Published in final edited form as:

Neurogastroenterol Motil. 2016 September ; 28(9): 1356–1369. doi:10.1111/nmo.12834.

Prolonged Esophageal acid exposures induce synaptic downscaling of cortical membrane AMPA receptor subunits in rats

Banani Banerjee¹, Bidyut K Medda¹, Jian Zhang¹, Vishwajit Tuchscherer³, Reji Babygirija², Pradeep Kannampalli¹, Jyoti N. Sengupta¹, and Reza Shaker¹

¹Gastroenterology & Hepatology, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, United States

²Gastroenterology & Hepatology, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, United States

³University of Wisconsin, Oshkosh, WI, United States

Abstract

Background—we recently reported the involvement of AMPA receptor subunit upregulation and phosphorylation in the rostral cingulate cortex (rCC) as the underlying mechanism of acute esophageal acid-induced cortical sensitization. Based on these findings we proposed to investigate whether prolonged esophageal acid exposures in rats exhibit homeostatic synaptic scaling through downregulation of AMPA receptor expression in rCC neurons. We intended to study further whether this compensatory mechanism is impaired when rats are preexposed to repeated esophageal acid exposures neonatally during neuronal development.

Methods—two different esophageal acid exposure protocols in rats were used. Since AMPA receptor trafficking and channel conductance depend on CaMKII α -mediated phosphorylation of AMPA receptor subunits, we examined the effect of esophageal acid on CaMKII α activation and AMPA receptor expression in synaptoneurosomes (SYNs) and membrane preparations from rCCs.

Key Results—in cortical membrane preparations, GluA1 and pGluA1Ser⁸³¹ expression were significantly downregulated following prolonged acid exposures in adult rats, this was accompanied by a significant downregulation of cortical membrane pCaMKII α expression. No

Corresponding Author: Reza Shaker, MD, Professor in Medicine, Chief of the division of Gastroenterology and Hepatology, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA, Tel: 414-955-6840, Fax: 414-955-6361, rshaker@mcw.edu.

Disclosures:

All authors agreed to participate in this study without any potential conflict of interest.

Author's contributions:

BB: Manuscript writing, study design, data acquisition and analysis.

BKM: surgical procedures, undertaking animal experiments.

JZ: assistance in molecular experiments, data acquisition and critical reading of the manuscript

VT: assistance in molecular experiments, data acquisition

RB: assistance in molecular experiments, data acquisition

PK: surgical procedures, undertaking animal experiments

JNS: critical reading of the manuscript

RS: scientific concept, critical revision of the manuscript and support of the work.

change in GluA1 and pGluA1Ser⁸³¹ expression was observed in rCC membrane preparations in rats preexposed to acid neonatally followed by adult rechallenge.

Conclusions & Inferences—this study along with our previous findings suggests that synaptic AMPA receptor subunits expression and phosphorylation may be involved bidirectionally in both esophageal acid-induced neuronal sensitization as well as acid-dependent homeostatic plasticity in cortical neurons. The impairment of homeostatic compensatory mechanism as observed following early-in-life acid exposure could be the underlying mechanism of heightening cortical sensitization and esophageal hypersensitivity in patients with GERD.

Keywords

Homeostatic plasticity; synaptic scaling; AMPA receptor subunits; esophageal hypersensitivity; cortical sensitization

Introduction

Homeostatic synaptic scaling is a form of plasticity by which neurons make compensatory adjustments to the strength of excitatory synapses according to their synaptic input. It can also be defined as the negative feedback mechanism to counter-balance Hebbian plasticity which is the positive feedback mechanism for the development of long-term memory and long-term potentiation in neural networks (1).

At the molecular level, synaptic scaling is a cellular process in which neurons identify activity-dependent changes in their own firing and then regulate AMPA receptor trafficking and accumulation at synapses to stabilize the synaptic strength (2–4). For example, blocking neuronal activity by incubating neuronal culture with TTX (Tetrodotoxin, Na⁺ channel blocker) or AMPA receptor antagonist (e.g. CNQX) for a long period (couple of hours to few days) causes an accumulation of AMPA receptors at synapses which correlates with increase in synaptic strength, whereas, increasing neuronal activity by GABA_A receptor antagonist (e.g. bicucullin or picrotoxin), decreases AMPA receptor content resulting in inhibition of synaptic strength. In *in vivo* system, depriving sensory input such as vision, scales up excitatory synapses in the visual cortex and, reexposing the visually deprived animals to light results in scaling down of synaptic strength (5–12). This sensory experience dependent synaptic scaling is mainly considered to be postsynaptic in nature at the level of AMPA receptor expression and activation globally or at individual synapses (3;13). The defect in synaptic scaling has been reported to contribute to several neurological disorders such as epilepsy and Alzheimer, however, the role of synaptic scaling, if any, in the development of chronic visceral hypersensitivity is not clearly defined.

Several studies emphasize the involvement of AMPA and NMDA receptor subunits activation and phosphorylation in cortical sensitization following visceral stimulations (14–16). In line with these findings, functional imaging studies in human have also implicated cingulate and insular cortices in the processing of pain of esophageal origin (17–19). In rat studies, early-in-life esophageal acid exposures demonstrated a long-term molecular alteration in NMDA subunits and postsynaptic density protein (PSD-95) expression in the neurons of rCC. However, rats exposed to acid in adulthood failed to exhibit this long-term

cortical alteration (15). On the other hand, acute acid exposure in adult rats exhibited a short-term upregulation of AMPA receptor subunits in the cingulate cortex which lasted only a couple of hours after the exposure (16). Based on these findings we intended to investigate whether prolonged esophageal acid exposures in adult rats exhibit synaptic scaling through downregulation of cortical membrane AMPA receptor subunit expression, and also to examine whether this mechanism is impaired in adult rats with esophageal acid exposures early in- life during neuronal development because of the underlying long-term molecular alteration and cortical plasticity.

Since AMPA receptor trafficking and channel conductance depend on CaMKII α -mediated phosphorylation of AMPA receptor subunits as well as activity-dependent AMPA receptor endocytosis and recycling (20–24), we planned to examine the effect of acid on CaMKII α and AMPA receptor subunits activation and coexpression of these receptor subunits with various synaptic proteins in the rCC.

Methods

Study Design

Experiments were undertaken in male Sprague Dawley rats (Harlan, Indianapolis, IN). The Institutional Animal Care and Use Committee of the Medical College of Wisconsin approved all experimental procedures in accordance with the guidelines of the International Association for the Study of Pain. Two different esophageal acid exposure protocols were used as shown in Fig 1. In Adult treatment (protocol 1), rats were exposed to either saline (Group 1) or 0.1N HCl, (Group 2) for 20 min at 0.1 ml/min under anesthesia starting at postnatal (P) day 60 (P60). The exposure was repeated for 7 days from P60–P66. In neonatal treatment and adult rechallenge (protocol 2); pups were exposed to either esophageal saline (Group 3) or 0.1N HCl (Group 4) at a dose of 200 μ l/animal/day from P7 through P13. Rats were allowed to grow and rechallenged either with saline or acid the same way as mentioned in adult treatment protocol from P60–P66. Tissues were collected from all groups on P66 after an interval of 4 hours following the last exposure. Brain tissues were sliced sagittally at 2mm from midline. The cingulate cortex obtained from the first slide on either side of the midline was transected rostro-caudally (rCC) as described previously (15) and in Fig. 1. For each animal, the rCC section from one side of the midline was used for membrane preparation and the section from the other side for synaptoneurosome (SYNs) isolation. The isolated SYNs which mainly comprised of presynaptic vesicles (synaptosomes) and postsynaptic densities (PSD) were specifically examined to establish that the acid-induced downregulation of membrane receptor expression resulted in increased accumulation in SYNs away from the membrane.

Cortical membrane protein extraction

rCC samples were ground in liquid nitrogen and resuspended in homogenization buffer (94 mM HEPES, pH 7.4; 5 mM EDTA, pH 8.0; 300 mM sucrose, 1 mM PMSF, Protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor (Pierce Biotechnology, Rockford, IL) at 2.0 ml per gram tissue. The homogenization was carried out at moderate speed for 20 sec and repeated three times at an interval of 5 sec. The

mixture was rotated at 4°C for 20 min and centrifuged at 20,000×g at 4°C for 30 min. The supernatant containing cytoplasmic proteins was removed and the pellet carrying the membrane proteins was resuspended in ice cold homogenization buffer and rotated at 4°C for 5 min and centrifuged at 20,000×g at 4°C for 10 min. The supernatant was discarded and the pellet was dissolved in lysis buffer (50 mM Tris pH 9.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 5 mM EDTA, 1 mM PMSF, protease inhibitor cocktail and phosphatase inhibitor) and incubated at 37°C for 30 min and centrifuged at 10,000×g for 20 min. The supernatant carrying membrane proteins was used for Western blot analysis.

Cortical synaptoneurosomes preparation

Synaptoneurosomes from rCC samples were isolated according to the method of Johnson et al (25). The tissues were collected immediately after dissection and individual rCC samples were gently homogenized in ice-cold solution containing 33% sucrose in 10 mM HEPES (pH-7.4), 0.5 mM EGTA, complete protease inhibitor and phosphatase inhibitor using five strokes of a glass/teflon homogenizer (Kontes Glass Company, Vineland, NJ). Nuclei and other debris were pelleted at 1000×g at 4°C for 10 min and the supernatant filtered through 3 layers of 100nm pore nylon mesh (Millipore, Bedford, MA), and subsequently through a 5µm pore PVDF syringe filter (Millipore). The filtrate was then centrifuged at 2000×g at 4°C for 10 min, and the supernatant was removed. The synaptoneurosome-containing pellet was then resuspended in the appropriate amount of lysis buffer. The ultrastructure of the isolated synaptoneurosomes was analyzed by electron microscopy using core facility of the Medical College of Wisconsin.

Western blot analysis of cortical membrane extracts and synaptoneurosome preparations

The protein content of the cortical membrane extracts and SYNs preparations was estimated using BCA method (Pierce). Various antibodies used for western blot analysis were rabbit anti-GluA1 (1:2500, Alomone Labs, Israel), rabbit anti-GluA2 (Alomone 1:5000), rabbit anti-pGluA1Ser⁸³¹ (Millipore, Billerica, MA, 1:1000), rabbit anti-pGluA1Ser⁸⁴⁵ (Millipore, 1:1000), rabbit anti-pGluA2Ser⁸⁸⁰ (Millipore, 1:1000), rabbit anti-CaMKIIα (Cell Signaling, Danvers, MA, 1:1000), rabbit anti-pCaMKIIThr²⁸⁶ (Cell Signaling, 1:1000), rabbit anti-PSD-95 (Cell Signaling, 1:1000), mouse anti-pERK (Cell Signaling, 1:1000), rabbit anti-ERK (Cell Signaling, 1:1000) and mouse anti-β actin (Sigma, St Louis, MO, 1:5000). To examine the specificity of antigen-antibody reaction, anti-GluA1 and anti-GluA2 antibodies were preabsorbed with respective blocking peptides (1µg peptide per 1 µg antibody) for 2 hr at room temperature and thereafter used for western blot. The intensity of protein expression for experimental and housekeeping gene (mouse anti β-actin) for individual tissue sample was measured by densitometric scanning using Alpha Image software program (Cell Biosciences Inc., Santa Clara, CA).

Immunofluorescence study using confocal microscopy

Rats were deeply anesthetized with sodium pentobarbital (50 mg/Kg, i.p.) and the chest was opened by mid-sternal incision. Animals were perfused transcardially with 300 ml of ice cold phosphate buffer solution followed by with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. rCC regions were collected and incubated in 4% paraformaldehyde for 2 hrs followed by overnight in 30% sucrose at 4°C. Thereafter, the tissues were embedded in O.C.T.

compound (Fisher Scientific, Pittsburgh, PA), and serial sections of 25µm thickness were collected on a cryostat. The immunostaining was carried out according to the method described previously (26). In brief, the cryosections were blocked with 10% normal goat serum (NGS), 0.5% Triton X-100 and 0.1% sodium azide for 60 min at room temperature. This was followed by incubation with primary antibody overnight at 4°C. Primary antibodies used for immunohistochemistry study were: PSD-95 (BD Bioscience, San Jose, CA, 1:300 dilution), pCamKIIα (cell signaling, 1:300 dilution), GRIP1 (BD Bioscience, 1:300 dilution), and GluA2 (Alomone, 1:200 dilution). Secondary antibodies were labeled with either Alexa-488 (Life Technology, 1:1,000) or Alexa-568 (Thermo Fisher Scientific, Carlsbad, CA, 1:2,000). DAPI (Sigma) was used to counter-stain the nucleus.

Three animals from acid-treated and saline-treated were used for quantifying the staining. Three cryosections of each animal were chosen from lateral to middle to represent the total 2mm ACC slice. Two different fields of each cryosection were imaged using a Zeiss LSM510 laser scanning confocal microscope at 63× magnification with z-stacks (3µm step-size). In each field, the PSD-95 & pCamKIIα or GRIP1 & GluA2 colocalization cells were counted. At the same time, pCamKIIα and GRIP1 translocation cells were counted also. Total cell number was counted by the Hoechst staining nuclei. We selected one section per animal from both groups and the image was opened under image J program (NIH, Bethesda, MD). Arbitrary grey scale units (in the range of 1–255) were assigned to quantify the intensity of staining of a given cell. The freehand tool was used to trace the outline of the selected cells and the intensity of staining was determined. The average intensity of staining from 3 sections per group was determined.

Data Analysis

For western blots, the intensity of expression for target protein and housekeeping β-actin for individual tissue sample was quantified by densitometric scanning using Image J program (NIH). The relative intensity of expression against β-actin for acid-treated group was compared with saline-treated control using student t test. The data were presented as mean ±SD and values with $p < 0.05$ were considered to be significant. For immunohistochemistry, the mean intensities of staining for acid-treated rats were compared with saline-treated control using student t test and presented as mean ±SD and values with $p < 0.05$ were considered to be significant.

Results

GluA1 and pGluA1Ser⁸³¹ expression in cortical membrane rapidly decrease following prolonged acid exposures in adult rats

We investigated two major AMPA receptor subunits GluA1 and GluA2 and their phosphorylation in rCC membrane preparations from rats receiving esophageal acid exposures at different time points during development as mentioned in Fig. 1.

In adult treatment protocol, relative expression of total GluA1 (100 KD protein) in cortical membrane preparation from acid-treated rats was significantly reduced compared to saline-treated controls (Fig. 2A, $*p < 0.001$ vs controls). Similarly, relative pGluA1Ser⁸³¹

expression in acid-treated rats was significantly downregulated compared to saline-treated controls (Fig. 2A, $*p<0.01$). On the other hand, repeated acid exposures in adult treatment protocol resulted in a small increase in relative GluA2 (100 KD) expression (Fig. 2B $*p<0.05$ vs saline control), whereas, no difference was observed in pGluA2Ser⁸⁸⁰ expression between acid and saline-treated rats. A significant downregulation of pCaMKII α (50 KD protein, alpha form), the enzyme involves in phosphorylation of GluA1 Ser⁸³¹ was also observed in acid-treated group (Fig. 2C, $*p<0.001$ vs saline controls).

No change in GluA1 and pGluA1Ser⁸³¹ expression in cortical membrane following neonatal treatment and adult rechallenge

In contrast to adult protocol, neonatal treatment and adult rechallenge demonstrated no significant difference in GluA1 and pGluA1Ser⁸³¹ expression between acid-treated and saline-treated rats (Fig. 3A, GluA1 $p=0.132$ and GluA1 Ser⁸³¹ $p=0.4$ vs saline controls respectively). There was a small but significant downregulation of membrane GluA2 in acid-treated group (Fig. 3B, $*p<0.05$ vs saline controls) with an increase in the expression of pGluA2Ser⁸⁸⁰ (Fig. 3B, $*p<0.05$ vs saline controls). Similarly, expression of pCaMKII α was also comparable between acid-treated and saline controls (Fig. 3C, $p=0.094$ vs saline controls).

Electron micrograph of cortical synaptoneurosomes (SYNs) preparation

Electron micrograph of SYNs preparation from rat cingulate cortex slice revealed the presence of various cellular matters such as vasicular structures, mitochondria and myelinated bodies but no whole cell structure (Fig. 4A & 4B). As shown, purified SYN carried presynaptic sac (synaptosome) attached to a resealed postsynaptic sac (neurosoma). The synaptic junction consisted of regions of presynaptic and postsynaptic plasma membranes closely associated within the synaptic cleft. The postsynaptic part was thick, lying closely adjacent to the postsynaptic membrane plus adherent sub-synaptic material (arrows). This fraction represented the postsynaptic density or PSD.

We examined the effect of acid treatment on the expression of a major protein PSD-95 (95 kD) in the isolated cortical SYNs in both protocols. In adult treatment, there was a significant decrease in PSD-95 expression in SYNs from acid-treated rats (Fig. 4C, $p<0.05$ vs saline controls), whereas, no difference in PSD-95 expression was observed between acid and saline-treated rats in neonatal treatment and adult rechallenge protocol (Fig. 4C, $p=0.5$ vs controls).

In the next set of experiments, we examined the expression of AMPA receptor subunits and pCaMKII α and pERK in the isolated SYNs from individual animals in both protocols.

Increased accumulation of GluA1 and pGluA1Ser⁸³¹ in isolated SYNs from rCCs following prolonged esophageal acid exposures in adult rats

In adult treatment protocol, the expression profiles of pGluA1Ser⁸³¹ and GluA1 in isolated SYNs were higher in acid-treated animals compared to saline controls ($p<0.05$ vs controls for GluA1 and $p<0.01$ vs controls for pGluA1Ser⁸³¹), indicating GluA1 trafficking away from the cortical membrane to postsynaptic density (Fig. 5). Similarly, higher postsynaptic

accumulation of two kinases, pERK and pCaMKII α (critical for AMPA receptor subunit GluA1 phosphorylation and downstream signaling) was observed in SYNs from acid-treated group compared to saline controls (Fig. 5, $p<0.01$ vs controls for pERK and $p=0.05$ vs controls for pCaMKII α). In contrast, a significant downregulation of SYN GluA2 accumulation was noticed in acid-treated animals (Fig. 5, $p<0.05$ vs controls) with no difference in expression of pGluA2Ser⁸⁸⁰ between two groups ($p=0.328$ vs saline controls).

No change in GluA1 and pGluA1Ser⁸³¹ expression in isolated SYNs from rCCs following neonatal treatment and adult rechallenge

In neonatal treatment and adult rechallenge protocol, the expression profiles of GluA1 and pGluA1Ser⁸³¹ in SYNs were comparable between acid-and saline-treated groups (Fig. 6, $p=0.06$ for GluA1, $p=0.42$ for pGluA1Ser⁸³¹). No difference in GluA2 expression was observed between the groups, whereas, there was a significant increase in pGluA2Ser⁸⁸⁰ accumulation in acid-treated group compared to saline controls (Fig. 6, $p=0.80$ vs saline controls for GluA2, $p<0.01$ vs saline controls for pGluA2Ser⁸⁸⁰). In the isolated SYNs, the expression of pCaMKII α and pERK was comparable between acid and saline-treated groups (Fig. 6 $p=0.52$ vs controls for pCaMKII α , $p=0.086$ vs controls for pERK).

Increased translocation (away from membrane) of pCaMKII α and GRIP1 in cortical neurons following prolonged esophageal acid exposures in adult rats

In immunohistochemistry (IHC) experiments, rCC tissue sections from rats in protocol 1 were double immunostained with 2 sets of antibodies; PSD-95/pCaMKII α and GRIP1/GluA2. The percentage of cells in cortical layers II & III exhibiting membrane colocalization of both PSD-95 and pCaMKII α was significantly higher in saline-treated controls compared to acid group (7C, $p<0.001$ vs saline controls, open arrows). Whereas, the double stained puncta in tissue section from acid-treated rat are scattered throughout the cell (7C, $p<0.01$ vs controls, open arrows). Since GRIP1 and GluA2 interaction is important for synaptic accumulation of AMPA receptors, we also examined the effect of esophageal acid on GRIP1 and GluA2 coexpression in cortical neurons. The percentage of GRIP1 positive cells coexpressing GluA2 was significantly reduced in acid-treated rats compared to saline controls (8B, $p<0.05$ vs saline controls). We also observe a distinct translocation of GRIP1 expression away from the membrane within the cell in acid-treated rats and the percentage of GRIP1 positive cells exhibiting this translocation was significantly higher in acid-treated group compared to saline controls (8B, $p<0.05$ vs control open arrows).

pNR2BSer¹³⁰³ but not total NR2B expression increases in cortical membrane in adult and neonatal treatment protocols

It has been well documented that AMPA and NMDA receptors are colocalized at individual synapses, and synaptic targeting of GluA1 is dependent on NMDA receptor activation. Therefore, we investigated the effect of prolonged esophageal acid exposures on membrane expression of three major subunits of NMDA receptors NR1, NR2A, NR2B and also pNR2BSer¹³⁰³ in rats in both protocols as described in Fig 1.

In adult treatment protocol, no difference was observed in total NR2B expression between the groups (Fig. S1, $p=0.28$ vs saline controls). Whereas, a significant increase in relative

expression of pNR2BSer1303 (pNR2BSer¹³⁰³) to β -actin ($p < 0.01$ vs saline) and also the ratio of pNR2BSer¹³⁰³ to total NR2B ($*p < 0.05$ vs saline) were observed in acid-treated rats compared to saline controls. The relative expression of NR2A subunits in acid-treated rats was significantly higher compared to saline-treated controls (Table 1, $p < 0.01$ vs saline controls). Since the basal level of membrane NR1 was very high, we failed to detect any significant difference in expression between acid-treated group and saline-treated controls (Table 1, $p = 0.42$ vs controls).

In neonatal treatment and adult rechallenge protocol, the expression of NR2B subunit was comparable between the groups (Fig. 2, $p = 0.11$ for NR2B). However, similar to the findings with adult treatment protocol, a higher expression of relative pNR2BSer¹³⁰³ expression to β -actin was observed in acid-treated group compared to saline controls (Fig. S2, $*p < 0.05$ vs controls), similarly, the ratio of pNR2BSer¹³⁰³ to total NR2B almost approached a significant increase compared to saline controls (Fig. S2B; $p = 0.056$ vs controls). The expression of NR2A and NR1 were comparable between the acid-treated and saline-treated groups (Table 1, $p = 0.62$ for NR2A and $p = 0.15$ for NR1 vs controls).

Discussion

Synaptic scaling is a form of homeostatic plasticity that compensates the perturbation in neuronal firing by scaling up or down the postsynaptic strength of excitatory synapses. At the cellular level, neurons detect the changes of their own firing through a set of calcium dependent sensors that then regulate AMPA receptor trafficking at synaptic sites to increase or decrease synaptic strength (27). Although recent interests in the role of synaptic scaling in various disease processes are emerging, its involvement in esophageal acid-induced cortical sensitization remains largely undefined. Here, we provide evidence that synaptic downscaling in cortical neurons indeed occurs following prolonged esophageal acid exposures in adult rats (protocol 1). We demonstrate for the first time that the esophageal acid-induced synaptic downscaling involves a significant reduction in membrane GluA1 and pGluA1Ser⁸³¹ expression in cortical neurons. This synaptic downscaling is also accompanied by a reduced membrane expression of pCaMKII α , the kinase involved in GluA1 phosphorylation and a simultaneous increase in GluA 2 surface expression. In contrast to the findings in protocol 1, no significant downregulation of membrane GluA1, pGluA1Ser⁸³¹ and pCaMKII α is observed in protocol 2, indicating that the synaptic downscaling mechanism is impaired in adult rats that are exposed to acid early-in-life during neuronal development.

We decided to focus on the rCC because in previous human fMRI studies we consistently demonstrated cerebral activity by esophageal acid stimulation in healthy individuals and exaggerated response in GERD patients (17;28). In addition, a significant increase in cingulate gyrus activity to distension following esophageal acid exposure was also recorded, suggesting its role in acid-induced central sensitization (19). In isolated synaptoneurosome in protocol 1, we demonstrate an acid-induced downregulation of PSD-95, the major scaffolding protein in post synaptic neurons responsible for membrane trafficking and anchoring of glutamate receptors. Interestingly, we also observe a higher accumulation of GluA1 and several kinases in synaptoneurosome in acid-treated group, suggesting that the

blockade in membrane trafficking results in accumulation of these molecules in the postsynaptic density away from the cortical membrane.

Recent evidence suggests that the surface AMPA receptor expression plays a critical role in synaptic scaling mechanism (29;30). Synaptic scaling in excitatory synapses is initially demonstrated in *in vitro* cultures by pharmacological manipulation of neuronal activity and one of the mechanisms underlying synaptic scaling was reported to be the altered synaptic expression of Ca²⁺ permeable (CP-), i.e. GluA1 subunit containing AMPA receptors (31–36). For instance, several studies reported that the inactivity-induced increase in GluA1 results in accumulation of CP-APMA receptors at synapses, which is detected as the polyamine sensitive AMPA receptor postsynaptic currents (32;37). Similarly, activity-dependent homeostatic synaptic plasticity in visual cortex is reported to be associated with selective regulation of GluA1 containing AMPA receptors at the synapses (7;8). In the present study, chronic acid treatment protocol in adult rats demonstrates a significant downregulation of membrane GluA1 expression suggesting its involvement in underlying mechanism of acid-induced homeostatic synaptic downscaling. We also observe concomitant decrease in membrane pCaMKII α and pGluA1Ser⁸³¹ expression in cortical neurons. Several biochemical studies have identified Ser⁸³¹ within C-terminal end of the GluA1 subunit as a phosphorylation target for the enzyme CaMKII (22;24;38). CaMKII α -mediated AMPA receptor subunit GluA1 phosphorylation has been implicated also in the mechanism of long term potentiation in a cellular model of learning and memory (22). Moreover, in a recent study, we have demonstrated GluA1 upregulation and CaMKII-mediated GluA1Ser⁸³¹ phosphorylation as the underlying mechanism of cortical sensitization during acute esophageal acid exposure in adult rats (16). Taken together, these findings emphasize that GluA1 and pGluA1Ser⁸³¹ are involved in both synaptic downscaling as well as synaptic transmission depending on the time and frequency of esophageal acid exposures.

Involvement of GluA2 in activity dependent synaptic scaling is well studied. The GluA2 interaction with synaptic scaffolding protein Glutamate receptor interacting protein 1 (GRIP1) is reported to play an essential role in synaptic accumulation of AMPA receptors during synaptic scaling up (39). At the same time, GluA2 interaction with synaptic proteins such as protein interacting with C kinase 1 (PICK1) or N-ethylmaleimide sensitive factor (NSF) is likely to mediate the removal of AMPA receptors from the synapse during homeostatic synaptic downscaling (40;41). Phosphorylation of GluA2 on amino acid 880 by protein kinase C (PKC) blocks GRIP1 interaction with GluA2 leaving PICK1 interaction unaffected (42). Moreover, GluA2 knockdown is reported to impair synaptic scaling up during activity blockage in dissociated cultures (43), suggesting a bidirectional role for GluA2 in activity-dependent synaptic transmission. Here, we have shown a small, but significant increase in membrane expression of GluA2 following esophageal acid exposures in protocol 1. In line with this finding, our immunohistochemistry study also demonstrated a significant reduction in percentage of cells with membrane GluA2 and GRIP1 coexpression along with an increase in GRIP1 translocation away from the cortical membrane indicating that esophageal acid-induced synaptic AMPA receptor downscaling also involved downregulation of GRIP1 and GluA2 interaction in cortical neurons.

It is well established that the increase in synaptic activity leads to new gene transcription and protein synthesis and recent evidence also suggests that both gene transcription and protein translation are required for synaptic downscaling (2;44). Although, most studied forms of synaptic downscaling are reported to be mediated by either glutamate receptors and/or L-type calcium channels, recent studies also implicated other classes of voltage gated calcium channels (VGCC) as well as beta-catenin signaling in the induction of synaptic downscaling (27;44–48). In line with these findings, we observe an upregulation of membrane NR2A and pNR2BSer¹³⁰³ expression in cortical neurons following prolonged esophageal acid exposures in adult rats (supplementary data). These data show that NMDA receptor mediated downstream signaling may also play a role in acid-induced AMPA receptor trafficking during synaptic downscaling in cortical neurons.

Among several scaffolding proteins, PSD-95 is the only MAGUK (membrane associated guanylate kinase) member protein implicated as critical for synaptic downscaling (49). There are several AMPA receptor interacting proteins that undergo polyubiquitination and subsequent degradation, thereby regulating the surface expression of AMPA receptors. This concept is supported by findings that NMDA triggers AMPA receptor endocytosis via proteosome-dependent degradation of PSD-95 (50). Accumulating evidence also suggests that a large repertoire of postsynaptic proteins undergo regulated proteosome dependent degradation during homeostatic plasticity (51;52). On the other hand, recent data demonstrate that the synaptic scaling down is accomplished through a regulated increase in the rate at which AMPA receptor subunits unbind from synaptic scaffolding complex and exit the synapse (53). In the present study, we consistently demonstrate acid-induced downregulation of PSD-95 in the isolated synaptoneurosomes as well as its colocalization with pCaMKII α in adult treatment protocol suggesting a possible involvement of proteosome dependent degradation of the major PSD-95, the major synaptic scaffolding protein in esophageal acid-induced homeostatic plasticity.

One important finding of this study is that the homeostatic synaptic downscaling of GluA1 and associated synaptic proteins is observed only in adult treatment (protocol 1), however, not present in rats that are preexposed to esophageal acid in neonatal period before subjecting to acid exposures in adulthood (protocol 2). In a previous study, we have shown that esophageal acid exposures early in life during the critical period of neuronal development result in a long-term alteration in NMDA receptor subunit composition and postsynaptic membrane protein expression in rCC neurons and this could be implicated as the underlying molecular mechanisms for the development of chronic esophageal hypersensitivity in adulthood (15). A potential impairment of homeostatic regulation is evident in this study in rats that were preexposed to acid early in life could be due to a shift in basal neuronal activity and altered synaptic plasticity, and may in part be involved in heightening cortical sensitization and acid-induced esophageal hypersensitivity in adulthood. One of the limitations of the present study is the lack of a quantifiable and reproducible behavioral model in experimental animals to verify the esophageal acid-induced visceral hyperalgesia to complement our molecular findings. However, in a recent study in adult rats with neonatally-induced cystitis, we have demonstrated the development of long-term visceral hyperalgesia with an increase in visceromotor response (VMR) indicating that the neonatal time point is important for the development of long-term neuronal sensitization and

visceral hyperalgesia (54). Recent clinical studies in humans also suggest that the early phase of childhood is a critical time in which trauma can induce visceral hypersensitivity and functional disorders later in life (55).

In conclusion, the present study demonstrates that prolonged esophageal acid exposures downregulate membrane GluA1 expression and phosphorylation in cortical neurons in rats. This synaptic downscaling may in part be involved in homeostatic compensatory mechanism that stabilizes neuronal excitability at the excitatory synapses. We further demonstrate that adult rats preexposed to acid at neonatal stage fail to exhibit this compensatory mechanism when subjected to repeated esophageal acid exposures later in life. This study along with our previous findings strongly suggest that synaptic AMPA and NMDA receptor subunits expression and phosphorylation may be bidirectionally involved in both esophageal acid-induced sensitization as well as activity-dependent homeostatic plasticity in cortical neurons. The functional consequence of synaptic scaling impairment has been well established in various neurological diseases such as Rett syndrome and epilepsy, however, its involvement in the development and maintenance of long-term visceral hypersensitivity is still evolving. Therefore, future studies on the complex interaction between synaptic scaling and various downstream signaling pathways may provide a better understanding of the esophageal pain mechanism in patients with gastroesophageal acid-induced disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study has been supported by NIH Grant R01DK025731 awarded to Reza Shaker.

Reference List

1. Vitureira N, Goda Y. Cell biology in neuroscience: the interplay between Hebbian and homeostatic synaptic plasticity. *J Cell Biol.* 2013 Oct 28; 203(2):175–86. [PubMed: 24165934]
2. Goold CP, Nicoll RA. Single-cell optogenetic excitation drives homeostatic synaptic depression. *Neuron.* 2010 Nov 4; 68(3):512–28. [PubMed: 21040851]
3. Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB. Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature.* 1998 Feb 26; 391(6670):892–6. [PubMed: 9495341]
4. Ibata K, Sun Q, Turrigiano GG. Rapid synaptic scaling induced by changes in postsynaptic firing. *Neuron.* 2008 Mar 27; 57(6):819–26. [PubMed: 18367083]
5. Desai NS, Cudmore RH, Nelson SB, Turrigiano GG. Critical periods for experience-dependent synaptic scaling in visual cortex. *Nat Neurosci.* 2002 Aug; 5(8):783–9. [PubMed: 12080341]
6. Maffei A, Nelson SB, Turrigiano GG. Selective reconfiguration of layer 4 visual cortical circuitry by visual deprivation. *Nat Neurosci.* 2004 Dec; 7(12):1353–9. [PubMed: 15543139]
7. Goel A, Jiang B, Xu LW, Song L, Kirkwood A, Lee HK. Cross-modal regulation of synaptic AMPA receptors in primary sensory cortices by visual experience. *Nat Neurosci.* 2006 Aug; 9(8):1001–3. [PubMed: 16819524]
8. Goel A, Xu LW, Snyder KP, Song L, Goenaga-Vazquez Y, Megill A, Takamiya K, Hugarir RL, Lee HK. Phosphorylation of AMPA receptors is required for sensory deprivation-induced homeostatic synaptic plasticity. *PLoS One.* 2011; 6(3):e18264. [PubMed: 21483826]

9. Goel A, Lee HK. Persistence of experience-induced homeostatic synaptic plasticity through adulthood in superficial layers of mouse visual cortex. *J Neurosci*. 2007 Jun 20; 27(25):6692–700. [PubMed: 17581956]
10. Maffei A, Turrigiano GG. Multiple modes of network homeostasis in visual cortical layer 2/3. *J Neurosci*. 2008 Apr 23; 28(17):4377–84. [PubMed: 18434516]
11. Petrus E, Anguh TT, Pho H, Lee A, Gammon N, Lee HK. Developmental switch in the polarity of experience-dependent synaptic changes in layer 6 of mouse visual cortex. *J Neurophysiol*. 2011 Nov; 106(5):2499–505. [PubMed: 21813745]
12. Gao M, Maynard KR, Chokshi V, Song L, Jacobs C, Wang H, Tran T, Martinowich K, Lee HK. Rebound potentiation of inhibition in juvenile visual cortex requires vision-induced BDNF expression. *J Neurosci*. 2014 Aug 6; 34(32):10770–9. [PubMed: 25100608]
13. Pozo K, Goda Y. Unraveling mechanisms of homeostatic synaptic plasticity. *Neuron*. 2010 May 13; 66(3):337–51. [PubMed: 20471348]
14. Cao Z, Wu X, Chen S, Fan J, Zhang R, Owyang C, Li Y. Anterior cingulate cortex modulates visceral pain as measured by visceromotor responses in viscerally hypersensitive rats. *Gastroenterology*. 2008 Feb; 134(2):535–43. [PubMed: 18242219]
15. Banerjee B, Medda BK, Schmidt J, Lang IM, Sengupta JN, Shaker R. Neuronal plasticity in the cingulate cortex of rats following esophageal acid exposure in early life. *Gastroenterology*. 2011 Aug; 141(2):544–52. [PubMed: 21616075]
16. Banerjee B, Medda BK, Pochiraju S, Kannampalli P, Lang IM, Sengupta JN, Shaker R. AMPA receptor subunits expression and phosphorylation in cingulate cortex in rats following esophageal acid exposure. *Neurogastroenterol Motil*. 2013 Dec; 25(12):973–e776. [PubMed: 24118589]
17. Kern MK, Birn RM, Jaradeh S, Jesmanowicz A, Cox RW, Hyde JS, Shaker R. Identification and characterization of cerebral cortical response to esophageal mucosal acid exposure and distention. *Gastroenterology*. 1998 Dec; 115(6):1353–62. [PubMed: 9834262]
18. Hobson AR, Furlong PL, Worthen SF, Hillebrand A, Barnes GR, Singh KD, Aziz Q. Real-time imaging of human cortical activity evoked by painful esophageal stimulation. *Gastroenterology*. 2005 Mar; 128(3):610–9. [PubMed: 15765397]
19. Lawal A, Kern M, Sanjeevi A, Antonik S, Mepani R, Rittmann T, Hussaini S, Hofmann C, Tatlo L, Jesmanowicz A, Verber M, Shaker R. Neurocognitive processing of esophageal central sensitization in the insula and cingulate gyrus. *Am J Physiol Gastrointest Liver Physiol*. 2008 Mar; 294(3):G787–G794. [PubMed: 18187518]
20. Derkach V, Barria A, Soderling TR. Ca²⁺/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc Natl Acad Sci U S A*. 1999 Mar 16; 96(6):3269–74. [PubMed: 10077673]
21. Kastning K, Kukhtina V, Kittler JT, Chen G, Pechstein A, Enders S, Lee SH, Sheng M, Yan Z, Haucke V. Molecular determinants for the interaction between AMPA receptors and the clathrin adaptor complex AP-2. *Proc Natl Acad Sci U S A*. 2007 Feb 20; 104(8):2991–6. [PubMed: 17289840]
22. Barria A, Muller D, Derkach V, Griffith LC, Soderling TR. Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. *Science*. 1997 Jun 27; 276(5321):2042–5. [PubMed: 9197267]
23. Kristensen AS, Jenkins MA, Banke TG, Schousboe A, Makino Y, Johnson RC, Huganir R, Traynelis SF. Mechanism of Ca²⁺/calmodulin-dependent kinase II regulation of AMPA receptor gating. *Nat Neurosci*. 2011 Jun; 14(6):727–35. [PubMed: 21516102]
24. Mammen AL, Kameyama K, Roche KW, Huganir RL. Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. *J Biol Chem*. 1997 Dec 19; 272(51):32528–33. [PubMed: 9405465]
25. Johnson MW, Chotiner JK, Watson JB. Isolation and characterization of synaptoneurosomes from single rat hippocampal slices. *J Neurosci Methods*. 1997 Dec 1; 77(2):151–6. [PubMed: 9489891]
26. Banerjee B, Medda BK, Schmidt J, Zheng Y, Zhang Z, Shaker R, Sengupta JN. Altered expression of P2X3 in vagal and spinal afferents following esophagitis in rats. *Histochem Cell Biol*. 2009 Dec; 132(6):585–97. [PubMed: 19784665]

27. Siddoway B, Hou H, Xia H. Molecular mechanisms of homeostatic synaptic downscaling. *Neuropharmacology*. 2014 Mar;78:38–44. [PubMed: 23911745]
28. Kern M, Hofmann C, Hyde J, Shaker R. Characterization of the cerebral cortical representation of heartburn in GERD patients. *Am J Physiol Gastrointest Liver Physiol*. 2004 Jan; 286(1):G174–G181. [PubMed: 14512287]
29. O'Brien RJ, Kamboj S, Ehlers MD, Rosen KR, Fischbach GD, Huganir RL. Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron*. 1998 Nov; 21(5):1067–78. [PubMed: 9856462]
30. Turrigiano GG, Nelson SB. Thinking globally, acting locally: AMPA receptor turnover and synaptic strength. *Neuron*. 1998 Nov; 21(5):933–5. [PubMed: 9856445]
31. Thiagarajan TC, Piedras-Renteria ES, Tsien RW. alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. *Neuron*. 2002 Dec 19; 36(6):1103–14. [PubMed: 12495625]
32. Thiagarajan TC, Lindskog M, Tsien RW. Adaptation to synaptic inactivity in hippocampal neurons. *Neuron*. 2005 Sep 1; 47(5):725–37. [PubMed: 16129401]
33. Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC. Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat Neurosci*. 2004 Mar; 7(3):244–53. [PubMed: 14770185]
34. Aoto J, Nam CI, Poon MM, Ting P, Chen L. Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron*. 2008 Oct 23; 60(2):308–20. [PubMed: 18957222]
35. Beique JC, Na Y, Kuhl D, Worley PF, Huganir RL. Arc-dependent synapse-specific homeostatic plasticity. *Proc Natl Acad Sci U S A*. 2011 Jan 11; 108(2):816–21. [PubMed: 21187403]
36. Clem RL, Anggono V, Huganir RL. PICK1 regulates incorporation of calcium-permeable AMPA receptors during cortical synaptic strengthening. *J Neurosci*. 2010 May 5; 30(18):6360–6. [PubMed: 20445062]
37. Sutton MA, Ito HT, Cressy P, Kempf C, Woo JC, Schuman EM. Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell*. 2006 May 19; 125(4):785–99. [PubMed: 16713568]
38. Barria A, Derkach V, Soderling T. Identification of the Ca²⁺/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. *J Biol Chem*. 1997 Dec 26; 272(52):32727–30. [PubMed: 9407043]
39. Gainey MA, Tatavarty V, Nahmani M, Lin H, Turrigiano GG. Activity-dependent synaptic GRIP1 accumulation drives synaptic scaling up in response to action potential blockade. *Proc Natl Acad Sci U S A*. 2015 Jul 7; 112(27):E3590–E3599. [PubMed: 26109571]
40. Brecht DS, Nicoll RA. AMPA receptor trafficking at excitatory synapses. *Neuron*. 2003 Oct 9; 40(2):361–79. [PubMed: 14556714]
41. Song I, Huganir RL. Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci*. 2002 Nov; 25(11):578–88. [PubMed: 12392933]
42. Chung HJ, Xia J, Scannevin RH, Zhang X, Huganir RL. Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *J Neurosci*. 2000 Oct 1; 20(19):7258–67. [PubMed: 11007883]
43. Gainey MA, Hurvitz-Wolff JR, Lambo ME, Turrigiano GG. Synaptic scaling requires the GluR2 subunit of the AMPA receptor. *J Neurosci*. 2009 May 20; 29(20):6479–89. [PubMed: 19458219]
44. Qiu Z, Sylwestrak EL, Lieberman DN, Zhang Y, Liu XY, Ghosh A. The Rett syndrome protein MeCP2 regulates synaptic scaling. *J Neurosci*. 2012 Jan 18; 32(3):989–94. [PubMed: 22262897]
45. Hou Q, Gilbert J, Man HY. Homeostatic regulation of AMPA receptor trafficking and degradation by light-controlled single-synaptic activation. *Neuron*. 2011 Dec 8; 72(5):806–18. [PubMed: 22153376]
46. Leslie KR, Nelson SB, Turrigiano GG. Postsynaptic depolarization scales quantal amplitude in cortical pyramidal neurons. *J Neurosci*. 2001 Oct 1; 21(19):RC170. [PubMed: 11567081]
47. Pak DT, Sheng M. Targeted protein degradation and synapse remodeling by an inducible protein kinase. *Science*. 2003 Nov 21; 302(5649):1368–73. [PubMed: 14576440]

48. Peng YR, He S, Marie H, Zeng SY, Ma J, Tan ZJ, Lee SY, Malenka RC, Yu X. Coordinated changes in dendritic arborization and synaptic strength during neural circuit development. *Neuron*. 2009 Jan 15; 61(1):71–84. [PubMed: 19146814]
49. Sun Q, Turrigiano GG. PSD-95 and PSD-93 play critical but distinct roles in synaptic scaling up and down. *J Neurosci*. 2011 May 4; 31(18):6800–8. [PubMed: 21543610]
50. Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron*. 2003 Oct 30; 40(3):595–607. [PubMed: 14642282]
51. Ehlers MD. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat Neurosci*. 2003 Mar; 6(3):231–42. [PubMed: 12577062]
52. Zhang D, Hou Q, Wang M, Lin A, Jarzylo L, Navis A, Raissi A, Liu F, Man HY. Na,K-ATPase activity regulates AMPA receptor turnover through proteasome-mediated proteolysis. *J Neurosci*. 2009 Apr 8; 29(14):4498–511. [PubMed: 19357275]
53. Tataavarty V, Sun Q, Turrigiano GG. How to scale down postsynaptic strength. *J Neurosci*. 2013 Aug 7; 33(32):13179–89. [PubMed: 23926271]
54. Miranda A, Mickle A, Schmidt J, Zhang Z, Shaker R, Banerjee B, Sengupta JN. Neonatal cystitis-induced colonic hypersensitivity in adult rats: a model of viscerovisceral convergence. *Neurogastroenterol Motil*. 2011 Jul; 23(7):683–e281. [PubMed: 21592255]
55. Chitkara DK, van Tilburg MA, Blois-Martin N, Whitehead WE. Early life risk factors that contribute to irritable bowel syndrome in adults: a systematic review. *Am J Gastroenterol*. 2008 Mar; 103(3):765–74. [PubMed: 18177446]

Key messages

The findings of this study provide a better understanding of the esophageal pain mechanisms for patients with gastroesophageal reflux disease (GERD). The main objective was to investigate whether prolonged esophageal acid exposures in Sprague Dowley rats resulted in homeostatic synaptic scaling through downregulation of AMPA receptor membrane expression in rCC neurons. Repeated esophageal acid exposures in adult rats demonstrated synaptic scaling by downregulation of GluA1, pGluA1Ser⁸³¹ and pCaMKII α expression in cortical membrane preparation. This neuronal compensatory mechanism was not observed in rats that were preexposed neonatally to repeated esophageal acid exposures during neuronal development.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Table 1

Effect of esophageal acid on cortical NMDA receptor subunits expression

NMDA receptor subunits Acid vs Saline (Change in expression)	NR1	NR2A	NR2B	pNR2B
Protocol 1 Adult treatment (Fig. 1)	↔	↑	↔	↑
Protocol 2 Neonatal treatment and adult challenge (Fig. 1)	↔	↔	↔	↑