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Identification and characterization of novel nicotinic receptor-associated proteins in *Caenorhabditis elegans*

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Nicotinic acetylcholine receptors (nAChRs) mediate fast excitatory neurotransmission in neurons and muscles. To identify nAChR accessory proteins, which may regulate their expression or function, we performed tandem affinity purification of the levamisole-sensitive nAChR from *Caenorhabditis elegans*, mass spectrometry of associated components, and RNAi-based screening for effects on *in vivo* nicotine sensitivity. Among the proteins identified was the calcineurin A subunit TAX-6, which appeared to function as a negative regulator of nAChR activity. We also identified five proteins not previously linked to nAChR function, whose inactivation conferred nicotine resistance, implicating them as positive regulators of nAChR activity. Of these, the copine NRA-1 colocalized with the levamisole receptor at neuronal and muscle plasma membranes, and, when mutated, caused reduced synaptic nAChR expression. Loss of SOC-1, which acts in receptor tyrosine kinase (RTK) signaling, also reduced synaptic levamisole receptor levels, as did mutations in the fibroblast growth factor receptor EGL-15, and another RTK, CAM-1. Thus, tandem affinity purification is a viable approach to identify novel proteins regulating neurotransmitter receptor activity or expression in model systems like *C. elegans*.

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Introduction

Nicotinic acetylcholine receptors are pentameric, ligand-gated ion channels (LGICs) that mediate excitatory neurotransmission in neurons and muscles (Changeux and Edelstein, 1998; Unwin, 2005). During biogenesis, synaptic function, and turnover, nAChRs need to interact with a plethora of proteins (Sanes and Lichtman, 2001). A limited number of such proteins is known; among them are the endoplasmic reticulum (ER) chaperones BiP (Blount and Merlie, 1991), calnexin (Keller *et al*, 1996), and the 14-3-3β ER forward transport protein (O'Kelly *et al*, 2002). At the cell surface, rapsyn stabilizes nAChRs at postsynaptic sites (Gautam *et al*, 1995), and a receptor tyrosine kinase, MuSK, induces clustering of nAChRs in response to neuron-secreted agrin (DeChiara *et al*, 1996; Gautam *et al*, 1996; Sanes and Lichtman, 2001). On cultured *Xenopus* muscle, also the FGF receptor can induce nAChR clustering (Peng *et al*, 1991). nAChR functional properties are regulated by kinases (Swope *et al*, 1999), and protein phosphatase 2B (PP2B; calcineurin) affects the rate at which nAChRs recover from desensitization (Khiroug *et al*, 1998; Liu and Berg, 1999).

Additional proteins that may interact with nAChRs have been identified by genetic analysis in *Caenorhabditis elegans*. The best studied nAChR in *C. elegans* is the levamisole-sensitive nAChR (levamisole receptor), which is expressed in muscles and (motor-) neurons (Fleming *et al*, 1997; Culetto *et al*, 2004; Towers *et al*, 2005). Five putative subunits, UNC-29, LEV-1 (non-α-subunits), UNC-38, UNC-63, and LEV-8 (α-subunits), were identified in genetic screens for resistance to the nematocidal cholinergic agonist levamisole (Lewis *et al*, 1980). However, *in vivo* interaction in *C. elegans* of those subunits in a single nAChR has not been shown, and the exact subunit composition of the levamisole receptor is not clear. By electrophysiology, UNC-29 and UNC-38 are required for levamisole receptor function at the neuromuscular junction (NMJ), but not for function of another, pharmacologically different nAChR (Richmond and Jorgensen, 1999). Three additional *C. elegans* genes have recently been shown to encode potential nAChR accessory proteins: (1) RIC-3 enhances the expression of multiple nAChRs in the cell periphery and on the plasma membrane of heterologous cells (Halevi *et al*, 2002). (2) LEV-10 facilitates clustering of levamisole receptors at the NMJ (Gally *et al*, 2004). (3) EAT-18 affects expression of pharyngeal nAChRs containing the subunit EAT-2 (McKay *et al*, 2004). It can be expected that other accessory proteins with important functional roles remain to be identified.

Evidence for physical association of accessory proteins with nAChRs is often indirect. For example, rapsyn was first identified simply as a major protein present in nAChR-rich membrane preparations (LaRochelle and Froehner, 1986). Ligand affinity chromatography of nAChRs and conventional peptide sequencing, however, allowed identification of only few accessory proteins (Briley and Changeux, 1977). Thus, such proteins may interact with nAChRs only with low affinity, and more advanced methods for isolation and identification, like tandem affinity purification (Rigaut *et al*,

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1999) and mass spectrometry, may be required to identify them. However, generic purification methods, in particular for integral membrane proteins of low abundance, like nAChRs, yet have to be established for multicellular organisms expressing them, including *C. elegans*. Though inherently more difficult, this approach is preferable over the use of heterologous expression systems, since it ensures that protein–protein interactions identified are as close as possible to the *in vivo* situation.

Here we report identification and characterization of proteins associated with the levamisole receptor. We used, for the first time in *C. elegans*, the tandem affinity purification to isolate the levamisole receptor, and identified copurified proteins by mass spectrometry. Potential roles in nAChR function were determined after RNAi and/or in mutants, revealing proteins whose inactivation conferred altered nicotine sensitivity *in vivo*. For three of those proteins, including the fibroblast growth factor receptor (FGFR) substrate adaptor SOC-1, the copine NRA-1, and the calcineurin TAX-6, we provide additional results showing functions in cell surface expression and regulation of nAChRs, as well as colocalization with the levamisole receptor *in vivo*.

Results

Purification of the levamisole receptor

To biochemically isolate the levamisole receptor and associated proteins, we employed the tandem affinity purification (TAP) (Rigaut *et al*, 1999). This method involves two genetically encoded affinity tags (Protein A attached to the tobacco etch virus (TEV) protease cleavage site and calmodulin-binding peptide (CBP)) to purify a transgenic protein and associated molecules through successive IgG- and calmodulin-chromatography steps. UNC-29, C-terminally fused with the TAP tag, could be detected in the detergent extract from whole animals and pulled down with IgG agarose (Figure 1A and B). The UNC-29::TAP protein was functional, since it restored normal nicotine sensitivity in *unc-29(x29)* loss-of-function mutants (Figure 1C). To avoid purification of proteins that associate only with UNC-29 monomers, we varied the TAP purification, using a 'split' TAP tag. We fused the Protein A/TEV tag to UNC-29 and the CBP tag to LEV-1; thus, only assembled complexes containing both subunits would be purified. Alternatively, we generated a strain that coexpressed UNC-29::TAP with hexa-histidine-tagged versions of LEV-1, UNC-38, and UNC-63, allowing Ni^{2+} chromatography as a third purification step. In total, four purifications were performed: two with the simple TAP tag on UNC-29, one with the third Ni^{2+} chromatography, and one with the split TAP tag. As a control, we also performed a mock purification from a wild-type (WT) extract containing no tagged protein (Figure 1D; Supplementary Figure 7 (for stronger contrast)).

We identified the purified proteins by analyzing tryptic peptides obtained from the whole sample using multi-dimensional protein identification technology (MudPIT; Link *et al*, 1999; Washburn *et al*, 2001) and searching the *C. elegans* proteome database (Tabb *et al*, 2002). Multiple peptides of each of the levamisole receptor subunits, UNC-29, UNC-38, UNC-63, LEV-8, and LEV-1, were identified in four different purifications (Table I and Supplementary data), verifying that the purification was successful, and showing that these subunits physically associate *in vivo*. Many other proteins were reproducibly represented by multiple peptides. For example, the BiP ortholog HSP-3 (Supplementary Table 1), which was shown previously to associate with nAChR subunits in vertebrates (Blount and Merlie, 1991), was identified all the four times. Together, these data indicate that our procedure indeed purified the levamisole receptor.

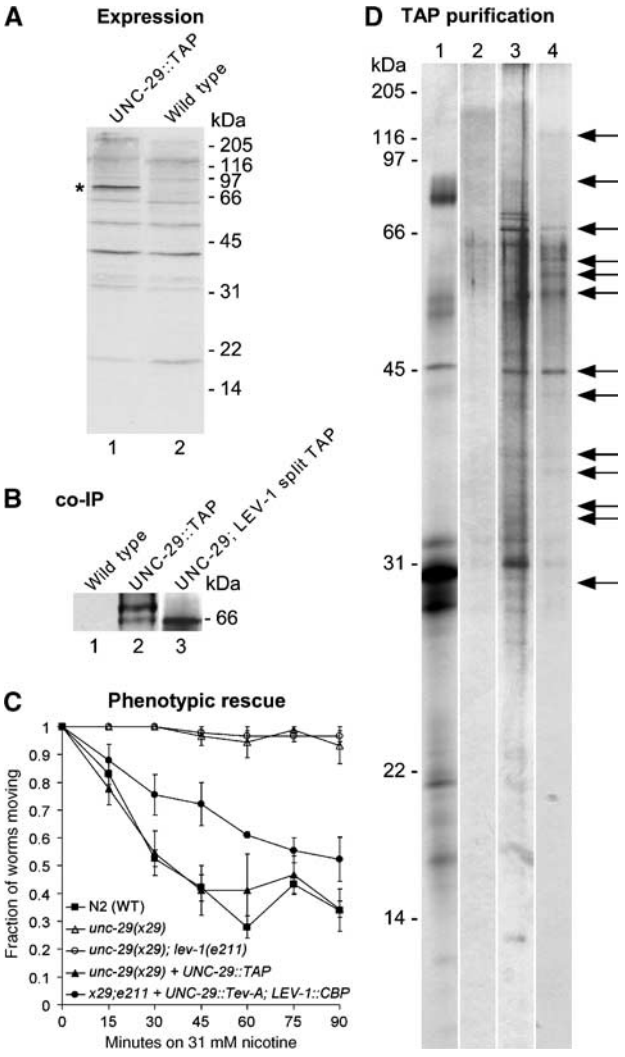

Figure 1 TAP of the levamisole receptor. (**A**) Immunodetection of the ProtA portion of UNC-29::TAP (lane 1, *) in detergent extracts of strain AQ748 and N2 (WT, lane 2). (**B**) UNC-29::TAP and UNC-29::TEV-ProtA were precipitated with IgG agarose from detergent extracts of strains AQ748 or AQ839, respectively, and detected by Western analysis directed against the ProtA portion of the respective tags. (**C**) Paralysis by nicotine (31 mM). WT (N2) or mutants (genotype as indicated) and the same mutants rescued with the respective integrated arrays (strains AQ748 and AQ839) were placed on nicotine plates and observed at the indicated times to determine the fraction of paralyzed animals (3–5 experiments, $n = 30$ each, error bars = s.e.m.). (**D**) Silver-stained proteins in fractions obtained by the TAP procedure: Lane 1: eluate after the first step (released from IgG matrix by TEV cleavage); lane 2: eluate after the second step (released from calmodulin matrix with EGTA) obtained from N2 extract (mock); lane 3: eluate obtained after the second step from AQ748 extract (UNC-29::TAP); lane 4: eluate obtained after the second step from AQ839 extract (UNC-29::TEV-ProtA, LEV-1::CBP; split TAP tag). Arrows indicate purified proteins not present in the mock purification. Two bands of ca. 70 kDa, possibly heat shock proteins associated with copurified UNC-29 monomers in lane 3, are absent in lane 4. Bands of 30 and 28 kDa correspond to TEV-protease.

Novel nAChR subunits copurified with the levamisole receptor

Two of the purified proteins were additional nAChR α-subunits not previously implicated in levamisole receptor function: ACR-8, and ACR-12 (Table I). We investigated whether these proteins, as well as ACR-13, that had just recently been identified as LEV-8 (Towers *et al*, 2005), functionally resemble levamisole receptor subunits, mutations which cause resistance to paralysis by cholinergic agonists (i.e. *unc-29(x29)*; Figure 2A and B). Mutants in *acr-8* (a Tc1 transposon insertion, *cxP821*, as well as a genomic deletion, *ok1240*; not shown), *acr-12*(*ok367*), and *acr-13/lev-8*(*x15*) were tested for sensitivity to nicotine and levamisole (Figure 2A and B). Interestingly, while *acr-8* and *acr-13* mutations conferred nicotine resistance, *acr-12(ok367)* did not. Moreover, only *acr-13/lev-8(x15)* animals were levamisole resistant. Thus, not all of the nAChR subunits copurified equally contribute to levamisole receptor function.

The finding that five nAChR α-subunits were copurified with the two non-α-subunits LEV-1 and UNC-29 could indicate that multiple distinct classes of levamisole receptors with differing α-subunit composition may exist. To address this possibility, we studied the expression patterns of these proteins. C-terminal GFP fusions of ACR-13 (truncated in the large cytoplasmic loop) and full-length ACR-8 were expressed in body muscles, few head and tail neurons, and nervecord synapses, but apparently not in ventral cord motor neurons (Supplementary Figure 1A and C). In contrast, full-length ACR-12::GFP was exclusively expressed in neurons, including ventral cord motor neurons (Supplementary Figure 1B). Thus, the expression domains of ACR-8, -12, and -13 collectively overlap with that of known levamisole receptor subunits in body muscles and motor neurons (Fleming *et al*, 1997; Culetto *et al*, 2004; Towers *et al*, 2005; data not shown).

To test for colocalization of ACR-8 and ACR-12 with the levamisole receptor subunit UNC-38 in individual postsynaptic receptor clusters, we developed a new method for *in vivo* labeling of cell-surface epitopes. We expressed nAChR subunits with epitope tags at the extracellular C-termini and detected them with fluorescent antibodies injected into the body cavity (Supplementary Figure 2). Thus, we could detect cMYC-tagged UNC-38 in puncta along nervecords. These were most likely postsynaptic receptor clusters, since they were juxtaposed to, but did not overlap with, presynaptic synaptobrevin (SNB-1::GFP; Figure 2C; Supplementary Figure 6C). UNC-38::3xMYC showed complete colocalization in the nervecord puncta with UNC-29::GFP (Figure 2D; Supplementary Figure 6D), as did HA-epitope-tagged LEV-1 (Supplementary Figure 5). However, HA-tagged ACR-8 or ACR-12 was present only in some of the clusters containing UNC-38::3xMYC (Figure 2E and F; Supplementary Figure 6E and F). Thus, ACR-8 and ACR-12 may contribute to levamisole receptors in only a subset of postsynaptic clusters.

RNAi reveals proteins affecting in vivo nicotine sensitivity

In addition to the nAChR subunits described above, more than 200 proteins were detected in the fractions containing the purified levamisole receptor. Though some of them were represented by multiple peptides, indicating high relative abundance, many others were identified by single peptides only. While the high sensitivity of our protein identification method facilitates discovery of low-affinity interactors, it probably also led to detection of nonspecific contaminants. Thus, we needed to identify those proteins likely to be genuine functional interactors. We included for further analysis all proteins isolated with the split TAP tag (i.e. the sample containing only assembled receptors), as well as proteins that were detected several times in the other three purifications (225 proteins; Table I and Supplementary Tables 1 and 2). As a means of judging the specific enrichment of proteins by our affinity purification over abundance in an extract, we compared our list of proteins with those found in a recent mass spectrometric analysis of the *C. elegans* proteome, which identified 1616 abundant proteins (including membrane proteins) in crude extracts by comparable identification methods (Mawuenyega *et al*, 2003). Based on this comparison, 69 of the 225 proteins were not clearly enriched by our TAP purification (Supplementary Tables 1 and 2). Since at least some of these proteins were probably nonspecific contaminants, we assigned low priority for further analysis to these proteins.

Next, we functionally assessed the identified proteins for effects on cholinergic neurotransmission. We depleted each protein by feeding the appropriate strain from a bacterial RNAi library (Kamath *et al*, 2003) to *rrf-3(pk1426)* mutant animals, which exhibit enhanced sensitivity to RNAi (Simmer *et al*, 2002), but show normal responses to nicotine (Figure 3A). Since levamisole receptor function is a major determinant of nicotine sensitivity in the paralysis assay (Figure 2A and B) and depletion of known levamisole receptor subunits causes strong nicotine resistance (Figure 3A and Table I), loss of a protein functionally associated with the levamisole receptor may confer altered sensitivity to nicotine. We could test 157 proteins this way; the remaining proteins were either not represented in the library (40 proteins), or RNAi caused lethal phenotypes or paralysis (28 proteins).

Through this RNAi screening, we identified 11 proteins whose depletion caused resistance to nicotine, including 6 nAChR subunits (Table I and Figure 3B). Depletion of 44 other proteins caused nicotine hypersensitivity (Table I; Supplementary Table 1 and Figure 3A); of these, 28 were more abundant in the purified sample than in a whole-proteome analysis (Mawuenyega *et al*, 2003) and therefore enriched by our purification. Thus, our results suggested potential functional interactions between 33 proteins and the seven nAChR subunits purified as part of the levamisole receptor (Table I).

Calcineurin sets the nicotine sensitivity of neurons and muscles

The most abundant protein whose depletion caused nicotine hypersensitivity was the calcineurin A subunit TAX-6 (Kuhara *et al*, 2002). Active calcineurin contains also the regulatory subunits calcineurin B (CNB-1) and calmodulin (also copurified; Supplementary Table 2). The regulatory subunits bind TAX-6 in a Ca^{2+}-dependent manner to replace its auto-inhibitory C-terminus from the active site (Hashimoto *et al*, 1990). We found that loss-of-function mutations in both *tax-6(p675)* and *cnb-1(jh103)* (Bandyopadhyay *et al*, 2002) conferred hypersensitivity to nicotine (Figure 3C). Further, we prepared a synthetic gain-of-function (g.o.f.) allele of *tax-6* by expressing constitutively active TAX-6 (lacking the

Table I nAChR subunits and proteins copurified with the levamisole receptor, that cause altered sensitivity to nicotine, when depleted by RNAi

Gene	No. of peptides identified	Identity/features	RNAi phenotype in *rrf-3(pk1426)*	Resistant (+) or sensitive (−) to nicotine after RNAi	No. of peptides whole proteome mass spec. (Mawuenyega *et al*, 2003)	Conservation to human homolog e-value over % length	
nAChR subunits							
T08G11.5	4	UNC-29	Slightly unc	+ +	0	1.9e–98	88.6
F09E8.7	4	LEV-1	Normal	+ + +	0	5.2e–94	84.2
Y110A7A.3	8	UNC-63	Normal	+ + +	0	1.4e–124	81.1
F21F3.5	3	UNC-38	Normal	+ +	0	2.3e–102	76.1
C35C5.5	5	ACR-13		+ + (mutant only)	0	7.9e–83	69.5
ZC504.2	4	ACR-8		+ + + (mutant only)	0	6.2e–81	58.4
R01E6.4	1	ACR-12	Normal	Normal (mutant, RNAi)	0	5.2e–95	74.3
RNAi confers resistance to nicotine							
T28F3.1	1	Copine, C2 domains	Few worms	+ + (RNAi and mutant)	0	5.9e–110	82.0
F41F3.2	1	SOC-1; pleckstrin homology domain	Normal	+ + (RNAi and mutant)	0	3.1e–4	75.1
Y71F9B.7*	1	PLK-2, POLO-like Kinase	Some unc, egl	+ (mutant only)	0	1.1e–163	90.5
C17G1.4	1	PHD (Zn-)finger	Normal	+ +	0	1.4e–20	76.4
T05F1.1	1	Nicalin homologue, membrane peptidase like	Few worms, slightly unc	+ + (RNAi and mutant)	1	3.0e–60	89.9
RNAi confers sensitivity to nicotine							
C02F4.2	4	TAX-6, Calcineurin A subunit	Small, slow growth, unc	− − − (RNAi and mutant)	1	3.6e–206	88.6
F22E10.1	2	P-glycoprotein; ABC transporter	Normal	−	0	9.1e–233	93.2
Y111B2A.22	2	PQN-81, prion-like	Strongly unc, short	− − (RNAi by soaking)	0	6.2e–13	46.0
T11F1.8	2	Receptor L-domain, as in EGF receptor tyrosine kinase	Normal	−	0	—	
R09H10.4	1	PTR-14: HMGCR/patched 5TM box	Normal	− − −	0	5.4e–27	68.0
W02A2.5	1	4 TM domain, ER associated ?	Normal	− −	0	3.7e–5	70.2
AH6.7	1	SRA-3, 7TM serpentine receptor	Normal	− −	0	—	
T05B11.2	1	SRR-9, 7TM serpentine receptor	Normal	− −	0	—	
T28F2.8	1	COL-51, collagen triple-helix repeat	Normal	− −	0	1.1e–52	74.5
C05C9.1	1	Lipid binding LBP/BPI/CETP family	Normal	−	0	1.9e–14	97.5
W08D2.5	1	E1–E2 ATPase, transport ATPase	Normal	− −	0	7.5e–203	81.4
M02G9.1	1	Keratin-like protein, cystein rich repeats	Normal	−	0	2.9e–30	82.4
Y76B12C.7	1	Probable CPSF 160 kDa subunit	Normal	− −	0	1.1e–221	99.1
F33E11.3	1	Zn-finger-like, PHD finger	Mostly normal, slightly unc	− −	0	3.0e–27	65.7
K01D12.15	1	Unknown	Normal, few worms	−	0	3.1e–2	81.4
D1081.7	1	Tudor domain	Normal, few worms	− −	0	6.0e–2	18.9
B0336.1	1	WRM-1; Armadillo repeat	Normal, few worms	− −	0	2.6e–11	27.9
F46F2.2	1	Casein kinase I	Small, low brood size, unc	− −	0	1.4e–133	61.4
T05D4.3	1	Seven TM domains; voltage-dep. Ca^{2+} channel?	Small adults, unc	− −	0	5.1e–2	36.4
W02B8.3	1	Unknown; TM domains	Few worms, unc	−	0	1.0e–6	38.8
C43E11.3	1	Similar to human NSD1	Few worms, slow growth, unc	− −	0	3.6e–43	43.3
Y48G1C.7	1	Unknown	Few progeny, pvu, unc, bli	− −	0	—	
ZC518.3a	1	Endo-/Exonuclease/phosphatase family	Few, sick, small, unc, rol	− − −	0	1.4e–142	85.8
ZK688.2	1	Putative membrane protein	Low brood size, sick, unc, small	− − −	0	5.8e–80	62.2
T20B12.3	1	Unknown	Few, sick, unc, small	− −	0	2.9e–37	51.6
W01F3.3	1	Kunitz domains	L1 arr., some escape, unc	− − −	0	1.5e–36	10.6
F32H2.1a	1	GEI-11, c-MYB domain	Few progeny, L1/L2 arrest, unc, sick	− − −	0	1.2e–46	44.2
F44E5.1	1	Unknown	Small, unc, few, slow growth	− − −	0	—	

The extent of the nicotine sensitivity phenotype is indicated by: + + + (strongly resistant) to − − − (strongly hypersensitive). Homologies to the best human candidate were taken from wormbase (http://www.wormbase.org). (*) The PLK-2 peptide identified could also originate from PLK-1. However, RNAi of PLK-1 is lethal (Kamath *et al*, 2003), but not genomic deletion of *plk-2(tm1395)*, which specifically caused resistance to nicotine (Figure 4A). Visible phenotypes abbreviated: unc—uncoordinated, egl—egg-laying defective, pvu—protruding vulva, bli—blisters, rol—roller.

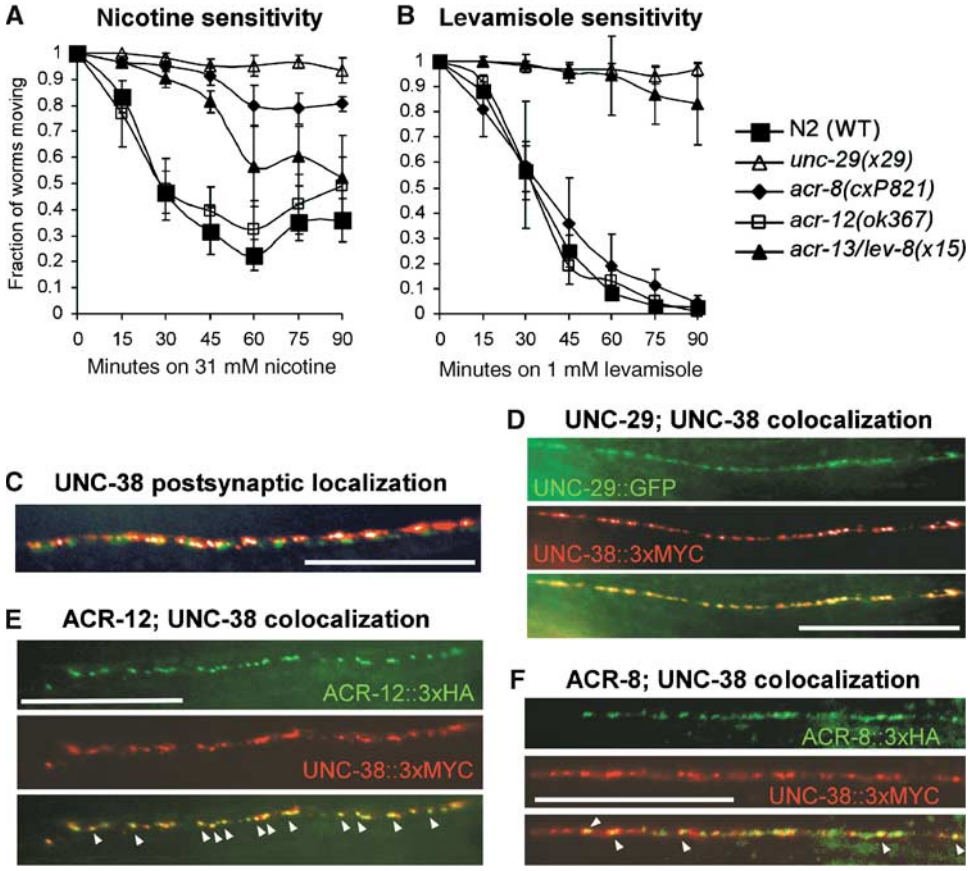

Figure 2 Analysis of novel nAChR subunits copurified with the levamisole receptor. (**A, B**) Paralysis of mutants lacking additional nAChR α-subunits that copurified with the levamisole receptor, *acr-8(cxP821)*, *acr-12(ok367)*, and *acr-13/lev-8(x15)*, was either observed on 31 mM nicotine (A) or 1 mM levamisole (B). For comparison, N2 (WT) or *unc-29(x29)* mutant animals were also analyzed (3–6 experiments, $n = 30$ each, error bars = s.e.m.). (**C**) Postsynaptic expression of UNC-38::3xMYC was analyzed *in vivo* with fluorescent (Cy3-labeled, red) antibodies directed against the extracellular cMYC epitope. Antibodies were injected into the pseudocoelom of animals expressing UNC-38::3xMYC and the presynaptic vesicle marker synaptobrevin SNB-1::GFP (green), driven by the *unc-4* promoter (strain AQ898). (**D**) Complete synaptic colocalization of UNC-29::GFP and UNC-38::3xMYC, labeled with anti-MYC-Cy3 antibodies injected into the pseudocoelom (strain AQ658). (**E, F**) Partial synaptic colocalization of either ACR-12::3xHA (E; strain AQ1013) or ACR-8::3xHA (F; strain AQ1011) with UNC-38::3xMYC, each labeled with injected fluorescent antibodies (anti-HA-Alexa488, green; anti-MYC-Cy3, red). Colocalization in the same nervecord cluster is indicated by arrowheads. In (C–E), the ventral nervecord of adult animals in the midbody region, within 1/4 body length anterior or posterior of the vulva is shown; anterior is left; scale bars = 20 μm.

auto-inhibitory C-terminus) in *tax-6(p675)* animals (Figure 3C; Supplementary Figure 3A). Significantly, these animals were nicotine resistant. Thus, calcineurin appears to negatively regulate levamisole receptor function.

TAX-6 is coexpressed with UNC-38 in muscles and neurons (Kuhara *et al*, 2002) (Supplementary Figure 3B). To study whether TAX-6 expression in muscle is sufficient to control nicotine sensitivity, we expressed TAX-6 in *p675* mutants either from its endogenous promoter or from the muscle-specific promoter *pmyo-3* (Supplementary Figure 3A). TAX-6 expressed in muscle rescued the nicotine sensitivity phenotype only partially, while expression from the *tax-6* promoter fully restored WT sensitivity (Figure 3C). Expression of the *tax-6* g.o.f. allele in muscles restored nicotine sensitivity to a level comparable to WT, but did not cause resistance (Figure 3C). These results suggest that calcineurin negatively regulates nicotine sensitivity to a certain degree by affecting nAChRs in body muscles; however, it is most likely required in neurons as well. Our findings parallel observations in rat chromaffin cells, where calcineurin inhibitors increased the rate at which nAChRs recover from nicotine-induced desensitization (Khiroug *et al*, 1998), while they increased the rundown of nicotine-induced peak currents mediated by chick ciliary ganglion α7 nAChRs (Liu and Berg, 1999). Our results suggest that a direct or indirect physical interaction, which (given the abundance of TAX-6 in the purified sample) may extend beyond the transient interaction expected for a phosphatase and its substrate, could mediate nAChR regulation by calcineurin in *C. elegans*.

Novel non-nAChR proteins reduce levamisole receptor function when depleted

Depletion or mutation of five non-nAChR proteins that had not previously been implicated in nAChR function conferred moderate resistance to nicotine (Figures 3B and 4A; Table I). These are: (1) a homolog of vertebrate Nicalin (encoded by T05F1.1), a putative type I ER-membrane protein that antagonizes Nodal signaling (Haffner *et al*, 2004); (2) a putative Ca^{2+}-dependent phospholipid-binding protein (T28F3.1) of the copine family (Creutz *et al*, 1998); (3) a protein containing a PHD Zn-finger motif and putative transmembrane domains (C17G1.4); (4) a POLO box-like serine/threonine kinase, PLK-2, whose mammalian homologs *Fnk* and *Snk* affect synaptic plasticity (Kauselmann *et al*, 1999; Pak and

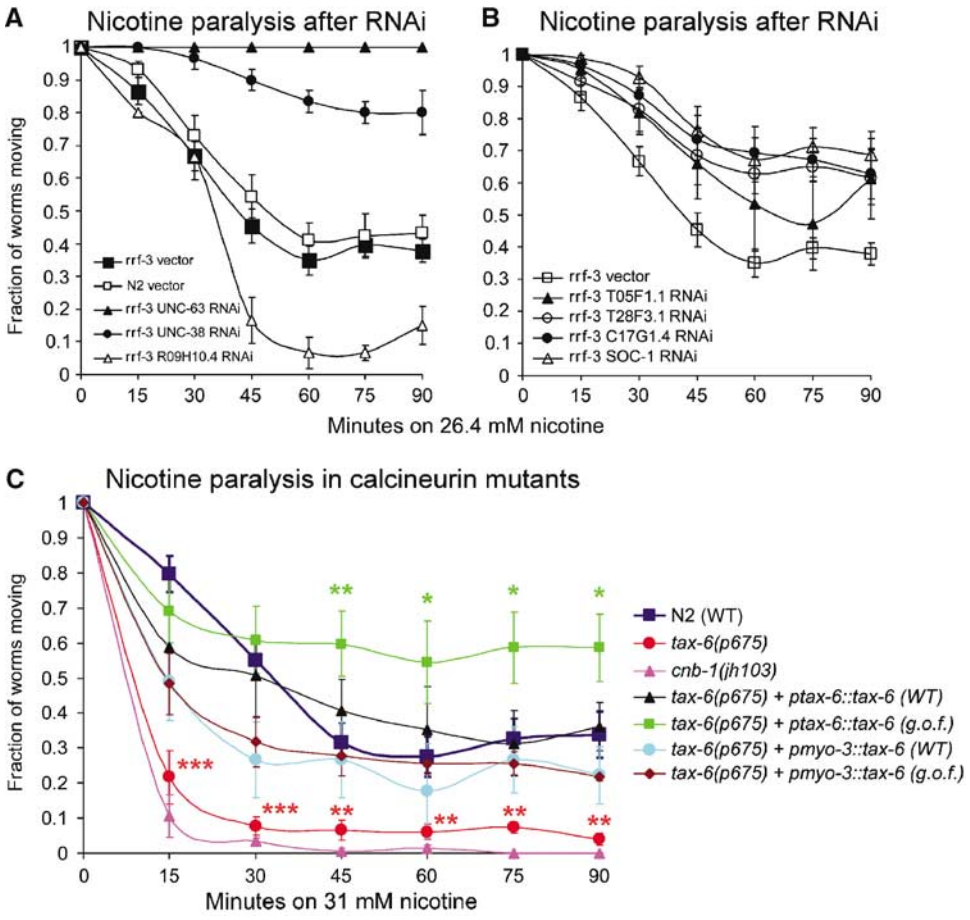

Figure 3 RNAi of proteins copurified with the levamisole receptor and mutation of TAX-6 calcineurin affect nicotine responses. (**A**) RNAi-sensitized animals of genotype *rrf-3(pk1426)* were depleted of either UNC-63, UNC-38, or a protein copurified with the levamisole receptor (R09H10.4) by feeding bacteria expressing double-stranded RNA specific for their genes. RNAi-treated animals were then subjected to paralysis by 26.4 mM nicotine, showing either resistance or hypersensitivity (two experiments, $n=30$ each). For comparison, *rrf-3(pk1426)* and N2 (WT) animals were fed with induced bacteria containing the empty vector L4440 (10–13 experiments, $n=30$ each). (**B**) RNAi (by feeding) of *nra-2* (T05F1.1), *nra-1* (T28F3.1), *nra-3* (C17G1.4), and *soc-1* induced significant resistance to 26.4 mM nicotine at all time points 30′ and later ($P<0.05$ or less, 5–6 experiments, $n=30$), except for the 75′ time point of *nra-2*. Control is *rrf-3(pk1426)* fed with bacteria containing empty vector. (**C**) Paralysis of calcineurin mutants induced by 31 mM nicotine. Mutants in the α and β subunits, *tax-6(p675)* and *cnb-1(jh103)*, are hypersensitive (compared to the WT, N2). *p675* mutants expressing *tax-6* WT cDNA show normal sensitivity to nicotine (strain AQ1015). Expression of a truncated *tax-6* cDNA (encoding a g.o.f. protein) in *p675* animals, driven by the *tax-6* promoter (strain AQ1016), causes significant resistance to nicotine. Expression of WT and g.o.f. TAX-6 from the muscle-specific *myo-3* promoter (strains AQ1017 and AQ1018, respectively) rescues nicotine sensitivity only partially (3–8 experiments, $n=30$ animals each; $^{**}P<0.01$, $^{*}P<0.05$, error bars = s.e.m.).

Sheng, 2003); and (5) SOC-1, which functions as a multi-substrate adaptor protein in FGF signaling (Schutzman *et al*, 2001). All five proteins are conserved in vertebrates (Table I). To further test whether these proteins are required for nicotinic receptor function, we obtained loss-of-function alleles for all of them: *soc-1(n1789)*, *plk-2(tm1395)*, *T28F3.1(ok1025)*, *T05F1.1(tm1453)*, and *C17G1.4(tm1649)*. In all cases, nicotine resistance paralleling the RNAi phenotype was observed (Figure 4A). On the basis of these mutant phenotypes, the latter three genes were designated *nra-1–3* (for nicotinic receptor associated).

To investigate the specificity of these genes' effects on nicotine sensitivity, we assayed sensitivity to other drugs, namely levamisole (a specific levamisole receptor agonist) and muscimol, an agonist of the inhibitory γ-aminobutyric acid receptor (GABAR) encoded by *unc-49*. We observed moderate levamisole resistance in *soc-1(n1789)*, *nra-1(ok1025)*, *nra-2(tm1453)*, and *nra-3(tm1649)* mutants, indicating that their nicotine resistance phenotype was due, at least in part, to effects on levamisole receptor function (Figure 4B). Second, of all of the mutants tested, only *soc-1(n1789)* showed resistance to muscimol in our assay (Figure 4C and D). These results indicated that the effects of *plk-2(tm1395)*, *nra-1(ok1025)*, *nra-2(tm1453)*, and *nra-3(tm1649)* were specific for nAChR function, while SOC-1 may have more general effects on the function of the body muscle NMJ and/or its receptors. Finally, all of the mutants except *nra-1(ok1025)* showed moderate to strong uncoordinated (*unc*) phenotypes in thrashing assays, further indicating effects on NMJ function (Figure 4C).

Loss of the copine NRA-1 causes reduced synaptic levamisole receptor expression

Copines possess two C2 domains, which can bind phospholipids in a Ca^{2+}-dependent manner, and a protein interaction domain (Tomsig *et al*, 2003), by which they may recruit other proteins to membranes. The levamisole resistance of *nra-1* mutants may, in principle, be due to reduction of either

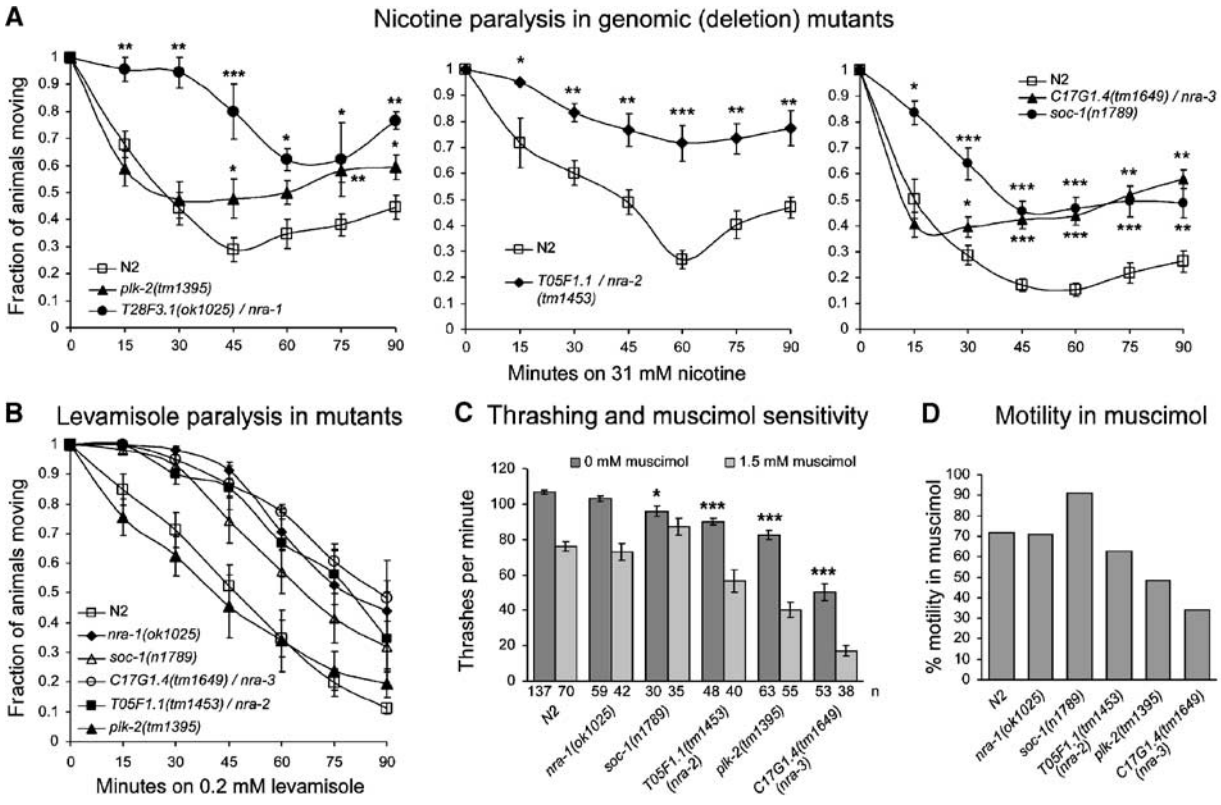

Figure 4 Pharmacological analysis of genomic mutants of levamisole receptor-associated proteins. (**A**) Genomic deletion or mutation of *plk-2(tm1395)*, *nra-1(ok1025)*, *soc-1(n1789)*, *nra-2(tm1453)*, and *nra-3(tm1649)* cause significant resistance to 31 mM nicotine, verifying the phenotypes previously observed after RNAi (3–13 experiments, $n=30$). (**B**) Mutations in *soc-1(n1789)*, *nra-1(ok1025)*, *nra-2(tm1453)*, and *nra-3(tm1649)* cause significant resistance to 0.2 mM levamisole in paralysis assays (4–7 experiments, $n=30$; $P<0.05$ or less for times 30′ and later for all mutants, except *plk-2(tm1395)*). (**C, D**) Thrashing assays: N2 (WT) and mutants in *soc-1(n1789)*, *nra-1(ok1025)*, *nra-2(tm1453)*, *nra-3(tm1649)*, and *plk-2(tm1395)* were placed in M9 solution in the absence or presence of 1.5 mM muscimol, a GABAR agonist, and body thrashes per minute were counted, showing significantly uncoordinated motilities of *soc-1*, *plk-2*, *nra-2*, and *nra-3* mutants (number of tested animals and *P*-values (*t*-test) indicated). All strains except *soc-1(n1789)* were significantly affected by muscimol ($P<0.05$ or less). Motility in muscimol relative to the untreated controls (D) confirmed the muscimol resistance of *soc-1(n1789)* mutants. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$; error bars = s.e.m. in all panels.

activity or (synaptic) expression of the levamisole receptor. We thus examined if loss of NRA-1 affected levamisole receptor expression, either throughout the cell or specifically at synaptic sites. We expressed LEV-1::GFP and UNC-38::3xMYC in *rrf-3(pk1426)* mutants and depleted them of NRA-1 by RNAi. LEV-1::GFP, as a marker for total cellular levamisole receptor expression, was not reduced after RNAi of *nra-1* (Figure 5A). However, when we probed synaptic expression of UNC-38::3xMYC with injected anti-MYC-antibodies, we found a significant reduction in synaptic UNC-38 expression levels (28% reduction, $P<0.0002$; Figure 5B). As a control for our assay's ability to detect changes in synaptic levamisole receptor levels, we tested *ric-3(md158)* mutants, which are defective for nAChR expression in the cell periphery (Halevi *et al*, 2002). Significantly, in *md158* animals expressing LEV-1::4xHA (Figure 5C), levels of surface levamisole receptor were largely reduced (by 65%, $P<2.3\times10^{-9}$; comparable results were obtained for the UNC-38::3xMYC transgene; data not shown). To verify the RNAi-based effects of NRA-1 depletion, we expressed and measured *in vivo* antibody-labeled LEV-1::4xHA in *nra-1* mutants. Significantly, these showed a reduction of synaptic levamisole receptor expression by 23%, $P<0.001$ (Figure 5C). In contrast, a MYC-epitope-tagged UNC-49 GABAR subunit showed normal synaptic expression (Figure 5D). Thus, the copine NRA-1 may, directly or indirectly, control expression levels of synaptic levamisole receptors, but not of GABARs.

To study the subcellular site of action of NRA-1, we looked at its expression pattern. Full-length NRA-1::GFP was expressed from its endogenous promoter in many cell types (Figure 6A–E), including neurons in the head and tail, ventral cord motor neurons, and body muscles. Importantly, NRA-1::GFP localized largely to plasma membranes in muscles and neurons (Figure 6B and D) and colocalized with antibody-labeled LEV-1::4xHA (Figure 6E), though it was also observed outside LEV-1 puncta. Thus, NRA-1 may function as a general membrane organizer that, however, specifically affects cell surface expression of levamisole receptors, but not of the GABAR UNC-49.

The FGFR pathway specifically affects synaptic levamisole receptor expression

Another levamisole receptor-associated protein, SOC-1, acts in RTK signaling, specifically downstream of the FGF receptor EGL-15 (Borland *et al*, 2001; Schutzman *et al*, 2001). Among its many biological activities, EGL-15 affects muscle protein degradation in starved animals (Szewczyk and Jacobson, 2003). This indicates that EGL-15 (and thus probably SOC-1) is expressed in body muscle, where also the antagonistic RTK phosphatase CLR-1 is expressed (Kokel *et al*, 1998),

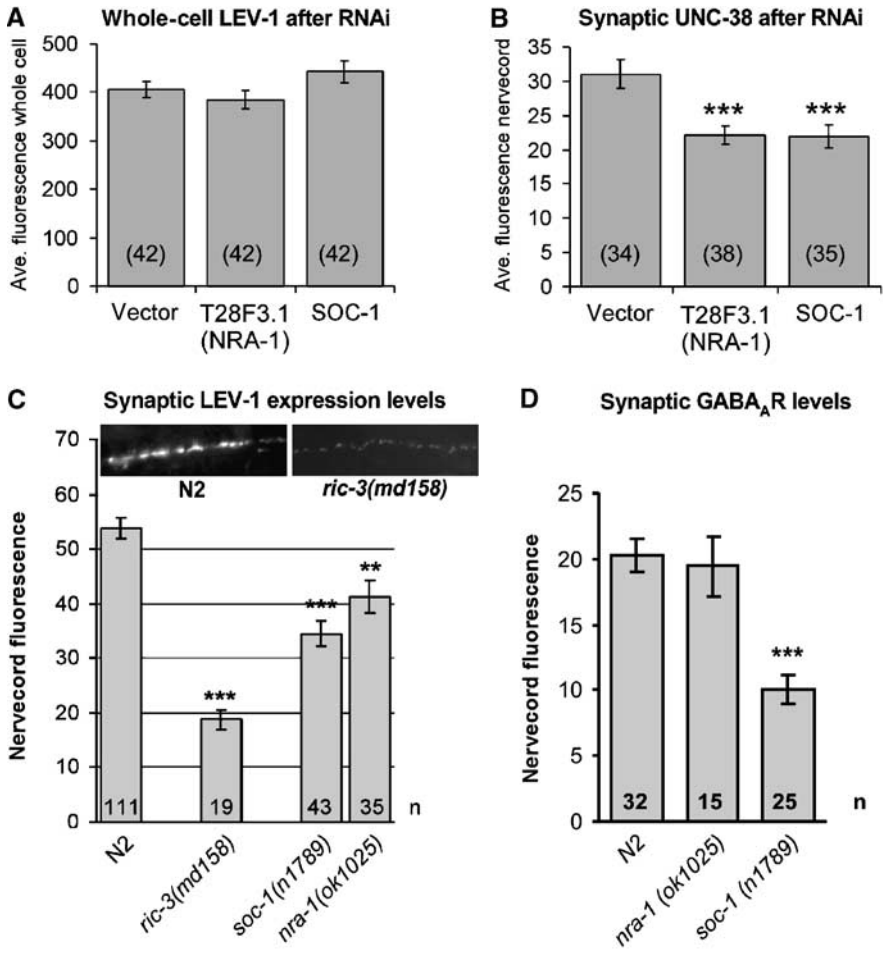

Figure 5 Reduced synaptic levamisole receptor expression in *nra-1* and *soc-1* mutants. (**A, B**) Muscular expression levels of LEV-1::GFP (A), and synaptic protein levels of UNC-38::3xMYC (B) in animals of strain AQ1019 depleted by RNAi of either NRA-1 or SOC-1. Fluorescence was quantified in body muscle, showing no significant change of total protein levels (A; $n=42$), or along nervecords in animals injected into the pseudocoelom with anti-MYC-Cy3 antibodies, revealing significant reduction in synaptic nAChR levels (B; $n=34$–38). (**C**) Synaptic LEV-1::4xHA levels, quantified after *in vivo* antibody staining, were compared in WT (N2), *ric-3(md158)*, *soc-1(n1789)*, and *nra-1(ok1025)* mutants (strains AQ881, AQ887, AQ1056, and ZX188, respectively) ($n=19$–111 animals). The inset shows representative images of ventral nervecords stained with anti-HA antibodies in WT and *ric-3(md158)* animals. (**D**) Synaptic 3xMYC::UNC-49 GABAR levels, quantified after *in vivo* antibody staining, were compared in WT (N2), *nra-1(ok1025)*, and *soc-1(n1789)* mutant backgrounds (strains FY386, ZX276, and ZX277, respectively) ($n=15$–32 animals). In (A), fluorescence of head muscles was quantified in young adult animals. In (B–D), fluorescence of the ventral nervecord of adult animals was quantified near the vulva. In all panels, $^{***}P<0.001$, $^{**}P<0.01$ (*t*-tests); error bars = s.e.m.

while EGL-15 is also found in other tissues (Bulow *et al*, 2004; Huang and Stern, 2004). SOC-1 is a homolog of the mammalian GAB multisubstrate adaptor proteins that, after phosphorylation by FGFR, recruits additional targets to the activated RTK; however, GAB proteins act downstream also of other RTKs (Liu and Rohrschneider, 2002). A second, parallel signaling pathway downstream of *egl-15* utilizes the adaptor protein SEM-5 (homologous to human GRB2), and activates the GTPase *ras* via the guanine nucleotide exchange factor SOS-1, assisted by the leucine-rich repeat protein SOC-2. Signaling through both pathways, either dependent on SOC-1 or SEM-5, is thought to activate a MAP kinase cascade (Schutzman *et al*, 2001).

Since loss of *soc-1* caused resistance to nicotine and levamisole (Figure 4A and B), we tested whether *soc-1(n1789)* mutants showed altered synaptic expression of levamisole receptors. This was the case: Synaptic LEV-1::4xHA expression was significantly reduced in *n1789* animals (by 36%; $P=3.6\times10^{-8}$; Figure 5C). Consistently, this was also found for epitope-tagged UNC-38::3xMYC, in *rrf-3(pk1426)* mutants depleted of SOC-1 by RNAi (reduced by 29%, $P=0.0005$; Figure 5B), while LEV-1::GFP expression was not altered (Figure 5A). Thus, synaptic targeting of levamisole receptors, rather than their general expression, appears to be specifically affected by SOC-1. Interestingly, *soc-1* mutants also exhibited reduced synaptic expression of the UNC-49 GABAR (51% reduction, $P<1e{-}6$; Figure 5D), consistent with the observed resistance of *soc-1* mutants to muscimol (Figure 4C and D). The loss of both GABARs and nAChRs from synaptic sites in *soc-1* mutants suggests a more general role of SOC-1 in assisting expression of LGICs at the NMJ, possibly acting downstream of EGL-15, and maybe also of other RTKs.

Of the proteins known to act in EGL-15 signaling, only SOC-1 was copurified with the levamisole receptor. Thus, to test if its involvement in synaptic nAChR expression correlated with its role in RTK signaling, as opposed to a secondary function not related to the FGF receptor, we studied nicotine sensitivity

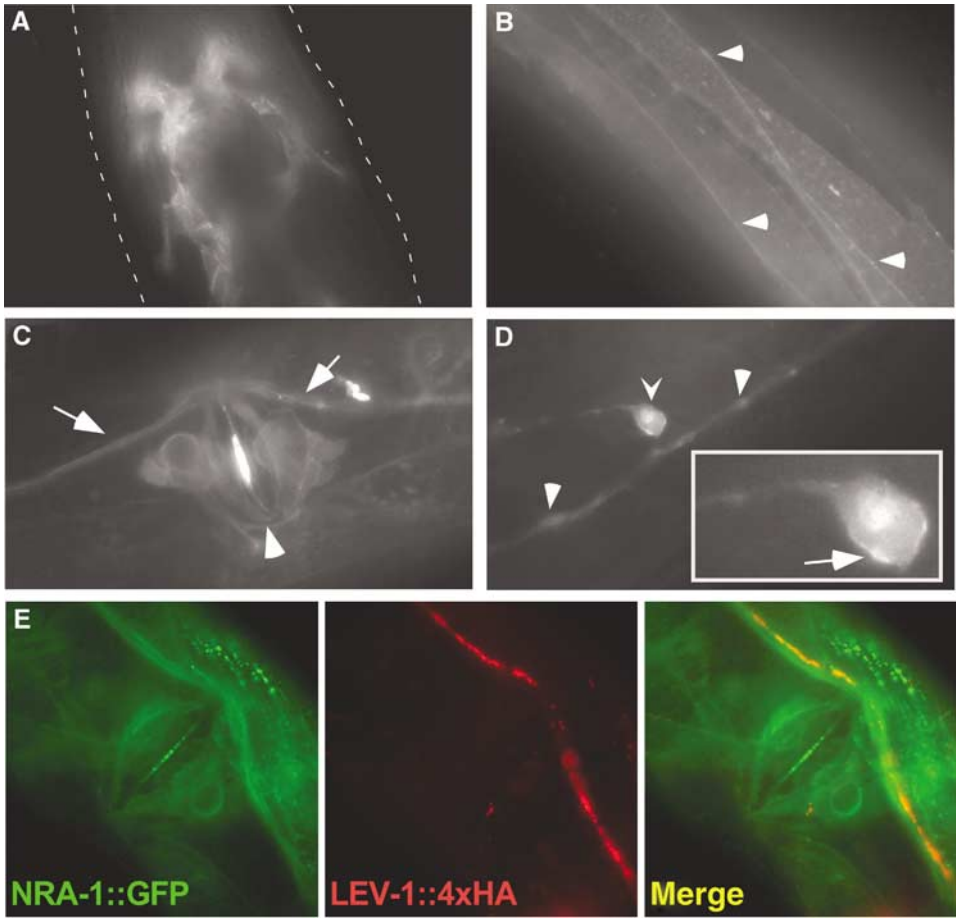

Figure 6 Coexpression of the copine NRA-1::GFP and LEV-1 at plasma membranes of neurons and muscles. (**A**) Expression of NRA-1::GFP in head neurons, in body wall muscles (**B**) (note the preferential expression at the plasma membrane), in hypodermal cells of the vulva (**C**), and at the plasma membrane of a midbody neuron (**D**). (**E**) NRA-1::GFP (green) was coexpressed with LEV-1::4xHA (red), which was then labeled *in vivo* with anti-HA antibodies (strains used in (A–D): ZX214, in (E): ZX206).

of other mutants affecting FGF signaling. Significantly, two partial loss-of-function alleles of *egl-15(n484* and *n1477*) (DeVore *et al*, 1995; Goodman *et al*, 2003), as well as mutations in three additional positive regulators of FGF signaling, *soc-2(ku167)*, *sos-1(c548)*, and *sem-5(n1779)*, caused moderate nicotine resistance, while mutation of the antagonistic phosphatase *clr-1(n1745)* caused hypersensitivity to nicotine (Figure 7A). *egl-15(n484)* mutants were also levamisole resistant (Figure 7B). Importantly, the drug response phenotypes observed for the mutants in *egl-15* and other positive FGF signaling regulators correlated with a reduction of synaptic LEV-1::4xHA expression, just like in *soc-1(n1789)* (Figure 7C; Supplementary Figure 4B). Our findings implicate SOC-1 and the FGFR cascade in levamisole receptor cell surface expression and/or clustering.

In vertebrates, such functions have been demonstrated for another RTK, namely MuSK (see introduction). We thus analyzed two mutations in CAM-1, which shows the highest homology to MuSK, and found that *cam-1(ak37)*, containing a premature stop before the TM domain (M Francis and A Maricq, personal communication), significantly reduced synaptic LEV-1::4xHA expression (by 22%, $P<0.0006$; Figure 5C) and conferred resistance to levamisole (Figure 5B). Also, deletion of the kinase domain alone (allele *ks52*; Koga *et al*, 1999) caused levamisole resistance (Figure 5B); however, *ks52* did not reduce synaptic levamisole receptor levels (Figure 5C), and neither *cam-1* allele was nicotine resistant (Figure 5A). These results indicate a complex involvement of *cam-1* also in levamisole receptor expression. To study whether CAM-1 and EGL-15 may cooperatively regulate synaptic expression of levamisole receptors, we depleted each RTK by RNAi either in *egl-15(n1477)* or *cam-1(ak37)* animals, respectively, and analyzed synaptic LEV-1::4xHA expression with injected antibodies (Figure 7D). Significantly, synaptic LEV-1 was reduced for both combinations of mutation and RNAi depletion relative to either mutant alone. Thus, EGL-15 and CAM-1 may act in parallel to govern synaptic levamisole receptor expression. Reduction of synaptic LEV-1 in both *egl-15* and *cam-1* mutants was further enhanced by SOC-1 RNAi, suggesting that loss of SOC-1 in *cam-1* mutants might reduce signaling also through EGL-15, and *vice versa* (Figure 7D). Like *soc-1(n1789)* (Figure 4C), *egl-15* and *cam-1* mutants exhibited uncoordinated phenotypes (Supplementary Figure 4C). However, in contrast to SOC-1, they did not affect muscimol sensitivity, indicating that they do not influence GABAR expression. Though many details of these putative signaling pathways remain to be investigated, our findings suggest that the functional interaction between nAChRs and SOC-1 may

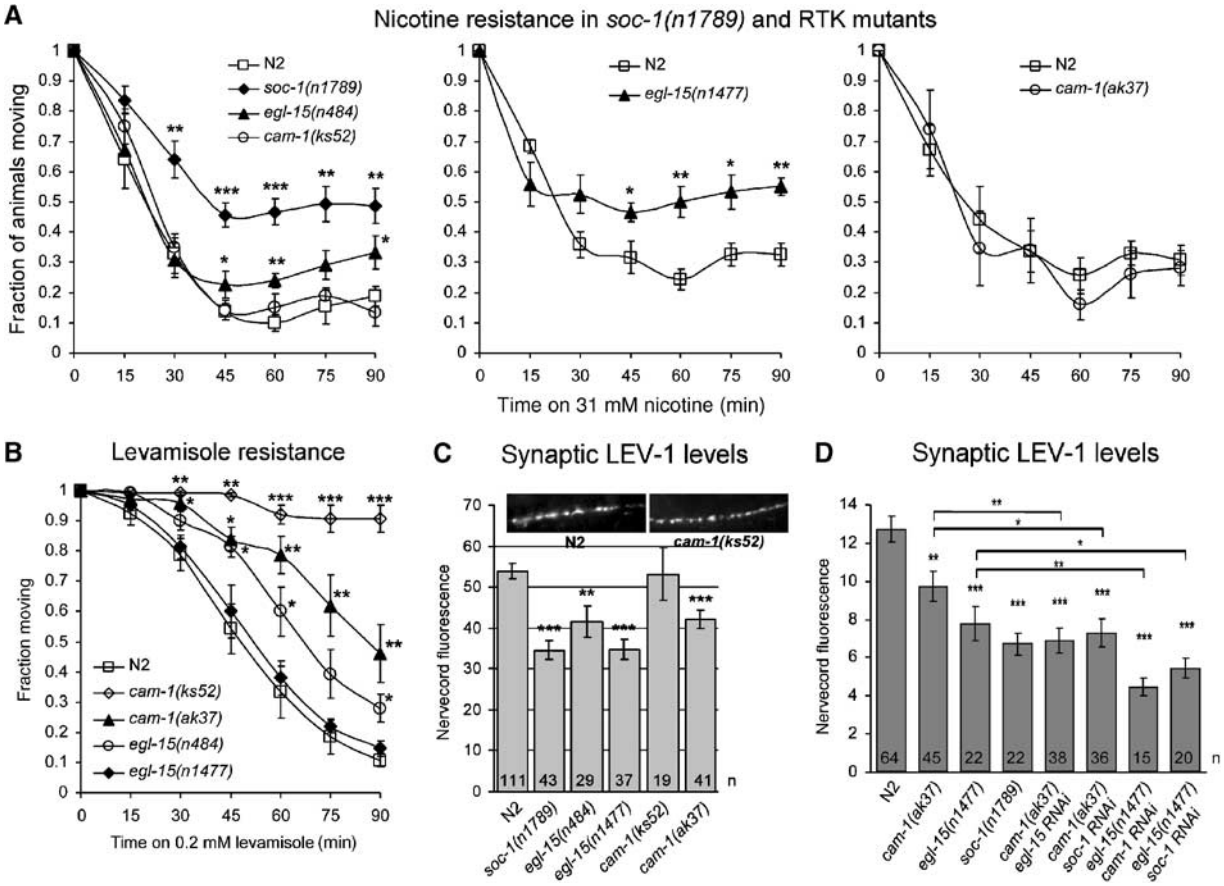

Figure 7 Mutations in *soc-1*, *egl-15*, and *cam-1* affect levamisole receptor function and synaptic expression. (**A**) Responses to 31 mM nicotine in paralysis assays were compared in WT (N2), *soc-1(n1789)*, *egl-15(n484* and *n1477)*, and *cam-1(ks52* and *ak37)* mutant animals, showing significant resistance for *soc-1* and *egl-15* mutants. (**B**) Paralysis by 0.2 mM levamisole was compared in WT (N2), *egl-15(n484* and *n1477)*, and *cam-1(ks52* and *ak37)* mutant animals, showing significant resistance for all mutants, except *n1477* (3–7 experiments, $n = 30$ each, in (A) and (B)). (**C**) Synaptic LEV-1::4xHA levels, quantified after *in vivo* antibody staining, were compared in WT (N2), *soc-1(n1789)*, *egl-15(e484* and *n1477)*, and *cam-1(ks52* and *ak37)* mutant backgrounds (strains AQ881, AQ1056, AQ1057, ZX174, AQ884, and ZX97, respectively) ($n = 19$–111 animals). The inset shows representative images of ventral nervecords stained with anti-HA antibodies. (**D**) Synaptic expression levels of LEV-1::4xHA, analyzed after *in vivo* antibody labeling in WT (N2), *cam-1(ak37)*, *egl-15(n1477)*, and *soc-1(n1789)* mutants (strains AQ881, ZX97, ZX174, and AQ1056, respectively), as well as in *cam-1(ak37)* and *egl-15(n1477)* mutants also depleted by RNAi of EGL-15, CAM-1, or SOC-1, as indicated ($n = 15$–64 animals). In all cases, significant reduction of synaptic LEV-1::4xHA relative to the WT was found. However, synaptic LEV-1 was further reduced in *ak37*/EGL-15 RNAi, *ak37*/SOC-1 RNAi, *n1477*/CAM-1 RNAi, and *n1477*/SOC-1 RNAi 'double' loss-of-function animals, relative to the *ak37* and *n1477* mutants, respectively. In (C) and (D), fluorescence of the ventral nervecord of adult animals was quantified near the vulva. $^{***}P < 0.001$, $^{**}P < 0.01$; $^{*}P < 0.05$, error bars = s.e.m. in all panels.

represent a mechanism for regulation of synaptic nAChR clustering and/or maintenance through RTK signaling.

Discussion

We have shown (to our knowledge for the first time) that tandem affinity purification can be used to isolate an integral membrane protein complex of low abundance, the *C. elegans* levamisole-sensitive nAChR, from its native environment in a multicellular organism. Even though the yields of purified receptor complex were low, we could identify novel proteins that functionally interact with this nAChR by combining proteomic analysis with genetic and behavioural assays. Most of these proteins have not previously been implicated in affecting the expression or modulation of nAChRs. Others, like calcineurin and BiP, were known to affect nAChR functional properties or expression (Blount and Merlie, 1991; Khiroug *et al*, 1998; Liu and Berg, 1999). Copurification of these proteins with the levamisole receptor may indicate direct interactions. Alternatively, some of the proteins identified may interact indirectly, perhaps through a protein scaffold containing this nAChR. Since the high sensitivity of mass spectrometry certainly led to the identification also of unspecific contaminants, especially among abundant proteins in *C. elegans* extracts (Mawuenyega *et al*, 2003), the use of RNAi to identify nicotine-resistance genes was an invaluable secondary screen that allowed us to focus on proteins that affect either the functional properties of nAChRs (like calcineurin), or their synaptic expression (like the copine NRA-1).

We identified five novel non-nAChR proteins that cause nicotine resistance when depleted. For two of those, the copine NRA-1 and the multisubstrate adaptor SOC-1, we could show a requirement for maintenance of normal levels of nAChRs at synaptic sites. Our findings indicate that RTK signaling, utilizing the multisubstrate adaptor SOC-1, promotes clustering and/or maintenance of both nAChRs and GABARs at synaptic sites in an intact animal, expanding previous observations made in cell culture (Peng *et al*,

1991). Our data further imply that SOC-1-like adaptor proteins may do so via direct or indirect physical interaction with neurotransmitter receptor proteins.

The association of the copine NRA-1 with the levamisole receptor gives new insight into the functions of this novel, widely occurring family of proteins (Creutz *et al*, 1998). Since copines, after binding Ca^{2+}, have the potential to direct other proteins to phospholipid membranes (Tomsig *et al*, 2003), NRA-1 may recruit proteins that interact with the levamisole receptor, possibly in an activity-dependent manner. Deletion of *nra-1* caused resistance to cholinergic agonists and reduced synaptic levamisole receptor levels, but did not affect synaptic levels of GABARs; thus, NRA-1 may play a relatively specific role in targeting or stabilizing the levamisole receptor at the plasma membrane.

Among the remaining proteins that we copurified are many whose known or inferred function may suggest a possible role in nAChR biology, for example, a casein kinase I homolog (F46F2.2), depletion of which caused nicotine hypersensitivity. Possibly even some of the copurified proteins lacking nicotine sensitivity phenotypes (Supplementary Table 2) may function in nAChR biology and have escaped detection due to ineffective RNAi or subtle phenotypes. Many of the proteins copurified with the levamisole receptor bear membrane-spanning domains, and may thus interact with it in the membrane environment. Future experiments, including coimmunoprecipitation, membrane-specific protein interaction assays, and electrophysiology, will allow us to define the exact functions of the novel nAChR interactors identified in our study.

Most of the proteins identified have vertebrate homologs; thus, their functions may be conserved in humans and could provide new insight into the molecular mechanisms underlying nicotine addiction. Our combined proteomic and genetic approach to define functional protein complexes in the multicellular organism *C. elegans* holds great potential for future applications, not only for the study of nAChRs, but also for analysis of other protein complexes in the nervous system and other tissues.

Materials and methods

Extract preparation, Western analysis, and TAP

Nematodes were resuspended in buffer D (20 mM Tris–HCl, pH 7.9; 150 mM NaCl; 10% glycerol). Then we added, adjusted to the total volume: 0.5 mM DTT; 0.5 mM PMSF; 1.0 mM EDTA; 1.0 μg/ml *N*α-*p*-tosyl-L-arginin-methyl-ester; 2.0 μg/ml of each pepstatin, leupeptin, and chymostatin (Sigma). The suspension was dripped into liquid N_2 and stored at −80°C. Frozen suspension, corresponding to 40 g of worms, was ground under liquid N_2 to a fine powder, which was slowly thawed on ice (as all subsequent steps). After dilution with buffer D and additives (see above) to 160 ml and homogenization in a glass tissue homogenizer (Kontes, New Jersey), the suspension was centrifuged at 22 500 *g* for 30′. The supernatant was centrifuged for 1 h at 125 000 *g*, the pellets of both centrifugations combined and resuspended in 120 ml buffer D with additives and 1% Triton-X 100. The suspension was homogenized and gently stirred for 2 h. After centrifugation at 22 500 *g* for 30′, the supernatant was centrifuged for 1 h at 125 000 *g*. The clear interphase was collected and dialyzed twice for 2 h against buffer D with 0.05% Triton-X 100. The TAP tag was immunochemically detected using peroxidase antiperoxidase soluble complex (PAP, Sigma), on blots of either total nematode lysate or IgG agarose immunoprecipitates from ca. 200 μl of extract. Tandem affinity purification was performed as described, substituting NP-40 by 0.05% Triton-X 100 in all buffers (Rigaut *et al*, 1999). Purification with a TAP tag on UNC-29 (strain AQ748) yielded all levamisole receptor subunits; however, UNC-29 was enriched (14 individual peptides identified, compared to 3–5 for the other subunits), indicating copurification of UNC-29 monomers. Purification using the split TAP tag (strain AQ839) yielded all subunits in similar abundance of 3–8 individual peptides.

RNAi screen

For RNAi, we used *rrf-3(pk1426)* mutants (Simmer *et al*, 2002), either by feeding bacterial strains from the Ahringer library (Kamath *et al*, 2003), or (in few cases) by soaking with *in vitro* transcribed double-stranded RNA (Maeda *et al*, 2001). For feeding, bacteria were grown on NGM plates containing 25 μg/ml carbenicillin for 48 h, then transcription was induced by spotting 100 μl of 100 mM IPTG onto the bacterial lawn. Three L4 animals were placed onto the dried plate, and transferred to a freshly induced plate after 24 h. Nicotine sensitivity of the progeny was scored 96 h later (see below). For candidates causing nicotine sensitivity phenotypes, the RNAi experiment was repeated to verify the finding.

Drug response and thrashing assays

A total of 30 young adult animals were transferred to NGM plates containing 0.425 or 0.5% (v/v) (−)-nicotine, or 1 or 0.2 mM levamisole. Paralysis was followed by visual inspection every 15′ and defined as lack of movement in response to prodding. For thrashing assays, animals were placed into M9 salt solution in 96-well plates, with a pad of NGM at the bottom of the wells, either without or with 1.5 mM muscimol for 1 h. Then, animals were filmed and body thrashes per minute were counted for 20 or more animals per genotype and drug condition and averaged.

In vivo antibody-binding assay

Animals expressing C-terminally epitope-tagged versions of nAChR subunits, or N-terminally tagged UNC-49 GABAR subunits, were mounted on dry agarose pads under halocarbon oil. They were injected into the pseudocoelom with fluorescently labeled monoclonal antibodies (anti-HA, clone 16B12, coupled to Alexa488, Molecular Probes; or anti-cMYC, clone 9E10, coupled to Cy3, Sigma) in a 1:200 dilution in injection buffer (20 mM K_3PO_4, 3 mM K citrate, 2% PEG 6000, pH 7.5). We injected until a few eggs were pushed out, assuming that this happens once a certain internal pressure is reached, and thus ensuring roughly equivalent concentration of antibody from animal to animal. Animals were recovered from the pads in M9 salt solution, and transferred to NGM plates seeded with OP50. Animals that moved normally, fed and laid eggs, were imaged after ca. 6 h (during this time, the coelomocytes took up excess antibody from the body fluid). Quantitative analysis of nervecord fluorescence was performed using ImageJ. Line scans were traced along fluorescent puncta of the ventral nervecord, which we analyzed in the midbody of the worm, within ca. 1/4 of the length of the animal either anterior or posterior of the vulva. After background correction, fluorescence values were averaged for individual line scans; then, the averaged values of line scans from 15–111 different animals of the same genotype and experimental conditions were averaged.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Note added in proof

While this paper was undergoing final revisions, Francis *et al* (2005) *Neuron* **46:** 581 reported that mutation of *cam-1* affects synaptic currents at the NMJ, specifically for a *C. elegans* nicotine-sensitive nAChR comprising the ACR-16 subunit.

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