Chronic glutamate treatment selectively modulates AMPA RNA editing, ADARs expression and activity in primary cortical neurons

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Conflict of interest

The authors have no conflict of interest

Abstract

Adenosine to Inosine RNA editing is a post-transcriptional process, catalyzed by ADAR enzymes, with an important role in diversifying the number of proteins belonging (deriving, secondo me)
from a single gene. In neurons, editing of ionotropic AMPA glutamate receptors has been shown to be altered under different experimental conditions, including severe pathologies, indicating the potential relevance of its modulation. In the current study, we treated rat primary cortical cell cultures with a sub-lethal dose of glutamate (10 μM), focusing on RNA editing process and ADAR activity. We found that chronic glutamate treatment down-regulates RNA editing levels at R/G site of GluA2-4 subunits of AMPA receptors and at the K/E site of CYFIP2. These changes are site-specific since they were not observed for the GluA2 Q/R site as well as for other non glutamatergic sites. Glutamate treatment also down-regulates the protein expression levels of both ADAR1 and ADAR2 enzymes, through a pathway that is Ca^{2+}- and calpain-dependent. Given that AMPA receptors containing unedited subunits show a slower recovery rate from desensitization as compared to those containing edited forms, the reduced editing at the R/G site may, at least in part, compensate glutamate over-stimulation perhaps through the reduced activation of postsynaptic receptors. In summary, our data provide direct evidence of the involvement of ADAR1 and ADAR2 activity as a possible compensatory mechanism for neuronal protection following glutamate over-stimulation.

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Running title: ADAR activity is modified by chronic glutamate treatment

Keywords: ADAR1; ADAR2; RNA editing; R/G site; AMPA receptors; Glutamate treatment

Introduction

Adenosine Deaminase catalyzes a post-transcriptional process called RNA editing {Bass, 2002 #98;Orlandi, 2012 #226} by acting through RNA (ADAR) enzymes. This peculiar molecular mechanism consists of the enzymatic deamination of specific adenosines (A) nucleosides into inosines (I). Since inosine has base-pairing properties similar to guanosine, it is read as guanosine by both splicing and translation machineries thus generating RNA molecules different from those coded by DNA {Nishikura, 2010 #161}. The RNA editing process contributes to the diversification of the information that is encoded in the genome of an organism thereby providing a greater degree of complexity {Tariq, 2012 #228}. Currently, the conversion of A to I is thought to be the most common RNA editing process in higher eukaryotic cells, especially in the brain {Paul, 1998 #235}.

In mammals, three members of the ADAR family have been characterized so far; ADAR1 and ADAR2 are active enzymes expressed in many tissues, while ADAR3 is expressed in the CNS{Chen, 2000 #262}, more abundantly in glial cells than in neurons {Kawahara, 2003 #263}; up to date, no functional RNA editing activity has been attributed to this enzyme {Orlandi, 2012 #226}. Current studies showed that the majority of RNA editing sites is located within intragenic non-coding sequences: the 5'UTR, 3′UTR and intronic retrotransposon elements, such as Alu and long interspersed elements {Levanon, 2004 #264;Li, 2009 #265}. However, A to I RNA editing in mammalian cells has been originally described for a number of protein-coding RNA sequences, resulting in dramatic changes of protein functions {Tariq, 2012 #228}. Given its critical role, ADARs expression and activity must be tightly controlled by cells; the subcellular distribution of ADARs {Sansam, 2003 #260} and their interaction with inhibitors{Tariq, 2013 #258} and activators{Garncarz, 2013

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#257;Marcucci, 2011 #259} have been shown to influence ADAR activities. Accordingly, knock-out of ADAR1 or ADAR2 in mice resulted in embryonic lethality or death shortly after birth, respectively {Hartner, 2004 #232;Higuchi, 2000 #233;Wang, 2000 #234}, clearly indicating that A-I RNA editing is essential for normal life and development.

RNA editing targets are abundant in the Central Nervous System (CNS){Paul, 1998 #235;Hoopengardner, 2003 #236} where proteins involved in synaptic transmission are recoded {Rosenthal, 2012 #237;Tariq, 2012 #228}. Dysregulation of this process might result in profound alterations in neuronal signaling and give rise to severe neurological disorders{Maas, 2006 #146} {Akbarian, 1995 #122} {Kawahara, 2004 #111;Silberberg, 2012 #238;Singh, 2012 #241}. In particular, RNA editing has profound effects on glutamate neurotransmission given that it recodes several glutamate receptor subunits {Seeburg, 2002 #94;Barbon, 2011 #204}. Glutamate is the most important excitatory neurotransmitter in the CNS and it is involved in cognitive functions like learning and memory. The neurotransmitter can potentially activate three types of ionotropic glutamate receptors, namely N-methyl-D-aspartate receptor (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) and Kainate receptors {Traynelis, 2010 #170}. RNA editing process might modify AMPA glutamate receptor subunits (GluAs) in several specific positions. The editing positions have been named on the basis of the amino acid substitution, such as the Q/R site in AMPA GluA2 and the R/G site in GluA2, GluA3, and GluA4{Seeburg, 2002 #94;Barbon, 2011 #204}. The amino acid changes alter channel properties {Lomeli, 1994 #104;Kamboj, 1995 #100}; for the GluA2 Q/R site, the presence of the positively charged amino acid arginine in the inner channel pore makes the receptor channel impermeable to Ca^{2+}, reduces its ion conductance and alters its current/voltage relationship {Hollmann, 1991 #266;Burnashev, 1992 #267} as well as affects receptor maturation and cellular trafficking {Greger, 2003 #102;Greger, 2002 #103}. Under physiological conditions, GluA2 is fully

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edited at the Q/R site and the resultant AMPA-GluA2-containing receptors are Ca^{2+}-impermeable, whereas AMPA receptors lacking GluA2 are permeable to Ca^{2+} {Isaac, 2007 #239;Liu, 2007 #240}. Whether AMPA receptors are permeable or impermeable to Ca^{2+} it might greatly affect neuronal plasticity and survival following injury or excitotoxicity events {Liu, 2007 #240}. The editing loss at the GluA2 Q/R site allows increased influx of Ca^{2+} leading to epileptic seizures and death as reported for ADAR2 null mice {Brusa, 1995 #89;Higuchi, 2000 #233}. In humans, transient ischemia reduced GluA2 subunit mRNA editing and decreased the abundance of ADAR2 mRNA, leading to cell death of pyramidal neurons {Peng, 2006 #163}. Furthermore, in the spinal motor neurons of sporadic Amiotrophic lateral Sclerosis (ALS) patients, editing at the GluA2 Q/R position was severely decreased, presumably through down-regulation of ADAR2 levels {Aizawa, 2010 #269;Hideyama, 2012 #268;Kawahara, 2004 #111;Kawahara, 2003 #224}, leading to highly Ca^{2+} permeable AMPA channels and in turn facilitating motoneurons death as reported in conditional ADAR2 knockout {Hideyama, 2010 #242}. Recently, it has been reported that exposure of cortical cells to excitotoxic levels of glutamate induces cleavage of ADAR2 and subsequent loss of GluA2 Q/R editing that would result in excitotoxic cell death{Mahajan, 2011 #243}.

Moreover, GluA2, 3, and 4 receptor subunits are edited at the R/G site; this site is located just before the sequences involved in the splicing events forming the Flip/Flop isoforms, and it seems to affect both the splicing events and the desensitisation properties of the AMPA receptor channels {Lomeli, 1994 #104;Krampfl, 2002 #150}. Particularly, edited receptors have an enhanced rate of recovery from desensitization, generating ion channels that are able to respond more rapidly to a train of impulses {Lomeli, 1994 #104}. We have recently shown that glutamate overstimulation, induced by spinal cord injury (SCI), reduced AMPA R/G editing levels{Barbon, 2010 #178}, presumably dampening post-synaptic excitatory responses to glutamate in an attempt to limit the progression of cell death.

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The over-activation of GluAs in several pathological conditions such as ALS, stroke, epilepsy and SCI is well established{Lau, 2010 #244}; however, GluAs functional state is tightly controlled by post-transcriptional modifications and the role of these regulatory events in neurological disorders has not yet been elucidated. Our work was aimed at evaluating the role of RNA editing of AMPA receptors and ADAR enzymes during glutamate over-stimulation. In particular, we report that chronic glutamate treatment in neuronal cells selectively affects the AMPA receptor R/G editing levels through a cellular cascade involving Ca^{2+} and calpain-dependent mechanisms.

Results

Effects of glutamate exposure on cell viability

We evaluated neuronal cell survival (DIV14 cortical cultures) 24 hours after exposure to different glutamate concentrations (10, 50, 100μM) by simultaneous staining with propidium iodide and antibody anti-NeuN protein. Figure 1 shows that 24h incubation promoted a dose-dependent reduction of cell viability, expressed as percentage of NeuN positive cells that present propidium iodide staining. The highest level of cell viability was found with the lowest glutamate concentration (10 μM). Based on these data, we chose 10μM of glutamate, i.e. the experimental condition showing the lowest cell death, for the following experiments.

Editing level quantification after chronic glutamate treatment

We exposed cortical neurons to glutamate for 24h and then collected the cells immediately or 72 h later, for editing analysis. The Q/R site of GluA2 that is almost fully edited under physiological conditions was not affected after 24h of chronic glutamate stimulus or 72h of washout (Fig. 2a), both at the pre-mRNA and mRNA level (Supplemental Fig.1). On the other hand, the R/G site was deeply affected and the changes were maintained even after washout. Concerning GluA2 R/G editing levels (Fig. 2b), two-way ANOVA showed an effect of treatment ($p<0.001$) in both Flip and Flop variants; in particular we observed an overall reduction of GluA2 R/G editing (-48.1%; $p < 0.001$), in the Flip variant as well as in the Flop variant (-41.1%; $p < 0.001$), after 24h of chronic treatment of mature

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neurons. Such glutamate-induced down-regulation persisted for at least 72h, with a decrease in editing level of -52.2% in the Flip ($p < 0.001$) and -44.5% in the Flop variant ($p < 0.001$).

A similar effect was observed for the GluA3 R/G site (Fig. 2c). Two-way ANOVA for the Flip variant showed both a treatment ($p < 0.001$) and a time ($p < 0.001$) effect, whereas for the Flop variant it showed an effect of treatment ($p < 0.001$), time ($p < 0.001$) and time x treatment interaction ($p < 0.05$). After glutamate chronic treatment, a robust decrease in the editing level of both isoforms was evident at both time points. After 24hr, the editing of GluA3 Flip and Flop variant decreased by -46.6% ($p < 0.001$) and by -45.6% ($p < 0.01$), respectively. Editing levels remained low even after 72hr of washout: -48.8% for the Flip variant ($p < 0.001$) and -61.1% for the Flop variant ($p < 0.001$).

Two-way ANOVA showed an effect of treatment ($p < 0.001$) for GluA4 editing level (Fig. 2d). Significant decreases were observed for the Flip transcript both after 24hr (-30.8%; $p < 0.001$) and after washout (-26.2; $p < 0.001$). Similarly, the Flop variant showed a significant decrease after 24hr (-36.4%; $p < 0.001$) and 72hr of washout (-47.5%; $p < 0.001$).

To distinguish whether the treatment effects on editing were selective for the AMPA receptor or instead were due to a general loss of editing capability, we evaluated the mRNAs encoding for the Cytoplasmic FMR1-interacting protein 2 (CYFIP2), the serotonin 5-HT2C receptor and the bladder cancer associated protein (BLCAP). We found that the editing site of CYFIP2 was affected by glutamate treatments. Two-way ANOVA showed both a treatment ($p < 0.001$) and a time ($p < 0.001$) effect. After 24hr, the editing of CYFIP2 K/E site was decreased after 24h of treatment (-56,4%; $p < 0.001$) and after the washout (-65,3 $p < 0,001$) (Fig. 3). In contrast, the five editing sites of 5-HT2C and the three of BLCAP were not affected by glutamate treatment (Table 1).

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Modulation of Glutamate Receptor subunit protein expression after glutamate treatment

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To evaluate the effects of glutamate treatment on AMPA subunit protein expression, we focus on GluA1 e GluA2 subunits that are the main expressed AMPA subunit. GluA1 protein expression was decreased of about 50% after glutamate treatment ($p < 0.001$) and remained down-regulated of about 40% after the washout period ($p < 0.01$). In contrast GluA2 protein expression was unaffected by glutamate treatment (Fig. 4).

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ADAR1 and ADAR2 expression after chronic glutamate treatment

To verify if the reductions of the editing level were related to changes in protein expression of the enzymes responsible for RNA editing, the expression level of ADAR1 and ADAR2 enzymes was evaluated (Fig. 3). Two-way ANOVA showed an effect of treatment ($p < 0.001$) for both ADAR1 and ADAR2 protein expression. Both enzymes were down-regulated both after 24hr of chronic glutamate treatment and after 72hr of washout. ADAR1 protein expression decreased by 58.6% after 24hr treatment ($p < 0.001$) and by 45.8% following washout ($p < 0.01$); the same was observed for ADAR2 protein which decreased by 63.3% after 24hr ($p < 0.001$) and by 57.9% after 72hr ($p < 0.001$).

ADAR2 RNA editing and alternative splicing after chronic glutamate treatment

One ADAR2 splicing event results in the inclusion of an additional 47nt cassette at the 5'end of the coding region, changing the reading frame of the mature ADAR2 transcript. The inclusion of this cassette depends on ADAR2 ability to edit its own pre-mRNA to generate a new intronic 3'acceptor site. In order to evaluate the potential change of ADAR2 catalytic activity, the self-editing level at position -1 were analyzed.

After chronic glutamate treatment, ADAR2 self-editing (Fig. 4) was reduced by -53.4% ($p < 0.001$); the level of ADAR2 self-editing was also down-regulated (-59.6%) even after 72hr of washout ($p < 0.001$). Two-way ANOVA showed an effect of treatment ($p < 0.001$), time ($p < 0.01$) and time/treatment interaction ($p < 0.05$). Following the editing variation, the splicing pattern of the canonical (-47nt) and alternative forms (+47nt) was changed (Supplemental Figures 2).

Glutamate-induced RNA editing and ADAR protein decrease is Ca^{2+}- and Calpain- dependent

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The following step was to investigate the potential mechanisms responsible for the modulation of ADARs expression and RNA editing level. To this end, we set up some experiments to evaluate the effect of Ca^{2+} influx, kinase activity as well as Calpain action, an important protease activated by Ca^{2+} cascade, as recently proposed {Mahajan, 2011 #243}. Cortical neurons were treated with BAPTA/am (Ca^{2+} chelator, 20μM), Calpain inhibitor (10μM), KN93 (CamKII inhibitor, 10 μM) in parallel with glutamate treatment. After glutamate treatment, the activation of Calpain was demonstrated by the cleavage of spectrin (Supplemental Figure 3).

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As in the previous experiments, the GluA2 Q/R editing level was not affected by glutamate; similarly, BAPTA/am, KN93 or Calpain inhibitor treatment did not modify the editing level at this site (Fig. 5a). As expected, chronic glutamate treatment induced GluA2 R/G editing down-regulation (-71.4%; $p<0.001$). Interestingly, both BAPTA/am and Calpain Inhibitor treatment blocked glutamate GluA2 R/G editing decrease (Fig. 5b), but KN93 did not (GluA2 R/G editing down-regulation: -47,7%; $p<0.001$). Of note, however the different treatments alone induced a partial down-regulation of GluA2 R/G comparing to untreated control.

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Considering the CYFIP2 K/E site (Fig.5c), the glutamate-dependent down-regulation (-66,96%, p<0,001) was completely abolished by BAPTA/am and Calpain Inhibitor treatments, but not by KN93 (CYFIP2 K/E editing down-regulation: -57,17%; p<0.001).

The analysis of ADARs protein expression revealed a similar pattern of changes seen for GluA2 R/G and CYFIP2 K/E editing variations (Fig. 6). After 24h, glutamate treatment caused a down regulation by -56.7% for ADAR1 (p<0.05) and by -67.5% for ADAR2 (p<0.01); in contrast, BAPTA/am and Calpain Inhibitor treatments prevented protein down-regulation, while KN93 did not prevent ADARs down-regulation (by -61.7% for ADAR1 (p<0.01) and by -62.9% for ADAR2 (p<0.01)).

Analysis of ADAR cofactors after chronic glutamate treatment

To further shed light on ADARs activity modulation, we analyzed the expression pattern of several recently reported stimulatory factor such as Split Hand/Foot Malformation 1 (SHFM1), and RNA binding protein hnRNP A2/B1 (hnRNPA2/B1), as well as inhibitory factors such as ribosomal protein S14 (RPS14) and Serine/Arginine-Rich Splicing Factor 9 (SRSF9) that can modulate ADAR activity (Fig. 7).

Chronic glutamate treatment induced an up-regulation of the inhibitory factor SRSF9 by a mean factor of 2.39 (log2R= 1.22 p<0.05), a trend of increase could be seen also for the inhibitory factor RPS14 although not statistically significant. The two tested stimulatory factors were not affected by chronic glutamate treatment. Inhibition of Ca2+ entry, CamKII activity or Calpain activity did not alter the expression level of the four ADAR cofactors (Supplemental Figure 4).

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GluA2 editing level quantification after acute glutamate treatment

We then investigated if acute glutamate treatment, as performed by Mahajan and collaborators {Mahajan, 2011 #243}, had the same effect of the chronic treatment on RNA editing level and ADAR protein expression. Cortical neurons were treated with 100 μM glutamate and the experiments performed at different time points. The editing level of GluA2 R/G and Q/R sites was examined immediately after 1hr of treatment and after 5hr of washout; moreover 6hr of continuous treatment was also performed.

Interestingly, the editing level of GluA2 Q/R site was not affected in acute treatments as previously observed in the chronic treatment (Fig 7a). Regarding the GluA2 R/G editing site (Fig. 7b), the editing level was unchanged 1hr after treatments whereas after 5hr of washout a robust down-regulation (-39.1%; $p < 0.001$) was observed, that persisted even after 6hr of treatment (-42.5%; $p<0.001$). The two-way ANOVA displayed an impact of treatment ($p<0.001$), time ($p<0.001$) and time/treatment interaction ($p<0.001$).

ADARs protein expression after acute glutamate treatment

Concerning ADAR1 (Fig. 8a), no difference in protein expression was observed after 1hr of treatment while a decrease was seen after 5hr of washout (-25.9% $p<0.05$). Following 6hr of continuous treatment, a reduction of ADAR1 by -42.1% ($p<0.01$) was observed.

With respect to ADAR2, a down-regulation was seen immediately after the acute treatment (-29.5%; $p<0.001$) whereas an increase was observed after wash-out (-66.4%; $p<0.001$). Following 6hr of continuous treatment, a deep ADAR2 protein expression reduction has been observed (-69.3%; $p<0.001$).

Discussion

We here report that, in primary cortical neurons, chronic glutamate treatment reduces the R/G editing levels of GluA2, GluA3, and GluA4 AMPA receptor subunits, without affecting the GluA2 Q/R editing level. Such decrease was accompanied by a parallel loss of ADAR1 and ADAR2 expression, through a signal cascade that is Ca2+- and Calpain- dependent. Of note, the protein expression of GluA1, but not GluA2, is also down-regulated.

One of the features of R/G editing site is to modulate the kinetic properties of AMPA receptor channels in association with alternative splicing at the Flip/Flop cassette, thus determining the time course for desensitization and resensitization{Lomeli, 1994 #104;Krampfl, 2002 #150}. AMPA receptors containing an edited (G) subunit show a faster recovery rate from desensitization compared with an unedited (R) form. Therefore, the reduced editing at the R/G site of the AMPA subunits would slow the kinetic of AMPA receptors resensitization, attenuating the response to the continuative glutamate stimulus. In turn, the reduced activation of post-synaptic receptors would limit calcium-induced activation of post-synaptic neurons, the relevant step mediating excitatory cell death{Choi, 1994 #253;Michaelis, 1998 #254}.

Notably and in contrast with a previous paper{Mahajan, 2011 #243}, the GluA2 Q/R editing level is not affected under our experimental conditions. The same occurs for the five sites of 5-HT_{2C}R and that of BLCAP. These results strongly point to a selective effect on R/G editing level of GluA2, A3, and A4 AMPA receptor subunits, despite the strong down-regulation of both ADAR enzymes.

Interestingly, glutamate treatment induced a robust down-regulation of CYFIP2 K/E site {Levanon, 2005 #275}. CYFIP2 mRNA is ubiquitously expressed with the K/E site edited to variable extents among human tissues {Nishimoto, 2008 #273}. In neurons CYFIP2 and its homolog CYFIP1 are localized at the synapse and have been described to interact with FMRP with a possible role in axon

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guidance and synapses formation {Abekhoukh, 2014 #274}. While it has been demostreted that the K/E editing site is ADAR2 specific {Riedmann, 2008 #276}{Nishimoto, 2008 #273} and its editing level is regulated in murine development {Wahlstedt, 2009 #277}, no data exist about the functional effect of the K/E editing substitution and its role in the field of glutamate transmission remains unknown.

Additionally, our finding that the GluA2 Q/R site is still fully edited indicates the continuous formation of GluA2 (R) containing AMPA receptors that, being impermeable to Ca^{2+}, may counteract the excitotoxic effects of glutamate. Our data suggest that an increased Ca^{2+} influx through unedited GluA2-containing AMPA channels is not involved in the cellular response to "in vitro" chronic glutamate treatment. Moreover, glutamate treatment strongly reduced the GluA1 subunits with no effects on GluA2 subunits indicating the generation (o formation?) of more AMPA channels impermeable to Ca2+ thus inhibiting the pathological effects of the treatment; accordingly, the regulatory changes seen here might represent a compensatory response set in motion by neuronal cells to attenuate, at least partially, glutamate excitotoxicity. This possibility is further reinforced by our recent observation that spinal cord injury produces a decrease in AMPA receptor R/G editing, perhaps reducing post-synaptic excitatory responses to glutamate and limiting the progression of cell death{Barbon, 2010 #178;Barbon, 2011 #204}. Thus, both our 'in vitro' and 'in vivo' evidence converge to indicate that a proper regulation in the editing levels is an important step in neuronal cell physiology and survival.

A recent work by Balik and associates {Balik, 2013 #270} reported that enhanced neuronal activity after treatment with the GABA-A channel blocker bicuculline (BIC) increased the level of R/G editing.in CA1, but not CA3, hippocampal cells, suggesting cell type / subfield specificity in neuronal response. These data are in apparent contrast with ours since we report a glutamate-induced down-

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regulation of R/G editing. Such discrepancy might be, at least in part explained, by the fact that we used glutamate to directly activate glutamate receptors in a sub-excitotoxic way, while Balik et al. enhance neuronal activity by inhibiting gabaergic interneurons: the intracellular pathways activated by the two paradigms might be different and so the amount of glutamate that activates glutamate receptors.. Alternatively, we use dissociated cortical neurons while Balik and collaborators used hippocampal slices observing modification only in CA1 subfield: the possibility exists that different neuronal cells differently respond to an exogenous stimulus. Nevertheless, both our and Balik's data show that the modulation of RNA editing is crucial for neuronal activity.

To further understand why glutamate treatment alters specific editing sites, we analyzed the expression pattern of recently found ADAR cofactors that might inhibit or activate ADAR activity {Tariq, 2013 #258}{Garncarz, 2013 #257}. We reported that the splicing factor SFRS9, an inhibitor of ADAR activity, is up-regulated after glutamate treatment and a trend toward an increase (although not statistically significant) could be seen also for the inhibitory factor RPS14. These data, could indicate that also these co-factors might be involved in the processes leading to the down-regulation of editing activity. It is worth mentioning that BIC treatment of hippocampal slices, besides inducing a partial up-regulation of R/G site editing and ADAR2 mRNA levels {Balik, 2013 #270}, also determined a strong down-regulation of SFRS9 mRNA {Tariq, 2013 #258}confirming a possible link between SFRS9 and ADAR2 activity.

As above mentioned, we found a profound reduction of both ADAR1 and ADAR2 as a consequence of chronic glutamate over-stimulation. To further investigate this effect, we evaluated ADAR activity by measuring the levels of ADAR2 self-editing and its splicing isoforms. ADAR2 edits its own pre-mRNA by introducing an alternative proximal 3′ acceptor site; this new acceptor site adds 47nt to the ADAR2 coding region{Rueter, 1999 #255}, giving rise to a frameshift that codes for a truncated

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ADAR2 protein lacking deaminase activity{Feng, 2006 #256}. Chronic glutamate stimulation reduces ADAR2 self-editing and, given that this editing position is a specific target of ADAR2, our results suggest a potential deactivation of ADAR2 enzymatic activity. If this holds true, the selectivity of ADAR2 action might be due to a site-specific cellular response to the treatment that, through changes in R/G editing only, leads to a specific modulation of AMPA receptor functions. However, the decrease in ADAR2 self-editing is not followed by the expected increase in the full length protein that is, instead, profoundly down-regulated. Moreover, chronic glutamate treatment induced an up-regulation of the splicing isoform of ADAR2 lacking a 30nt cassette in the deaminase domain, which is characterized by a low editing activity {Rueter, 1999 #255}.

Taken together our data indicate that glutamate treatment induced a down-regulation in the expression of ADAR1 and ADAR2 proteins, together with a decrease of ADAR2 enzymatic activity. Interestingly, such reduction seems to be dependent on Ca^{2+} inflow since the treatment with extracellular Ca^{2+} chelator BAPTA/am reverted ADAR1, ADAR2{Mahajan, 2011 #243} as well as GluA2 R/G and CYFIP2 K/E editing level reductions.

Recent data reported that ADAR activity might be regulated by phosphorylation{Orlandi, 2012 #226} {Marcucci, 2011 #261}; to this end, we investigated the contribution of CamKII, one of the most important kinases of the glutamatergic system, by inhibiting its activation. We found that the reduction of R/G editing levels was not prevented by CamKII inhibition, suggesting that other modifications or other kinases, take place to regulate ADARs activity.

It has been reported that the protease Calpain might cleave ADAR2 after glutamate excitotoxicity treatment {Mahajan, 2011 #243}. Our results confirm and extend these data indirectly showing that the same mechanism takes place after chronic glutamate treatments and it might be responsible for the changes of ADAR1 activity. Further analyses are needed to confirm ADAR1 cleavage by Calpain.

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However discrepancy exists with the work of Mahajan and collaborators{Mahajan, 2011 #243} since they showed a down-regulation of GluA2 Q/R editing that was not observed in our experiments, neither at the mRNA or pre-mRNA level. Notably, although in our acute treatments we exactly reproduced the same experimental conditions of Mahajan and collaborators{Mahajan, 2011 #243}, we did not observe GluA2 Q/R editing reductions at any of the points analyzed, otherwise we confirmed the decrease in R/G editing and ADAR1 and ADAR2 protein expression also after acute glutamate treatment.

Taken together, our findings show that chronic treatment with sub-excitotoxic doses of glutamate causes a decrease in the R/G editing site of all AMPA receptors, presumably in an attempt to dampen potential excitotoxic effects of the continuous stimulation. Of note, CYFIP2 K/E is down-regulated in a pattern similar to that found for the R/G site. The functional consequence of this modulation remains unknown due to the lack of information regarding the role of the K/E site in the physiology of the protein. The reported effects are selective for these specific editing sites and occur through reduction of ADAR1 and ADAR2 protein levels in a Ca^{2+}- and Calpain-dependent fashion further pointing to a finely tuning of these mechanisms as pivotal under both physiological and pathological conditions.

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Material and Methods

Primary neuronal cell cultures and treatments

Our experiments complied with guidelines for the use of experimental animals issued by the European Community Council Directive 86/609/EEC and were approved by the Italian Ministry of Health (Project ID: 320/2010). Rat cortical cultures were prepared as previously described {Orlandi, 2011 #247;Lesuisse, 2002 #249}. In brief, cerebral cortices from day 18 Sprague-Dawley rat embryos (E18, Charles River Laboratories Inc., Wilmington, MA, USA) were mechanically dissociated by trituration

in cold HBSS (Invitrogen) containing 10 mM HEPES (pH 7.4). The suspension was allowed to settle for 5 min, and the top fraction was collected. The neurons were centrifuged for 5 min at 200 g and resuspended in serum-free Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 30 U/ml penicillin (Sigma-Aldrich), 30 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, United States of America) and 0.5 mM Glutamax (Invitrogen). Neurons were plated at a density of 30,000 cells/cm^2 on poly-D-lysine (Sigma-Aldrich) coated Petri dishes {de Lima, 1997 #245;Rao, 1998 #246}. Three days after plating, 50% of the medium was replaced with fresh medium. Subsequently, half of the medium was replaced once a week.

Cell death analysis

Glutamate concentration for the following analyses was chosen after propidium iodide analysis (PI) of DIV14 rat cortical cultures incubated for 24hr with different concentration of glutamate (10, 50 and 100µM). After treatment, neuronal cultures were incubated with 5µM/ml of propidium iodide (Sigma-Aldrich) in HBSS for 15min at room temperature to stain nuclei of dead cells. The propidium iodide is incorporated by dying or dead cells exclusively, emitting red light; NeuN protein staining, a neuronal specific marker, was used to detect neuronal cells. Apoptotic cells present either condensed or fragmented nucleus and are labeled and necrotic cells appear as characteristic red dots. Neuronal death (Fig.1) was expressed by the percentage of NeuN positive cells that present prodidium iodide staining.
The coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (4% PFA-PBS, Invitrogen) for 15 min at room temperature and before mounting, DAPI nuclear staining was performed.

Treatment of neuronal cultures

Cortical neuron cells for western blot and RNA editing analysis were chronically treated with 10µM Glutamate (Sigma-Aldrich) and harvested after 24hr of treatment{Qian, 2011 #250;Ha, 2009 #252}; moreover after treatment cells were maintained in normal culture medium for additional 3 days (washout). BAPTA/am calcium chelator (Sigma-Aldrich) 20µM and Calpain inhibitor (Sigma-Aldrich) 10µM was added 30 min prior to addiction of Glutamate and maintained for the 24h of chronic treatment {Bordji, 2010 #271}.

Acute treatments were carried out in three different time points: 100µM Glutamate for 1hr; 100µM Glutamate for 1hr and cells harvested after 5hr of washout; 100µM Glutamate for 6hr of continuous treatment. Each experiment was performed using three independent preparations of DIV14 neurons.

Western blot analysis

Cells harvested from primary cortical cultures were solubilized with modified RIPA (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA630, 0.25% NaDOC, 0.1% SDS, 1% NP-40 and Roche protease inhibitor tablets) and then sonicated. A portion of the lysate was used for the Bicinchoninic Acid (BCA) protein concentration assay (Sigma-Aldrich). Equal amounts of protein were applied to precast SDS polyacrylamide gels (4–12% NuPAGE Bis-Tris gels; Invitrogen) and the proteins were electrophoretically transferred to a Nitrocellulose Transfer Membrane (GE Healthcare, Waukesha, WI, USA) for 2 h. The membranes were blocked for 60 min with 3% nonfat dry milk in TBS-T (Tris-buffered saline with 0.1% Tween-20, Sigma-Aldrich) and then incubated overnight at 4°C in the blocking solution with the mouse monoclonal anti-ADAR1 (1:350; Santa-Cruz Biotechnologies, Dallas, Texas, USA. Cod: sc-73408), rabbit polyclonal anti-ADAR2 (1:350; Abcam Cambridge, England, United Kingdom; cod: AB-64830), rabbit polyclonal GluA1 (1:200, Millipore, Billerica, MA,

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USA cod: AB1504), rabbit polyclonal GluA2 (1:2500, Millipore cod: AB1768-25UG) and mouse monoclonal anti-GAPDH (1:10000, Millipore Billerica, MA 01821; cod: MAB374) primary antibodies.

For detection, after 3 washes in TBS-T, the membranes were incubated for 1 h at room temperature with IR-Dye secondary antibodies. Signals were detected using Odyssey Infrared Imaging System (LI-COR Biosciences) and quantified using Odyssey Software Version 1.1 (LI-COR Biosciences).

Data are presented as the ratio of the intensity band of the investigated protein to that of the GAPDH band and are expressed as a percentage of controls. Each condition was carried out and analyzed in three independent primary culture dishes.

RNA extraction and retro-transcription reaction

Total RNA from cultured neurons was extracted using the ZymoResearch™ kit (Irvine, CA 92614, USA), according to the manufacturer's instructions. Reverse transcription was carried out using Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) provided by Invitrogen. Briefly, 2 µg of total RNA were mixed with 2.2 µl of 0.2 ng/µl random hexamer (Invitrogen), 10 µl of 5× buffer (Invitrogen), 10 µl of 2 mM dNTPs, 1 µl of 1 mM DTT (Invitrogen), 0.4 µl of 33 U/µl RNasin (Promega, Madison, WI, USA) and 2 µl MMLV-RT (200 u/µl) in a final volume of 50 µl. The reaction mixture was incubated at 37°C for 2 h, and then the enzyme was inactivated at 95°C for 10 min.

RNA editing quantification

The editing level quantification for AMPA receptor (GluA2, GluA3, GluA4), 5-hydroxytryptamine receptor 2C (5-HT_{2C}R) transcripts, Bladder Cancer Associated Protein (BLCAP), CYFP2 and ADAR2 pre- mRNA was done by sequence analysis using the same approach as previously described{Barbon,

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2003 #7;Barbon, 2011 #219;Barbon, 2010 #178}. Briefly, in the electropherogram obtained after RT-PCR and sequencing analysis of a pool of transcripts that might be edited or not, the nucleotide that undergoes the editing reaction appears as two overlapping peaks: A from unedited transcripts and G from the edited ones. The editing level might be reliably calculated as a function of the ratio between the G peak area and A plus G peaks areas. The areas representing the amount of each nucleotide were quantified using Discovery Studio (DS) Gene 1.5 (Accelrys Inc., San Diego, CA, USA), and the means and standard errors (N > 3) for each experimental group were calculated and used for subsequent statistical analysis.

ha eliminato: ~~Relative quantification of ADAR2 mRNA isoforms~~¶
~~In the present study we used the Agilent 2100 bioanalyzer for semi-quantitative RT-PCR analysis of alternatively spliced ADAR2 isoforms{Barbon, 2010 #178} {Gottwald, 2001 #251}. The bioanalyzer uses lab-on-a-chip technology to perform gel electrophoresis. Samples were separated electrophoretically in a polymer solution, similar to capillary electrophoresis. This instrument detects laser-induced fluorescence using an intercalating dye, which is added to the polymer. The bioanalyzer software automatically calculates the size and concentration of each separate band. The PCR products were analyzed using DNA 500 Lab kit chips (Agilent Technologies, Waldbronn, Germany). All the chips were prepared according to the manufacturer's instructions.~~ ¶

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Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was performed to analyses the expression variation of several recently reported ADAR cofactors {Tariq, 2013 #258;Garncarz, 2013 #257}: Split Hand/Foot Malformation 1 (SHFM1), RNA binding protein hnRNP A2/B1, ribosomal protein 14 (RPS14) and Serine/Arginine-Rich Splicing Factor 9 (SRSF9).

The RNA expression pattern of the genes of interest was analyzed using Applied Biosystems 7500 real-time PCR system (Life Technologies, Foster City, CA, USA). PCR was carried out using TaqMan Universal PCR Master Mix (Life Technologies), which contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference and optimized buffer components. AmpErase UNG treatment was used to prevent the possible reamplification of carry-over PCR products. Thermal cycling was started by incubation at 50 °C for 2 min and at 95 °C for 10 min for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95 °C for 15 s for melting and 60 °C for

1 min for annealing and extension. Then, 20 ng of sample was used in each real-time PCR reaction in a final volume of 20 µl The expression ratio of the target genes (SHFM1: Rn01455913_m1, Invitrogen; hnRNPA2/B1: Rn.PT.58.24173291 Integrated DNA Technologies; RPS14: Rn00821130_g1, Invitrogen; SRSF9: Rn.PT.58.18075228 Integrated DNA Technologies) in the treated cells, in comparison with the non treated cells, was calculated as described by Pfaffel and Colleagues {Pfaffl, 2002 #105}, using the geometric mean of two housekeeping genes (GAPDH: Rn99999916_m1; H2AFZ: Rn00821133_g1). Each individual determination was repeated in triplicate.

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Statistical analysis

Each experiment was performed at least in three independent preparations of rat cortical cultures. Statistical analysis of the editing and splicing data as well as of protein levels was performed using two-way ANOVA followed by Bonferroni's post-test. One-way ANOVA was used for the statistical analysis of protein inhibitors experiments and of qPCR experiments. Bonferroni correction was used after ANOVA.

Acknowledgement

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Conflict of interest

The authors have no conflict of interest

References

Figure Legends

Fig. 1: Kinetics of Glu mediated excitotoxicity. (A) Cortical neurons were treated with different doses of glutamate for 24h. The graph reports the percentage of NeuN positive cells (a neuronal cell marker) that present prodidium iodide staining (cell death marker). (B) Representative image of propidium iodide staining used to detect dying neurons.

Fig. 2: Editing levels of AMPA receptors subunits after chronic glutamate treatment. The measurements were taken after 24h of continuous treatment (Ctr 24h and Glu 24h) and after 72h of glutamate wash-out (Ctr+WO and Glu+WO). (A) GluA2 Q/R editing level; (B) GluA2 R/G editing level for the Flip and Flop versions; (C) GluA3 R/G editing level for the Flip and Flop versions; (D) GluA4 R/G editing level for the Flip and Flop versions. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig. 3: Editing levels of CYFIP2 K/E site after chronic glutamate treatment. The measurements were taken after 24h of continuous treatment (Ctr 24h and Glu 24h) and after 72h of glutamate wash-out (Ctr+WO and Glu+WO). Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

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Fig. 4: GluA1 and GluA2 protein expression pattern after chronic glutamate treatment. The measurements were taken after 24h of continuous treatment (Ctr 24h and Glu 24h) and after 72h of glutamate wash-out (Ctr+WO and Glu+WO). The graphs report expression data normalized on GAPDH expression and relative to control samples. Representative western blots are reported below the graphs. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig 5: ADAR1 and ADAR2 protein expression pattern after chronic glutamate treatment. The measurements were taken after 24h of continuous treatment (Ctr 24h and Glu 24h) and after 72h of glutamate wash-out (Ctr+WO and Glu+WO). The graphs report expression data normalized on GAPDH expression and relative to control samples. (A) ADAR1 protein expression and (B) ADAR2 protein expression. Representative western blots are reported below the graphs. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig. 6: Effect of chronic glutamate treatment on ADAR2 catalytic activity and on its splicing pattern. (A) Estimation of ADAR2 self-editing at the intronic position -1. The measurements were taken after 24h of continuous treatment and after 72h of glutamate wash-out. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig. 7: Expression level of RPS14, hnRNPA1B2, SRSF9, SHF1 mRNA by qPCR after 24h of glutamate treatment. Data are reported as Log2 of the expression ratio R= 2-□□ct (expression level of

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control sample is equal to 0) as in {Pfaffl, 2002 #105} and represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after one-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig. 8: Editing levels of GluA2 Q/R (A) R/G sites (B) and CYFIP2 K/E site (C) in DIV14 cortical neuron chronically treated with glutamate (24h, 10 □M – gray bar) or not (white bars) in parallel with BAPTA/am (Ca2+ chelator), Calpain inhibitor and KN93 (CAmKII inhibitor) treatment. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after one-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig. 9: Protein expression analysis of ADAR1 (A) and ADAR 2 (B) in DIV14 cortical neuron chronically treated with glutamate (24h, 10 μM – gray bar) or not (white bars) in parallel with BAPTA/am (Ca^{2+} chelator), Calpain inhibitor and KN93 (CAmKII inhibitor) treatment. Representative western blots are reported below the graphs. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after one-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Fig. 10: Analysis of RNA editing in DIV14 cortical neurons acutely treated with 100 μM glutamate for 1h; for 1h of Glu and 5h of wash out; for 6h of continuous Glutamate treatment. (A) Editing level of GluA2 Q/R site and (B) editing level of GluA2 R/G site. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

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Fig. 11: Protein expression analysis of ADAR1 (A) and ADAR 2 (B) in DIV14 cortical neuron acutely treated with 100 μM glutamate for 1h; for 1h of Glu and 5h of wash out; for 6h of continuous Glutamate treatment. Data represent mean values and standard errors obtained from at least 3 independent evaluations. Bonferroni correction was used after two-way ANOVA (*p<0,05; **p<0,01; ***p<0,001).

Table 1: Editing levels of the five $5HT_{2C}R$ editing sites and the three BLCAP sites

	Ctr 24h	**Glu 24h**	**Ctr + WO**	**Glu +WO**
5HT2CR Site A	84.4±1.17	80.5±0.92	81.9±0.79	78.7±0.96
5HT2CR Site B	86.8±0.32	85.0±0.35	85.5±0.56	83.1±0.34
5HT2CR Site C'	7.5±0.20	6.9±0.67	7.7±0.01	7.4±0.93
5HT2CR Site C	26.5±0.10	23.5±1.00	22.3±0.37	19.6±1.05
5HT2CR Site D	72.3±0.67	70.8±2.32	70.4±0.15	68.0±0.93
BLCAP Y/C Site	51.3±0.56	51.9±0.60	52.8±0.39	52.7±0.19
BLCAP Q/R Site	2.9±0.65	3.8±0.18	4.0±0.65	2.2±0.11
BLCAP K/R Site	5.9±0.10	6.2±0.45	6.7±0.60	5.1±0.40