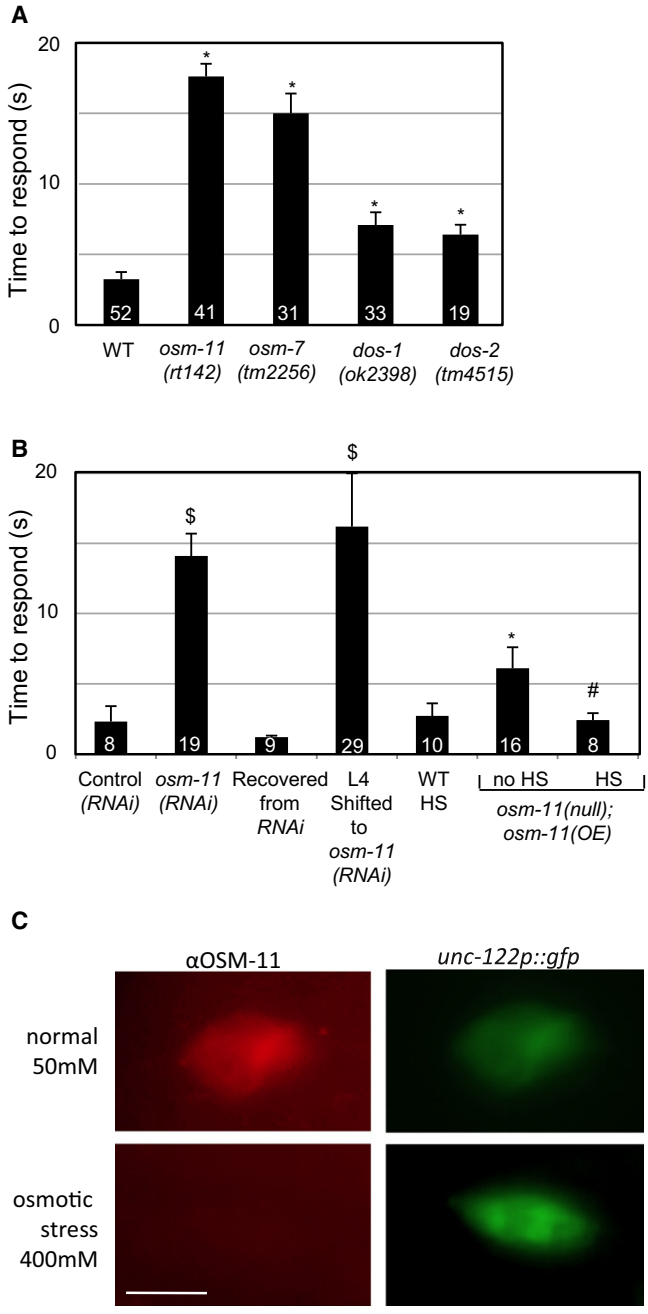

Figure 1. *C. elegans* DOS-Motif Proteins Are Required for Response to Octanol

(A) Octanol responses of animals carrying the likely null alleles *osm-11(rt142)*, *osm-7(tm2256)*, *dos-1(ok2398)*, and partial loss-of-function allele *dos-2(tm4515)*. *p < 0.0005 versus wild-type (WT).

(B) *osm-11* function is not required during development for octanol response in adults. $p < 10^{-4} versus control *(RNAi)*; *p > 0.05 versus WT; #p > 0.05 versus *osm-11(null);osm-11(OE)* no heat shock (HS).

(C) OSM-11 protein accumulates in coelomocytes. *osm-11p::gfp* reporter constructs do not drive GFP expression in coelomocytes [4]. Top left: representative image of OSM-11 accumulation in a coelomocyte using αOSM-11 antiserum [4]. Bottom left: osmotic stress likely results in diminished OSM-11 secretion by seam cells into pseudocoelom. Top and bottom right: GFP expression (*unc-122p::gfp*) in coelomocytes [53]. Scale bar represents 6 μm.

Time to respond to octanol in (A) and (B) is reported as mean time to initiate backward locomotion (s) ± standard error of the mean (SEM). See Supplemental Experimental Procedures for details.

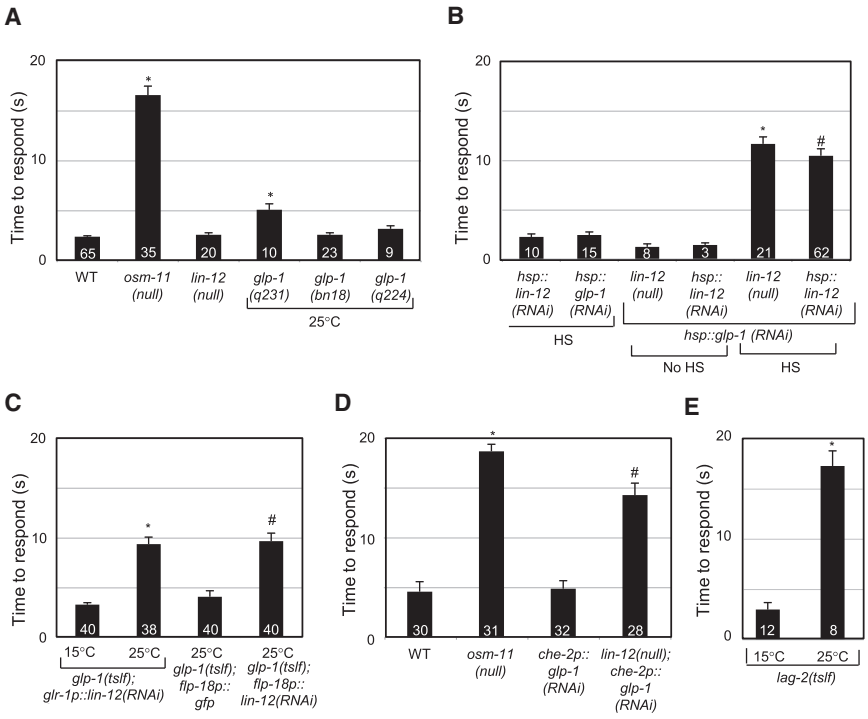

Figure 2. *C. elegans* Notch Receptors and DSL Ligand Are Required for Normal Octanol Response

(A) Decreasing function of either of the Notch receptor genes *lin-12* or *glp-1* alone does not dramatically impair octanol response. *p < 10^{-11} versus wild-type (WT).

(B) Knockdown of both Notch receptors impairs octanol response in adult animals. #p < 0.05 versus *hsp::lin-12(RNAi)* HS; *p < 0.05 versus *lin-12(null)*.

(C) Notch receptors are required in neurons. *lin-12* function is required in a subset of interneurons for normal octanol response. *lin-12(RNAi)* in *glr-1*- and *flp-18*-expressing neurons impairs octanol response. *p < 10^{-11} versus animals at 15°C; #p < 10^{-4} versus *glp-1(tslf);flp-18p::gfp*.

(D) *glp-1* functions in ciliated sensory neurons to regulate octanol response. *glp-1(RNAi)* in *che-2*-expressing ciliated sensory neurons impairs octanol response in *lin-12(null)* animals. *p < 10^{-12} versus WT; #p < 10^{-8} versus *che-2p::glp-1(RNAi)*.

(E) *lag-2* function is required in adult animals for octanol response. *p < 10^{-7}.

Time to respond to octanol is reported as mean time to initiate backward locomotion (s) ± SEM. See Supplemental Experimental Procedures for details.

animals remain unexplored. Expression of *osm-11* cDNA in seam cells and hypodermal tissues [21] restored octanol response in *osm-11(null)* animals (*wrt-6p::osm-11*, Figure S1C). In tissue culture studies, OSM-11 is a secreted protein. Furthermore, in vivo OSM-11 can diffuse within the body cavity to activate Notch signaling at distant sites in developmental contexts [4]. We detected OSM-11 immunoreactivity in coelomocytes (Figure 1C), which are endocytic cells that clear secreted proteins from the pseudocoelom. Coelomocyte clearance of secreted proteins requires the function of the *cup-4* and *cup-5* genes. Loss of *cup-4* function dramatically decreases coelomocyte endocytosis, whereas loss of *cup-5* function results in aberrant accumulation of internalized proteins in enlarged endocytic vacuoles [22, 23]. Based on the OSM-11 immunoreactivity, the OSM-11 protein did not accumulate in the coelomocytes of *cup-4* animals but accumulated to excessive levels in *cup-5* animals. However, OSM-11 immunoreactivity in seam cells was unchanged (data not shown). Because the *osm-11* promoter does not drive expression in coelomocytes [4], OSM-11 is likely secreted into the pseudocoelom, diffuses, and accumulates in coelomocytes.

Prolonged exposure to high external osmolarity, loss of *osm-11*, or loss of *osm-7* results in increased levels of the osmolyte glycerol and behavioral changes [5]. We found that when *C. elegans* were reared on standard plates containing 51 mM NaCl, all coelomocytes contained OSM-11 immunoreactivity (Figure 1C), but rearing *C. elegans* on 400 mM NaCl plates decreased or eliminated OSM-11 immunoreactivity, whereas immunoreactivity in the seam cells was not altered (data not shown). This suggests that osmotic stress may diminish OSM-11 secretion by the seam cells into the body cavity. OSM-11 likely modulates octanol response, and perhaps physiological changes, in a non-cell-autonomous manner. Additional rescue experiments support this conclusion (Figure S1C). Taken together, these results suggest that OSM-11 is a secreted, diffusible protein released into the body cavity that modulates octanol response in a non-cell-autonomous manner.

Notch Receptors Are Required for Octanol Avoidance

Because OSM-11 is required for octanol response, the canonical Notch signaling pathway might play a hitherto unsuspected role in *C. elegans* chemosensory behavior. Therefore, we examined the role of the *C. elegans* Notch receptors LIN-12 and GLP-1 in octanol avoidance using animals harboring the *lin-12(n941)* null allele or any of three *glp-1* temperature-sensitive, partial loss-of-function (*tslf*) alleles. *lin-12(null)* animals responded robustly to octanol. Two strains of animals carrying *glp-1(tslf)* alleles that were shifted to the restrictive temperature as adults had normal responses, and animals carrying the third allele had only slightly impaired response (Figure 2A). *lin-12* and *glp-1* are tightly linked (<22 kb apart), and loss of both Notch receptors results in embryonic lethality [24]. Therefore, a combination of transgene-based RNAi and/or mutant alleles was used to simultaneously decrease activity of both *C. elegans* Notch receptors.

Induction of either *glp-1* or *lin-12* RNAi knockdown using a heat-shock promoter in otherwise normal adult animals [*hsp::glp-1(RNAi)* and *hsp::lin-12(RNAi)*, respectively] alone did not alter octanol response (Figure 2B), consistent with the relatively normal response of *lin-12* and *glp-1* loss-of-function animals. However, RNAi knockdown of *glp-1* in *lin-12(null)* animals dramatically impaired octanol response after heat-shock induction. Similarly, RNAi knockdown of *lin-12* in *glp-1(tslf)* animals impaired octanol response when animals were shifted to the restrictive temperature (Figure 2C; data not shown). Additionally, transgenic animals in which *lin-12* and *glp-1* could be simultaneously knocked down in adults [*hsp::glp-1(RNAi)*; *hsp::lin-12(RNAi)*] were defective in octanol

response several hours after heat-shock induction (Figure 2B). These results suggest that both LIN-12 and GLP-1 Notch receptors play a role in adult animals in modulating response to octanol but that the two receptors function redundantly.

Notch Receptors Act in Nonoverlapping Subsets of Neurons

Where might Notch receptors function to regulate octanol response? LIN-12 is required for proper development of the somatic gonad, and GLP-1 is required for germ cell proliferation, but laser ablation of the gonad did not result in octanol response defects (Figure S1D). We considered whether *C. elegans* Notch receptors might function in the nervous system to regulate behavior. *lin-12p::gfp* is expressed in RIG interneurons, and *glr-1p::lin-12(RNAi)* knockdown of *lin-12* in *glr-1*-expressing interneurons (which include RIG) is sufficient to replicate *lin-12* loss-of-function defects in reversal rates during locomotion [11]. *glr-1p::lin-12(RNAi)* in *glp-1(tslf)* animals at the permissive temperature (15°C) did not perturb octanol avoidance. However, *glr-1p::lin-12(RNAi)* significantly impaired octanol response in *glp-1(tslf)* animals raised at the restrictive temperature (25°C), consistent with *lin-12* acting in *glr-1*-expressing neurons (Figure 2C) [25, 26]. To further narrow down the site of *lin-12* action, we used the *flp-18* promoter to drive *lin-12(RNAi)* in AVA, RIM, AIY, and RIG interneurons along with M2 and M3 pharyngeal neurons. Whereas the control *flp-18p::gfp* transgene had no effect in *glp-1(tslf)* animals, the *flp-18p::lin-12(RNAi)* transgene perturbed octanol response in *glp-1(tslf)* animals raised at the restrictive temperature (25°C). Combined, these results indicate that *lin-12* is likely required for octanol response in AVA, RIM, and/or RIG interneurons, which express both *glr-1* and *flp-18* (Figure 2C).

Redundant function of the two Notch receptor genes could be explained by their expression in the same cells or by action in different parts of the response circuit. Our results suggest that the GLP-1 Notch receptor is required in a different set of neurons from LIN-12 for octanol response. We generated a *glp-1p::gfp* reporter construct that was expressed in many neurons, including the ciliated ASH, AWB, and ADL sensory neurons that detect octanol (Figure S2A). To determine the site of *glp-1* function, we knocked down *glp-1* by RNAi using the *osm-10* promoter (for ASH, ASI, PHA, and PHB), the *gpa-11* promoter (for ASH and ADL [27]), and the *che-2* promoter (for all ciliated sensory neurons including ASH, ADL, and AWB [28]). None of these promoters drive expression in AVA, RIM, or RIG interneurons where *lin-12* likely acts (see Figure S6). RNAi knockdown of *glp-1* in *osm-10*- and *gpa-11*-expressing neurons only modestly affected octanol response in *lin-12(null)* animals (Figure S2B). However, RNAi knockdown in the larger set of *che-2*-expressing neurons dramatically impaired octanol response in *lin-12(null)* animals (Figure 2D). These results suggest that *glp-1* normally acts in *che-2*-expressing ciliated neurons to modulate octanol response and that *lin-12* is primarily required in AVA, RIM, and/or RIG interneurons. The redundant function of Notch receptors is likely due to properties of the neural circuit and not due to coexpression of the two genes in the same neurons.

Notch Ligands act via Notch Receptors to Regulate Octanol Avoidance

If LIN-12 and GLP-1 Notch receptors are activated by OSM-11 to regulate octanol response, then increasing Notch signaling should ameliorate *osm-11* loss-of-function defects. We utilized two Notch gain-of-function alleles: *lin-12(n137n460)*, a cold-sensitive gain-of-function (csgf) allele, and *glp-1(ar202)*, a temperature-sensitive gain-of-function (tsgf) allele, to test this hypothesis. The gain-of-function alleles had little or no effect on octanol response in otherwise normal animals regardless of temperature (Figure S2C). Both of the receptor gain-of-function alleles partially suppressed the octanol response defect of *osm-11(null)* animals at the restrictive temperature (Figure S2C), suggesting that OSM-11 acts via the Notch receptors.

Canonical DSL-domain Notch ligands, such as LAG-2, activate Notch receptors in concert with DOS-motif proteins in developmental contexts [4]. The cellular expression pattern of a *lag-2p::gfp* reporter [29] suggested that *lag-2* is expressed in many neurons of adult *C. elegans*, including the AVA command interneurons that regulate locomotion and help initiate reversals in response to aversive stimuli (Figure S2D). We found that octanol response requires *lag-2* function. *lag-2(q420tslf)* animals were defective in their response to octanol at the restrictive temperature but responded normally when reared at the permissive temperature (Figure 2E). Taken together, these results suggest that the DSL-domain ligand LAG-2 and the DOS-motif coligand OSM-11 both contribute to activation of neuronal Notch receptors in adult animals to modulate octanol response.

Identification of Neuronal Notch Targets

Direct transcriptional targets of the Notch pathway that are expressed in neurons would be excellent candidates for downstream targets of Notch signaling in octanol avoidance pathways, but few functional targets of Notch receptors beyond helix-loop-helix transcription factors have been identified. A previous study identified a list of 163 likely Notch target genes with four or more LAG-1 consensus binding sites in upstream or intronic regulatory sequences [30]. Of these, only 22 were likely or definitely expressed in neurons based on known expression patterns or predicted protein function. Two of these genes, *egl-4* and *lst-1*, acted downstream of Notch receptors based on genetic criteria.

egl-4 encodes a cGMP-dependent kinase (PKG) that is expressed in many head neurons and has been previously implicated in several *C. elegans* behaviors [31–34]. *egl-4(n479)* loss-of-function animals respond normally to octanol (data not shown). However, the gain-of-function *egl-4(ad450)* allele partially suppressed the octanol response defect of *osm-11(null)* animals in double-mutant animals (Figure S2E). This result is consistent with *egl-4* acting, directly or indirectly, as one of several downstream targets of Notch receptor activation.

lst-1 is a direct transcriptional target of *lin-12* Notch signaling in vulval cell fate specification and encodes a protein lacking vertebrate orthologs [30]. *lst-1p::gfp* is expressed in many *C. elegans* head neurons, including the octanol-sensing AWB neurons [35]. Animals lacking *lst-1* were as defective in their octanol response as *osm-11(null)* animals (Figure S2E). Because the *lin-12(csgf)* allele suppressed the octanol response defect of *lst-1(null)* animals, we conclude that *lin-12* likely acts via additional target genes to regulate octanol response.

OSM-11 Overexpression Induces Quiescence in Adults via Notch Receptors

Adult *C. elegans* overexpressing *osm-11* [*osm-11(OE)*] via the *hsp::osm-11* transgene exhibited uncharacteristic

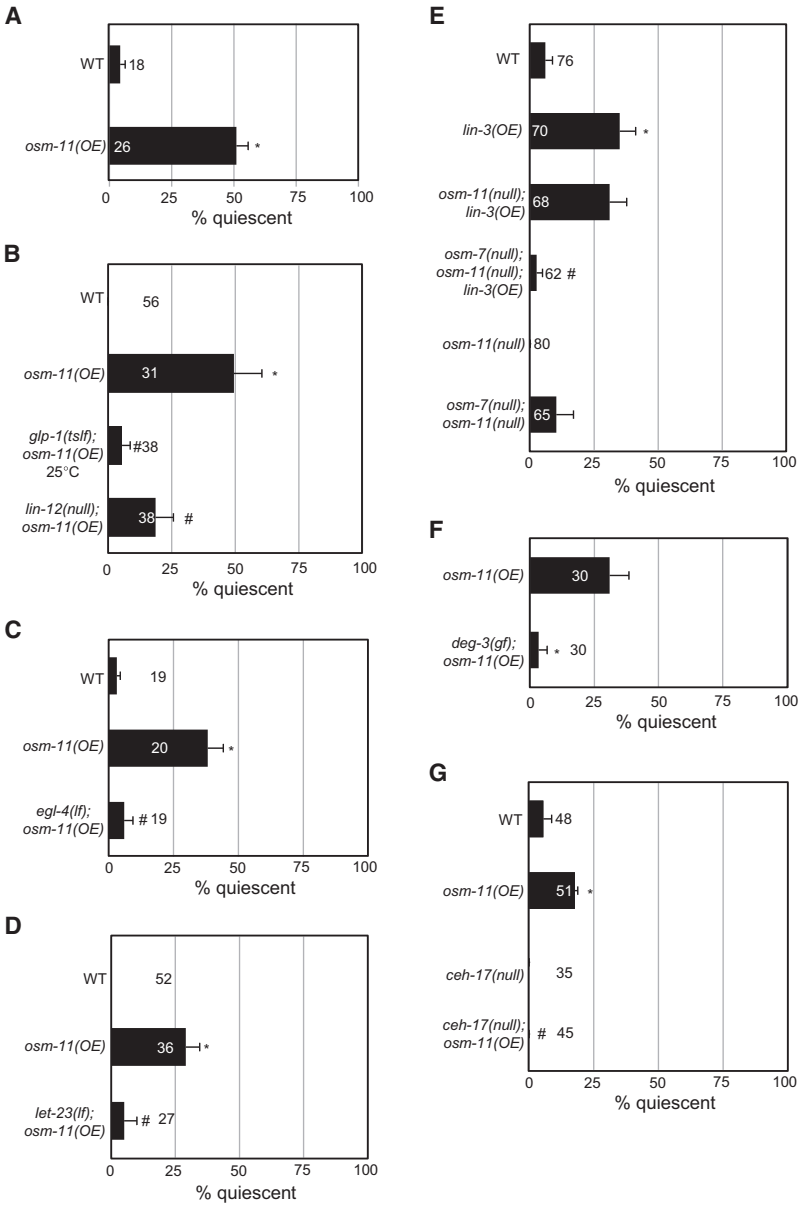

Figure 3. OSM-11 Overexpression Induces Quiescence in Adults

In all panels, *osm-11* was expressed under the heat-shock promoter (*hsp::osm-11*).

(A) OSM-11 overexpression (OE) induces spontaneous, transient, and anachronistic quiescence in adult animals. *$p < 10^{-5}$ versus wild-type (WT).

(B) *osm-11(OE)*-induced adult quiescence requires Notch receptor function. *glp-1(tslf)* or *lin-12(null)* suppresses *osm-11(OE)*-induced quiescence. *p < 0.05 versus WT; #p < 0.05 versus *osm-11(OE)*.

(C) OSM-11 overexpression-induced quiescence is suppressed by *egl-4 (lf)*. *p < 0.0001 versus WT; #p < 0.05 versus *osm-11(OE)*.

(D) Loss of LET-23 EGF receptor function [*let-23(lf)*] suppresses *osm-11(OE)*-induced quiescence. *p < 0.001 versus WT; #p < 0.05 versus *osm-11(OE)*.

(E) Simultaneous loss of *osm-7* and *osm-11* suppresses LIN-3 EGF overexpression [*lin-3(OE)*]-induced adult quiescence. *p < 0.01 versus WT; #p < 0.05 versus *lin-3(OE)*.

(F) *deg-3*-induced neurodegeneration suppresses *osm-11(OE)*-induced quiescence. *p < 0.05. Because the locomotion of *deg-3(u662)* animals is severely uncoordinated, pharyngeal pumping was used to assess quiescence.

(G) Defective axon outgrowth in *ceh-17*-expressing neurons suppresses *osm-11(OE)*-induced quiescence. *ceh-17(null)* affects processes of ALA and a subset of other neurons. *p < 0.05 versus WT; #p < 0.001 versus *osm-11(OE)*.

Quiescence behavior is reported as percent of quiescent adult animals after OSM-11 overexpression ± SEM. See Supplemental Experimental Procedures for details.

bouts of spontaneous and transient quiescence after heat-shock induction, during which locomotion and pharyngeal pumping ceased for several minutes regardless of the presence of food (Figure 3A). Control animals did not display noticeable changes in behavior after a 1 hr recovery from heat shock. Between quiescent bouts, *osm-11(OE)* animals moved with coordinated sinusoidal locomotion and had normal pharyngeal pumping rates.

The quiescence of *osm-11(OE)* animals was similar to the sleep-like quiescence observed in *C. elegans* during larval molting lethargus [36, 37] in four ways. First, the quiescent state was transient. Second, animals entered and exited the quiescent state spontaneously. Third, quiescent *osm-11(OE)* animals could be "woken" by prodding with a metal wire. This perturbation elicited short bursts of coordinated sinusoidal locomotion. Fourth, *osm-11(OE)* animals exhibited increased arousal thresholds to mechanosensory stimulation during quiescent bouts, but they responded normally between quiescent bouts. During ectopic quiescence, *osm-11(OE)* animals failed in 47% ± 9% of trials to respond to body touch. By contrast, only 10% ± 0% of heat-shocked wild-type or 8% ± 4% of non-heat-shocked *osm-11(OE)* animals failed to respond [n = 30, p < 0.01 for *osm-11(OE)* heat shock versus WT or no heat shock]. Similar results were observed using dilute octanol as a stimulus (data not shown). The diminished sensory responses during quiescence suggest that overexpression of OSM-11 might anachronistically activate the quiescence observed during *C. elegans* molting lethargus.

We did not observe inappropriate quiescence in transgenic adult animals overexpressing Notch receptors. To confirm that *osm-11* overexpression induced inappropriate quiescence by activating Notch receptors, we examined *osm-11(OE)* quiescence in animals lacking Notch receptor function. We found that either loss of the *lin-12* Notch receptor or diminished function of the *glp-1* Notch receptor suppressed *osm-11(OE)*-induced locomotion and pumping quiescence (Figure 3B). These results suggest that increasing *osm-11* levels inappropriately activates Notch signaling, resulting in behavioral quiescence.

OSM-11-Induced Quiescence Requires Previously Defined Quiescence Pathways

Does *osm-11* overexpression induce quiescence through signaling pathways previously implicated in *C. elegans* molting lethargus quiescence? *C. elegans* quiescence requires the

function of EGL-4 PKG [36, 37]. We found that loss of *egl-4* suppressed *osm-11(OE)*-induced quiescence (Figure 3C); this is consistent with OSM-11 overexpression leading to inappropriately increased *egl-4* activity with consequent quiescence in adult animals.

Endogenous *C. elegans* molting quiescence also requires LIN-3 EGF and LET-23 EGF receptor function [37]. LET-23 receptor function is required in the ALA neuron. Loss of *let-23*, the ALA neuron, or ALA processes is sufficient to decrease quiescence induced by LIN-3 EGF ligand overexpression [37]. Loss of the LET-23 EGF receptor suppressed *osm-11(OE)*-induced locomotion and pumping quiescence in adult animals (Figure 3D), suggesting that EGF receptor signaling is required for Notch-induced quiescence. We also examined the relationship between *osm-11* and *lin-3*. Overexpression of *lin-3* [*lin-3(OE)*] in transgenic adult animals using the heat-shock promoter (*hsp::lin-3*) causes anachronistic quiescence [37] reminiscent of quiescence induced in *osm-11(OE)* animals. We found that loss of *osm-11* was not sufficient to suppress *lin-3OE*-induced quiescence, but simultaneous loss of both *osm-11* and *osm-7* dramatically suppressed *lin-3OE*-induced quiescence (Figure 3E). Thus, *osm-7* and *osm-11* may play redundant roles in *lin-3(OE)*-induced anachronistic quiescence.

The role of the ALA neuron in *osm-11* overexpression quiescence was examined using two strategies. First, the ALA neuron (among others) was genetically ablated in *deg-3(u662)* animals [38]. Inappropriate *osm-11*-induced quiescence was suppressed in *deg-3(u662);osm-11(OE)* animals (Figure 3F). Second, ALA (and SIA) processes were perturbed in the *ceh-17(np1)* background [39], which also suppressed *osm-11(OE)*-induced quiescence (Figure 3G). Combined, these results suggest that Notch-induced quiescence in adult *C. elegans* requires the ALA neuron and EGF signaling that normally regulate quiescence during molting lethargus.

Notch Signaling Alters Quiescence during Molting Lethargus

To determine whether altering Notch signaling affects quiescence during L4-to-adult (L4/A) molting lethargus, we developed a simple microfluidics-based approach to assess quiescence in multiple animals simultaneously during L4/A lethargus (Figure S3). Total quiescence was determined for L4/A lethargus by loading mid-L4-stage larvae into microfluidic chambers and recording activity into adulthood (Figure 4A). Both *lin-12(null)* and *glp-1(tslf)* animals had increased total quiescence versus control animals (Figure 4B). Also, Notch receptor gain-of-function alleles resulted in increased total quiescence [*glp-1(ar202tsgf)* and *lin-12(n137n460csgf)* animals, Figure 4C]. Similar results were observed in animals overexpressing LIN-12 (*lin-12p::lin-12*, Figure S4A). Increasing GLP-1 receptor activity in ciliated sensory neurons was sufficient to increase total quiescence in wild-type animals [*che-2p::glp-1(IC)*, Figure 4D], and expression of GLP-1(IC) in ciliated neurons partially restored quiescence in *glp-1(tslf)* animals (Figure 4D).

Next, we examined the impact of Notch ligands. Decreasing *lag-2* function increased total quiescence [*lag-2(q420tslf)*, Figure 4E]. Similarly, loss of either *osm-7* or *osm-11* increased total quiescence (Figure 4E). However, simultaneous loss of function of both *osm-7* and *osm-11* resulted in decreased total quiescence (Figure 4E), which was unexpected given the increased quiescence of single-mutant animals. Combined, these results suggest that Notch receptors and ligands regulate L4/A quiescence in a complex fashion.

Notch Signaling Impacts the Duration of L4/A Lethargus

The decreased total quiescence of *osm-7(null);osm-11(null)* animals could be caused by a shorter L4/A lethargus. Therefore, the impact of Notch signaling on the duration of lethargus was determined. The L4/A lethargus was significantly shorter for *osm-7(null);osm-11(null)* animals (Table S1A). The decreased lethargus period of *osm-7(null);osm-11(null)* animals suggested that they were defective in lethargus entry or maintenance. There was no difference in the time at which *osm-7(null);osm-11(null)* animals entered lethargus (Figure S4D). However, *osm-7(null);osm-11(null)* animals exited lethargus early (Figure 4F; Figure S4E), suggesting that premature exit from L4/A lethargus causes decreased total quiescence.

To reconcile the complex effects of Notch pathway manipulation on L4/A quiescence, we delineated the impact of Notch signaling on two other components of quiescence: basal locomotion activity (hereafter referred as basal activity) and arousal threshold.

Notch Signaling Regulates Basal Activity and Arousal Thresholds

One factor that could influence quiescence maintenance is basal activity, which was assessed as body bends per minute [40]. Animals lacking both *osm-7* and *osm-11* had twice as many body bends per minute compared to control animals either at the adult stage or between L4/A quiescent bouts (Table S1B). The dramatically increased basal activity likely contributes to the decreased total quiescence of *osm-7; osm-11* animals.

During quiescence, animals have high arousal thresholds because they respond slowly or less robustly to sensory stimulation [36]. To assess arousal threshold, we used the mechanosensory body touch assay [41]. Wild-type adult animals responded to light touch in 100% of trials, but quiescent L4/A animals responded in roughly 50% of trials (Table 1), reflecting an increased arousal threshold. Decreasing Notch signaling lowered arousal thresholds during L4/A quiescence; quiescent *lin-12(null)* or *glp-1(tslf)* animals responded more frequently than control quiescent animals (Table 1). Conversely, increasing Notch receptor function increased arousal thresholds; *glp-1(tsgf)*, *lin-12(csgf)*, *che-2p::glp-1(IC)*, and *lin-12(OE)* animals had increased arousal thresholds compared to their respective controls (Table 1). Also, decreasing *lag-2* function or loss of *osm-7* and/or *osm-11* decreased arousal thresholds. Overall, Notch signaling correlated with arousal thresholds during L4/A quiescence (Table 1; Table S1C). Low arousal thresholds may reflect poor-quality quiescence. We speculate that poor-quality quiescence leads to compensatory increases in quiescence, as observed in both total L4/A quiescence and duration of lethargus (Figure 4; Table S1A). Overall, our results indicate that the Notch signaling pathway regulates basal activity, duration of lethargus, arousal thresholds, and quiescence in *C. elegans*.

Discussion

Like sleep, quiescence is a complex behavior dependent on multiple processes including arousal threshold and cessation of activity. We propose a model (Figure 5) wherein Notch signaling levels directly correlate with arousal levels during the L4/A molt. Increased arousal thresholds resulted in either inappropriate or increased quiescence. The consequences of decreased Notch activity are more complicated. Increased

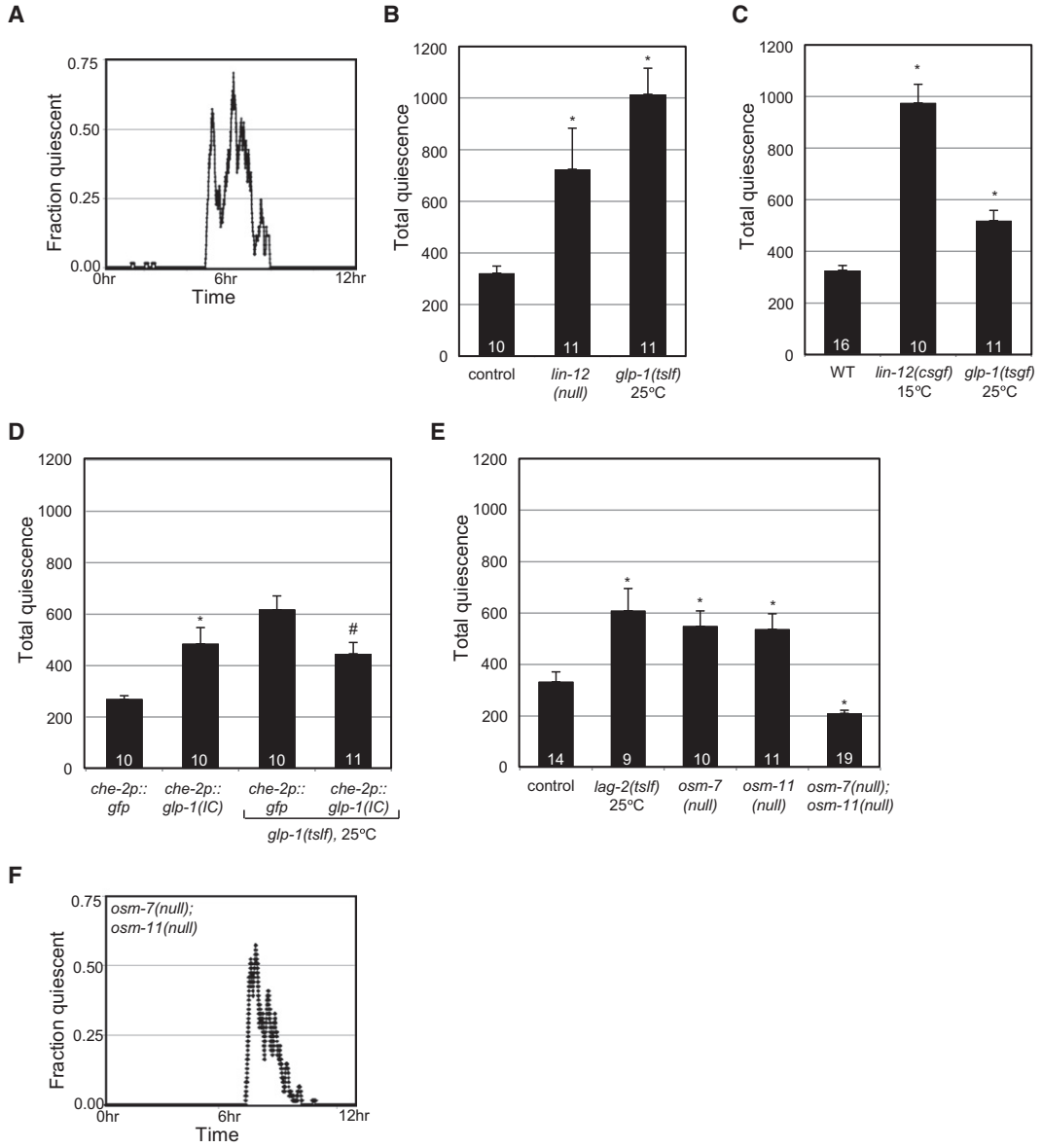

Figure 4. Notch Signaling Regulates L4-to-Adult Molting Quiescence

(A) Representative fractional quiescence graph. Animals exhibit L4/A molting lethargus quiescence for a few hours before adulthood.

(B) Loss of LIN-12 receptor or decreased GLP-1 receptor function increases total L4/A quiescence. *p < 0.02 versus control (*mgIs49*).

(C) Increased LIN-12 or GLP-1 function increases total L4/A molting quiescence. *p < 10^{-4} versus wild type (WT).

(D) Increasing GLP-1 receptor activity in ciliated sensory neurons increases total L4/A quiescence in wild-type animals [*che-2p::glp-1(IC)*]. Expression of *che-2p::glp-1(IC)* transgene restores total quiescence in *glp-1(tslf)* animals to normal levels. *p < 0.003 versus *che-2p::gfp*; #p < 0.02 versus *glp-1(tslf);che-2p::gfp*.

(E) Notch ligands and coligands regulate total L4/A quiescence. *p < 0.002 versus control (*mgIs49*).

(F) Representative fractional quiescence graph of *osm-7(null);osm-11(null)* for comparison with (A).

Total L4/A quiescence in (B)–(E) is calculated as area under the curve ± SEM. See Supplemental Experimental Procedures for details.

basal activity and/or decreased arousal thresholds resulted in homeostatic compensation in which animals increased quiescence to compensate for poor-quality quiescence. Further decreases in Notch signaling left *osm-7(null);osm-11(null)* animals unable to maintain quiescence as a result of dramatically increased basal activity and decreased arousal thresholds. The results presented here are consistent with Notch signaling playing a major, albeit complex, role in the regulation of behavioral quiescence. Notch pathway regulation of L4/A quiescence is specific, because the Notch signaling pathway genes have no impact on satiety quiescence (Y.-j. You and L. Avery, personal communication).

The presence of sleep-like states in *Drosophila* and *C. elegans* permits the dissection of conserved pertinent pathways [42]. In *Drosophila*, CREB has been implicated in sleep homeostasis [43] and a novel GPI-anchored protein (with a possible homolog in *C. elegans*) encoded by *sleepless* regulates sleep via an interaction with the Shaker K^+ channel [44]. cAMP and EGFR signaling have been implicated in sleep regulation in both *Drosophila* and *C. elegans* [36, 37, 43, 45].

Table 1. Notch Activity Regulates Arousal during L4/A Molting Quiescence

	% Failed to Respond	n
Wild-type	50 ± 2	221
osm-11(null)	29 ± 5[a]	71
osm-7(null)	25 ± 5[a]	71
osm-7(null);osm-11(null)	44 ± 4[a]	76
lag-2(tslf), 25°C	31 ± 1[b]	35
glp-1(tslf), 25°C	27 ± 3[a]	49
glp-1(tsgf), 25°C	70 ± 5[a]	31
lin-12(null)	40 ± 1[a]	51
lin-12(csgf), 15°C	65 ± 1[a]	37
lin-12p::gfp	45 ± 2	40
lin-12p::lin-12	62 ± 3[c]	43
che-2p::gfp	46 ± 2	31
che-2p::glp-1(IC)	61 ± 3[d]	35

Notch activity regulates arousal thresholds during L4/A molting quiescence. % of animals failing to respond to body touch with a hair during quiescent bouts is reported. Notch activity correlates with arousal thresholds. High Notch activity results in animals with high arousal thresholds, and vice versa.

[a] $p < 10^{-3}$ by analysis of variance.

[b] $p < 0.002$ versus wild type.

[c] $p < 0.003$ for *lin-12p::lin-12* versus *lin-12p::gfp* in *pha-1* rescue.

[d] $p < 0.008$ for *che-2p::glp-1(IC)* compared to transgenic control animals expressing *che-2p::gfp* in wild type background. See supplemental methods for Table 1 for details. *osm-11(OE)*-induced quiescence in adult animals also results in increased arousal thresholds. (See text.)

Interestingly, the mammalian OSM-11 ortholog DLK1 is expressed in the ventral tegmental area, substantia nigra pars compacta, and Raphe nuclei of adult rat and human brains [46], regions of the brain that have been implicated in sleep-wake cycle regulation [47].

Previous studies identified two signaling pathways that regulate quiescence in *C. elegans*: EGF and PKG [36, 37]. *egl-4* PKG functions downstream of EGF signaling, because loss of *egl-4* prevents inappropriate quiescence caused by *lin-3* EGF overexpression [37]. We found that loss of *egl-4* PKG function also prevents OSM-11-induced anachronistic quiescence, suggesting that *egl-4* also acts downstream of Notch signaling. However, loss of *osm-7* and *osm-11* suppressed LIN-3 EGF-induced quiescence, suggesting that there may be crosstalk and/or feedback regulation between the Notch and EGF signaling pathways.

Multiple lines of evidence suggest that OSM-11 is expressed in hypodermal seam cells [4] but is secreted and acts non-cell autonomously. OSM-11 likely acts on sensory neurons in the head, including those that coexpress GLP-1 and EGL-4 PKG, and on the RIG interneurons that express LIN-12. However, other neurons are also likely involved. The ALA neuron is required for quiescence [37]; interestingly, ALA is one of three *C. elegans* neurons with processes directly underlying seam cells. OSM-11 secreted from the seam cells may act directly on the ALA neuron. Or, because Notch receptor expression has not been observed in ALA, the ALA neuron may regulate OSM-11 secretion from seam cells. There are two other classes of neurons with processes underneath the seam cells: CAN and PVD. The function of the CAN neurons remains unclear, although they associate with the excretory canal cell that regulates *C. elegans* osmotic balance [48]. The PVD neurons are involved in mechanical touch response [49], which might occur during osmotic swelling and/or shrinking. A functional interaction of PVD and/or CAN with the seam cells would be consistent with a role for OSM-11 in osmotic stress [7].

Our results support an ethological model in which OSM-11 and Notch signaling modulate neuronal function, behavior, and adaptation to environmental stress. Adaptation to high external osmolarity or loss of DOS coligands *osm-7* or *osm-11* causes both physiological and behavioral changes in *C. elegans.* Increased external osmolarity decreases OSM-11 levels in the pseudocoelom, consistent with osmotic regulation of OSM-11 secretion from the seam cells. Either osmotic adaptation or loss of DOS coligands causes physiological adaptation to high environmental osmolarity via an unknown pathway that induces *gpdh-1* expression and consequent glycerol osmolyte synthesis [50]. Additionally, either osmotic adaptation or DOS gene loss causes behavioral changes including decreased response to high osmolarity and octanol. These behavioral changes are dependent on Notch receptor signaling in the neurons of adult animals and are not dependent on *gpdh-1* upregulation. It is tempting to speculate that these physiological and behavioral changes are adaptive and are coordinated by global regulation of Notch receptor signaling. Humoral OSM-11 may play a key role in regulating Notch signaling in various tissues to coordinate physiological and behavioral adaptation to osmotic stress. Because Notch receptor signaling has been recently been implicated in regulation of *C. elegans* heterochronic genes [51, 52], humoral OSM-11 acting on Notch receptors may also play a pivotal role in the temporal regulation of physiological and behavioral events at the larval molt, including quiescence.

Supplemental Information

Supplemental Information includes six figures, one table, Supplemental Discussion, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.04.010.

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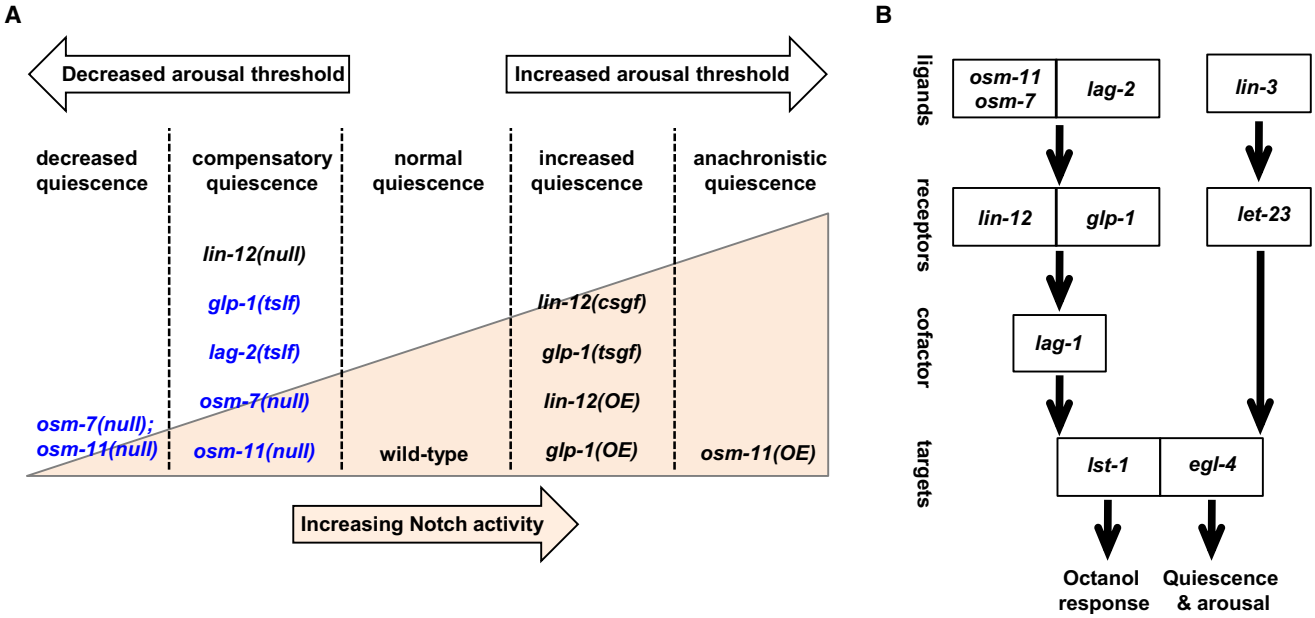

Figure 5. Model for Notch Signaling Regulating Multiple Aspects of L4-to-Adult Molting Quiescence

(A) Schematic model summarizing the impact of various Notch pathway genes. Gene name and type of allele is listed; Notch activity increases left to right (illustrated by peach wedge). For each genotype, the impact of altered Notch signaling on total L4/A quiescence is listed above the wedge; OSM-11 coligand overexpression also induced anachronistic quiescence in adult animals. Decreased Notch signaling decreased arousal threshold during L4/A molting lethargus, whereas increased Notch signaling increased arousal thresholds (white arrows). Decreased *glp-1* Notch receptor function, decreased DSL ligand function, or loss of DOS coligands increased *C. elegans* basal locomotion activity (blue text), whereas loss of *lin-12* Notch receptor decreased basal activity.

(B) Genetic pathway illustrating relationships between *C. elegans* genes regulating octanol response and quiescence. *egl-4* PKG also contributes to, but is not required for, octanol response; the role of the EGF pathway in octanol response has not been addressed. Neuronal Notch receptors act redundantly and in different populations of neurons in this behavior: *lin-12* in a subset of interneurons, and *glp-1* in ciliated sensory neurons. Notch receptors may also act elsewhere to regulate quiescence. Activation of either EGF or Notch pathways by ligand overexpression induces anachronistic quiescence, and these pathways are both required for normal L4/A molting quiescence. EGF and Notch pathways may act in parallel, converging on *egl-4* to regulate molting quiescence (illustrated), and/or there may be functional interrelationships between these signaling pathways.

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