Published in final edited form as:
Curr Biol. 2008 July 8; 18(13): 1010–1015. doi:10.1016/j.cub.2008.05.051.

Memory in *Caenorhabditis elegans* is Mediated By NMDA-Type Ionotropic Glutamate Receptors

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Summary

Learning and memory are essential processes of both vertebrate and invertebrate nervous systems that allow animals to survive and reproduce. The neurotransmitter glutamate signals via ionotropic glutamate receptors (iGluRs) that have been linked to learning and memory formation [1,2]; however, the signaling pathways that contribute to these behaviors are still not well understood. We therefore undertook a genetic and electrophysiological analysis of learning and memory in the nematode *Caenorhabditis elegans*. Here we show that two genes, *nmr-1* and *nmr-2*, are predicted to encode the subunits of an NMDA-type (NMDAR) iGluR that is necessary for memory retention in *C. elegans*. We cloned *nmr-2*, generated a deletion mutation in the gene and show that like *nmr-1* [3], *nmr-2* is required for *in vivo* NMDA-gated currents. Using an associative learning paradigm that pairs starvation with the attractant NaCl [4], we also show that the memory of a learned avoidance response is dependent on NMR-1 and NMR-2, and that expression of NMDARs in a single pair of interneurons is sufficient for normal memory. Our results provide new insights into the molecular and cellular mechanisms underlying the memory of a learned event.

Results and Discussion

Associative learning in *C. elegans*

A number of learning paradigms have been developed in *C. elegans* [5–10], including salt chemotaxis learning where wild-type worms learn to avoid normally attractive NaCl if it is first paired with starvation [4] (Fig. 1). Thus, when tested in a chemotaxis assay, the chemotaxis index (CI) (see Experimental Procedures) of conditioned worms (starved in the presence of NaCl) 10 minutes after conditioning was approximately −0.5 compared to 0.6 observed for mock-conditioned worms (starved in the absence of NaCl). This learned avoidance behavior weakened with time, with most of the worms reaching the source of NaCl two hours post conditioning (CI ~ 0.75) (Fig. 1B). Interestingly, naive worms initially showed a greater

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avoidance of NaCl (CI ~ 0.2 at 10 minutes) compared to mock-conditioned worms ($p<0.01$), suggesting that starvation in the absence of salt enhances the attraction to NaCl.

Several gene products have been implicated in the learning of salt avoidance, including HEN-1, a protein with an LDL motif that is expressed in the bilateral pair of ASE salt-sensing neurons (ASER and ASEL) [11], CASY-1, the orthologue of calsyntenins/alcadeins that is specifically required in ASER [12], as well as proteins involved in the insulin-like signaling pathway [13] and the Go (GOA-1) and Gq (EGL-30) pathway [14]. However, no genes have been described that contribute to the memory of the learned event.

AMPA receptors (AMPARs) and NMDARs have been implicated in learning and memory in many organisms [1,2]. In vertebrates, neural activity influences the cycling of AMPARs in and out of synapses. This dynamic behavior is thought to modify synaptic strength and may underlie cellular mechanisms of learning and memory, such as long-term potentiation (LTP) and long-term depression (LTD) [15,16]. Modification of AMPAR trafficking is also thought to regulate synaptic plasticity and thus learning and memory in *C. elegans* [17] and *Aplysia* [18]. In addition, NMDARs have been implicated in associative learning and memory in *Drosophila* [19,20] and *Apis mellifera* (honeybee) [21], and disrupting NMDAR function prevents LTP and leads to changes in learning and memory in mice [22–24] and *Aplysia* [25,26]. However, linking iGluRs and memory formation to specific cells and neural circuits that control behavior is limited by the tremendous complexity of most nervous systems and the relative difficulty of achieving specific genetic perturbations. To overcome these difficulties, we undertook a genetic analysis of associative learning and memory in *C. elegans*.

nmr-2 encodes a protein with greatest sequence identity to vertebrate NR2 subunits

To determine whether glutamatergic signaling is required for salt chemotaxis learning in *C. elegans*, we first tested the role of the GLR-1 [27,28] and GLR-2 [29] AMPAR subunits, and the NMR-1 NMDAR subunit [3]. In addition, we cloned and characterized a second gene, *nmr-2*, encoding a putative NMDAR subunit. The full-length *nmr-2* cDNA is predicted to encode a 990 amino acid protein and includes an additional 513 bp compared to that predicted by GENEFINDER analysis [30]. NMR-2 is predicted to have a membrane topology similar to that of other iGluR subunits and greatest sequence identity to vertebrate NR2 subunits (Fig. 2A, B). To study the contribution of NMR-2 to neuronal function, we generated a deletion mutation in *nmr-2* using standard techniques; first, screening for insertion of the Tc1 transposon in the *nmr-2* locus, then identifying a rare imprecise excision event (Fig. 2C). The *nmr-2 (ak7)* deletion removes approximately 2.5 kb of genomic sequence, including that predicted to encode transmembrane domains II and III and the S2 domain that forms part of the ligand binding pocket (Fig. 2C). Similar to *nmr-1(ak4)* mutants [3], *nmr-2(ak7)* mutants were viable and showed no gross defects in locomotion (data not shown).

nmr-1 and *nmr-2* mutants are defective in salt chemotaxis learning

To test the role of both non-NMDA and NMDA iGluRs in learning and memory, we characterized salt chemotaxis learning in *glr-1(n2461)* [27], *glr-2(ak10)* [29], *nmr-1(ak4)* [3], and *nmr-2(ak7)* mutants. All single mutants showed normal chemotaxis to NaCl in mock-conditioned assays and avoided NaCl just after conditioning (Fig. 3A–D). Interestingly, the *nmr-1* and *nmr-2* mutants were unable to fully retain the memory of the learned behavior and recovered from the avoidance state (CI = 0 at 30 min) more rapidly than either wild type animals (CI = 0 at 60 min) (Fig. 1B) or AMPAR mutants (CI = 0 at 50 min). We also examined memory retention in double mutants. Worms with either the *glr-1(n2461)* or *glr-2(ak10)* mutation in combination with either *nmr-1(ak4)* or *nmr-2(ak7)* were not different from either the *nmr-1* or *nmr-2* single mutants (data not shown). Similarly, the *nmr-1(ak4); nmr-2(ak7)* double mutant was not significantly different from either single mutant (Fig. 3E). These data suggest that

NMDARs, but not AMPA-type non-NMDARs, are required for the memory component of salt chemotaxis learning and that NMR-1 and NMR-2 may combine to form a functional heteromeric NMDAR.

nmr-1 and *nmr-2* mutants can sense food and starvation

To ensure that the memory defects observed in *nmr-1* and *nmr-2* mutants were not due to an inability to sense starvation, we tested the behavior of both well-fed and starved mutants in the basal and enhanced slowing response. Sawin et al. [31] showed that well-fed animals move more slowly in the presence of food than in the absence of food (basal slowing response). Furthermore, when starved animals encounter food the slowing response is even greater (enhanced slowing response). Both the basal and enhanced slowing response was normal in the *nmr-1(ak4)* and *nmr-2(ak7)* mutants (data not shown) indicating that these worms can normally sense starvation.

NMDA-gated currents are dependent on both *nmr-1* and *nmr-2*

To test the hypothesis that functional NMDARs in *C. elegans* require both NMR-1 and NMR-2, we measured glutamate- and NMDA-gated currents in AVA interneurons of wild-type worms and *nmr-2* mutants. In wild-type worms, glutamate elicited a rapidly activating inward or outward current that quickly desensitized when the membrane potential was held at either −60 mV or +40 mV, respectively (Fig. 4A). This rapid current component is mediated by GLR-1/GLR-2 AMPARs [29]. A smaller, more slowly desensitizing current component that is known to be dependent on NMR-1 [3] was also observed. The slower, outwardly rectifying current could be isolated using the specific agonist NMDA (Fig. 4B). Similar to that found for *nmr-1* mutants [3], glutamate elicited a rapidly activating and inactivating current in *nmr-2 (ak7)* worms (Fig. 4C); however, NMDA-gated currents were not observed (Fig. 4D). These data further support the notion that NMR-1 and NMR-2 form a heteromeric NMDAR.

NMDARs function in the RIM interneurons to facilitate memory retention

We next determined in which neurons NMDARs function to facilitate the retention of avoidance memory. *nmr-1* and *nmr-2* are co-expressed in a limited number of neurons [32], including the command interneurons AVA, AVD, AVE and PVC that form part of the neural circuit that regulates both forward and backward movement [33], the RIM interneurons, and the AVG pioneer neuron. We expressed wild-type *nmr-1* in a subset of these neurons in transgenic *nmr-1(ak4)* mutants using cell-specific promoters and tested these worms in the salt chemotaxis learning assay. The behavior was restored in transgenic *nmr-1* mutants that expressed *nmr-1* under the regulation of the *glr-1* promoter that drives expression in all cells that normally express NMDARs (Fig. 5A). However, expressing *nmr-1* in either AVA (*rig-3* promoter), AVD (*tol-1* promoter), or AVG (*odr-2* promoter) did not rescue the memory defect of *nmr-1(ak4)* worms (Fig. 5A). Interestingly, avoidance behavior in transgenic mutants that expressed *nmr-1* in the RIM interneurons using the *tdc-1* promoter was not significantly different than wild-type worms (Fig. 5A). Together, these data suggest that NMDARs expressed in the RIM interneurons play a crucial role in memory retention in the salt chemotaxis-learning paradigm.

To determine whether RIM synaptic activity is important for memory retention, we assessed salt chemotaxis learning in *tdc-1(n3420)* mutants. *tdc-1* encodes a tyrosine decarboxylase that is expressed in RIM and necessary for both tyramine and octopamine biosynthesis and neurotransmission [34]. Interestingly, *tdc-1* mutants showed the same memory retention defects as NMDAR mutants (Fig. 5B), suggesting that signaling downstream of RIM occurs via neurotransmission rather than electrical coupling through gap junctions.

NMR-1 and NMR-2 are essential for memory retention of a learned avoidance behavior

Using *C. elegans*, we have taken a genetic approach to identify the cellular and molecular requirements for an associative learning behavior. Interestingly, we showed that the NMDAR subunits NMR-1 and NMR-2, but not the GLR-1 and GLR-2 AMPAR subunits, are required for the memory of a learned avoidance response. Thus, in salt chemotaxis learning [4], *nmr-1* and *nmr-2* single mutants learned to avoid NaCl after starvation conditioning; however, their memory of this association was impaired and chemotaxis toward NaCl recovered more rapidly than in wild-type animals. This is the first evidence that NMDARs are required for memory retention in *C. elegans* and provides insight into the genes and neural circuits that regulate a fundamental process that is conserved across species.

The NMR-1 and NMR-2 subunits are co-expressed in the same subset of neurons and are predicted to form a functional heteromeric NMDA-type iGluR [32]. In support of this hypothesis, we showed that memory defects of the *nmr-1; nmr-2* double mutant were identical to both single mutants, and that like *nmr-1* [3], *nmr-2* is required for NMDA-gated currents in the AVA interneurons. NMR-1 and NMR-2 are expressed in 5 pairs of interneurons [3,32] and are required in only one of these, the RIMs, for memory retention of salt avoidance. The RIM interneurons receive input from the ASE salt-sensing neurons via the AIY interneurons. Ablating either AIY or RIM changes the behavior of worms in starved conditions. Wild-type worms transferred to a food free environment initially execute a high frequency of direction changes (reversals), which gradually diminishes over time [35]. In contrast, worms lacking either AIY or RIM maintain a high reversal frequency under starved conditions [36]. Furthermore, modifying reversal behavior has been implicated in navigation processes during taxis behaviors [37–39]. Thus, NMDARs in the RIM interneurons may maintain the association between NaCl and starvation by experience dependent modification of the reversal frequency. We also showed that *tdc-1* mutants have the same memory defects as *nmr-1* and *nmr-2* mutants, suggesting that the signaling pathway downstream of RIM involves either tyramine or octopamine neurotransmission. Interestingly, octopamine has been shown to modulate associative learning in insects [40–43] and our results suggest that similar mechanisms may exist in *C. elegans*.

NMDARs are thought to facilitate associated learning and memory by acting as coincidence detectors [44]. Thus, activation of vertebrate NMDARs requires two events to happen simultaneously: depolarization of the postsynaptic cell that relieves a voltage dependent Mg^{2+} block of the channel pore; and ligand-binding to the receptor causing channel opening. NMDA-gated currents in *C. elegans* are outwardly rectifying consistent with a voltage dependent Mg^{2+} block on the receptor [3]. Interestingly, although GLR-1 and GLR-2 are expressed in the same neurons as NMR-1 and NMR-2, the GLR-1/GLR-2 AMPARs do not appear to have a central role in salt chemotaxis learning and memory. This suggests that other non-NMDA-type iGluR subunits, e.g., GLR-4 or GLR-5 that are co-expressed with NMR-1 and NMR-2 [32], may have critical roles in these processes. Contrary to salt chemotaxis learning, GLR-1 is necessary for long-term habituation to vibration stimuli, but a role for NMDARs in this form of learning has not been described [17]. Our findings suggest that two independent signaling pathways regulate the memory of these different learning processes – habituation and associative learning. Further genetic analyses of salt chemotaxis learning, including the identification of interacting molecules acting upstream or downstream in the pathway, will help elucidate the neuronal mechanisms of learning and memory acquisition in *C. elegans* and may lead to a better understanding of these important behaviors in more complex organisms.

Experimental Procedures

General methods and strains

Animals were grown at 20 °C unless otherwise noted. All strains were derivatives of the Bristol strain N2 (wild-type). The mutants used in this study were *glr-1(n2461), glr-2(ak10), nmr-1 (ak4), nmr-2(ak7),* and *tdc-1(n3420).* Transgenic strains were generated by microinjection to achieve germ-line transformation as previously described [45]. The *nmr-2(ak7)* deletion mutation was generated by imprecise excision of the Tc1 transposon from the *nmr-2* locus. PCR was used to identify Tc1 insertion and subsequent excision. Electrophysiological recordings *in vivo* from the interneuron AVA were made as previously described [3,46]. The paired glutamate- and NMDA-gated currents for wild-type and *nmr-2(ak7)* were recorded from the same AVA neuron.

Salt chemotaxis learning assay

Details of the learning assay have been previously described [4]. The animals were washed with 10 mM MOPS buffer, placed on a conditioning plate (10 mM MOPS-NH_{40} [pH 7.2], 50 mM NaCl, 3% agar) or a mock-conditioned plate (10 mM MOPS-NH_4 [pH 7.2], 3% agar) and incubated at 20 °C for 4 hr. The animals were again collected and chemotaxis was assayed by placing them at the center of a 6 cm plate on chemotaxis agar (10 mM MOPS-NH_4 [pH 7.2], 3% agar) on which a salt gradient had been formed for 19–23 hr by placing an agar plug containing 50 mM NaCl at one end of the plate. Thereafter, the number of animals was counted every 10 min for a total of 4 hrs. The chemotaxis index was calculated as previously described [13], (A−B)/(A+B) where A was the number of animals on the NaCl side of the plate and B was the number of animals on the opposite side (Fig. 1A). To account for worms that died or were not able to move, animals that remained at the starting point were not counted. Student's *t* test or ANOVA was used to determine statistical significance. Error bars throughout represent the SEM.

Plasmids

The various promoter fusions to *nmr-1* coding sequences were constructed using the GATEWAY system (Invitrogen). To construct entry vectors carrying a promoter sequence, the promoter regions were amplified by PCR from *C. elegans* genomic DNA and then inserted into the pDONR201 vector by site specific recombination. Promoter fragments were: 5.3 kb *glr-1*; 4 kb *rig-3*; 5.5 kb *tol-1*; 5 kb *odr-2*; and 4.5 kb *tdc-1*. To generate destination vectors, *nmr-1* coding sequences were amplified from first strand cDNA and subcloned into the *Kpn*I sites of the pPDDEST vector. The oligonucleotides used to amplify *nmr-1* were 5′-CAGATATGTTCCGAATATCAGTTA-3′ (sense) and 5′-CACATAAAATCTAGTTGATCTTGCT-3′ (antisense). The cosmid T01C3 contains an open reading frame predicted to encode an NMDAR subunit (NMR-2). We identified the authentic 5′ end of *nmr-2* by PCR amplification from first strand *C. elegans* cDNA using spice leader SL1-specific oligonucleotides. Analysis of the predicted NMR-2 protein sequence was done using the ExPASy Proteomics suite of programs [47]. Sequence data for the *nmr-2* cDNA is available in GenBank accession number EU588979.

Acknowledgements

We thank the *Caenorhabditis* Genetics Center for strains, which is funded by the National Institutes of Health: National Center for Resources. We thank A. Fraser and A. Coulson for providing cosmid clones. We are appreciative of the Worm Genome Consortium for providing the *C. elegans* genome sequence and proteome for its rapid annotation. This research was made possible by support from NIH Grant NS35812 (A.V.M.), by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture and Science of Japan (R.H.), and by KAKENHI (Grant-in-Aid for Scientific Research) on Priority Areas "Systems Genomics" from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Y.I.).

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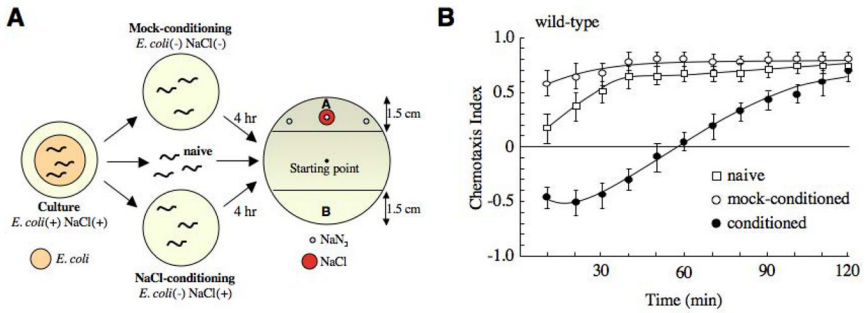

Figure 1.

Salt chemotaxis learning in wild-type worms. (A) Schematic of the salt chemotaxis learning assay. The starting point of naive, mock-conditioned and conditioned worms at the beginning of the chemotaxis assay is indicated. Sodium azide (NaN_3) was used to paralyze animals once they reached the source of NaCl. (B) Salt chemotaxis learning behavior in naive (n=5), mock-conditioned (n=5) and conditioned (n=6) wild-type worms.

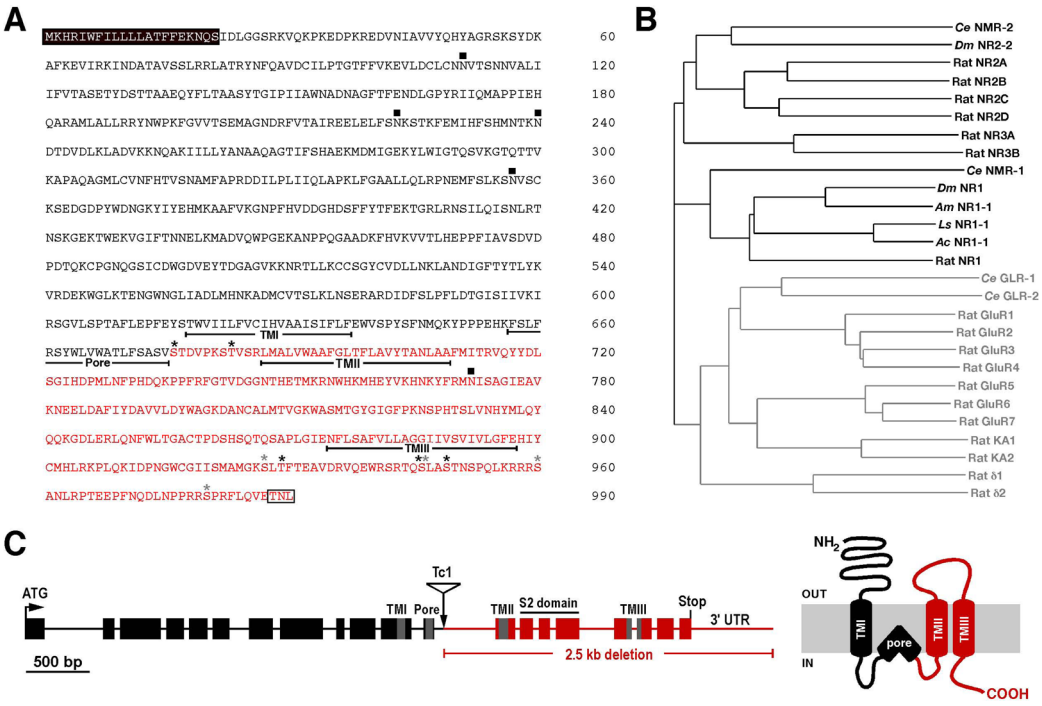

Figure 2.

nmr-2 encodes a 990 a.a. protein with greatest sequence identity to vertebrate NR2 subunits. (A) Predicted protein sequence encoded by the *nmr-2* gene. Indicated are the putative transmembrane domains (underlined); N-linked glycosylation sites (filled squares); PKA (gray asterisks) and PKC (black asterisks) phosphorylation sites; the putative signal sequence (black box); the region deleted by the *ak7* mutation (red text); and the putative PDZ-domain binding motif (white box). (B) Phylogenetic tree of *C. elegans* (*Ce*), *Rattus norvegicus* (Rat), *Drosophila melanogaster* (*Dm*), *Apis mellifera* (*Am*), *Aplysia californica* (*Ac*) and *Lymnaea stagnalis* (*Ls*) iGluRs. NMDARs are highlighted in black and non-NMDARs in gray text. (C) Genomic organization of the *nmr-2* locus with exons and introns represented as boxes and lines, respectively (left). The site of the Tc1 insert is indicated and the region deleted by its imprecise excision is shown in red. The approximate location of the sequence encoding the pore region and TMI-TMIII are highlighted in grey, and the S2 domain coding sequence is shown (black line). The predicted membrane topology of NMR-2 with the region deleted by the *ak7* mutation shown in red (right).

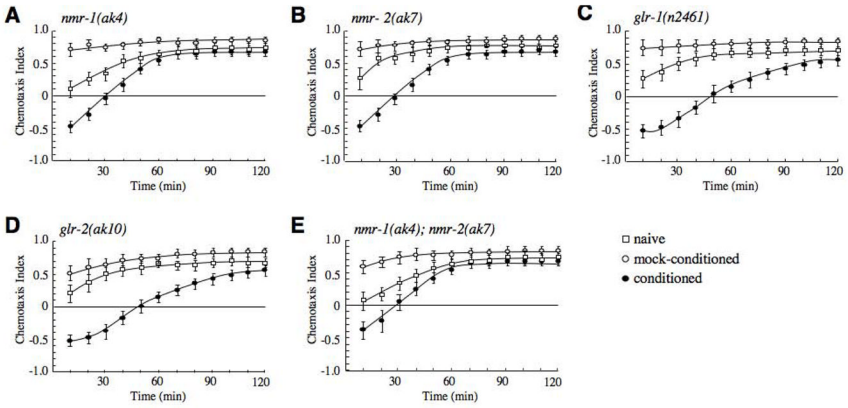

Figure 3.

Retention of avoidance memory is impaired in *nmr-1* and *nmr-2* mutants. (A–E) Chemotaxis learning in *nmr-1(ak4)* (n=6) (A), *nmr-2(ak7)* (n=6) (B), *glr-1(n2461)* (n=4) (C), *glr-2(ak10)* (n=5) (D) and *nmr-1(ak4); nmr-2(ak7)* (n=4) (E). *nmr-1* and *nmr-2* single mutants, and the *nmr-1; nmr-2* double mutant were statistically different from wild-type at 40 min ($p<0.001$).

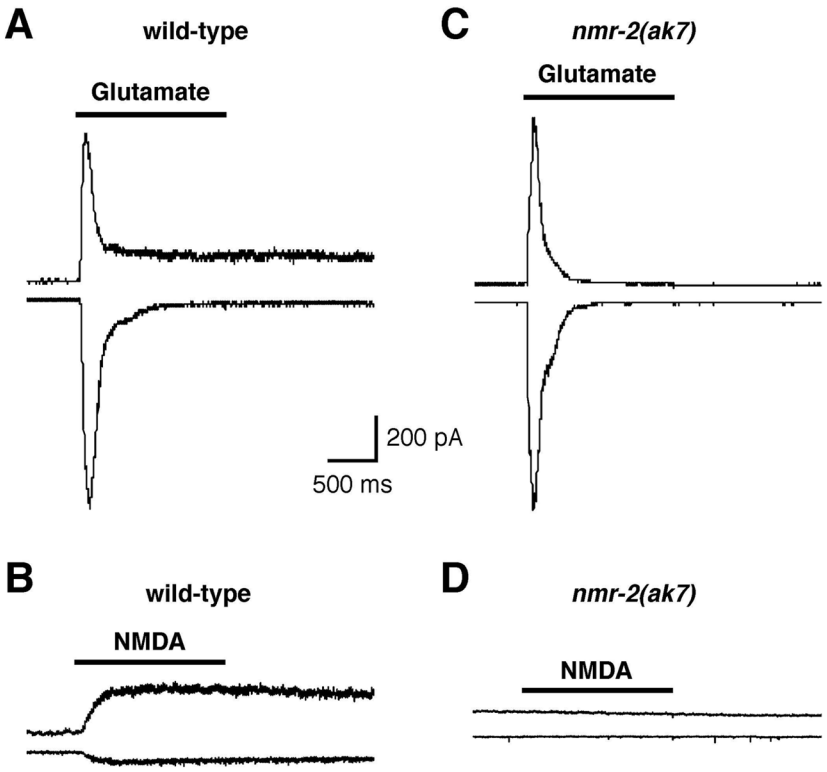

Figure 4.
NMR-2 is required for NMDA-gated currents in the AVA interneuron. Currents in response to 1 mM glutamate (A, C) or 1 mM NMDA (B, D) recorded from the AVA interneuron held at either −60 or +40 mV in either wild-type (A, B) or *nmr-2(ak7)* (C, D) worms.

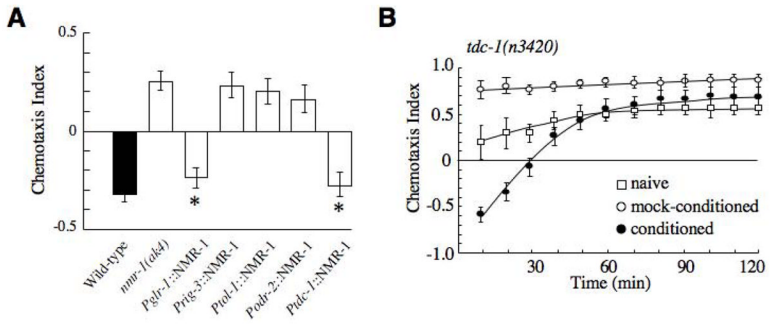

Figure 5.

NMDARs are required in the RIM interneurons to facilitate memory retention. (A) Chemotaxis index 40 minutes post conditioning in wild-type worms (black), *nmr-1(ak4)* mutants and transgenic *nmr-1* mutants (white) that expressed wild-type *nmr-1* under the regulation of various cell specific promoters (n=4–5). * Significantly different from *nmr-1(ak4)*, $p<0.001$. (B) Chemotaxis learning in *tdc-1(n3420)* mutants (n=6). *tdc-1* mutants were statistically different from wild-type at 40 min ($p<0.005$).