A role for α -adducin (ADD-1) in nematode and human memory

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Identifying molecular mechanisms that underlie learning and memory is one of the major challenges in neuroscience. Taken the advantages of the nematode *Caenorhabditis elegans*, we investigated α -adducin (*add-1*) in aversive olfactory associative learning and memory. Loss of *add-1* function selectively impaired short- and long-term memory without causing acquisition, sensory, or motor deficits. We showed that α -adducin is required for consolidation of synaptic plasticity, for sustained synaptic increase of AMPA-type glutamate receptor (GLR-1) content and altered GLR-1 turnover dynamics. ADD-1, in a splice-form- and tissue-specific manner, controlled the storage of memories presumably through actin-capping activity. In support of the *C. elegans* results, genetic variability of the human *ADD1* gene was significantly associated with episodic memory performance in healthy young subjects. Finally, human *ADD1* expression in nematodes restored loss of *C. elegans add-1* gene function. Taken together, our findings support a role for α -adducin in memory from nematodes to humans. Studying the molecular and genetic underpinnings of memory across distinct species may be helpful in the development of novel strategies to treat memory-related diseases.

The EMBO Journal (2012) 31, 1453–1466. doi:10.1038/emboj.2012.14; Published online 3 February 2012

Subject Categories: neuroscience

Keywords: actin cytoskeleton; adducin; *C. elegans*; episodic memory; glutamate

Introduction

Dynamic changes including the formation of new synapses, morphological changes of dendrites, and the redistribution of synaptic proteins during long-term potentiation (LTP), as well as regulation of long-term depression (LTD), are hypothe-

sized to underlie the remarkable plasticity of the nervous system (Matus, 2000; Okamoto *et al*, 2004). Stimulation of the neuronal network among others can trigger sustained changes in size of existing synaptic areas through the remodelling of the actin cytoskeleton (Matsuzaki *et al*, 2004). An elegant study by Honkura *et al* (2008) demonstrated the existence of at least three pools of F-actin in synaptic spines. These different actin pools tightly regulate synapse morphology. In addition, actin filaments are particularly dynamic near the post-synaptic density (PSD) (Frost *et al*, 2010), suggesting that an active process of continuous turnover of the actin network confers stability of the PSD.

Adducin in vertebrates is a ubiquitously expressed membrane cytoskeletal protein localized at the spectrin-actin junctions (Bennett *et al*, 1988; Kuhlman *et al*, 1996; Li *et al*, 1998) where it promotes assembly of the spectrin-actin cytoskeleton. Three closely related genes termed α , β , and γ encode adducins in vertebrates. These forms are differentially expressed; α and γ forms are abundant in most tissues including the nervous system, while the β form is mainly present in the erythrocytes and the brain (Citterio *et al*, 2003). All adducin forms share a similar domain structure, composed of an N-terminal aldolase domain, and a tail region that contains critical phosphorylation sites and a lysine-rich region at the extreme C-terminal end of the protein. The head and tail regions are connected by the neck domain that is critical for the function of adducins (Li *et al*, 1998). The native adducin is a mixture of heterodimers and higher oligomers comprised of α/β or α/γ subunit combinations. Oligomerized adducin caps the fast growing barbed ends of actin filaments (Kuhlman *et al*, 1996), recruits spectrin to actin filaments (Gardner and Bennett, 1987; Bennett *et al*, 1988; Hughes and Bennett, 1995), and bundles actin (Mische *et al*, 1987). In vertebrates, adducin activity is inhibited by protein kinase C (PKC) (Matsuoka *et al*, 1998), cyclic AMP-dependent protein kinase (PKA) (Matsuoka *et al*, 1996), and Ca^{2+} -calmodulin (Gardner and Bennett, 1987; Kuhlman *et al*, 1996; Porro *et al*, 2010) that blocks interactions of adducin with F-actin and spectrin, resulting in exposure of the free barbed ends (Matsuoka *et al*, 1998). In contrast, Rho-associated kinase (ROCK) enhances adducin-actin interactions (Kimura *et al*, 1998; Fukata *et al*, 1999). Adducin family proteins have been shown to regulate learning and memory. β -Adducin knockout mice show deficits in Morris water-maze test but not in LTP formation (Rabenstein *et al*, 2005). However, further experiments demonstrated that knockdown of β -adducin in mice alters LTP and LTD, causes motor and exploratory behaviour deficits, and alters the expression of α and γ forms (Porro *et al*, 2010). Therefore, the role of specific adducin forms in learning and memory remains unclear. Additionally, recent studies demonstrated the role of adducin in the degradation and assembly of new synapses in mice hippocampus, and at the *Drosophila* neuromuscular junctions (Bednarek and Caroni, 2011; Pielage *et al*, 2011).

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Received: 27 October 2011; accepted: 5 January 2012; published online: 3 February 2012

Caenorhabditis elegans reacts among others to olfactory (Colbert and Bargmann, 1995; Nuttley *et al*, 2002), gustatory (Saeki *et al*, 2001), and thermal (Mori *et al*, 2007) cues. In addition, the relatively simple nervous system of *C. elegans* composed of 302 neurons allows associative learning between a variety of volatile or soluble chemoattractants, or cultivation temperature, and food (Morrison and van der Kooy, 1997, 2001; Tomioka *et al*, 2006). Previous studies have also shown that regulators of learning and memory are conserved between mammals and *C. elegans* (Morrison and van der Kooy, 1997, 2001; Rose *et al*, 2003; Kuhara and Mori, 2006; Stetak *et al*, 2009). Therefore, the analysis of genes in *C. elegans* can provide important insights into the mechanisms of learning and memory, including humans.

Given the advantages of the nematode *C. elegans*, in the current work we investigated the role of the single worm orthologue of α -adducin *add-1*, in synaptic plasticity during aversive associative learning (defined here as the acquisition of the aversive behaviour; tested immediately following conditioning) and memory (defined here as the retention of the conditioned behaviour over time; tested after short- or long-term delay). *add-1* was previously identified in regulating germline transgene silencing (Robert *et al*, 2005). Here, we found that *add-1* loss-of-function mutant worms show normal chemotaxis, locomotor behaviour, and aversive olfactory associative learning, but they have impaired short- and long-term memory. Specifically, adducin is required *in vivo* for consolidation of changes in the PSD, and sustained increase of AMPA-type glutamate receptor (GLR-1) content in the synapses. ADD-1 also plays an important role in changes of GLR-1 turnover dynamics at the synapse. ADD-1 presumably functions through capping the fast growing barbed end of actin filaments. The role of ADD-1 in synaptic plasticity is splice-form specific, and the lysine-rich C-terminal end of the protein is essential for ADD-1 function. Finally, using tissue-specific rescue experiments, we demonstrate that α -adducin likely controls the storage of memories cell-autonomously in the AVA command interneuron by consolidating altered synaptic structures, and through the maintenance of increased amount of AMPA-type glutamate receptor at the synapses. Our results suggest that exposure to olfactory cues in combination with food withdrawal modifies the olfactory neural network. This may increase the responsiveness of the command interneuron AVA, which is an important regulator of backward movements. Thus, conditioned worms will show an increased escape behaviour.

In addition to the *C. elegans* experiments, data obtained in humans also support a role of α -adducin in memory. Genetic variability of the *ADD1* gene (encoding human adducin- α) was significantly associated with episodic memory performance. Finally, expression of human α -adducin in *C. elegans* efficiently compensated for loss of nematode *add-1* gene, suggesting that, despite the differences in the amino-acid sequences between worms and humans, the molecular function of α -adducin is conserved.

Taken together, our findings support a role for α -adducin in memory in such diverse species as nematodes and humans. Furthermore, we demonstrate that capping of actin filaments at the fast growing barbed end is likely required for long-term consolidation of synaptic plasticity. This suggests that dynamic remodelling of the actin cytoskeleton in synapses

during learning has to be followed by stabilization of actin filaments for an efficient memory storage.

Results

Loss of adducin (*add-1*) causes impairment of short- and long-term memory

To study the physiological function of worm α -adducin (ADD-1) orthologue (Supplementary Figure S1), we analysed the defects in aversive associative learning and memory using an *add-1* deletion allele (*tm3760*) (National BioResource Project, Japan). The *tm3760* deletion removes 312 bp of the *add-1* coding region that covers exon 10 and exon–intron boundary, which causes insertion of the remaining intronic sequences and gives an in frame deletion/insertion (Supplementary Figure S2B). The deletion in *tm3760* mutants alters all *add-1* splice forms and removes the conserved neck region including the dimerization sequence (Supplementary Figures S1A, B and S2A–C), which has been shown to be essential for the function of vertebrate adducins (Li *et al*, 1998). Furthermore, the deletion affects the correct processing or stability of the mRNA as we observed a reduction of *add-1* mRNA levels compared with wild-type expression (Supplementary Figure S2D). Finally, removal of one copy of the *add-1(tm3760)* using a deficiency shows no additional defects (Supplementary Figure S3), which suggests that the *tm3760* deletion is likely a loss-of-function (*lf*) mutant of the *C. elegans* α -adducin (*add-1*) gene. *add-1(tm3760)* mutants appear healthy, fertile and display no obvious morphological or locomotory defects (Figure 1B and data not shown).

To test the role of *add-1* in aversive olfactory associative learning, we first analysed the chemotaxis behaviour of *add-1(tm3760)* mutant worms towards three chemoattractants (diacetyl, benzaldehyde, and isoamyl-alcohol) and a repellent (octanol) as described previously (Bargmann *et al*, 1993). As shown in Figure 1A, *add-1(tm3760)* mutant worms exhibited efficient chemotaxis similar to wild-type animals. Furthermore, both wild-type and *add-1(tm3760)* mutants showed the same motility and responded similarly to food starvation (Sawin *et al*, 2000; Mohri *et al*, 2005), indicating that *add-1(tm3760)* mutants have no sensory or motor defects (Figure 1B).

Next, we tested the role of ADD-1 in aversive olfactory associative learning, using established context-dependent starvation conditioning protocol (Cassata *et al*, 2000; Wicks *et al*, 2000; Kuhara and Mori, 2006). In this assay, combination of a 1-h starvation period in the presence of diacetyl, or benzaldehyde (conditioned) dramatically reduced the attraction towards chemoattractants in both wild-type animals and *add-1(tm3760)* mutants to a similar extent. In the same assay, starvation alone had no effect, and adaptation (chemoattractant alone) showed only a minor decrease in chemotaxis. These results suggest that *add-1* function is not required for the acquisition process (Figure 1C and D).

We also tested short-term aversive memory (Stetak *et al*, 2009) by conditioning the animals with diacetyl or benzaldehyde, and letting the animals recover for 30 min in the absence of the chemoattractant prior to testing. Interestingly, in wild-type animals, the negative association of diacetyl or benzaldehyde in combination with starvation persisted during the 30-min delay period, but we observed a dramatic memory defect in *add-1(tm3760)* mutant worms (Figure 1C and D).

Figure 1 ADD-1 regulates short- and long-term memory. (A) Chemotaxis of wild-type or mutant worms was assayed towards 1 or 0.1% diacetyl, benzaldehyde, or isoamyl-alcohol volatile chemoattractant, and 1 or 0.1% octanol as repellent. Chemotaxis index was calculated as CI = (worms at the attractant spot – worms at the solvent spot)/total number of worms. (B) Locomotor behaviour and response to starvation of wild-type and *add-1(tm3760)* mutant worms was tested by counting body bends of well-fed (fed) or starved (starved) young adults on empty (empty) or seeded (food) NGM agar plates (C–E) Starvation, adaptation, associative learning (conditioned), and memory after a 30-min recovery period in the absence of attractant (30 min delay) of *add-1(tm3760)* and wild-type animals was tested with starvation conditioning assay using (C) diacetyl, (D) benzaldehyde, or (E) NaCl. (F) Long-term memory was tested as shown in Supplementary Figure S4A. Conditioned wild-type and *add-1(tm3760)* mutant worms were tested for their preference towards diacetyl immediately after the conditioning (conditioned), after a 16-h (16-h delay), or after a 24-h delay (24-h delay). All experiments were done in triplicate and repeated at least three times. Error bars indicate mean $\pm$ s.e.m. Significance between data sets as indicated was tested with two-tailed Student's *t*-test (NS $P > 0.05$).

We obtained similar results using NaCl as attractant, in a gustatory starvation conditioning assay (Wicks *et al*, 2000) (Figure 1E). In order to test the role of ADD-1 during long-term aversive associative memory (LTAM), we performed multiple conditioning trainings consisting of food withdrawal in combination with diacetyl (Supplementary Figure S4A). Worms were tested for chemotaxis towards DA immediately following the conditioning phase, after 16- or 24-h delay. This repetitive conditioning induced long-term memory that per-

sisted for at least 24 h (Figure 1F), and similar to previous findings (Squire and Barondes, 1970; Segal *et al*, 1971; Kauffman *et al*, 2010) required transcription and translation (Supplementary Figure S4B). While the learning was effective and comparable in both genotypes, we observed significant decrease in long-term memory in *add-1* mutant compared with wild-type worms after 16- or 24-h delay period (Figure 1F). Taken together, these results show that loss of *add-1* function impairs both short-term and long-term aver-

sive associative memory but not learning, regardless of the sensory input.

Adducin expression in neurons overlaps with AMPA-type glutamate receptor, GLR-1

To identify the cellular focus of ADD-1, we first investigated the expression pattern of a rescuing translational *add-1* reporter construct, by using the endogenous 3.1 kb promoter region fused to ADD-1 minigene with an N-terminal tRFP (Supplementary Figure S2E). ADD-1 was expressed throughout the life cycle of *C. elegans* in several tissues, including the intestine and rectal epithelia (Figure 2C), the coelomocytes (Figure 2E), the seam cells (Figure 2F), as well as the nervous system (Figure 2A, B, D, and G–L). In the head and tail ganglia, ADD-1 expression largely overlapped with the ionotropic glutamate receptor, GLR-1 (Figure 2G–I) among others in AVA, AVE, and AVD command interneurons and the PVC neuron (Figure 2J–L and P). These neurons project their axons along the ventral nerve cord (VNC) where they are interconnected through GLR-1 containing synapses. In order to test the subcellular localization of ADD-1, we simultaneously observed the localization of tRFP labelled ADD-1, and GFP-tagged GLR-1 in the VNC. ADD-1 colocalized with GLR-1 (Figure 2M–O) and ADD-1 accumulated at the synaptic areas. This subcellular distribution suggests a structural role of ADD-1 in the GLR-1 containing synapses along the VNC.

In order to further investigate the cellular requirement of ADD-1, we performed tissue-specific rescue experiments by using *add-1* minigene, which consisted of a cDNA encoding the N-terminal part of the *add-1* gene, fused to a 1-kb C-terminal genomic piece encoding all splicing forms (Supplementary Figure S2E). The *add-1* minigene was reintroduced into *add-1(tm3760)* mutant worms under the control of the *nmr-1*, the *lim-4*, the *rig-3*, the *odr-2*, or the *tdc-1* promoters (Figure 2P and Q). The activity of these promoters overlaps with certain ADD-1 expressing neurons (Figure 2P). In the short-term aversive memory test, *Pnmr-1*, and *Prig-3*-driven *add-1* minigene rescued the memory defects of *add-1(tm3760)* mutants, while no rescue was observed with *Plim-4*-, *Podr-2*-, or *Ptdc-1*-driven *add-1* minigene (Figure 2Q).

The *C. elegans add-1* locus encodes several splice forms that differ in their C-terminal end (Supplementary Figure S2E and F). The *add-1a* splice form contains an extreme C-terminal PDZ-binding motif, while the *add-1b* and *add-1c* forms include the lysine-rich region that is important for binding of vertebrate α -adducin to spectrin and actin *in vitro* (Li et al, 1998). Interestingly, similar alternative splice forms of the α -adducin mRNA are present in the vertebrate genomes that differ in their C-terminal part including the lysine-rich region. We therefore wondered which of the splice forms are required for synaptic plasticity in nematodes. We generated isoform-specific rescue constructs that contained the cDNA encoding *add-1a*, *b*, or *c* isoform fused to *add-1* promoter region, and reintroduced them into *add-1(tm3760)* mutant worms. In the olfactory memory test, both *add-1b* and *add-1c* splice forms rescued the memory defects of *add-1(tm3760)* mutants, while no rescue was observed with the *add-1a* form (Supplementary Figure S2E and R). We further confirmed these results by introducing *add-1a*, *b*, or *c* isoforms under the control of the *rig-3* promoter. Similar to the endogenous promoter, both *add-1b* and *add-1c* splice forms

rescued the memory defects of *add-1(tm3760)* mutants when expressed under the control of the *rig-3* promoter (Supplementary Figure 2S). Finally, in order to exclude possible regulatory elements in the intronic sequences of the *add-1* minigene, we fused the longest *add-1b* isoform to *nmr-1*, *rig-3*, or *tdc-1* promoters. In conjunction with the results observed for the minigene construct, *Pnmr-1*, and *Prig-3*-driven *add-1b* rescued the memory defects of *add-1(tm3760)* mutants, while no rescue was observed with *Ptdc-1*-driven *add-1b* gene (Supplementary Figure 2S).

In summary, ADD-1 expression was detected among others in AVA. Furthermore, neuron-specific rescue studies demonstrate that the function of *add-1* in memory is essential predominantly in AVA command interneuron. The AVA neuron requires specific ADD-1 splice forms that contain the lysine-rich region for efficient memory.

Sustained consolidation of synaptic plasticity depends on adducin

Previously, we demonstrated that GLR-1-positive synapses in *C. elegans* VNC change their size upon associative learning and memory consolidation (Stetak et al, 2009). Furthermore, persistent alteration in synaptic size correlates with memory retention capability. Therefore, we investigated the role of ADD-1 in synaptic structure, first by analysing the subcellular localization of ADD-1 during associative learning. Quantitative analysis of the tRFP signal intensity showed a three-fold increase in ADD-1 content upon conditioning (Figure 3B) while the number of punctae was not affected (Figure 3A). The observed change in fluorescence intensity persisted for the 30-min delay phase (Figure 3B).

Since ADD-1 function is required in AVA neurons for memory consolidation, and we found an increase in the amount of ADD-1 at the GLR-1-positive synapses in the VNC, we next asked whether remodelling of these synapses during associative learning and memory require ADD-1 function. Therefore, we investigated GLR-1 fluorescence intensities and GLR-1 punctae volume posterior to the vulva in naive, diacetyl conditioned, and memory consolidated wild-type and *add-1(tm3760)* mutant worms (Figure 3C and D). Loss of adducin had no effect on GLR-1 punctae number and morphology (data not shown). We observed an increase in the fluorescence intensity of GLR-1::GFP signal upon associative learning both in wild-type and in *add-1(tm3760)* animals but not following starvation or DA treatment alone (Figure 3C). While conditioning induced a similar increase in GLR-1::GFP intensity in both genotypes, GLR-1::GFP intensity in *add-1(tm3760)* animals reverted after 30 min to nearly the level observed in unconditioned animals, but persisted in wild-type animals (Figure 3C). We found similar results when we analysed GLR-1::GFP-positive punctae volumes (Figure 3D) where changes in *add-1(tm3760)* mutant worms reverted to the level of unconditioned animals after 30 min of delay. Thus, ADD-1 consolidates changes in GLR-1 content and punctae volume during memory formation.

Changes of GLR-1 dynamics during associative learning is regulated by adducin

Our results demonstrate that ADD-1 stabilizes changes in GLR-1 containing synaptic structures and the increase in glutamate receptor abundance along the VNC. We then asked whether adducin regulates GLR-1 diffusion and traf-

Figure 2 Expression pattern and subcellular localization of ADD-1. (A) *tRFP::add-1* (red) expression in adult worm (blue: *myo-3::gfp* coinjection marker). (B) Localization of ADD-1::tRFP in the head ganglia. (C–F) *add-1* expression was also detected in (C) the gut, (D) the CAN neurons, (E) coelomocytes, and (F) seam cells. Colocalization of (G) tRFP::ADD-1 with (H) the glutamate receptor (GLR-1) fused to GFP driven by endogenous promoter. Merged image of (G, H) is shown in (I). Arrow points to AVA command interneuron. (J) tRFP::ADD-1 expression overlaps with (K) GLR-1 in PVC neuron. Merged image of (J, K) is shown in (L). Asterisk labels the intestine. (M–O) The subcellular localization of (N) ADD-1 was observed along the VNC, where it colocalizes with (M) GLR-1-positive putative synapses. Merged image of (M, N) is shown in (O). Scale bars represent 50 μ m on (A), 10 μ m on (F) 5 μ m on (I), and 2 μ m on (O). (P) Expression pattern of the different neural promoters used in (Q, S). Overlap with ADD-1 expressing neurons are highlighted in bold. (Q) Tissue-specific rescue of the memory defect of *add-1(tm3760)* mutant worms carrying the *add-1* minigene under the control of different promoters. Young adult worms of each transgenic line were conditioned with DA and their preference towards DA was tested immediately (conditioned) or following 30 min recovery in the absence of DA (30 min delay). (R) Splice-form-specific rescue of the memory defect was tested in *add-1(tm3760)* mutant worms carrying different arrays for their preference towards DA immediately (conditioned) or following 30 min recovery in the absence of DA (30 min delay). (S) Splice-form-specific rescue of the memory defect in *add-1(tm3760)* mutant worms carrying arrays as indicated using DA immediately (conditioned) or following 30 min recovery in the absence of DA (30 min delay). All experiments were done in triplicate and repeated in three independent experiments. At least two independent transgenic lines were tested for each construct. Error bars indicate mean $\pm$ s.e.m. Data sets were compared as indicated using two-tailed Student's *t*-test (NS $P > 0.05$).

ficking at the synapses, as this could lead to an increase in receptor density during associative learning. Indeed, we observed a significant decrease of GLR-1::GFP mobility following conditioning with DA using fluorescence recovery

after photobleaching (FRAP) in the synapses along the VNC (Figure 4A and B; Supplementary Table S1). A single training with DA paired with starvation evoked a long-lasting change in GLR-1 mobility that remained for at least 4 h after the

Figure 3 ADD-1 regulates consolidation of structural plasticity of GLR-1 distribution in the ventral nerve chord. (A) Number of ADD-1 containing punctae along the ventral nerve chord, in naive, conditioned with DA, and after 30 min recovery in wild-type worms. (B) Average fluorescence intensity of trFP::ADD-1 in the VNC posterior to the vulva in naive, upon conditioning with DA (conditioned), or conditioning followed by 30 min recovery in the absence of DA (30-min delay) in wild-type adults. (C) Average fluorescence intensity of GLR-1::GFP in the posterior VNC in naive, upon starvation (starved), conditioning with DA (conditioned), upon treatment with DA in the presence of food (adapted), or after conditioning followed by 30 min recovery in the absence of DA (30 min delay) in wild-type and *add-1(tm3760)* animals. (D) Average volume of the GLR-1::GFP signal in the VNC in naive, upon starvation without (starved), conditioning with DA (conditioned), upon treatment with DA in the presence of food (adapted), or after conditioning followed by 30 min recovery in the absence of DA (30 min delay) in wild-type and *add-1(tm3760)* animals. GLR-1::GFP volumes were measured using ImageJ on confocal images (voxel size: $0.11 \times 0.11 \times 0.44 \mu$ m³). Error bars indicate mean $\pm$ s.e.m. Significance between data sets as indicated was tested with two-tailed Student's *t*-test (NS $P > 0.05$).

conditioning phase (Figure 4B; Supplementary Table S1). Finally, we could not detect any change in GLR-1 mobility after starvation, or after conditioning wild-type animals with DA in the presence of abundant food (adaptation) (Figure 4C; Supplementary Table S1). These results demonstrate that associative learning specifically causes a long-lasting decrease in GLR-1 mobility, which could lead to an activity-dependent receptor accumulation at synapses. In contrast to wild-type animals, deletion of *add-1* showed a marginal decrease in the amount of the mobile GLR-1 fraction in naive worms (suggesting a possible minor role of ADD-1 in GLR-1 receptor turnover), but we could not detect further change in GLR-1 mobility upon conditioning in *add-1(tm3760)* mutants (Figure 4D; Supplementary Table S1). These results suggest that associative learning causes a long-

lasting decrease in GLR-1 mobility, which could lead to receptor accumulation at synapses.

GLR-1 function in AVA is essential for memory formation

In our study, we found that the cellular focus of adducin is in AVA interneuron, which projects its axon along the ventral nerve chord. Furthermore, we showed that *add-1* regulates GLR-1-positive punctae structure and GLR-1 turnover along the VNC specifically during memory formation. Thus, we wondered if selective deletion of GLR-1 function in AVA could lead to memory defects. A GLR-1::GFP transgene (Rongo et al, 1998) (*nuls25*) fully rescued both the nose touch defect and the learning deficiency of the *glr-1* loss-of-function mutant (Hart et al, 1995) (Figure 5D and E). In order to get AVA-

Figure 4 GLR-1 dynamics changes in ADD-1-dependent manner upon associative learning and memory. (A) FRAP of GLR-1 was monitored in wild-type naive (upper panels) or conditioned (lower panels) worms. Scale represents the relative fluorescence intensity. (B) FRAP signal of GLR-1 in wild-type animals was quantitatively analysed in naive, in conditioned worms, or in animals after the indicated recovery phase following conditioning (naive: $n = 12$; conditioned: $n = 13$; 60 min delay: $n = 6$; 120 min delay: $n = 5$; 240 min delay: $n = 5$). (C) FRAP signal of GLR-1::GFP in wild-type animals starved for 1 h, or upon treatment with DA without starvation (adapted) (naive: $n = 12$; starved: $n = 7$; adapted: $n = 9$). (D) FRAP signal of GLR-1::GFP in naive, or conditioned *add-1(tm3760)* animals (naive: $n = 9$; conditioned: $n = 6$). Error bars indicate mean $\pm$ s.e.m.

specific knockdown of GLR-1, we introduced an interfering GFP hairpin (GFPhp) under the control of the *rig-3* promoter in *glr-1(lf); nuls25* mutant worms. We monitored the efficient and specific knockdown of the rescuing GLR-1::GFP in otherwise *glr-1*-deficient worms, by measuring the GFP signal in different neurons (Figure 5A–C). In such a genetic background, the strong reduction in the amount of GLR-1::GFP from AVA neuron resulted in a nose touch defect in agreement with previous findings (Figure 5D) (Hart *et al*, 1995). Next, we tested short-term aversive memory by conditioning the animals with diacetyl and testing chemotaxis immediately or following a 30-min delay. In conjunction with our hypothesis, we observed a specific memory defect in worms with reduction of GLR-1 function in AVA neurons (Figure 5E). Thus, memory formation requires the function of GLR-1 in AVA interneuron that is likely dependent on ADD-1 mediated stabilization of the synapses.

Stabilization of actin filaments by ADD-1 is essential for memory

Stabilization of the synaptic area may be achieved through the modification of the actin cytoskeleton that could be regulated by the barbed-end capping activity of ADD-1. Cytochalasin B (CCB) is a well-characterized fungal metabo-

lite that inhibits actin polymerization by binding to the barbed end of actin filaments in a broad variety of species from plants to vertebrates including *C. elegans* (Flanagan and Lin, 1980; MacLean-Fletcher and Pollard, 1980; Cowan and McIntosh, 1985; Goldstein, 1995) and may act analogous to actin-capping proteins. Therefore, we tested if pharmacological inhibition of actin polymerization could compensate for loss of *add-1* gene function. Application of different concentrations of CCB had no significant toxic side effect on chemotaxis (Figure 6A), or aversive olfactory associative learning (Figure 6B). Next, we applied increasing concentrations of CCB following conditioning and tested the associative memory after a 30-min delay period (Figure 6C). In accordance with our hypothesis, 10 or 20 μ M CCB fully rescued the memory defect of *add-1(tm3760)* mutant worms, while it had no effect on memory in wild-type animals (Figure 6C). We obtained similar results when we analysed the effect of CCB on GLR-1 punctae volumes along the VNC, where 10 μ M CCB had no effect on synaptic structures in wild-type animals, but restored the defect in consolidation of altered GLR-1 punctae observed in *add-1(tm3760)* mutant worms (Figure 6D). Thus, efficient memory and consolidation of synaptic plasticity likely requires the barbed-end capping activity of ADD-1.

Figure 5 The function of GLR-1 in AVA is essential for memory. (A, B) Representative expression pattern of the rescuing GLR-1::GFP protein in the head ganglia in (A) *glr-1(n2461)*, *nuls25*, or (B) in *glr-1(n2461); nuls25; Ex[prig-3::GFP]* transgenic worms. AVA neuron is highlighted with dotted line and asterisk labels the nerve ring. Scale bar represents 5 μ m. (C) Quantification of the GLR-1::GFP fluorescence intensities in different neurons with or without *rig-3* promoter driven GFP construct ($n = 10$ for each genotype). (D) Percentage of nose touch response of different mutant worms as indicated ($n = 61$ –124). (E) Chemotaxis (naive, N), associative learning (conditioned, C), and memory after a 30-min recovery period in the absence of the attractant (30 min delay, D) in wild-type and different mutants as indicated were tested using aversive olfactory conditioning assay with diacetyl. All experiments were done in triplicate and repeated in three independent experiments. Two independent transgenic lines were tested for the *prig-3::GFP* extrachromosomal array. Error bars indicate mean $\pm$ s.e.m. Significance between data sets as indicated was tested with two-tailed Student's *t*-test (NS $P > 0.05$).

Behavioural genetic studies support a role for α -adducin in human memory

In order to demonstrate a general evolutionary role of adducin in memory, we used a behavioural genetics approach to investigate the impact of genetic variability in the human α -adducin homologue on human hippocampus-dependent episodic memory. Human α -adducin (adducin 1, MIM:102680) is encoded by *ADD1*, which is located on chromosome 4p16.3 and spans an 86-kb large genomic region. To capture *ADD1*-related genetic variability, we selected 21 tagging SNPs in Hardy–Weinberg equilibrium (HWE; $P_{HWE} \geq 5\%$) with minor allele frequency $\geq 5\%$ that located within or very close to *ADD1* (Figure 7A–C). Tagging SNPs were selected using Haploview (<http://www.broad.mit.edu/mpg/haploview>) to cover the genomic region harbouring *ADD1* at $r^2 \geq 0.85$, while the mean pairwise r^2 was 0.95. Genotype–phenotype correlations were run under the additive and dominant genetic models. SNP rs10026792 was significantly associated with episodic memory performance as quantified by a picture-based, delayed free recall task. A allele carriers recalled significantly more pictures 10 min after presentation than

non-carriers ($P = 0.0005$) (Table I). This result remained significant also after conservative Bonferroni correction for multiple comparisons (21 SNPs, 2 genetic models, $P_{Bonf} = 0.021$) (Figure 7A). The genetic effect on memory performance was more pronounced for pictures with high emotional content, but was also observed for neutral pictures (Table I). Five additional *ADD1* SNPs in linkage disequilibrium (LD) with rs10026792 were also associated with episodic memory performance, at least at a nominal, uncorrected significance level. In addition to the 10-min delayed free recall task, participants also performed a free recall task 24 h after learning. Again, rs10026792 was significantly associated ($P = 0.0005$) with performance in this task, which reflects, among others, protein synthesis-related memory consolidation. Interestingly, we observed no association of *ADD1* SNPs with cognitive phenotypes other than hippocampus-dependent episodic memory (i.e., attention and working memory; all P -values > 0.05 ; Supplementary Table S2).

In order to further support our findings, we generated a ‘humanized’ *C. elegans add-1(tm3760)* strain carrying cDNA

Figure 6 CCB suppresses memory defects of *add-1(tm3760)* mutant worms. (A) Chemotaxis of wild-type or mutant worms was assayed towards diacetyl in the absence or presence of CCB as indicated. (B) Associative learning (conditioned) of *add-1(tm3760)* and wild-type animals was tested with starvation conditioning assay towards diacetyl in the absence or presence of CCB as indicated. (C) Associative learning (conditioned) and memory after a 30-min recovery period in the absence of attractant (30 min delay) of *add-1(tm3760)* and wild-type animals in the absence or presence of CCB. (D) Average volume of GLR-1::GFP punctae in the posterior VNC in naive, after conditioning with DA (conditioned), or conditioning followed by 30 min recovery in the absence of DA (30 min delay) in wild-type and *add-1(tm3760)* animals in the absence or presence of CCB as indicated. Bars indicate mean $\pm$ s.e.m. Significance between data sets as indicated was tested with two-tailed Student's *t*-test (NS $P > 0.05$).

of the longest splice form of the human *add1* gene under the control of worm *add-1* regulatory sequences. We tested the worms carrying the extrachromosomal array for short-term memory, and demonstrated that expression of the human *ADD1* in *add-1(tm3760)* mutants efficiently substituted the worm *ADD-1* protein function (Figure 7D), suggesting that despite the divergence in the amino-acid composition, human adducin likely plays a similar molecular role in worms and in humans.

Discussion

Here, we investigated the role of the actin network regulating protein α -adducin in aversive olfactory associative learning and memory in *C. elegans*. Phenotypic analysis of the α -adducin (*add-1*) knockout mutant worms demonstrated the selective role of the gene in short- and long-term memory but not in the learning (acquisition) process. Previous study of β -adducin knockout mice showed a deficit in fear conditioning and Morris water-maze paradigms; however, the test setup did not discriminate between, acquisition, short-term and long-term memories (Rabenstein *et al*, 2005). Our results

show that memory, the retention of the conditioned behaviour over time, likely needs *ADD-1* function in the GLR-1 expressing AVA command interneuron (Figure 2). Previously, GLR-1 was demonstrated to play a role in associative as well as non-associative olfactory learning (Morrison and van der Kooy, 2001). Our results using a cell-specific downregulation of the GLR-1 activity in AVA on the other hand, demonstrate a selective role of the ionotropic glutamate receptor GLR-1 in memory without affecting olfactory learning (Figure 5). Thus, GLR-1 is likely essential for both associative learning and memory that involves GLR-1 activity in distinct neurons. The AVA command interneuron is an important regulator of backward movement and ablation of AVA was previously found to abolish long reversals (Chalfie *et al*, 1985). Therefore, following conditioning, a sustained increase in glutamatergic synaptic sensitivity in AVA neuron could lead to increased reversals and backward movement upon repeated exposure to the chemoattractant. Thus, the increased sensitivity of AVA towards diacetyl could be the direct cause of avoidance behaviour. In good correlation with our previous findings (Stetak *et al*, 2009), and recent work of Ha *et al* (2010), the neural network of associative learning does not involve AVA

Figure 7 Human α -adducin, *ADD1* associates with memory performance. **(A)** Significances (y axis, $-\log_{10}P$) are shown for individually genotyped *ADD1* SNPs that were tested for association with episodic memory performance in 1085 healthy Swiss young adults. The red horizontal line indicates the Bonferroni-corrected significance level. **(B)** LD structure (r^2 values) of the chromosomal region harbouring *ADD1* as calculated in the sample of 1085 healthy Swiss young adults. Red squares represent areas of tight LD ($r^2 > 0.8$), bright and dark yellow squares represent areas of moderate LD (r^2 ranging between 0.6 and 0.4), blue squares represent areas of low LD ($r^2 < 0.3$). Two blocks of tight LD could be identified (2825–2850 kb and 2874–2890 kb). These two blocks were also in pairwise LD. **(C)** Visualization of known transcripts in the *ADD1* region. Chromosomal positions were retrieved from the March 2006 UCSC genome browser assembly. **(D)** Rescue of the memory defect of *add-1(tm3760)* mutant worms carrying the human *ADD1* under the control of *C. elegans add-1* promoter. Worms were conditioned with DA and their preference towards DA was tested immediately (conditioned) or following 30 min recovery in the absence of DA (30 min delay). All experiments were done in triplicate and repeated in three independent experiments. Two independent transgenic lines were tested. Error bars indicate mean $\pm$ s.e.m. Data sets were compared as indicated using two-tailed Student's *t*-test.

Table 1 Genotype-dependent memory performance^a ($n = 1086$)

Genotype (rs10026792)	Emotional pictures, mean $\pm$ s.e.m.	Neutral pictures, mean $\pm$ s.e.m.	All pictures, mean $\pm$ s.e.m.
GG ($n = 515$)	-0.11 ± 0.04	-0.04 ± 0.04	-0.10 ± 0.04
GA/AA ($n = 571$)	0.10 ± 0.04	0.09 ± 0.04	0.11 ± 0.04
	$P = 0.0004$	$P = 0.038$	$P = 0.0005$

^aNote: Values are z-transformed to allow direct comparison between phenotypes.

for the learning process (acquisition). On the other hand, the data presented here together with our previous results (Stetak *et al*, 2009), suggests that the AVA command interneuron likely plays a central role in memory, and persistent modulation of synaptic strength in AVA is responsible for the formation of memories towards negative cues.

Analysis of the *ADD-1* splice forms in *C. elegans* revealed the importance of the conserved C-terminal Lys-rich MARCKS-related domain in the formation of memory (Figure 2). In vertebrates, the MARCKS-related domain is required for all adducin functions identified up to now, and likely needed for association of adducin with spectrin and actin (Matsuoka *et al*, 2000). Interestingly, both human and

C. elegans add-1 locus encode for splice forms that differ in their C-terminal end with some forms lacking the MARCKS-related domain. These splice forms may therefore have functions unrelated to the organization of the actin cytoskeleton. The activity of vertebrate *ADD1* is regulated *in vitro* by PKC and PKA through phosphorylation of residues in the neck and in the MARCKS-related domains, furthermore, by binding of Ca^{2+} /Calmodulin to the C-terminal end of the protein (Matsuoka *et al*, 2000). PKC, PKA, as well as Ca^{2+} signalling have long been known to be critical for LTP formation and learning in several species including *Drosophila* and *C. elegans* (Drain *et al*, 1991; Nakazawa *et al*, 1995; Li *et al*, 1996; Mehren and Griffith, 2004), our results therefore sug-

gest that these kinases may exert their role in memory at least in part through regulation of ADD-1 activity.

Previously, it has been shown in vertebrates that LTP induces increase in volume of existing dendritic spines (Matsuzaki *et al*, 2004; Okamoto *et al*, 2004), a feature that is strongly correlating with AMPARs content. Such structural plasticity requires the modification of the actin cytoskeleton. In contrast to habituation, associative learning in *C. elegans* increases GLR-1 content and causes a dynamic remodelling of putative synapses without formation of new synapses (Rose *et al*, 2003; Stetak *et al*, 2009). Here, we show that olfactory associative learning regulates the average size and receptor content but not the number of GLR-1-positive synapses in ADD-1-independent manner. On the other hand, the persisting changes in GLR-1 punctae size along the VNC that correlate with memory consolidation require the presence of ADD-1. These results are in accord with our previous findings, with the *C. elegans* behavioural results presented here (Figure 1), and with the hypothetical role of adducin in vertebrates (Matsuoka *et al*, 2000). Furthermore, our findings expand recent results on the role of β -adducin in the formation and elimination of synapses in mice and in *Drosophila* (Bednarek and Caroni, 2011; Pielage *et al*, 2011). Besides the proposed role of adducins in the degradation and assembly of new synapses, ADD-1 could be an important element in existing GLR-1 containing synapses where it controls persistent changes in actin cytoskeleton and thereby could contribute to the consolidation of changes in synapse structure and composition, both of which is required for memory.

Besides the function of α -adducin in the maintenance of increased synapse volume upon associative learning, we also demonstrate that ADD-1 plays a role in AMPA-type glutamate receptor mobility. It was previously shown in vertebrates that LTP induction *in vitro* results in a stimulation-dependent decrease of the GluR1 recovery rate, suggesting that the reduction of the synaptic receptor exchange ultimately allows synaptic trapping of AMPA receptors (Tardin *et al*, 2003; Sharma *et al*, 2006). Our *in vivo* FRAP results in synapses along the VNC (Figure 4) are consistent with the vertebrate findings, as we observed a reduced recovery rate and decreased GLR-1 mobility upon learning in worms that could lead to the long-term accumulation of AMPA-type glutamate receptor GLR-1 at the synapses. A single training results in long-lasting changes on GLR-1 dynamics that is specific for aversive olfactory associative learning. In contrast, loss of α -adducin prevents the decrease of GLR-1 recovery rate and may interfere with the long-term accumulation of GLR-1 at the synapses. Upon associative learning, insertion of GLR-1 from intracellular pools into the PSD may occur largely independent of ADD-1 function, as we observed increase in the amount and volume of GLR-1 signals in add-1 mutants. However, stabilization of the actin cytoskeleton and consolidation of membrane-bound GLR-1 likely requires the function of ADD-1. This hypothesis is further supported by computational modelling, which suggests that slight local shifts in receptor density could lead to large changes in the post-synaptic response. According to these models (Earnshaw and Bressloff, 2006; Lisman and Raghavachari, 2006), the actin cytoskeleton mediated rapid and continuous changes of the synaptic architecture. Upon associative learning, a transient signal could activate effectors, such as ADD-1, that

induces long-lasting modification of the synaptic structure required for the formation of memory.

As predicted from our hypothesis, loss of ADD-1 function was fully rescued by the application of an actin filament-stabilizing drug. While CCB was not toxic, we observed a dose-dependent rescue of the memory defect at the behavioural level. Furthermore, the drug consolidated the increase of GLR-1 punctae volumes otherwise abrogated in *add-1(tm3760)* mutants (Figure 6). Altogether, our results strongly suggest that barbed-end capping of F-actin is critical for memory consolidation. In accord with this result, disruption of actin filaments in vertebrates by depolymerizing agents, such as latrunculin A, inhibits LTP (Krucker *et al*, 2000; Lang *et al*, 2004; Chen *et al*, 2007). In a similar way, latrunculin A inhibits the long-lasting synapse enlargement that takes place after LTP induction in vertebrates (Matsuzaki *et al*, 2004). Thus, integrity of the actin cytoskeleton is necessary for functional and structural plasticity. Hence, ADD-1 could be one of the evolutionary conserved molecular determinant that indirectly controls glutamate receptor signalling by consolidating rearrangement of the actin cytoskeleton through the barbed-end capping activity of ADD-1. Thereby, ADD-1 maintains the changes in glutamatergic synapse structure and composition following learning.

Finally, the behavioural genetics findings support an association of genetic variability in the human homologue of α -adducin (*ADD1*) with memory-related phenotypes. *ADD1* SNPs were significantly associated with performance in delayed free recall tasks (i.e., 10 min and 24 h after learning), suggesting that in humans, *ADD1* plays a role in hippocampus-dependent memory. Interestingly, no association was found between *ADD1* SNPs and performance in attention and working memory tasks, which predominantly engage prefrontal cortical areas and fronto-parietal cortical networks. Although it is not possible to directly relate human and nematode phenotypes at the behavioural level, our findings suggest a similar molecular function of *ADD1* in nematodes and humans.

In conclusion, this study might contribute to a better understanding of the conserved molecular mechanisms regulating structural plasticity in synapses that are crucial for memory formation. Furthermore, the development of specific inhibitors of *ADD1* function may provide a powerful clinical approach to block transient traumatic memories, and thus prevent post-traumatic stress disorders. Similarly, drugs enhancing *ADD1* function may be helpful in treating disorders associated with loss of episodic memory capacity, such as Alzheimer's disease.

Materials and methods

General methods and strains used

Common reagents were obtained from Sigma (Sigma-Aldrich, St Louis, MO) unless otherwise indicated. Standard methods were used for maintaining and manipulating *C. elegans* (Brenner, 1974). Experiments were conducted at 20°C. The *C. elegans* Bristol strain, variety N2, was used as the wild-type reference strain in all experiments. Alleles and transgenes used were *add-1(tm3760)*, *add-1(tm3760); nuls25[glr-1::gfp]*, *nuls25[glr-1::gfp]*, *glr-1(n2461)*, *glr-1(n2461); nuls25[glr-1::gfp]*, *add-1(tm3760); utrEx20[padd-1::add-1minigene, psur-5::dsred]*, *utrEx26[padd-1::trFP::add-1minigene, rol-6d]*, *add-1(tm3760); utrEx21 [plim-4::add-1minigene, psur-5::dsred]*, *add-1(tm3760); utrEx22[pnmr-1::add-1minigene, psur-5::dsred]*, *add-1(tm3760); utrEx23[podr-2::add-1minigene, psur-*

5::dsred], *add-1(tm3760)*; *utrEx29[ptdc-1::add-1minigene, psur-5::dsred], add-1(tm3760)*; *utrEx28[prig-3::add-1minigene, psur-5::dsred], add-1(tm3760)*; *utrEx30[padd-1::add-1a, psur-5::dsred], add-1(tm3760)*; *utrEx31[padd-1::add-1b, psur-5::dsred], add-1(tm3760)*; *utrEx38[padd-1::add-1c, psur-5::dsred], utrEx26[padd-1::human add-1, psur-5::dsred], add-1(tm3760)*; *utrEx45[ptdc-1::add-1b, psur-5::dsred], add-1(tm3760)*; *utrEx46[prmr-1::add-1b, psur-5::dsred], add-1(tm3760)*; *utrEx47[prig-3::add-1b, psur-5::dsred], add-1(tm3760)*; *utrEx48[padd-1::trfp::add-1minigene, pmyo-3::gfp], add-1(tm3760)*; *utrEx49[prig-3::add-1a, psur-5::dsred], add-1(tm3760)*; *utrEx50[prig-3::add-1c, psur-5::dsred], glr-1(n2461)*; *nuls25[glr-1::gfp]; utrEx51[prig-3::GFPhp, psur-5::dsred]*.

Transgenic lines were generated by injecting the indicated DNA at a concentration of 100 ng/ μ l, together with transformation marker, and 100 ng/ μ l λ phage/*HindIII* carrier DNA into both arms of the syncytial gonad as described (Mello *et al*, 1991). The transformation markers *psur-5::mdsred*, *pmyo-3::gfp* were used at 10 ng/ μ l concentration, and *pRF4(rol-6D)* was used at 5 ng/ μ l concentration.

Molecular biology

α -Adducin minigene was constructed by fusing a 3.1-kb promoter region with the N-terminal *add-1* cDNA fragment (encoding amino acids 1–534) that is common in all *add-1* isoforms (see Supplementary Figure S2E) together with the C-terminal genomic region of the *add-1* gene using a unique *SacI* site. Introducing *tRFP* sequence after the start codon of the *add-1*minigene generated *tRFP* translational reporter construct. Splice-form-specific constructs were generated by replacing the genomic C-terminal end of the *add-1*minigene with the appropriate cDNA. For the tissue-specific rescue experiments, the coding region of the *add-1*minigene or cDNA for specific *add-1* splice form, were placed under the control of a 940-bp fragment of the *nmr-1* promoter, a 2.6-kb piece of the *lim-4*, a 3.2-kb fragment of the *rig-3*, a 3.4-kb fragment of the *odr-2*, or a 780-bp of the *tdc-1* promoters.

Real-time RT-PCR

Total RNA was isolated from synchronized adult worms and 400 ng total RNA was reverse transcribed using a mix of Random Decamers (Ambion) and Anchored Oligo(dT)₂₀ Primer (Invitrogen). Real-time PCR was performed using the SyBr Fast Kit (Kapa Biosystems) according to the manufacturer's recommendations in a Corbett Research RG-6000A instrument. Expression levels were normalized to *tba-1* and *cdc-42* using a geometric mean of their level of expression.

C. elegans behaviour assays

Chemotaxis to different compounds was assessed as described previously (Bargmann *et al*, 1993). A population of well-fed, young adults was washed three times with CTX buffer (5 mM KH₂PO₄/K₂HPO₄ pH 6.0, 1 mM CaCl₂, and 1 mM MgSO₄) and 100–200 worms were placed at the middle of a 10-cm test plate. Worms were given a choice between a spot of attractant or repellent in ethanol with 20 mM sodium-azide and a counter spot with ethanol and sodium-azide. The distribution of the worms over the plate was determined after 1 h and the chemotaxis index was calculated as described (Bargmann *et al*, 1993).

Olfactory conditioning was assessed as described (Nuttley *et al*, 2002) with some modifications. Starvation conditioning was performed without food in the presence of 2 μ l undiluted chemoattractant spotted on the plate for 1 h on 10 cm CTX plates (5 mM KH₂PO₄/K₂HPO₄ pH = 6.0, 1 mM CaCl₂, 1 mM MgSO₄, 2% agar). Naive and conditioned worms were tested for their chemotaxis towards DA as described above. All rescue experiments were performed in a blinded manner.

Long-term associative memory was tested as shown in Supplementary Figure S4A using two cycles of conditioning with DA with 30 min rest between trainings. Cycloheximide (800 μ g/ml) and actinomycin D (100 μ g/ml) treatments were performed in all washing steps before and during the conditioning phases. Following conditioning, worms were kept on NGM plates in the presence of abundant food for 16 or 24 h and tested for chemotaxis to DA after the recovery phase.

Chemotaxis to water-soluble compounds was assessed as described (Wicks *et al*, 2000). Pairs of opposite quadrants of four-quadrant Petri plates (Falcon X plate, Becton Dickinson Labware)

were filled with buffered agar (2% agar, 5 mM KH₂PO₄/K₂HPO₄ pH 6.0, 1 mM CaCl₂, and 1 mM MgSO₄), either containing 25 mM NaCl or no salt. Adjacent quadrants were connected with a thin layer of molten agar. A population of well-fed, young adult worms was washed three times with CTX buffer (5 mM KH₂PO₄/K₂HPO₄ pH 6.0, 1 mM CaCl₂, and 1 mM MgSO₄) and 100–200 worms were placed at the intersection of the four quadrants. The distribution of the worms over the four quadrants was determined after 10 min. For NaCl conditioning, animals were exposed to 25 mM NaCl in CTX buffer for 4 h.

Locomotory rate assays were performed on a bacterial lawn as described (Sawin *et al*, 2000; Mohri *et al*, 2005). Briefly, worms were grown under uncrowded conditions with or without food on CTX plates for 1 h. Two minutes after transfer on 6 cm assay plates seeded with OP50, the number of body bends of eight animals from each strain was counted for 1 min.

Microscopy

GFP (or tRFP)-tagged proteins were detected with a Zeiss Axiovert 200M LSM 510Pascal confocal microscope. Animals were immobilized with 20 mM sodium-azide and GLR-1::GFP was recorded posterior to the vulva, along the z-axis. Quantification of GLR-1::GFP cluster volume was performed using the ImageJ Object Counter 3D plugin. Total fluorescence intensities in a 50- μ m long part of the VNC posterior to the vulva were measured on the projected z-sections using ImageJ. FRAP was conducted on young adult animals that were immobilized by putting worms in 4 μ l 10 μ m polystyrene microspheres solution (Polysciences GmbH, Germany) on a 4% agarose pad. GLR-1::GFP was recorded in punctae posterior to the vulva every 5 s for 4 min (at 9% laser intensity). Bleaching of the region of interest was done with 488 nm laser using 30 iterations (~1 s total illumination) at 100% laser energy. Following recording, images were aligned using the ImageJ Stackreg plugin to compensate for movements of the animals and intensity of the ROI was measured with the Time Series Analyser plugin. Normalization of the fluorescence signal was performed as described (Phair *et al*, 2004).

Human studies

The study sample ($n = 1085$) was recruited in two sites in the cities of Zurich and Basel. The ethics committees of the Cantons of Zurich and Basel, Switzerland, approved the study. Genetic analysis was performed in the combined sample.

Zurich site. We recruited 466 young Swiss subjects at the University of Zurich (351 females and 115 males, with a mean age of 21.6 years, s.d. $\pm$ 2.6). After complete description of the study, all subjects gave written informed consent. Subjects performed a picture-based episodic memory task as described previously (de Quervain *et al*, 2007). In short, 30 picture stimuli (10 positive, 10 neutral, 10 negative) from the International Affective Picture System (IAPS) were presented (Lang *et al*, 2008). International affective picture system (IAPS): Affective ratings of pictures and instruction manual. Technical Report A-8. University of Florida, Gainesville, FL). After a delay of 10 min, subjects were unexpectedly instructed to recall the pictures. The phenotype of interest was episodic memory for visual information, measured as the number of recalled pictures, irrespective of emotional quality. Additionally, concentration and attention were measured with the d2 cancellation test, and working memory was assessed with the digit span test.

Basel site. We recruited 620 young Swiss subjects at the University of Basel (403 females and 217 males, with a mean age of 22.7 years, s.d. $\pm$ 3.6). After complete description of the study, all subjects gave written informed consent. Subjects were presented 72 IAPS pictures (24 positive, 24 negative and 24 neutral) and, after a 10-min delay, they were instructed to write down a short description of the previously seen pictures. The number of correctly recalled pictures served as phenotype.

Array-based SNP genotyping

Samples were processed as described in the Genome-Wide Human SNP Nsp/Sty 6.0 User Guide (Affymetrix). Briefly, 250 ng of DNA was digested in parallel with *StyI* and *NspI* restriction enzymes (New England Biolabs, Beverly, MA). Enzyme-specific adaptor oligonucleotides were then ligated onto the digested ends and

diluted ligation reactions were subjected to PCR with Titanium Taq DNA Polymerase. Three PCR reactions of 100 μ l were performed for *StyI*-digested products and four PCR reactions for *NspI*. PCR products were combined and purified with the Filter Bottom Plate (Seahorse Bioscience, North Billerica, MA) using Agencourt Magnetic Beads (Beckman Coulter, Fullerton, CA). Around 250 μ g of purified PCR products were fragmented and following fragmentation, the DNA was end labelled with 105 units of terminal deoxynucleotidyl transferase at 37°C for 4 h. The labelled DNA was then hybridized onto Genome-Wide Human SNP 6.0 Array at 50°C for 18 h. The hybridized array was analysed according to the manufacturer's (Affymetrix) instructions on an Affymetrix GeneChip Command Console (AGCC, version 3.0.1.1214). Generation of SNP calls and Array quality control were performed using the command line programs of the Affymetrix Power Tools package (version: apt-1.12.0). Contrast QC was chosen as QC metric, using the default value of ≥ 0.4 . Mean Call Rate for all samples averaged 98.5%. All samples passing QC criteria were subsequently genotyped using the Birdseed (v2) algorithm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Acknowledgements

We thank Anne Spang and Jean Pieters for generously sharing methods, reagents, and instruments. We also like to thank the National Bioresource Project, Japan and the *Caenorhabditis* Genetic Center (supported by NIH-NCRR) for providing nematode strains. This work was supported in part by the Swiss National Science Foundation (SNSF) Sinergia Grants CRSIKO_122691 and CRSI33_130080 (DQ and AP). This work was also part of the National Centre of Competence in Research (NCCR) Synapsy and of the 7th framework program of the European Union (ADAMS, project number 242257, FP7-HEALTH-2009) (DQ and AP). VV was supported by the Werner Siemens Foundation PhD grant and Opportunities for Excellence PhD Program of the Biozentrum.

Author contributions: VV performed the experiments, evaluated the results, and wrote the manuscript; LG and PD performed the experiments; CV performed the experiments and evaluated the results; DQ and AP designed and evaluated the experiments and wrote the manuscript; AS designed, performed, and evaluated the experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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