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ORIGINAL PAPER

Glutamate-Dependent BMAL1 Regulation in Cultured Bergmann Glia Cells

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Abstract Glutamate, the major excitatory amino acid, activates a wide variety of signal transduction cascades. This neurotransmitter is involved in photic entrainment of circadian rhythms, which regulate physiological and behavioral functions. The circadian clock in vertebrates is based on a transcription-translation feedback loop in which Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1 (BMAL1) acts as transcriptional activator of others clock genes. This protein is expressed in nearly all suprachiasmatic nucleus neurons, as well as in the granular layer of the cerebellum. In this context, we decided to investigate the role of glutamate in the molecular mechanisms involved in the processes of transcription/translation of BMAL1 protein. To this end, primary cultures of chick cerebellar Bergmann glial cells were stimulated with glutamatergic ligands and we found that BMAL1 levels increased in a dose- and time dependent manner. Additionally, we studied the phosphorylation of serine residues in BMAL1 under glutamate stimulation and we were able to detect an increase in the phosphorylation of this protein. The increased expression of BMAL1 is most probably the result of a stabilization of the protein after it has been phosphorylated by the cyclic AMP-dependent protein kinase and/or the Ca^{2+}/diacylglycerol dependent protein kinase. The present results strongly suggest that glutamate participates in regulating BMAL1 in glial cells and that these cells might prove to be important in the control of circadian rhythms in the cerebellum.

Keywords Glutamate receptors · Bergmann glia · Clock genes

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate
BGC	Bergmann glia cells
Bmal1	Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1
EAAT	Excitatory amino acid transporter
GLAST	Glutamate-aspartate transporter
Glu	Glutamate
KA	Kainate
KB-R7943	2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate
NMDA	N-methyl-D-aspartate

Introduction

L-Glutamate (Glu) is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS). It is present

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in virtually all areas of the brain, its receptors are widely distributed and expressed in the majority of the cells in the brain. This neurotransmitter exerts its actions through the activation of two main subtypes of receptors classified according to their structure and signalling into: ionotropic (iGluRs) and metabotropic receptors (mGluRs). According to their pharmacological and molecular properties, iGluRs are divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoaxazolepropionate (AMPA) and kainate (KA) receptors [20]. Based on sequence homology, pharmacology and signal transduction mechanisms, mGluRs are categorized into three groups. Group I receptors are coupled to stimulation of phospholipase C with the consequent release of intracellular Ca^{2+}, while Groups II a nd III are coupled to inhibition of adenylate cyclase. These three groups are activated by specific agonists, (RS)-3, 5-dihydroxyphenylglycine (DHPG) acts upon Group I, (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG) activates Group II, while L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) is selective for Group III [9].

Removal of the excitatory amino acid from the synaptic cleft is carried out by a family of sodium-dependent Glu transports [10]. To date, five subtypes of Glu transporters have been characterized, known as excitatory amino acid transporters (EAAT-1 to 5). These EAATs have a different pattern of distribution and kinetic properties in different regions of the brain. It is known that more than 80 % of the total brain removal of Glu from the synaptic cleft, is carried out by EAAT1 or GLAST (Glu/aspartate transporter) and/or EAAT2 or Glt-1 (Glu transporter 1) [30]. Within the cerebellum, the bulk of the Glu uptake takes place in Bergmann glia cells (BGC), which express GLAST [42].

BGC span the entire cerebellar molecular layer in vertebrates, their soma is aligned within the Purkinje cell layer, in fact these cells cover completely excitatory and inhibitory synapses [48]. BGC are involved in neurotransmitter uptake and turnover, K^+ homeostasis, lactate supply and pH regulation due to the expression of a set of receptors and transporters [32]. In language of glutamatergic transmission, BGC are in a very neighbouring proximity to the parallel fiber-Purkinje cell synapses, and participle in the Glu/glutamine shuttle that assures Glu supply to the presynaptic terminals. In this sense, BGC respond to glutamatergic stimulation, as we have been able to characterize over the years [2]. Because of it, it has become an excellent model in which the role of glia cells as partners of neurons can be documented.

Glu is involved in photic entrainment of circadian rhythms, which regulate physiological and behavioral functions, are synchronized to 24-h day period and are found in every kingdom of life [31] and are generated endogenously through genetic control [26]. In vertebrates, the suprachiasmatic nucleus (SCN) of the hypothalamus is the master clock that drives the circadian system to synchronize multiple peripheral clocks, which function in the variety of tissues, presumably through combination of neural and humoral signaling [8, 46, 49]. At the molecular level, circadian rhythms are regulated by the so-called *"clock genes"* that oscillate in a circadian manner [11, 40, 51]. The proteins products of clock genes are transcription factors that control their own and *clock-controlled genes* transcription. One of the clock genes, *bmal1*, is a positive component of the handle of the machinery of the circadian system [21]. It is known that defects in this gene, results in loss of circadian rhythms [4], impaired glucose homeostasis [41], infertility [24], altered sleep pattern [29], increased sensitivity to chemotherapy and radiation [16], among others. Furthermore, in the SCN, *bmal1* is an intracellular regulator for the generation of circadian Ca^{2+} rhythms [22]. Nowadays, the clock genes discovery has enabled the identification of brain areas that express the molecular machinery necessary for the generation of circadian rhythms. Accordingly, daily oscillations in gene expression of the clock genes have been identified in several regions of the brain [6, 13], including amygdala, olfactory bulb, lateral habenula, a variety of nuclei in the hypothalamus [17], and interestingly cerebellum [1, 38]. Within this structure, Namihira and colleagues reported patterns of expression of *clock* and *bmal1* genes which are differentially expressed. Furthermore, the cerebellum is been proposed as a peripheral oscillator, which can be desynchronized of SCN by food [35]. Purkinje cells play an important role in the generation of cerebellar circadian rhythms, whether glial cells participate in the generation of this rhythms is unknown.

Glu is the most prevalent excitatory neurotransmitter in the CNS [25] and glial cells are essential regulators of the formation, maintenance and function of glutamatergic synapses [25]. The molecular mechanisms involved in BMAL1 regulation are far from been established, and no role for Glu has been documented either in neurons or glia cells. As a step forward in the characterization of the regulation of such an important transcription factor, we demonstrate here using the established model of cultured cerebellar BGC, Glu participates in the regulation of BMAL1 protein levels in glia cells through the activation of its receptors and transporters.

Materials and Methods

Animals

Chick embryos (10 days old) were obtained from Avi-Mex and maintained at 37 °C until used. All experiments were performed according to International Guidelines on the

Ethical Use of Animals and had the specific approval of the Animal Ethics Committee of Cinvestav-Mexico. All efforts were made to reduce the number of embryos used and their suffering.

Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). A23187 (5-(methylamino)-2-({(2R,3R,6S,8S,9R, 11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl) ethyl]-1,7-dioxaspiro[5.5] undec-2-yl}methyl)-1,3-benzoxazole-4- carboxylic acid), KB-R7943 (2-[2-[4-(4-Nitrobenzyloxy) phenyl] ethyl] isothiourea-mesylate), DHPG ((RS)-3,5-dihydroxyphenilglicine), AMPA, NMDA, THA (Threo-b-hydroxyaspartate), DNQX (6,7-Dinitroquinoxaline-2,3-dione), L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid), CPCCOEt (7-(Hidroxyimino)-cyclopropa-[b]-chromen-1a-carboxylate ethyl ester), Asp (D-aspartate) and L-Glu were all obtained from Tocris-Cookson (St. Louis, MO, USA). KA was obtained from Ocean Produce International (Shelburne, Nova Scotia, Canada). Polyclonal anti-BMAL1 was purchased from Santa Cruz Biotech., (Santa Cruz, CA, USA; E-19: sc-8614). Horseradish peroxidase-linked anti-rabbit antibodies, and the enhanced chemiluminescence reagent (ECL), were obtained from Amersham Biosciences (Buckinghamshire, UK). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell Culture and Stimulation Protocol

Primary cultures of cerebellar BGC were prepared from 14-day-old chick embryos as previously described [39]. Cells were plated in 6-well plastic culture dishes in DMEM containing 10 % fetal bovine serum, 2 mM glutamine, and gentamicin (50 µg/mL) and used on the fourth–seventh days after culture. Before any treatment, confluent monolayers were washed twice to remove all non-adhering cells with assay buffer (25 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2 6H_2O$, 33.3 mM glucose, 1 mM Na_2HPO_4 and 1.8 mM $CaCl_2$ at pH 7.4) and then treated as indicated. Inhibitors were added 30 min before agonists. The cells were treated with Glu analogues added to culture medium for the indicated time periods.

SDS-PAGE and Western Blots

Cells from confluent monolayers were harvested with phosphate-buffer saline (PBS) (10 mM K_2HPO_4/KH_2PO_4, 150 mm NaCl, pH 7.4) containing phosphatase inhibitors (10 mM NaF, 1 mM Na_2MoO_4 and 1 mM Na_3VO_4). The cells were lysed for the extraccion of nuclear proteins with buffer A (1 M HEPES, pH 7.9; 1 M KCl, 50 mM EDTA, 10 mM EGTA, 100 mM DTT, 100 mM PMSF, 1 mg/mL aprotinin and 1 mg/mL leupeptin) and buffer B (1 M HEPES, pH 7.9; 4 M NaCl, 50 mM EDTA, 10 mM EGTA, 100 mM DTT and 50 µL/mL complete). Cell lysates were denaturalized in Laemmli's sample buffer, and equal amount of protein (50 µg as determined by the Bradford method) were resolved through 10 % SDS-PAGE and then electroblotted to nitrocellulose membranes. Blots were stained with Ponceau S stain to confirm that protein content was equal in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated in TBS containing 5 % dried skimmed milk and 0.1 % Tween 20 for 2 h to block the excess of non-specific protein binding sites. Membranes were then incubated overnight at 4 °C with the particular primary antibody, BMAL1, followed by secondary antibody. Immunoreactive polypeptides were detected by chemiluminescence and exposed to X-ray films. Densitometry analyses were performed and data analyzed with Prism, GraphPad Software (San Diego, CA, USA).

Immunoprecipitation

Cells lysates were pre-absorbed with 3 µL of G protein coupled agarose for 4 h at 4 °C. The cleared lysates (1 mg of protein) were incubated with anti BMAL1 antibody overnight, 4 °C and then immunoblotted as described above.

Statistical Analysis

Data are expressed as the mean (average) ±standard error mean (SEM). One-way analysis of variance (ANOVA) was performed to determine significant differences between conditions. When this analysis indicated significance (at the 0.05 level), post hoc Student–Newman–Keuls test analysis was used to determine which conditions were significantly different from each other with the Prism, GraphPad Software (San Diego, CA, USA).

Results

BMAL1 is Expressed in Bergmann Glia Cells

BMAL1 is a clock protein that forms the core of the circadian molecular machinery. It is needed to generate a proper circadian gene expression pattern, and by these means controls almost all the functions of an organism. Accordingly, we asked ourselves whether this protein is present in glia cells and if so, would it be modulated by the major excitatory amino acid in the vertebrate brain. Using the system of chick cerebellar Bergmann glia cultures, we could detect the characteristic 69 kDa band (Fig. 1). We

then decided to study the role of Glu in the expression of BMAL1 in BGC cultures. As a first approach, we exposed confluent BGC monolayers to 1 mM Glu for 30 min and a significant increase in the expression of BMAL1 was found (Fig. 1a). Because of the variability of the detection levels of actin to normalize the results of each experiment, we decided to normalize all subsequent blots with the Ponceau S staining (1b).

To further characterize the Glu effect, we established the time dependence of BMAL1 increase. To this end, we performed treatments of BGC with 1 mM Glu for 3, 6 and 12 h. A 6 h Glu treatment is sufficient to increase the expression of this protein (Fig. 2a), therefore, all subsequent experiments were done after 6 h of glutamatergic stimulation. To establish a physiological relevance of this expression, we treated BGC cultures with increasing Glu concentrations, and as can be seen in panel b of Fig. 2, a clear dose-dependency could be established with an EC_{50} of 451 μM, suggesting a receptor-mediated effect (Fig. 2c). However, as has been reported, the concentration of Glu in the synaptic cleft has been estimated to be in the low millimolar range [10]; therefore subsequent experiments were performed with a concentration 1 mM Glu.

Pharmacological Characterization of Glu-dependent Increase in BMAL1

Through the use of pharmacological tools, the profile of the Glu response was determined. Confluent BGC monolayers were exposed to different concentrations of the specific ionotropic receptor agonists 250 μM KA, 500 μM AMPA or NMDA plus glycine (10 μM), a 25 μM concentration of the group I metabotropic receptor agonist DHPG or 500 μM concentration of the group III mGluRs agonist L-AP4 for 6 h. As shown in panel a of Fig. 3, the iGluRs agonist AMPA, as well as the group I mGluRs agonist DHPG augments BMAL1 expression in BGC cultures. Neither, KA, NMDA nor L-AP4 were capable to mimic the Glu effect (Fig. 3a).

To evaluate the ionotropic and metabotropic contribution to Glu response, we took advantage of specific Glu receptor antagonists. BGC cultures were treated for 30 min with 50 μM DNQX (an AMPA receptors antagonist) or 100 μM CPCCOEt (a metabotropic Glu receptors antagonist of group I) prior to a 1 mM Glu or 25 μM DHPG stimulation for 6 h; the results are presented in Fig. 3b and c. As expected, both antagonists, DNQX and CPCCOEt were capable to completely reduce the Glu and DHPG response, suggesting the involvement of AMPA and metabotropic Glu (group I) receptors in the Glu effect (Fig. 3 panels b, c). Taking into consideration that Glu transporters have been described as signal transducers [34], we exposed BGC cultures to a 1 mM concentration of D-aspartate and to the transportable inhibitor THA. The results are presented in Fig. 3 (panel d). Although a discrete increase in BMAL1 is present upon D-aspartate, the fact that THA could not reproduce this response effect suggest that Na^{+} influx through the transporter is not necessary for the Glu-dependent increase in BMAL1 expression.

Next, we decided to explore if the Na^{+}/Ca^{2+} exchanger is involved in Glu-dependent BMAL expression. Confluent BGC monolayers were incubated for 30 min with 15 μM 2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R7943, a blocker of the Na^{+}/Ca^{2+} exchanger) prior to 1 mM Glu or 10 μM A23187 (a Ca^{2+} ionophore) stimulation for 6 h. The results shown in Fig. 3 indicate that the selective Na^{+}/Ca^{2+} exchanger blocker KB-R7943, completely abolished the Glu response. These results suggest that the co-transport of Glu and Na^{+} leads to the activation of the Na^{+}/Ca^{2+} exchanger. In summary, Glu-dependent BMAL1 expression is mediated through ionotropic (AMPA) and metabotropic (group I) Glu receptors. Furthermore, the Na^{+}/Ca^{2+} exchanger is also involved in the expression of this clock protein.

At this stage, it was important to establish if the Glu effect was the result of an increase in BMAL1 synthesis or, if it was due to an increase in BMAL1 half-life time. Therefore, BGC cultures were treated with 100 μg/mL cycloheximide (CHX) for 30 min to block de novo protein synthesis before a 1 mM Glu treatment for 15, 30, 60 and 120 min and BMAL1 levels were determined (Fig. 4a). The results obtained provide us information on the likely involvement of Glu in regulating BMAL1. Therefore, we decided to evaluate the effect of each stimulus, separately, on the expression of BMAL1 considering the time where Glu-dependent effect is presented (6 h). To this end, BGC cultures were pre-exposed for the indicated time periods

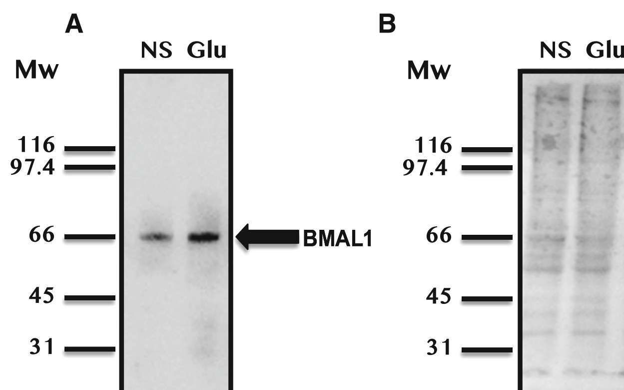

Fig. 1 BMAL1 is expressed in Bergmann Glia Cells. BGC monolayers were treated with vehicle (NS) or 1 mM Glu for 30 min. Levels of BMAL1 expression were detected via Western blots with anti-BMAL1 antibody (**a**) and were normalized with to Ponceau S staining (**b**). An autoradiography of a typical experiment is shown

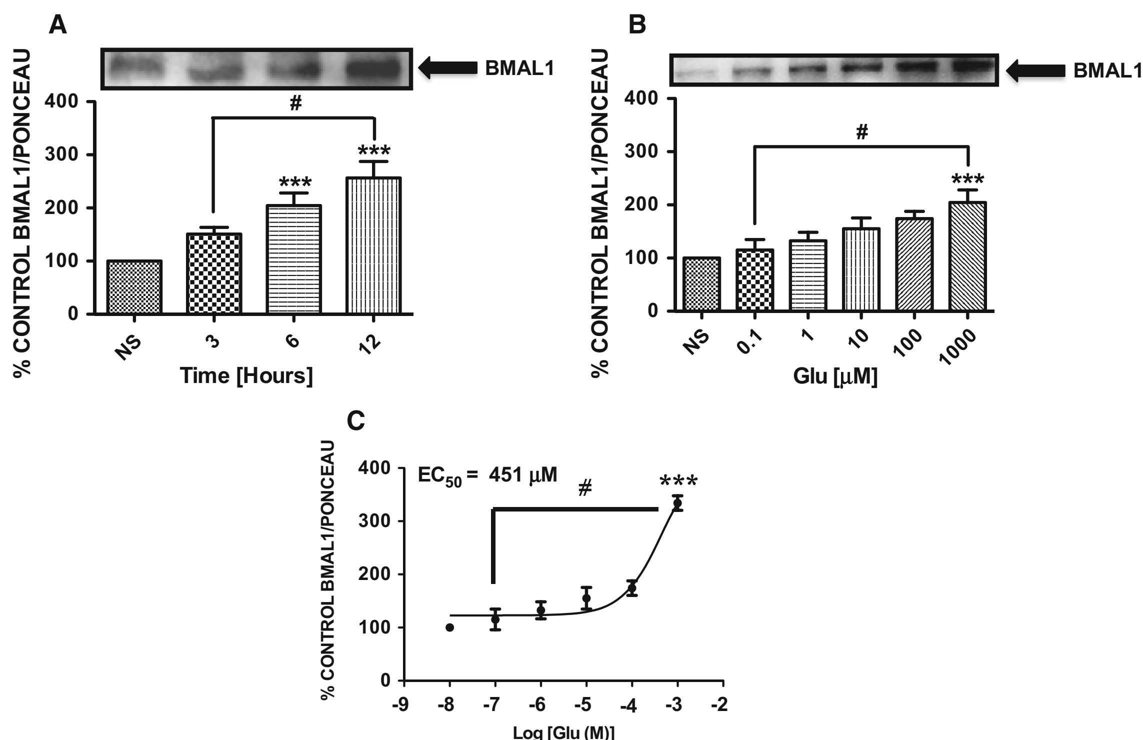

Fig. 2 BMAL1 expression is Glu time- and dose-dependent. **a** BGC monolayers were treated with vehicle (NS) or 1 mM Glu for different time periods. **b** BGC monolayers were treated with vehicle (NS) or increasing Glu concentrations for 6 h. Levels of BMAL1 expression were detected as described in Fig. 1. **c** Nonlinear regression of the incubation with increased Glu concentrations for 6 h. Data are expressed as mean ± SEM from at least three independent experiments. An autoradiography of a typical experiment is shown. One-way ANOVA was performed comparing against data obtained from non-stimulated (NS) cells (***$P < 0.001$). In *panels* a and b a Newman–Keuls Multiple Comparison Test was performed to analyze the effect of Glu at different time periods and with different Glu concentrations, respectively ($^{\#}P < 0.05$)

(0, 1.5, 3 and 6 h) with 1 mM Glu, 100 µg/mL CHX or 1 mM Glu plus 100 µg/mL CHX. These data were indicative of an increase in BMAL1 half-life time since CHX treatment was not capable to decrease the levels of BMAL1 when the Glu stimulus was present or not, suggesting that Glu is likely to be regulating BMAL1 stability (Fig. 4b).

BMAL1 Expression is Regulated Through PKA and PKC

Zhang et al. [52] reported that the Ca^{2+}/diacylglycerol-dependent protein kinase (PKC) facilitates ubiquitin cleavage and prevents the formation of polyubiquitinated chains on BMAL1, which then leads to a stabilization of BMAL1. Based on these data, we decided to analyze if in our culture system of BGC, BMAL1 protein is phosphorylated by PKC leading to BMAL1 stabilization. To this end, we performed immunoprecipitation assays coupled to Western blot identification (Fig. 5a). The blots presented in Fig. 5b show that immunoprecipitation of BGC lysates with anti-phosphoserine (PSer) antibodies and Western blot analysis with anti-BMAL1 antibodies enabled us to appreciate that BMAL1 is serine phosphorylated under 1 mM Glu. Interestingly, exposure of the cultured cells to 500 nM dibutyryl-cAMP (dbcAMP), a PKA activator, or 100 nM of the diacylglycerol analogue tetradecanoylphorbol 13-acetate (TPA) for 6 h results in an increase in BMAL1 levels similarly to the described effect of Glu (Fig. 5c).

Discussion

Glu is the major excitatory amino acid in the vertebrate CNS, which activates a wide variety of signal transduction cascades. Several lines of evidence suggest that Glu is the main photic signal for the circadian clock. Moreover, it has been shown that Glu can also play a role in SCN output to other hypothalamic structures, suggesting that this neurotransmitter might be a signaling molecule of suprachiasmatic cells [53]. Interestingly, Glu can also be synthesized in SCN glia or neurons and is recycled through the glutamate/glutamine cycle [18, 36]. On the other hand, it is

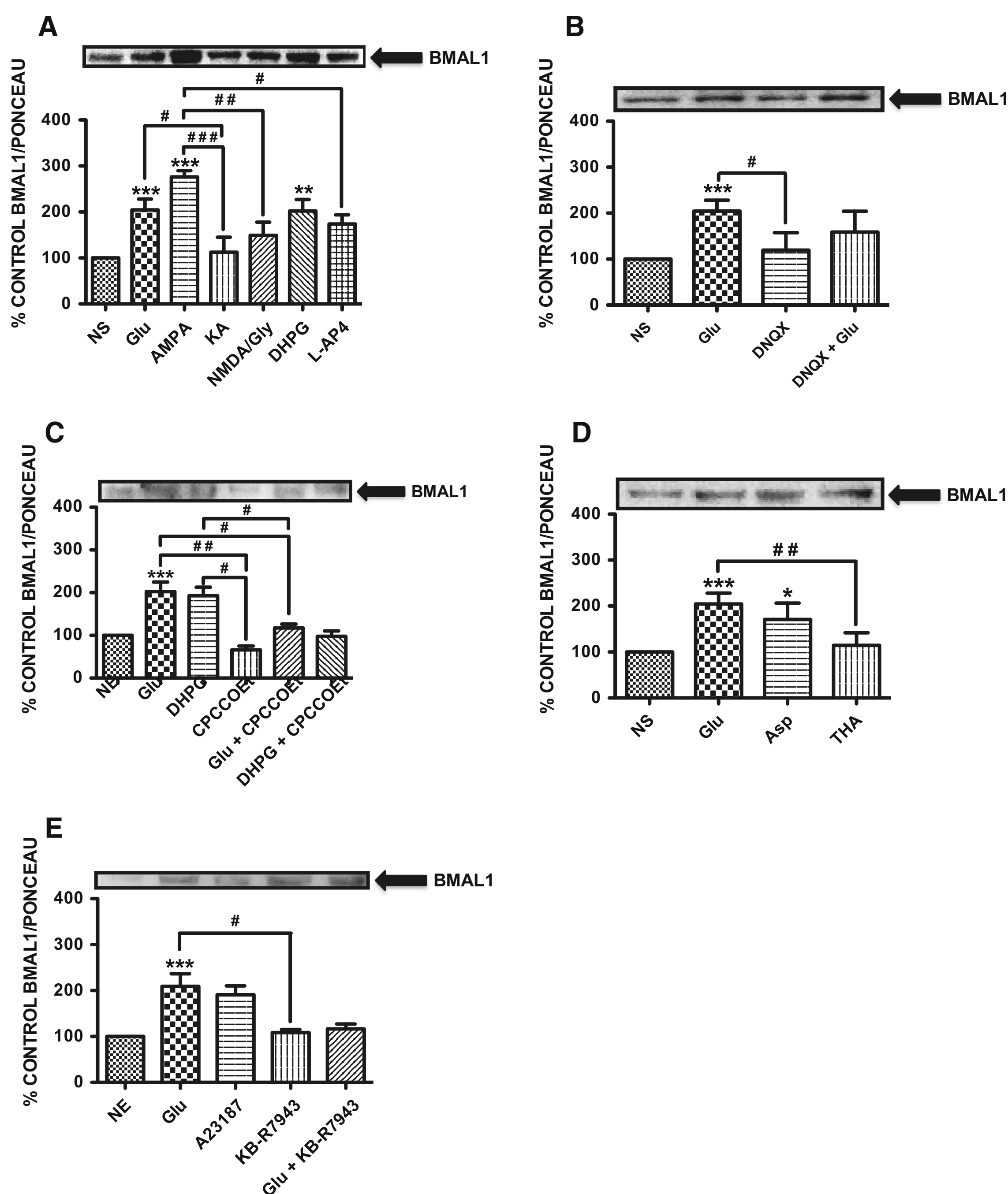

Fig. 3 Pharmacological profile of Glu-induced BMAL1 expression. **a** BGC monolayers were treated for 6 h with vehicle (NS), 1 mM Glu or the indicated Glu agonists; 500 μM AMPA, 250 μM KA, 500 μM NMDA plus 10 μM Gly, 25 μM DHPG and 500 μM L-AP4. **b** BGC monolayers were incubated for 30 min with 50 μM DNQX (AMPA receptor antagonist) before the 1 mM Glu treatment for 6 h. **c** BGC monolayers were incubated with the metabotropic Glu receptors antagonist of group I CPCCOEt (100 μM) for 30 min before 1 mM Glu or 25 μM DHPG exposure (6 h). **d** BGC monolayers were incubated with vehicle (NS), 1 mM Glu, 1 mM aspartic acid (Asp), or the Glu transporter blocker, 100 μM THA for 6 h. **e** BGC monolayers were pre-exposed for 30 min with 15 μM KB-R7943 (a blocker of the Na^+/Ca^{2+} exchanger) and then to 1 mM Glu or 10 μM A23187 (Ca^{2+} ionophore) for 6 h. Levels of BMAL1 expression were detected as described in Fig. 1. Data are expressed as mean ± SEM from at least three independent experiments. An autoradiography of a typical experiment is shown. One-way ANOVA was performed comparing against data obtained from non-stimulated (NS) cells ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). In all five panels Newman–Keuls Multiple Comparison Test was performed to analyze AMPA effect, Glu antagonists effect, DHPG effect, Glu transporter blocker effect and blocker of the Na^+/Ca^{2+} exchanger effect, respectively ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$)

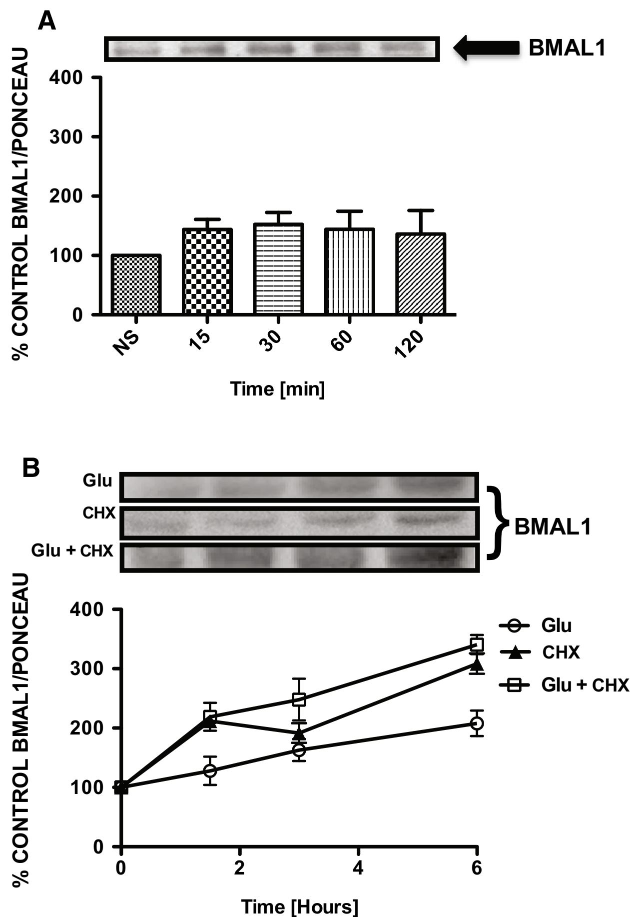

Fig. 4 Glu stabilizes to BMAL1 protein. **a** BGC monolayers were incubated for 30 min with 100 µg/mL CHX before the 1 mM Glu treatments in the indicated time periods. **b** BGC monolayers were incubated with 1 mM Glu (*white circles*), 100 µg/mL CHX (*white squares*) or 1 mM Glu plus 100 µg/mL CHX (*black triangles*) in the indicated time periods. Levels of BMAL1 expression were detected as described in Fig. 1. Data are expressed as mean ± SEM from at least three independent experiments. An autoradiography of a typical experiment is shown. One-way ANOVA was performed comparing against data obtained from non-stimulated (NS or 0 h) cells

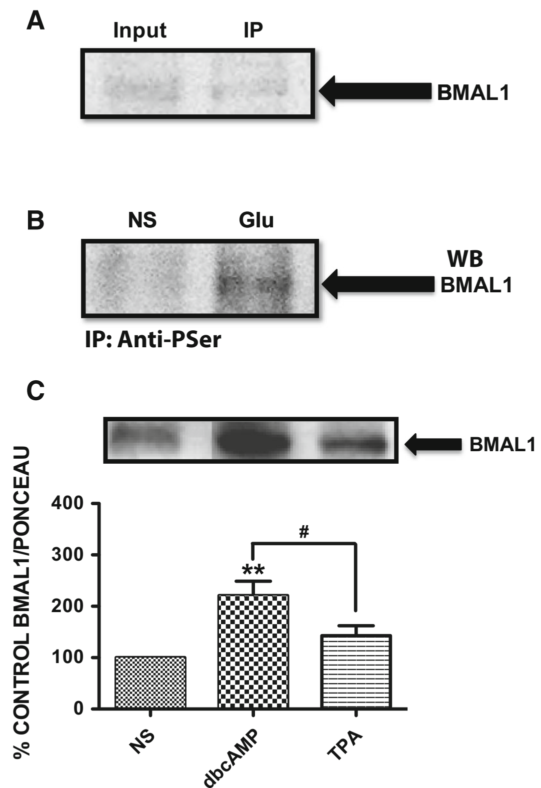

Fig. 5 Protein kinases A and C participate in the regulation of BMAL1 expression. **a** BGC monolayers were immunoprecipitated with anti-phosphoserine antibodies and the immunoprecipitation assay was analyzed by Western blot with anti-BMAL1 antibodies. **b** BGC monolayers were exposed to vehicle (NS) or 1 mM Glu. Total extracts were immunoprecipitated with anti-phosphoserine and then subjected to Western blot analysis with anti-BMAL1 antibodies. **c** BGC monolayers were treated for 6 h with vehicle (NS), 500 µM db-cAMP or 100 nM TPA. Levels of BMAL1 expression were detected as described in Fig. 1. Data are expressed as mean ± SEM from at least three independent experiments. An autoradiography of a typical experiment is shown. One-way ANOVA was performed comparing against data obtained from non-stimulated (NS) cells (**$P < 0.01$). In panel c a Newman-Keuls Multiple Comparison Test was performed to analyze db-cAMP effect versus TPA effect ($^{\#}P < 0.05$)

noteworthy that although Glu uptake is not regulated in glia in a circadian manner, is known it is modulated by clock gene expression [3].

The role of Glu as a transmitter at retinohypothalamic tract (RHT)/SCN synaptic connections is important and critical for the mediating photic regulation of circadian rhythmicity. In 1993, Castel and colleagues demonstrated that RHT terminals innervating the SCN show Glu immunoreactivity associated with synaptic vesicles, which confirms the role of Glu as a neurotransmitter in the SCN. Since then, different types of Glu receptors were identified and localized in the SCN [15].

As in SCN cells, BGC also express different subtypes of Glu receptors. In our study, the increase in BMAL1 expression is due to the interaction of Glu with AMPA receptors, which in these cells are basically composed of GRIA1, GRIA3 and GRIA4 and thus Ca^{2+}-permeable [33, 37]; and also to the interaction of Glu with group I mGluRs, which are coupled to phosphatidylinositol metabolism and the release of the Ca^{2+} from the endoplasmic reticulum. In contrast, NMDA receptors appear not related to the regulation of BMAL1 levels in BGC; however, the Na^{+}/Ca^{2+} exchanger seems to be involved in the expression of this clock protein in these cells. A discrete effect was detected upon BGC treatment with Glu transporters ligands (Fig. 3). Whether the signaling mechanisms used by Glu receptors and transporters to stabilize BMAL1 are the same, is not known at the moment but one can

speculate that an increase in intracellular Ca^{2+} levels, triggers a series of events that augment BMAL1 [34].

Clock proteins are necessary for the generation and regulation of circadian rhythms within individual cells throughout the organism. These rhythms influence nearly all aspects of physiology and behavior, including rest-wake cycle, hormone secretion, body temperature and metabolism. For example disruption of BMAL1 results in infertility [24], loss of circadian rhythms [27], defective glucose homeostasis [41], among other dysfunctions.

It has been shown that control of circadian clock components operates not only at the post-transcriptional level [7], but also at the level of protein stability [43]. Interestingly, post-translational modifications of clock proteins are important for ensuring the maintenance of circadian rhythms, as they can modulate the activity and turnover of major clock components [14]. In particular, BMAL1 is a critical regulator of the circadian clock, which undergoes various post-translational modifications, including phosphorylation [12, 43, 45, 50], acetylation [19], sumoylation [5] and ubiquitination [28].

Of particular interest, is known that BMAL1 phosphorylation by distinct kinases appears to regulate its unique activities [28] and can be a common mode of regulation. Specially, it has been reported that phosphorylation by Casein Kinase Iε (CKIε) activates BMAL1-mediated transcription [12], while phosphorylation by Mitogen-activated Protein Kinases (MAPKs) inhibits it [45]. BMAL1 is also targeted by other kinases, for example by Casein Kinase 2α (CK2α), which is involved in BMAL1 intracellular localization [50]; and Glycogen Synthase Kinase 3β (GSK3β), which controls stability and activity of BMAL1 [43]. However, the phosphorylation status of several of these residues changes in a time-specific manner and regulates period length [14]. All these post-translational modifications, make BMAL1 a highly stable protein, with a half-life time is probably higher than 12 h (Fig. 4), which is higher than the to the half-life time of other clock proteins [17]. Accordingly, the mammalian PKC family is involved in phase resetting of circadian clock [23, 47] and it has been reported that PKC stabilizes BMAL1, thus preventing its degradation by the 26S proteasome [44]. These facts are in line with the results depicted in Figs. 4 and 5.

Concerning PKA, little is known about its involvement in circadian rhythms and clock proteins, specifically its

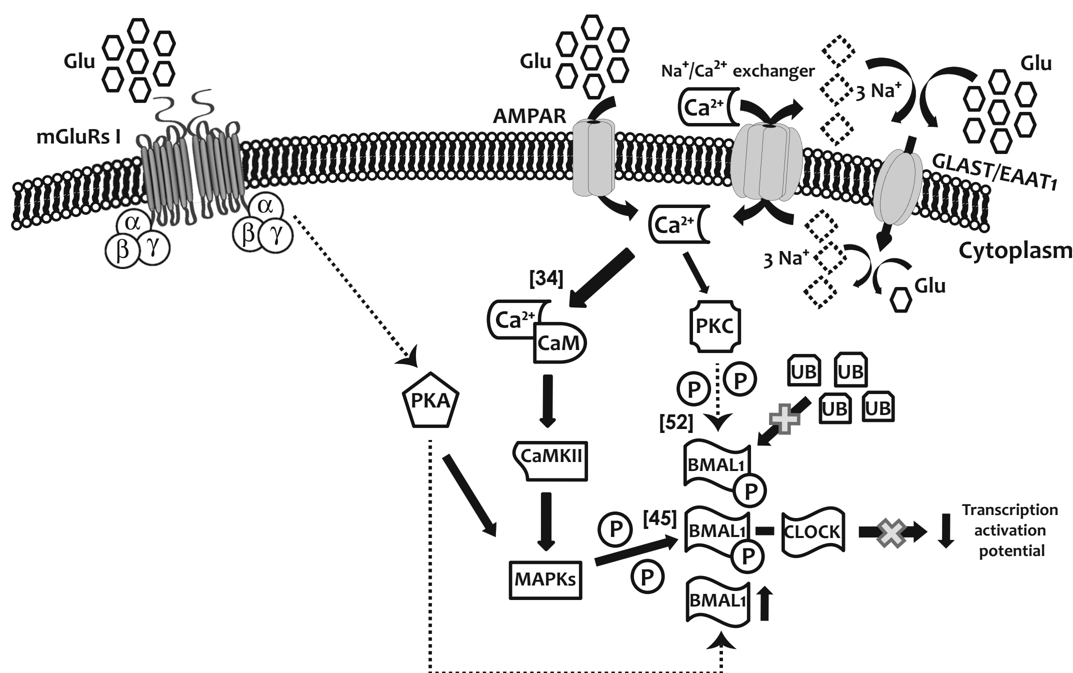

Fig. 6 Current model for Glu-dependent BMAL1 regulation in cultured Bergmann Glia Cells. Other PKA substrates include BMAL1, favoring the increase of BMAL1 levels. Stimulation of Bergmann glia AMPA receptors induces to Ca^{2+} influx; that successively binds to calmodulin (CaM) activating Ca^{2+}/Calmodulin-dependent Protein Kinase II (CaMKII) [34], which in turn activates MAPKs. Besides, Ca^{2+} also activates Protein Kinase C (PKC). Subsequently, PKC phosphorylates to BMAL1 at S269 site impeding BMAL1 ubiquitination (UB) and therefore stabilizes the protein [52]. On the other hand, Glu uptake leads to Na^+ influx, which activates the Na^+/Ca^{2+} exchanger, increasing in intracellular Ca^{2+} levels, and inducing the activation of CaMKII and PKC. Activation of adenylate cyclase (AC) cleaving Protein Kinase A (PKA). Among the substrates of PKA are Mitogen-activated Protein Kinase (MAPKs) that phosphorylates (P) BMAL1 protein in Thr-534 site resulting in a decrease of CLOCK-BMAL1 transcription activation potential [45]

relationship with BMAL1, nonetheless it would be important to dissect the mechanism involved in the stabilizing effect of PKA activation.

In summary, we report here a Glu-dependent regulation of BMAL1 in BGC that might be linked to a PKA/PKC-mediated stabilization. These results should pave the way of our understanding of the role of glial Glu receptors in the molecular regulation of circadian rhythms. A schematic representation of the major findings of this work is presented in Fig. 6. Work currently in progress in our lab is aimed as to dissect the signalling cascades involved in Glu-dependent BMAL1 stabilization.

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Conflict of interest The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this manuscript.

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