

Clathrin-mediated endocytosis is required for compensatory regulation of GLR-1 glutamate receptors after activity blockade

Maria E. Grunwald*[†], Jerry E. Mellem[‡], Nathalie Strutz^{*§}, Andres V. Maricq[‡], and Joshua M. Kaplan*^{†¶}

*Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114; and [†]Department of Biology, University of Utah, Salt Lake City, UT 84112

Edited by Corey S. Goodman, Renovis, South San Francisco, CA, and approved January 9, 2004 (received for review September 24, 2003)

Chronic changes in neural activity trigger a variety of compensatory homeostatic mechanisms by which neurons maintain a normal level of synaptic input. Here we show that chronic activity blockade triggers a compensatory change in the abundance of GLR-1, a *Caenorhabditis elegans* glutamate receptor. In mutants lacking a voltage-dependent calcium channel (*unc-2*) or a vesicular glutamate transporter (VGLUT; *eat-4*), the abundance of GLR-1 in the ventral nerve cord was increased. Similarly, the amplitude of glutamate-evoked currents in ventral cord interneurons was increased in *eat-4* VGLUT mutants compared with wild-type controls. The effects of *eat-4* VGLUT mutations on GLR-1 abundance in the ventral cord were eliminated in double mutants lacking both the clathrin adaptin protein *unc-11* AP180 and *eat-4* VGLUT. In contrast, mutations that decreased ubiquitination of GLR-1 did not prevent increased ventral cord abundance of GLR-1 in *eat-4* VGLUT mutants. Taken together, our results suggest that GLR-1 is regulated in a homeostatic manner and that this effect depends on clathrin-mediated endocytosis but does not require ubiquitination of GLR-1.

Regulation of glutamate receptor (GluR) abundance at synapses has been proposed as a mechanism for expressing activity-dependent changes in synaptic strength. There are two categories of activity-dependent plasticity. Hebbian forms of plasticity, such as long-term potentiation and long-term depression, are triggered by acute changes in activity, whereas homeostatic plasticity is triggered by chronic changes in activity. Induction of long-term potentiation increases the synaptic abundance of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (1, 2), whereas long-term depression decreases their abundance (3). Likewise, some forms of homeostatic plasticity are associated with changes in the abundance of AMPA receptors at synapses (4–6). Manipulations that chronically suppress activity increased AMPA receptor abundance at synapses, whereas those that enhance activity decreased their synaptic abundance. This homeostatic change in receptor abundance has been termed synaptic scaling. Synaptic scaling has been studied primarily in cultured rodent neurons (4–6); however, a similar phenomenon has also been described at the *Drosophila* neuromuscular junction (7).

AMPA receptors at synapses are derived from a pool of rapidly recycling receptors. AMPA receptors are inserted into synaptic membranes by exocytosis (8, 9) and are removed by clathrin-mediated endocytosis (3, 10–13). Endocytosis of AMPA receptors can be stimulated by GluR agonists and by insulin (11, 12, 14–18). In *Caenorhabditis elegans*, conjugation of ubiquitin to a GluR, GLR-1, triggers its endocytosis and degradation (13). Clathrin-mediated endocytosis is required for induction of long-term depression in rat neurons (9, 10, 12, 18–20). The role of endocytosis in homeostatic forms of plasticity has not been determined.

We have used the nematode *C. elegans* as a genetic model to study regulation of GluR abundance at synapses after chronic changes in activity. The *C. elegans* genome encodes two *N*-

methyl-D-aspartate (NMDA) and eight non-NMDA receptor subunits (21), one of which is encoded by the *glr-1* gene (22, 23). The *glr-1* GluRs are localized at sensory–interneuron and interneuron–interneuron synapses in the ventral cord (13, 24). Here we show that the abundance of GLR-1 in the ventral cord is regulated by a homeostatic compensatory mechanism and that this process depends on clathrin-mediated endocytosis.

Materials and Methods

Strains. Standard methods were used to culture the following alleles and transgenes: *eat-4(n2474)*, *eat-4(ky5)*, *eat-4(ak75)*, *unc-11(e47)*, *nuls25* GLR-1::GFP, *nuls108* GLR-1(4KR)::GFP, *akIs3 nmr-1::gfp* (transcriptional fusion), and *nuls1 glr-1::gfp* (transcriptional fusion).

Transgenes and Germ-Line Transformation. Plasmids were constructed by standard techniques, and sequences were verified where appropriate; full details are available on request. Transgenic strains were isolated by microinjecting various plasmids (typically at 20–100 ng/ μ l) by using either *ttx-3::gfp* (O. Hobert, Columbia University, New York) or GLR-1::GFP as a marker. The plasmid KP#749 contains a CFP::SNB-1 fusion gene (24) expressed with the *eat-4* promoter. The plasmid pSB110, containing the *snb-1* synaptobrevin coding sequence, was kindly provided by M. Nonet (Washington University, St. Louis). The plasmid KP#747 contains a 5.9-kb *eat-4* promoter driving the expression of a cyan fluorescent protein-tagged EAT-4 protein. Expression of this EAT-4::CFP transgene is sufficient to rescue the nose touch sensitivity defect of *eat-4* vesicular glutamate transporter (VGLUT) mutants.

Quantitative Fluorescence Microscopy. All imaging was done on a Zeiss Axiovert 100 microscope using an Olympus Planapo \times 100 objective (numerical aperture, 1.4) and an ORCA digitally cooled charge-coupled device camera (Hamamatsu, Middlesex, NJ). Animals were immobilized with 10 mM levamisole. Maximum intensity projections of Z-series stacks of ventral cord images (with no thresholding) were obtained by using META-MORPH 4.5 software (Universal Imaging, Media, PA). Line scans of ventral cord fluorescence were obtained with META-MORPH 4.5 and analyzed by using IGOR software (WaveMetrics, Lake Os-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GluR, glutamate receptor; VDCC, voltage-dependent calcium channel; VGLUT, vesicular glutamate transporter; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate.

[†]Present address: Institute for Neurodegenerative Disease, Massachusetts General Hospital, Harvard Medical School, 114 16th Street, Charlestown, MA 02129.

[§]Present address: Institute of Physiology I, University of Tübingen, Gmelinstrasse 5, D-72076 Tübingen, Germany.

[¶]To whom correspondence should be addressed at: Department of Molecular Biology, Massachusetts General Hospital, Wellman 8, 50 Blossom Street, Boston, MA 02114. E-mail: kaplan@molbio.mgh.harvard.edu.

© 2004 by The National Academy of Sciences of the USA

wego, OR). Each line scan corresponds to $\approx 80 \mu\text{m}$ of the ventral cord. For the sake of consistency, all images were taken in a single region of the ventral cord (lying just posterior to the vulva). Puncta amplitudes ($\% \Delta F/F$), widths (at half-maximal peak fluorescence), and densities were estimated in an automated manner in IGOR, using custom written algorithms as described in ref. 13. Statistical significance was determined by two-tailed Student's *t* test. In some cases, cumulative probability distributions are shown for all puncta of a given genotype (i.e., pooled from all line scans). Values represent averaged data from all animals examined. We also compared averaged values from experiments performed on specific days and observed the same changes.

Electrophysiology. Voltage clamp recordings of glutamate-evoked currents in the interneuron AVA were done as described in ref. 25. To identify AVA, we used a strain (*akIs3*) that expresses a transcriptional fusion of the *nmr-1* promoter and GFP and shows fluorescent staining in all ventral cord interneurons except AVB. Adult hermaphrodites were glued to Sylgard-coated coverslips and manually dissected with glass needles. Adherent tissue was removed by rinsing with extracellular fluid (ECF) and by digestion with collagenase. Membrane currents from AVA cell bodies were recorded at room temperature in the whole-cell configuration using a patch-clamp amplifier (EPC-9, HEKA Elektronik, Lambrecht, Germany). Currents were evoked by a 0.75-s pulse of 1 mM glutamate in ECF applied with a Picospritzer II (General Valve, Fairfield, NJ) to the nerve-ring region containing the interneuron cell bodies. The standard pipette solution contained 115 mM K-gluconate, 25 mM KCl, 0.1 mM CaCl_2 , 50 mM Hepes, 5 mM MgATP, 0.5 mM Na-GTP, 0.5 mM Na-cAMP, and 1 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate, pH adjusted to 7.35 with KOH. The standard ECF contained 150 mM NaCl, 5 mM KCl, 5 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, and 15 mM Hepes, pH adjusted to 7.35 with NaOH. Osmolarity of the pipette solution (340 mOsm) and the ECF (345 mOsm) were adjusted with sucrose.

Western Blots. Mixed-stage worms from enriched worm plates were washed with M9, lysed, and homogenized by sonication. This whole-worm homogenate was used for estimating expression levels. Proteins were separated by SDS/PAGE, and GFP-fusion proteins were detected by quantitative Western blotting with mouse monoclonal anti-GFP antiserum (Roche) using enhanced chemiluminescence (Pierce). Peroxidase-coupled anti-mouse antisera were from Amersham Biosciences. Abundance of GLR-1::GFP in different transgenic strains was expressed as a fraction of the total protein concentrations in worm extracts. Protein concentrations in worm extracts were determined by using the bicinchoninic acid reagent (Pierce).

Results

Chronic Activity Blockade Increases GLR-1 Abundance at Synapses. Mutations in the *unc-2* gene, which encodes an α -subunit of a voltage-dependent calcium channel (VDCC), greatly reduce evoked synaptic release of neurotransmitter and are predicted to decrease excitability of neurons and muscles (26, 27). We examined the distribution of GLR-1 receptors in mutants lacking *unc-2* VDCC to determine whether chronic blockade of neural activity altered receptor abundance. We visualized the distribution of GLR-1 receptors in the ventral nerve cord of living animals by expressing a GFP-tagged form of GLR-1 (GLR-1::GFP) (13, 24, 28). GLR-1::GFP is localized in punctate structures in the ventral nerve cord. We previously showed that $>80\%$ of these puncta were closely apposed to presynaptic markers (synaptobrevin or a VGLUT) (13), suggesting that a large fraction of GLR-1::GFP puncta correspond to postsynaptic elements. We measured the abundance of GLR-1::GFP (24) at

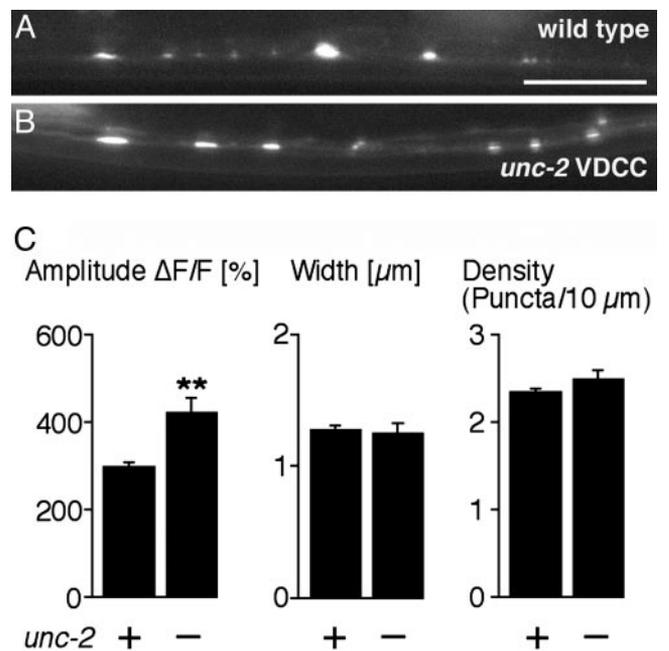


Fig. 1. Mutants lacking *unc-2* VDCC had increased abundance of GLR-1::GFP in the ventral cord. Representative images of GLR-1::GFP (*nuls25*) in the ventral cords of wild-type (A) and *unc-2*(*e55*) (B) animals. In a region posterior to the vulva, GLR-1::GFP puncta were brighter in *unc-2*(*e55*) VDCC animals (B) than those observed in wild type (A). A 10- μm scale bar for A and B is shown in A. (C) Puncta amplitudes, widths, and densities are compared in wild-type ($n = 180$) and *unc-2*(*e55*) VDCC ($n = 29$) animals. Values that differ significantly from wild type are indicated as follows: *, $P < 0.01$; **, $P < 0.001$.

puncta in the ventral nerve cord by quantitative fluorescence microscopy, as described in ref. 13, and found that puncta amplitudes were significantly increased in *unc-2* VDCC mutants compared with wild-type animals (Fig. 1). The width and density of GLR-1::GFP puncta in *unc-2* VDCC mutants were not significantly different from those of wild-type animals (Fig. 1C). These results suggested that chronic activity blockade increased the synaptic abundance of GLR-1.

Chronic Blockade of Glutamate Release Increases GLR-1 Abundance at Synapses.

The effects of *unc-2* VDCC mutations on GLR-1 could be mediated by changes in cell excitability or by changes in presynaptic transmitter release. To distinguish between these possibilities, we examined the distribution of GLR-1::GFP in *eat-4* mutants, which are defective for glutamatergic synaptic transmission (29, 30). The *eat-4* gene encodes a VGLUT (29, 31–33), which fills synaptic vesicles with glutamate. We found that GLR-1::GFP puncta amplitudes and widths were significantly increased in *eat-4* VGLUT mutants compared with wild-type animals (Fig. 2 A, B, and D), indicating that the synaptic abundance of GLR-1 was increased. The density of GLR-1::GFP puncta in *eat-4* VGLUT mutants was not significantly different from that in wild-type animals (Fig. 2D).

We performed two experiments to confirm that the changes in GLR-1::GFP distribution were caused by the *eat-4* VGLUT mutation. First, we showed that similar changes in GLR-1::GFP synaptic fluorescence were observed in strains carrying two independent *eat-4* alleles (*n2474* and *ky5*) (Fig. 2 and data not shown). The allele *n2474* corresponded to a nonsense allele (W199Stop), and *ky5* corresponded to a small deletion (≈ 300 nt) in the 5' half of the gene; therefore, both alleles were likely to severely reduce the function of the *eat-4* VGLUT. Second, we showed that the increased puncta amplitudes in *eat-4* VGLUT

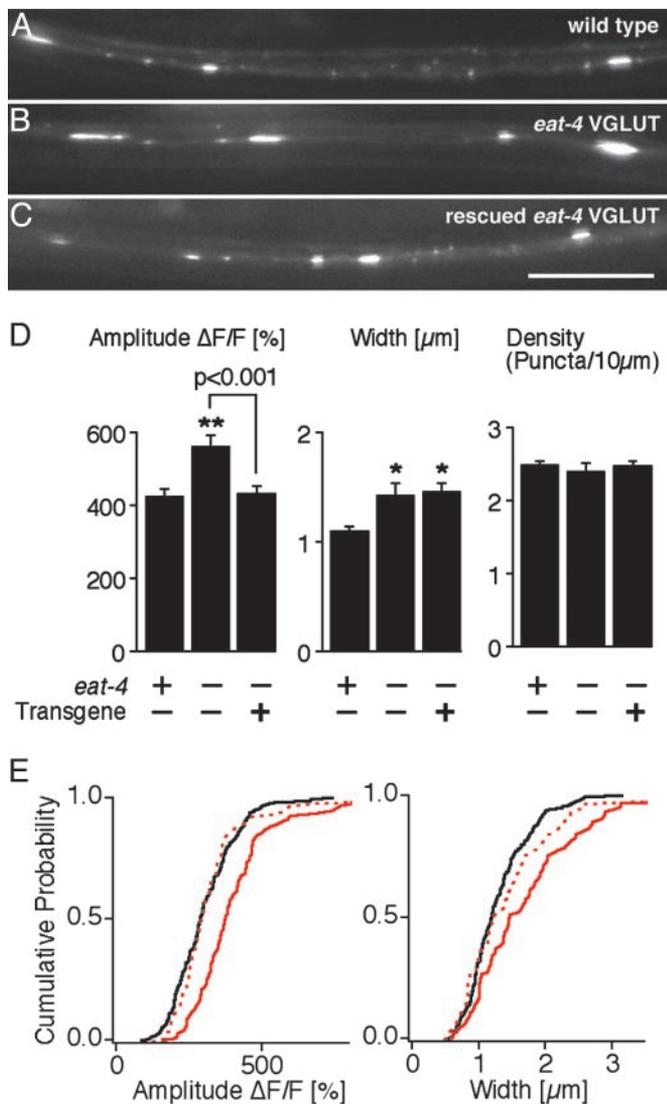


Fig. 2. Mutants lacking *eat-4* VGLUT had increased abundance of GLR-1::GFP in the ventral cord. Representative images of GLR-1::GFP (*nuls25*) in the ventral cords of wild-type (A), *eat-4(n2474)* (B), and transgenic *eat-4(n2474)* VGLUT animals expressing a wild-type *eat-4* VGLUT genomic construct (KP#747) (C). In a region posterior to the vulva, GLR-1::GFP puncta were wider and brighter in *eat-4(n2474)* VGLUT animals (B) than those observed in wild type (A) and in mutants rescued with the *eat-4* transgene (C). A 10- μm scale bar for A–C is shown in C. (D) Puncta amplitudes, widths, and densities are compared in wild type ($n = 46$), *eat-4(n2474)* VGLUT ($n = 37$) mutants, and *eat-4(n2474)* VGLUT mutants rescued with the *eat-4* transgene ($n = 52$). Values that differ significantly from wild type are indicated as follows: *, $P < 0.01$; **, $P < 0.001$. (E) Cumulative probability distributions of puncta amplitude and width of wild type (black) and *eat-4(n2474)* mutant (red) puncta are compared. The amplitudes and widths of *eat-4(n2474)* are uniformly shifted to larger values than those observed in wild type. Scaled reductions of 30% and 27% in the *eat-4(n2474)* amplitude and width, respectively, are shown as dashed red lines.

mutants were rescued by transgenes expressing wild-type *eat-4* VGLUT (Fig. 2 C and D). Increased puncta widths were not rescued, which could reflect incomplete rescuing activity of the transgene or a dominant effect of overexpressing the *eat-4* VGLUT.

It remained possible that *eat-4* VGLUT mutations caused an overall increase in the levels of GLR-1::GFP in the ventral cord, i.e., increasing both synaptic and nonsynaptic receptors. To

estimate the levels of nonsynaptic GLR-1 receptors, we measured the level of diffuse GLR-1::GFP fluorescence in the ventral cord (interpunctal fluorescence) as the ratio of the ventral cord fluorescence between puncta to the fluorescence measured on a region of the coverslip that lacked animals. The interpunctal fluorescence was not significantly increased in *eat-4* mutants ($51.4 \pm 1.5\%$ $\Delta F/F$ WT, $45.8 \pm 2.6\%$ $\Delta F/F$ *n2474*, and $54.1 \pm 1.9\%$ $\Delta F/F$ *ky5*), suggesting that chronic blockade of glutamate release at synapses caused an increased accumulation of GLR-1::GFP at postsynaptic elements rather than a uniform increase in ventral cord fluorescence.

In synaptic scaling, AMPA receptor levels are globally regulated at all synapses and synaptic strengths are multiplicatively scaled (4–6). To determine whether the increased GLR-1::GFP fluorescence observed in *eat-4* VGLUT mutants reflected a global change in all synapses, we compared the distributions of puncta amplitudes and widths in the posterior ventral cords of *eat-4* VGLUT mutants versus wild-type animals (Fig. 2E). We found that the wild-type puncta amplitude distribution was well fit by a multiplicatively scaled version of the *eat-4* VGLUT distributions. In the case of puncta widths, the *eat-4* VGLUT mutation had a slightly disproportionate effect on larger puncta. These results suggest that synaptic release of glutamate uniformly regulates the abundance of GLR-1 receptors across the entire population of puncta in the posterior ventral cord.

Blocking Glutamate Release Does Not Alter the Expression Level of GLR-1 Receptors.

The increased abundance of GLR-1::GFP at synapses in *eat-4* VGLUT mutants could be explained by an increase in the expression level of GLR-1 receptors. We tested this possibility using two separate experimental approaches. First, we analyzed expression of a transcriptional reporter construct (*nuls1*) in which the *glr-1* promoter drives expression of GFP. We found that the expression of GFP produced by this construct in *eat-4* VGLUT mutants [$98,803 \pm 3,505$ arbitrary units (AU)/ μm] was not significantly different from that observed in wild-type controls ($100,797 \pm 2,459$ AU/ μm ; $P = 0.6$). Second, we measured the total abundance of GLR-1::GFP in wild type and *eat-4* VGLUT mutants by quantitative Western blots and found that they were indistinguishable (wild type, 5.0 ± 0.4 AU; *eat-4*, 4.4 ± 0.4 AU; $P = 0.3$). Therefore, the increased puncta fluorescence observed in *eat-4* VGLUT mutants was not caused by increased expression of GLR-1.

Chronic Blockade of Glutamate Release Increases the Amplitude of Glutamate-Evoked Currents.

The increased GLR-1::GFP fluorescence in *eat-4* VGLUT mutants could reflect an increase in the abundance of receptors in the plasma membrane or in internal organelles (e.g., endosomes). To distinguish between these possibilities, we compared the amplitudes of glutamate-activated currents in wild-type and *eat-4* VGLUT mutant interneurons in whole-cell voltage-clamp recordings. We found significantly higher glutamate-activated current amplitudes in *eat-4* VGLUT mutants compared with those observed in wild-type controls ($P < 0.05$, ANOVA; Fig. 3). These results are consistent with the idea that chronic suppression of glutamate release at synapses results in a compensatory increase in the abundance of GLR-1 in the plasma membrane. However, we cannot exclude the possibility that changes in posttranslational modification of GLR-1 contribute to the difference in glutamate-evoked currents.

Clathrin-Mediated Endocytosis Is Required for Increased GLR-1::GFP Abundance in *eat-4* VGLUT Mutants.

Synaptic GLR-1 receptors are likely to be derived from a pool of recycling receptors undergoing exocytosis, endocytosis, and postendocytic degradation, as has been shown for rodent AMPA receptors (3, 10–12). Consistent with this idea, we previously showed that GLR-1::GFP is

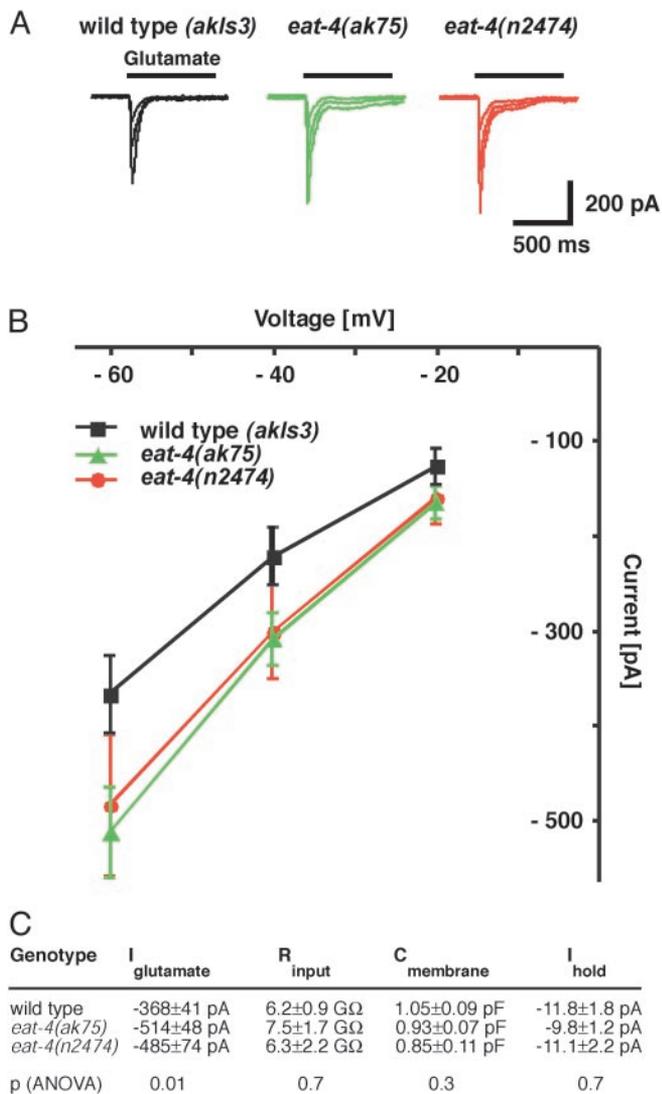


Fig. 3. Glutamate-gated current is increased in *eat-4* mutants. (A) Representative current traces from the three strains. Glutamate (1 mM) was pressure-applied onto the interneuron AVA, which was voltage-clamped at the indicated potential. Each set of current traces corresponds to holding potentials of -20 , -40 , and -60 mV. Glutamate-evoked currents had higher amplitudes in mutants lacking *eat-4* VGLUT. (B) Current-voltage relationship for glutamate-gated currents in wild type ($n = 10$) and in *eat-4(ak75)* ($n = 8$) and *eat-4(n2474)* ($n = 7$) mutants. Data shown are the mean current evoked at each potential and SEM. ANOVA indicates that the currents from either allele of *eat-4* are significantly different from wild type ($P < 0.05$). The parameters input resistance (R_{input}), membrane resistance ($C_{membrane}$), and holding current (I_{hold}) were indistinguishable among genotypes (C). Shown are values for glutamate-evoked currents ($I_{glutamate}$), R_{input} , $C_{membrane}$, and I_{hold} and corresponding P values from ANOVA.

removed from postsynaptic elements by clathrin-mediated endocytosis (13). If the accumulation of GLR-1::GFP at synapses in *eat-4* VGLUT mutants was caused by a change in recycling of GLR-1, then mutations that block endocytosis should prevent membrane recycling and thereby block the effects of the *eat-4* VGLUT mutation on synaptic abundance of GLR-1. On the other hand, if glutamate release regulates GLR-1 assembly or anterograde trafficking, we would expect that mutations in the endocytic machinery and *eat-4* VGLUT mutations would have additive effects on the synaptic abundance of GLR-1::GFP. We tested this idea by comparing puncta amplitudes and widths in *eat-4* VGLUT single mutants to those in *unc-11;eat-4* VGLUT

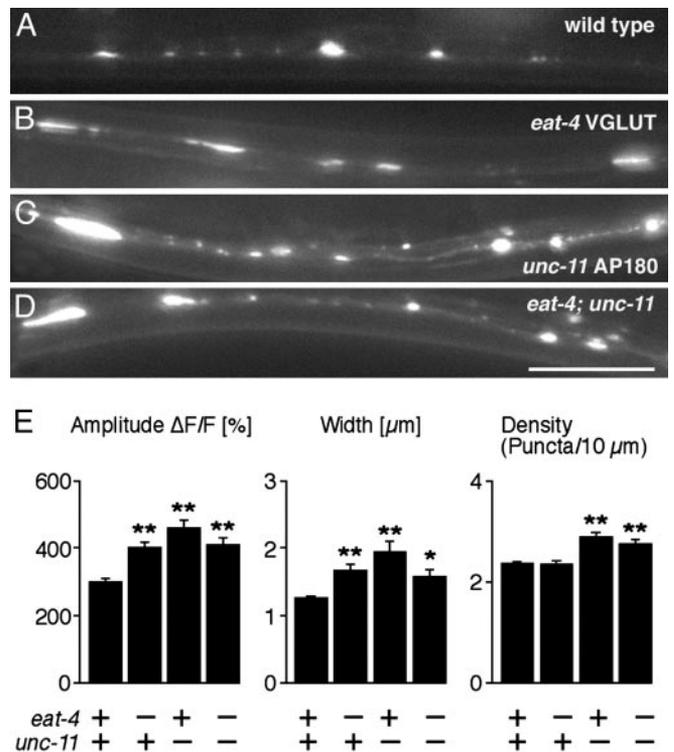


Fig. 4. Increased GLR-1::GFP abundance in *eat-4* VGLUT mutants depended on *unc-11* AP180. Shown are representative images of GLR-1::GFP (*nuls25*) in the ventral cords of wild type (A), *eat-4(n2474)* (B), *unc-11(e47)* (C), and *eat-4(n2474);unc-11(e47)* double mutants (D). A 10- μ m scale bar for A–D is shown in D. Puncta amplitudes and widths in *eat-4;unc-11* double mutants (D) were not different from those in *eat-4* (B) or *unc-11* (C) single mutants. (E) Puncta amplitudes, widths, and densities are compared in wild type ($n = 180$), *eat-4(n2474)* ($n = 95$), *unc-11(e47)* ($n = 45$), *eat-4(n2474);unc-11(e47)* double mutants ($n = 36$). Values that significantly differ from wild type are indicated as follows: *, $P < 0.01$; **, $P < 0.001$.

double mutants. The *unc-11* gene encodes the clathrin adaptin protein AP180 (34), which is required for endocytosis of several cargo proteins in *C. elegans*, flies, and yeast (13, 34–36). We previously showed that *unc-11* AP180 is required for removing GLR-1::GFP from synapses (13). We found that puncta amplitudes and widths in *unc-11* AP180;*eat-4* VGLUT double mutants were not significantly different from those in *eat-4* VGLUT single mutants, as would be predicted if the increased puncta fluorescence in *eat-4* VGLUT mutants were caused by a change in recycling of GLR-1 (Fig. 4).

An alternative interpretation of these results is that the failure to recycle presynaptic proteins in *unc-11* AP180 mutants (34) decreased glutamate release, thereby causing increased GLR-1 accumulation at synapses. However, expression of *unc-11* AP180 in presynaptic cells failed to restore normal GLR-1::GFP distribution in *unc-11* mutants (data not shown). Therefore, our results are most consistent with the idea that endocytosis in the ventral cord interneurons is required for regulating the synaptic abundance of GLR-1 after activity blockade.

Ubiquitination of GLR-1 Is Not Required for Changes in Synaptic Abundance Caused by *eat-4* VGLUT Mutations. Recently, we showed that ubiquitin is directly conjugated to the cytoplasmic tail of GLR-1::GFP and that ubiquitination of GLR-1 promotes endocytic removal of receptors from postsynaptic elements (13). Consistent with this idea, a mutation that prevents ubiquitination of GLR-1, GLR-1(4KR), increases the synaptic abundance of GLR-1. Therefore, a decrease of ubiquitination of GLR-1 after

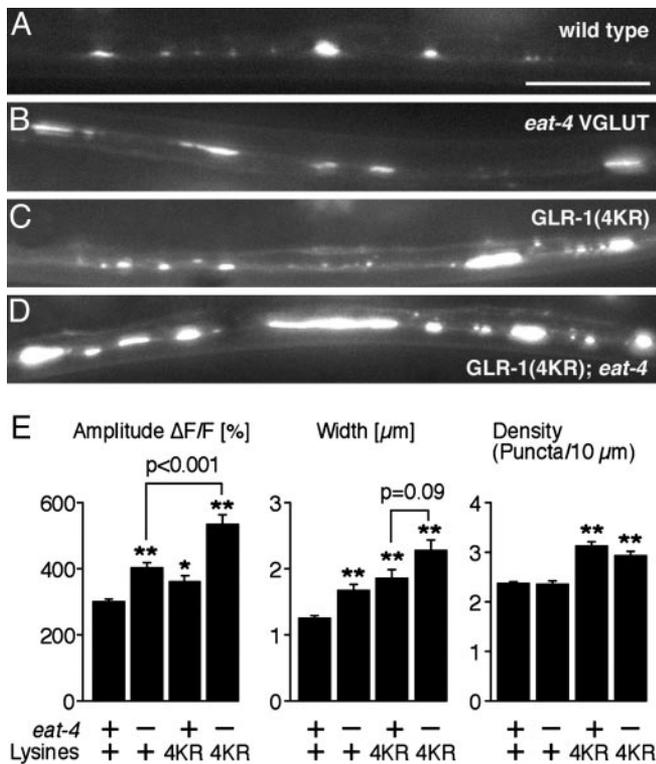


Fig. 5. Increased GLR-1::GFP abundance in *eat-4* VGLUT mutants was independent of ubiquitination of GLR-1. Representative images of GLR-1::GFP (*nuls25*) in the ventral cords of wild type (A), *eat-4(n2474)* (B), *nuls108* GLR-1(4KR)::GFP (C), and *nuls108* GLR-1(4KR)::GFP;*eat-4(n2474)* mutants (D). A 10- μm scale bar for A–D is shown in D. GLR-1(4KR)::GFP puncta were wider and brighter in *eat-4(n2474)* VGLUT animals (D) than those observed in wild-type (C) and GLR-1::GFP puncta in *eat-4(n2474)* (B) animals. (E) Puncta amplitudes, widths, and densities are compared in wild type ($n = 180$), *eat-4(n2474)* ($n = 95$), *nuls108* GLR-1(4KR)::GFP ($n = 29$), and *nuls108* GLR-1(4KR)::GFP;*eat-4(n2474)* ($n = 26$). Values that significantly differ from wild type are indicated as follows: *, $P < 0.01$; **, $P < 0.001$.

activity blockade may lead to an increase in the synaptic abundance of GLR-1::GFP. To test whether changes in the ubiquitination of GLR-1 are required for the compensatory increase in GLR-1::GFP after activity blockade, we examined the abundance of GLR-1(4KR) in *eat-4* VGLUT mutants. We found that puncta amplitudes and widths in *eat-4* VGLUT;GLR-1(4KR)::GFP double mutants were significantly greater than those found in either single mutant (Fig. 5). These results suggest that ubiquitination of GLR-1 was not required for the accumulation of GLR-1::GFP in the ventral cord caused by activity blockade.

Discussion

Here we examined how abundance of the *C. elegans* GLR-1 receptor is altered after chronic blockade of activity. Our work leads to three conclusions. First, blockade of synaptic release of glutamate increased the abundance GLR-1 receptors at ventral cord puncta and increased the amplitude of glutamate-evoked currents. These results suggest that GLR-1 abundance at postsynaptic elements is regulated by a compensatory homeostatic mechanism. Second, the increase in GLR-1 abundance at postsynaptic elements did not occur when clathrin-mediated endocytosis was blocked. This result suggests that the homeostatic regulation requires active recycling of GLR-1 receptors. Third, this phenomenon did not require changes in ubiquitination of GLR-1.

Our results are similar in some respects to earlier work done on cultures of rodent neurons (4–6) and on the *Drosophila*

neuromuscular junction (7). After activity blockade in *C. elegans*, we found that total receptor fluorescence at postsynaptic elements was globally increased in a multiplicative manner. The amplitude of glutamate-evoked currents was increased by a similar scaling factor, suggesting that compensation resulted in increased levels of GLR-1 in the plasma membrane. Our studies do not address one hallmark of homeostatic plasticity. In both cultured rodent neurons and in *Drosophila*, chronic activity blockade triggers pre- and/or postsynaptic modifications that restore synaptic efficacy (6, 7, 37–39). Because we are unable to directly measure synaptic efficacy and quantal size, we cannot make explicit comparisons of compensatory regulation of GLR-1 to synaptic scaling or homeostatic plasticity in *Drosophila*. Nonetheless, it remains possible that the biochemical mechanisms underlying compensatory regulation of GLR-1 may be similar to those occurring in quantal scaling in other systems.

Our work differs in some respects from a prior study done on the *Drosophila* neuromuscular junction. Featherstone *et al.* (40) showed that increased presynaptic glutamate levels in motor neurons decreased the abundance of GluRs in postsynaptic muscles. Thus, this result and our studies support the idea that glutamate secretion down-regulates postsynaptic GluR abundance. However, our results suggest that vesicular release of glutamate regulates postsynaptic abundance of GLR-1. By contrast, Featherstone *et al.* (40) argue that the source of glutamate regulating GluRs during development was nonvesicular. It is possible that this discrepancy reflects biological differences between the two systems. For example, nonvesicular secretion of glutamate may be more prevalent at neuromuscular junctions than at neuron–neuron synapses.

Our results suggest that changes in the expression of *glr-1* cannot explain changes in GLR-1 abundance after activity, consistent with a prior study of cultured rat hippocampal neurons (4). Interestingly, in cultured rat spinal neurons, activity blockade increased the half-life of GluR1, thereby leading to increased steady-state abundance (5). The latter results are consistent with experiments showing that chronic activity blockade alters the stability of many postsynaptic proteins (41). Thus, it is possible that different cellular mechanisms lead to homeostatic compensation in different preparations. Earlier work on rodent neurons showed that clathrin-mediated endocytosis of AMPA receptors is involved in several forms of long-term depression, a form of Hebbian plasticity (10, 12, 18–20). Our results suggest that clathrin-mediated endocytosis is also involved in homeostatic forms of plasticity.

A simple model to explain our results is that synaptically released glutamate binds to GLR-1 and alters local recycling of GLR-1 receptors. It is also possible that changes in the abundance of GLR-1 are prompted by failure to ligand another class of GluRs, e.g., NMDA or metabotropic receptors. It seems unlikely that this is caused by failure to ligand NMDA receptors, because GLR-1 abundance at synapses is not significantly altered in mutants lacking NMDA receptors (ref. 25 and M.E.G. and J.M.K., unpublished observations). Similarly, chronic blockade of NMDA receptors does not alter the synaptic abundance of AMPA receptors in cultured rodent neurons (5, 6). However, NMDA receptor activation is required for a decrease in AMPA receptor abundance upon chronic activity enhancement (4). Further experiments are required to explore the role of other GluRs in compensatory regulation of GLR-1.

Our results suggest that accumulation of GLR-1::GFP in *eat-4* VGLUT mutants reflects an increased abundance of GLR-1 receptors at postsynaptic elements. The *eat-4* VGLUT mutations significantly increased puncta fluorescence but had no measurable effect on interpuncta (i.e., nonsynaptic) fluorescence in the ventral cord. Thus, blockade of glutamate release resulted in increased accumulation of GLR-1::GFP in puncta. We previously showed that $\approx 80\%$ of the GLR-1::GFP puncta in the

ventral cord colocalize with presynaptic markers and therefore correspond to postsynaptic elements (13, 24, 28). It is unlikely that the effect of activity blockade on the distribution of GLR-1::GFP was caused by a disproportionate effect on a subpopulation of nonsynaptic puncta because *eat-4* VGLUT mutations had a significant effect on the entire population of puncta in the posterior ventral cord. These results all support the idea that activity blockade led to accumulation of GLR-1::GFP at postsynaptic elements.

The dependence on clathrin-mediated endocytosis implies that some aspect of GLR-1 recycling is required to produce compensatory changes in GLR-1 abundance. In principle, the increased fluorescence of GLR-1::GFP puncta after activity blockade could be caused by either increased surface GLR-1 or increased receptors residing in an intracellular subsynaptic organelle, e.g., an endosome. Our results favor the former hypothesis. First, the amplitude of glutamate-evoked currents was increased in *eat-4* VGLUT mutants. These results do not distinguish whether the increased surface GLR-1 receptors are localized at synapses, because it is likely that pressure ejection of glutamate activates both synaptic and nonsynaptic receptors. Second, the *eat-4* VGLUT effect was occluded by mutations in *unc-11* AP180, suggesting that the higher receptor abundance in the plasma membrane was caused by a defect in GLR-1 recycling. Third, trafficking of endosomal cargo to lysosomes requires formation of ubiquitin conjugates in the limiting membrane of late endosomes (42–44). However, we found that expression of GLR-1(4KR) receptors, which cannot be ubiquitinated, did not occlude the increase in puncta fluorescence. Therefore, our results are most consistent with the idea that the increased puncta fluorescence observed in *eat-4* VGLUT mu-

tants was caused by increased abundance of GLR-1 in the plasma membrane. Increased surface expression could be caused by either increased exocytosis or decreased endocytosis of GLR-1. Further experiments are required to distinguish between these possibilities.

A variety of homeostatic mechanisms regulating synaptic transmission have been described (4–7, 38, 45). These compensatory mechanisms have been proposed to play various roles in the development and function of neuronal networks. Synaptic scaling has been proposed as a mechanism to counteract the tendency of Hebbian mechanisms to produce excessive synaptic potentiation, thereby stabilizing network activity (39). However, its physiological significance has remained uncertain because synaptic scaling has been documented primarily in cultures of dissociated neurons. Several recent studies suggest that homeostatic regulation of GluRs also occurs in intact animals (7, 37, 40, 46). Thus, our results provide further support for the idea that homeostatic regulation of GluRs may also occur *in vivo* and therefore play a role in shaping the activity of neural networks in intact animals. Our results suggest that *C. elegans* provides a useful model to study the genetic basis for homeostatic mechanisms regulating synaptic function.

We thank A. Fire, O. Hobert, M. Nonet, the *C. elegans* Genetic Stock Center, and A. Coulson for strains and reagents. We also thank members of the Kaplan laboratory and an anonymous reviewer for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants NS32196 (to J.M.K.) and NS35812 (to A.V.M.). M.E.G. was supported by a fellowship from the American Heart Association (California Division). N.S. was supported by the Deutsche Forschungsgemeinschaft.

- Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C. & Malinow, R. (2000) *Science* **287**, 2262–2267.
- Shi, S. H., Hayashi, Y., Petralia, R. S., Zaman, S. H., Wenthold, R. J., Svoboda, K. & Malinow, R. (1999) *Science* **284**, 1811–1816.
- Carroll, R. C., Lissin, D. V., von Zastrow, M., Nicoll, R. A. & Malenka, R. C. (1999) *Nat. Neurosci.* **2**, 454–460.
- Lissin, D. V., Gomperts, S. N., Carroll, R. C., Christine, C. W., Kalman, D., Kitamura, M., Hardy, S., Nicoll, R. A., Malenka, R. C. & von Zastrow, M. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 7097–7102.
- O'Brien, R. J., Kamboj, S., Ehlers, M. D., Rosen, K. R., Fischbach, G. D. & Hagan, R. L. (1998) *Neuron* **21**, 1067–1078.
- Turrigiano, G. G., Leslie, K. R., Desai, N. S., Rutherford, L. C. & Nelson, S. B. (1998) *Nature* **391**, 892–896.
- Davis, G. W. & Goodman, C. S. (1998) *Nature* **392**, 82–86.
- Passafium, M., Piech, V. & Sheng, M. (2001) *Nat. Neurosci.* **4**, 917–926.
- Lu, W., Man, H., Ju, W., Trimble, W. S., MacDonald, J. F. & Wang, Y. T. (2001) *Neuron* **29**, 243–254.
- Luscher, C., Xia, H., Beattie, E. C., Carroll, R. C., von Zastrow, M., Malenka, R. C. & Nicoll, R. A. (1999) *Neuron* **24**, 649–658.
- Carroll, R. C., Beattie, E. C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R. A., Malenka, R. C. & von Zastrow, M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 14112–14117.
- Man, H. Y., Lin, J. W., Ju, W. H., Ahmadian, G., Liu, L., Becker, L. E., Sheng, M. & Wang, Y. T. (2000) *Neuron* **25**, 649–662.
- Burbea, M., Dreier, L., Dittman, J. S., Grunwald, M. E. & Kaplan, J. M. (2002) *Neuron* **35**, 107–120.
- Lin, J. W., Ju, W., Foster, K., Lee, S. H., Ahmadian, G., Wyszynski, M., Wang, Y. T. & Sheng, M. (2000) *Nat. Neurosci.* **3**, 1282–1290.
- Beattie, E. C., Carroll, R. C., Yu, X., Morishita, W., Yasuda, H., von Zastrow, M. & Malenka, R. C. (2000) *Nat. Neurosci.* **3**, 1291–1300.
- Ehlers, M. D. (2000) *Neuron* **28**, 511–525.
- Liang, F. & Hagan, R. L. (2001) *J. Neurochem.* **77**, 1626–1631.
- Snyder, E. M., Philpot, B. D., Huber, K. M., Dong, X., Fallon, J. R. & Bear, M. F. (2001) *Nat. Neurosci.* **4**, 1079–1085.
- Carroll, R. C., Beattie, E. C., von Zastrow, M. & Malenka, R. C. (2001) *Nat. Rev. Neurosci.* **2**, 315–324.
- Wang, Y. T. & Linden, D. J. (2000) *Neuron* **25**, 635–647.
- Brockie, P. J., Madsen, D. M., Zheng, Y., Mellem, J. & Maricq, A. V. (2001) *J. Neurosci.* **21**, 1510–1522.
- Hart, A. C., Sims, S. & Kaplan, J. M. (1995) *Nature* **378**, 82–85.
- Maricq, A. V., Peckol, E., Driscoll, M. & Bargmann, C. I. (1995) *Nature* **378**, 78–81.
- Rongo, C., Whitfield, C. W., Rodal, A., Kim, S. K. & Kaplan, J. M. (1998) *Cell* **94**, 751–759.
- Brockie, P. J., Mellem, J. E., Hills, T., Madsen, D. M. & Maricq, A. V. (2001) *Neuron* **31**, 617–630.
- Richmond, J. E., Weimer, R. M. & Jorgensen, E. M. (2001) *Nature* **412**, 338–341.
- Schafer, W. & Kenyon, C. (1995) *Nature* **375**, 73–78.
- Rongo, C. & Kaplan, J. M. (1999) *Nature* **402**, 195–199.
- Lee, R. Y., Sawin, E. R., Chalfie, M., Horvitz, H. R. & Avery, L. (1999) *J. Neurosci.* **19**, 159–167.
- Berger, A., Hart, A. & Kaplan, J. (1998) *J. Neurosci.* **18**, 2871–2880.
- Bellochio, E. E., Reimer, R. J., Freneau, R. T., Jr., & Edwards, R. H. (2000) *Science* **289**, 957–960.
- Bellochio, E. E., Hu, H., Pohorille, A., Chan, J., Pickel, V. M. & Edwards, R. H. (1998) *J. Neurosci.* **18**, 8648–8659.
- Takamori, S., Rhee, J. S., Rosenmund, C. & Jahn, R. (2000) *Nature* **407**, 189–194.
- Nonet, M. L., Holgado, A. M., Brewer, F., Serpe, C. J., Norbeck, B. A., Holleran, J., Wei, L., Hartwig, E., Jorgensen, E. M. & Alfonso, A. (1999) *Mol. Biol. Cell* **10**, 2343–2360.
- Zhang, B., Koh, Y. H., Beckstead, R. B., Budnik, V., Ganetzky, B. & Bellen, H. J. (1998) *Neuron* **21**, 1465–1475.
- Wendland, B. & Emr, S. D. (1998) *J. Cell Biol.* **141**, 71–84.
- Davis, G. W. & Goodman, C. S. (1998) *Curr. Opin. Neurobiol.* **8**, 149–156.
- Davis, G. W. & Bezprozvanny, I. (2001) *Annu. Rev. Physiol.* **63**, 847–869.
- Turrigiano, G. G. (1999) *Trends Neurosci.* **22**, 221–227.
- Featherstone, D. E., Rushton, E. & Broadie, K. (2002) *Nat. Neurosci.* **5**, 141–146.
- Ehlers, M. D. (2003) *Nat. Neurosci.* **6**, 231–242.
- Katzmann, D. J., Babst, M. & Emr, S. D. (2001) *Cell* **106**, 145–155.
- Reggiori, F. & Pelham, H. R. (2001) *EMBO J.* **20**, 5176–5186.
- Urbanowski, J. L. & Piper, R. C. (2001) *Traffic* **2**, 622–630.
- Paradis, S., Sweeney, S. T. & Davis, G. W. (2001) *Neuron* **30**, 737–749.
- Desai, N. S., Cudmore, R. H., Nelson, S. B. & Turrigiano, G. G. (2002) *Nat. Neurosci.* **5**, 783–789.