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Calcium-Dependent Eukaryotic Initiation Factor 2 α Phosphatase Complexed with Calcineurin Restores Control to Anterior Piriform Cortex Neural Circuitry after Activation by Essential Amino acid Deficiency

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Abstract

Essential (dietary-indispensable) amino acids (IAA)s are vital precursors for protein synthesis; they cannot be synthesized in metazoans but must be obtained from food to survive. In sensing a reduction of an IAA, the mammalian anterior piriform cortex (APC) is rapidly activated. The initial behavioral response is an abrupt end to an IAA deficient meal about 20 min after meal onset. IAA depletion in the APC activates the conserved eukaryotic initiation factor 2 α (eIF2 α) kinase, GCN2, via uncharged tRNA. GCN2 kinase activity increases phosphorylation of eIF2 α (P-eIF2 α), which blocks global protein synthesis. The result is that APC inhibitory elements with short half-lives cannot be replaced. Without their inhibition in this highly sensitive brain area, activation of the glutamatergic output cells ensues. Following APC activation, P-eIF2 α must be reduced to release the blockade on protein synthesis and allow recovery of inhibition in the circuit. This completes the homeostatic response, restoring control in the APC. A role for calcium (Ca²⁺) in regulating P-eIF2 α was explored here using Ca²⁺ blockers, immunoblotting and electrophysiology in APC brain slices. The responses to IAA depletion in the APC were Ca²⁺ dependent, showing a role for Ca²⁺ in the system. Yet, the kinase activity of GCN2 was unaffected by intracellular Ca²⁺ chelation. Thus, control must be accomplished by phosphatase activity. We suggest that regulation of P-eIF2 α , and neuronal stability in the APC, require the activity of protein phosphatase 1, a Ca²⁺-dependent subunit of the phosphatase acting on P-eIF2 α . This implicates the Ca²⁺/calmodulin-dependent calcineurin (Protein Phosphatase 2B/PP2B) as acting with the constitutive repressor of eIF2 phosphorylation (PPP1R15B/CReP) after GCN2 activation in the brain.

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Abbreviations: APC: Anterior Piriform Cortex; CaN: Calcineurin (Protein phosphatase 2B[PP2B]); CReP: Constitutive Repressor of eIF2 α Phosphorylation/PPP1R15b; Dev: IAA devoid medium; eIF2 α ; α -subunit of the Eukaryotic Initiation Factor 2; EPSP: Excitatory Postsynaptic Potential; GCN2: General Control Nonderepressible 2 kinase (EIF2AK4); IAA: Indispensable Amino Acid; KCC2: Neuronal Potassium Chloride Cotransporter; LOT: Lateral Olfactory Tract; Norm U: Normalized Volume Density Units; P-: Phosphorylated; PEK/PERK: Pancreatic eIF-2 α Kinase (EIF2AK3); PP1: Protein Phosphatase 1; PPP1R15: PP1's Regulatory Subunit 15

Introduction

Essential (indispensable) amino acids (IAA) are the critical precursors for protein synthesis that must be obtained from food; their deprivation is first sensed in the highly excitable anterior piriform cortex (APC), a paleocortex in the vertebrate brain [1,2]. Omnivorous mammals and birds show rapid sensing of IAA deficient food during the first meal; they reject a deficient diet within 20 min [3]. This adaptive behavioral response is associated with activation of neurons both *in vivo* [4] and *in vitro* [5], found in the ventro-rostral area of the APC (APCvr) [5-7]. The APCvr has few inhibitory elements, rendering it highly excitable [6]. The activated APC projects to appropriate feeding/foraging circuits in the brain, initiating the behavioral responses [8].

The first six steps of this conserved signaling mechanism are found in the APC [9], reviewed in Gietzen et al. and Anthony and Gietzen [2, 10]: leading to interruption of global protein synthesis. Without protein synthesis to restore the rapidly-turning-over potassium-chloride cotransporter (KCC2) and the GABA_A receptor, these crucial control elements are quickly depleted [11], activating the APC circuitry. The mechanism for restoration of control to APC circuits after excitation by IAA deficiency is the final step to be revealed in this homeostatic system.

Several lines of evidence suggest that Ca²⁺ plays a

role in the APC's responses to IAA depletion. There is increased Ca²⁺ in APC slices *in vitro* [12] upon repletion after the slices had been deprived of an IAA for 2-6 h. Phosphorylated-CaMKII (P-CaMKII), an accepted indicator both of increased intracellular Ca²⁺ [Ca²⁺]_i and activated calmodulin (CaM), was increased in the APC of rats 20 min after introduction of an IAA deficient diet [13]. Moreover, Ca²⁺ is required for sensing AAs (but not exclusively IAA) by a Ca²⁺ receptor [14]. Finally, the class III phosphatidylinositol 3-kinase, hVps34, which requires BAPTA-sensitive [Ca²⁺]_i in order to activate mTOR via CaM [15], is also implicated in the APC's response to an IAA deficient food [16]. These several findings implicate Ca²⁺ in the APC's responses to IAA depletion, albeit in various ways.

To investigate how Ca²⁺ is involved in the signaling of IAA deficiency in the APC we subjected APC slices to several Ca²⁺-modulating treatments, in either an IAA (threonine) deficient (Dev) or a corrected (COR, i.e., control (Cont)) medium, and also studied microchip array results in APC slices from rats fed either Dev or Cont. The results are consistent with a role for a Ca²⁺-dependent phosphatase complex in restoring inhibitory control to the APC after activation by IAA deficiency.

Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Livermore CA, weighing 180-250 g) were paired and housed in plastic cages in a vivarium at room temperature (22°C $\pm$ 2°C) with an inverted 12:12 dark:light cycle in an artificially illuminated 22 $\pm$ 2°C laboratory. Animal care protocols followed NIH guidelines (NIH *Guide for the Care and Use of Laboratory Animals*) and were approved by our Institutional Animal Care and Use Committee. Fresh water and diet were freely available. A threonine basal diet (BAS), or 6% casein (each contains about 40% of the requirement for the limiting IAA) was fed for at least a week. For the *in vitro* studies, the tissue was taken without further dietary changes. For the gene-chip experiments, animals were fed BAS for the week prior to the experiment and then given a fresh food cup with either BAS, threonine devoid (Dev) or an IAA complete diet (corrected, COR i.e., Control, Cont) for 2 hr prior to

collection of the APC tissue; for details and rationale for use of these diets see Koehnle et al. [3].

Brain slices: For each slice experiment the brain was quickly removed and prepared as in Rudell et al. [5]. APC slices (400 μm) were dissected as described in detail [5], and kept in aerated control medium for recovery from slicing. The most responsive area of the APC as determined previously [5,7] was included in the slices, which were bisected to provide bilateral experimental and control hemislices. The samples were switched to either Cont or Dev, with or without Ca^{2+} inhibitors (see below). Replacement of media required 2 min for complete mixing, determined in preliminary experiments using colored media. After the buffer was switched, the sections remained in the new buffer for an additional 3 min. Thus, the slices were exposed to the test media for approximately 5 min, according to the recommendation of Hou and Klann [17], and because the electrophysiological response to Dev can take as little as 6 min [5].

Western blots: Tissues were then prepared for immunoblotting: Brain tissue was homogenized in extraction buffer (0.25 M Tris-HCl, pH 6.8; 2.5% sodium dodecyl sulfate [SDS]; 2 mM EDTA; 2 mM EGTA; 10% glycerol; 5% 2-mercaptoethanol) with protease and phosphatase inhibitor cocktails I and II diluted at 1:100 (Sigma, St. Louis, MO). The homogenate was heated for 10 minutes at 100°C and centrifuged at 3,000 rpm for 10 minutes, and the supernatant was stored at -80°C until analyzed. Protein levels in the brain samples were analyzed via the Bio-Rad (Hercules, CA) DC Protein Assay, a modification of the Lowry assay. Colorimetric absorbencies were read on a Multiskan Ascent 96-well plate reader (Labsystems Oy, Helsinki, Finland). Samples were diluted 1:2 or 1:4 in an SDS reducing buffer and electrophoresed at 200 V for approximately 50 minutes in Invitrogen (Carlsbad, CA) 10% BisTris gels. Volumes of samples added to wells were adjusted so that equal amounts of protein were added to each well. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were immunoblotted using the following antibodies: phosphorylated eIF2 α (rabbit polyclonal IgG (Phospho-eIF2 α [Ser51], Cell Signaling Technology, Beverly, MA) diluted 1:1000), phosphorylated CaMKII (rabbit polyclonal IgG [Anti-

ACTIVE CaM KII pT286, Promega, Madison, WI] diluted 1:2000), phosphorylated pancreatic eIF-2 α kinase PEK/PERK (EIF2AK3) (Thr 981, Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:200). Antibodies were diluted as listed above into 5% nonfat dry milk dissolved in TBS-T (Trisbuffered saline with 0.1% Tween-20, pH 7.5) for 1 hour at 24°C and then overnight at 4°C. Biotinylated protein standards (Cell Signaling) were diluted according to the manufacturer's protocol and visualized with horseradish peroxidase (HRP)-conjugated anti-biotin antibody (Cell Signaling) diluted 1:2,000. The secondary antibodies, an anti-rabbit IgG conjugated with HRP (Cell Signaling) or an anti-mouse IgG conjugated with HRP (Cell Signaling) were diluted 1:2,000. The HRP-conjugated secondary antibody and the HRP-conjugated anti-biotin antibody were mixed into 10 ml of 5% milk (w/v) in TBS-T and incubated for 1 hour with the PVDF membrane. The membrane was then washed three times with TBS-T and incubated with the chemiluminescent reagent (0.5 ml of 20 times LumiGLO [Cell Signaling], 0.5 ml of 20 times peroxide [Cell Signaling], into 9.0 ml of double-distilled H_2O) for 1 minute at 24°C. Membranes were placed on Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) for 15 seconds to 30 minutes. Quantitative analysis of phosphoproteins in the digitized Western blot images was done using ImageQuant5.1 (Molecular Dynamics, Piscataway, NJ). Western blot bands were evaluated for volume density; this was the integrated intensity of all pixels excluding the background. After scanning for the protein of interest, the same blots were stained with Coomassie Blue, re-scanned and protein levels were determined for each band, again using ImageQuant.

For electrophysiology: After APC slice dissection as described in Rudell et al. [5], excitatory post synaptic field potentials (EPSP) were evoked by stimulating either the lateral olfactory tract (LOT) or layer III and recorded from layer III of the APC using standard electrophysiological techniques. See Rudell et al. [5] for details and examples of the electrophysiological responses. Experimental media were Cont or Dev with and without inhibitors as in the Western blot studies described above.

Inhibitors: Calcium chelators were: BAPTA-AM (30 μM), EGTA (4 mM), and EGTA-AM (30 μM); inhibitors

were: ryanodine (0.1, 1 or 5 μ M, 9, 12 de-hydrorryanodine) and Ruthenium Red, a mitochondrial uncoupler that is also a nonselective ryanodine receptor blocker (10 μ M), from Calbiochem, San Diego CA. Inhibitors from Sigma were: the L-type Ca^{2+} channel blocker, nifedipine (15 μ M, 1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester), thapsigargin, a selective inhibitor of the sarco/endoplasmic reticulum calcium ATPase, (0.1, 0.3 or 10 μ M), and FCCP (10 nM), a selective mitochondrial uncoupler [18].

Data analysis: Data for the phosphoprotein of interest were normalized to the protein level determined by Coomassie Blue staining in the same band as described above and are reported as normalized density units (NormU). Because increased P-eIF2 α indicating the response of GCN2 to IAA deprivation is well established [19] as seen in the APCvr area [7], slices that failed the positive control criteria: Dev>Cont for P-eIF2 α , indicating misplaced dissections, were excluded. Data sets were analyzed by 2-way ANOVA using the anatomical location of the slice (in mm anterior to Bregma) and the contents of the media as independent variables and the normalized phosphoprotein units (Norm U) as the dependent variable. After a significant ANOVA, differences among means were determined by the Fischer's LSD test. For comparing 2 samples, we used the Student's *t* test. Where control and test samples were bilateral sections from the same slice, a paired *t* test was used.

For electrophysiological data, we report either EPSP amplitude (mV), or normalized responses, the ratio, EPSP amplitude in Dev to the baseline in Cont, Mean $\pm$ standard error. The EPSP response to IAA depletion $\pm$ inhibitors was compared with one way ANOVA. Differences between slopes were determined by a Z score.

Microchip analysis: The tissues were processed as reported previously [20] and hybridized to the Affymetrix Rat Neurobiology U34 chip. This microarray contains sequences from approximately 1300 known genes and will reliably detect at least a 2-fold difference in mRNA expression. Results were re-analyzed using the April 2015 revision of the Affymetrix annotation tables and subjected to Gene Ontology analysis (GO

Ontology database, released August 6, 2015). For the GO analysis, the Bonferroni correction for multiple testing was used. Significance was assumed at $P < 0.05$.

Results

Extracellular Ca^{2+}

P-eIF2 α : After 5 min in media containing 4 mM EGTA and 0 mM Ca^{2+} (EGTA- Ca^{2+}) to remove extracellular calcium $[\text{Ca}^{2+}]_o$ from the Dev slice medium, P-eIF2 α was slightly, but non-significantly lower relative to Dev alone, while P-eIF2 α in both Dev and Dev+EGTA- Ca^{2+} remained higher than in Cont (media effect, $F_{2,18}=3.74$, $P=0.044$), Figure 1a and b.

P-CaMKII: There was no difference due to slice location within the APC. Removing $[\text{Ca}^{2+}]_o$ from the Dev medium reduced P-CaMKII to the control level ($F_{8,27}=3.22$, $P=0.011$; Figure 1a and b).

Electrophysiology: When $[\text{Ca}^{2+}]_o$ was removed from the medium, the EPSP was abolished, showing the well known requirement for Ca^{2+} in synaptic transmission, Figure 1c. Nifedipine: When L-type Ca^{2+} channels, which allow $[\text{Ca}^{2+}]_o$ entry into the cell, were blocked by nifedipine (15 μ M), the EPSP response to Dev was blunted for about 40 min (more than enough time for the behavioral response at 20 min, see above), Figure 1d. Clearly, both P-CaMKII, and increased amplitude of the EPSP in the Dev medium (showing activation of the APC), require $[\text{Ca}^{2+}]_o$. The lack of significance for P-eIF2 α suggests that extracellular $[\text{Ca}^{2+}]_o$ is not required. Therefore, we studied intracellular calcium $[\text{Ca}^{2+}]_i$ using BAPTA/AM as in Gulati et al. [15].

Intracellular Ca^{2+} : The membrane-permeable form of BAPTA, BAPTA/AM (BAP), is an intracellular Ca^{2+} chelator acting on fast $[\text{Ca}^{2+}]_i$ events [21]. BAP was used at 10 or 30 μ M; results were consistent over 10 studies.

P-eIF2 α : In 2 trials BAP (10 μ M) reduced eIF2 α phosphorylation in both Dev and Cont media (effect of BAP, $F_{3,6}=5.04$, $P=0.04$). Thereafter we used BAP at 30 μ M. There were no differences between the results at these doses, so the data were combined. Overall, the effect

Figure 1: Effects of EGTA on P-eIF2 α and P-CaMKII. (a): Segments of western blots for P-CaMKII (top), P-eIF2 α (middle) and Coomassie-stained protein in the same blots (bottom). Treatments: Cont, TDev or Dev/EGTA-Ca²⁺ (EGTA), are indicated across the top. Slice locations in mm anterior to Bregma are shown below the bar graphs. (b): Normalized density units (Norm U) for P-eIF2 α and P-CaMKII, means $\pm$ SE. Stars “*” indicate significance at $P < 0.05$. (c): Normalized amplitude (Norm Amp) of the EPSP in APC layer III. Media changes indicated above the timeline indicate times of exposure to the slice. (d): Amplitude (mV) of EPSP over time, with additions to the media as indicated.

of slice location was significant ($F_{2,132} = 9.75$, $P = 0.0001$), Figure 2a. BAP reduced P-eIF2 α in both Cont and Dev (for the effect of reduced $[Ca^{2+}]_i$; $F_{3,132} = 6.46$; $P = 0.0004$, indicated by * in Figure 2b, compare the black bars for

BAPTA vs lighter bars for Vehicle). Importantly, BAP failed to block the effect of IAA depletion. The increases due to Dev, with or without BAP, were highly significant, $P < 0.005$. In duplicate studies with all 4 groups run

Figure 2: *Effects of BAPTA/AM (BAP).* Lanes in the western blot (a) are indicated across the top as C: Cont, CB: Cont+BAP, D: Dev, DB: Dev+BAP. “Protein” is the Coomassie Blue stained band, as in Figure 1. Figure 2(b): P-eIF2α in samples as described in Figure 1(b). Here, control (Cont, dark bars) and threonine devoid medium (Dev, grey bars); treatments, BAPTA or Vehicle, are given on the X axis. Figure 2(c): P-CaMKII, as for Figure 2(b). Stars “*” indicate significance at $P < 0.05$ for BAP effect. The symbol “§” indicates significance of IAA depletion in the Dev slices, showing intact GCN2 activity. (d): EPSP in APC, see text for sequence of responses.

simultaneously, P-eIF2α was increased in both BAP and Vehicle conditions, indicating similar GCN2 kinase activity, unaffected by BAP. The increases in P-eIF2α the Dev medium, in vehicle or BAP were: 1.15 ± 0.06 or 1.35 ± 0.12 , respectively; there was no difference, $P = \text{NS}$; for the intact GCN2 kinase (Dev) effect see the “§” over the Dev bars in Figure 2b.

P-CAMKII: Both medium and slice location showed significant effects: (medium $F_{3,132} = 13.2$, $P < 0.001$; slice location $F_{2,132} = 8.25$, $P = 0.0004$), Figure 2c. Here the effects of BAP (*) reduced P-CaMKII in both Dev and Cont media, and blocked the Dev effect, which was

only seen in the vehicle group (§).

Electrophysiology: The EPSP in APC slices mirrored the P-eIF2α blot results, Figure 2d. When the medium was changed from Cont to Dev (after approx. 50 min of baseline recording) the rapid increase in EPSP amplitude is seen clearly, as expected [5]. When BAP (30 μM) was added to the Dev medium, the EPSP amplitude was reduced to Cont levels. Similar results in 7 studies showed an average increase due to Dev of $10 \pm 2\%$, paired t , $P = 0.02$. Addition of BAP to the medium reduced the EPSP to $-4 \pm 4\%$, i.e., returned to baseline. There was no consistent effect seen when

Figure 3: Means and SE for (a) Effect of BAP on Norm U for P-PERK in slices prepared as in Figures 1 and 2. Conditions are as in Figures 1 and 2, again Protein is shown as the Coomassie stain, here the control medium was Dev; there were no significant differences.

Figure 4: Effects of ryanodine (Ryano, Ry). Dev1 (1 μ M Ryano in Dev) or Dev 5 (5 μ M Ryano in Dev) on (a), P-eIF2 α and (b), P-CaMKII, Norm U as described for Figure 1. The star “*” indicates $P < 0.05$, $N = 5$ studies. (c): EPSP amplitudes (mV) in APC slices bathed in Control, Devoid or Dev+Ry (ryanodine, 5 μ M). Timing and durations of media applications are indicated above waveform numbers on the X axis.

BAP was added to the Cont medium for slice recording (data not shown).

EGTA/AM blocks slow intracellular Ca^{2+} events, but here it had no effect on the increased amplitude of the EPSP response to the Dev medium (ratio Dev+vehicle:Cont: 1.11 ± 0.13 vs Dev+EGTA/AM:Cont:

1.12 ± 0.18 , NS). This shows that slow Ca^{2+} events are not involved in the responses studied here, and provides an important control to show that the results with BAPTA/AM were not due to the AM moiety.

Other eIF2 kinases: There are four eIF2 α kinases (EIF2AK1-4), including GCN2 (EIF2AK4) [19]. PKR

(EIF2AK2) has been shown to be dispensable for sensing IAA deficiency [22] and HRI (EIF2AK1) has not been reported in brain tissue; these 2 were not tested here. To learn whether BAP was reducing P-eIF2 α via an effect on eIF2 α activated by EIF2AK3/PEK/PERK in the APC, we measured P-PERK in Dev $\pm$ BAP. Rather than a decrease, as we saw with P-eIF2 α in the Dev+BAP medium, there was a small increase in P-PERK (Control: 0.36 ± 0.06 vs Dev+BAP: 0.46 ± 0.08 , $P=0.055$, NS via paired t test), Figure 3. Clearly BAP did not *reduce* the expression of P-PERK. Thus the effects of BAP in *lowering* P-eIF2 α were not due either to kinase or phosphatase activity involving PEK/PERK.

Thapsigargin is known to cause ER stress and activate PEK/PERK [23]. To provide a further test for PEK/PERK we used thapsigargin (10 μ M) in Dev. There were no changes in either P-eIF2 α or P-CaMKII expression with thapsigargin ($F_{1,18}=0.491$, $P=NS$ and $F_{5,17}=0.253$, $P=NS$) at any of the slice levels. Similarly, the EPSP study with thapsigargin (0.1 μ M), the response to Dev medium was unaffected.

Sources of $[Ca^{2+}]_i$: Ryanodine receptors:, *9,21-Dehydro-Ryanodine* blocks Ca^{2+} induced- Ca^{2+} release from the endoplasmic reticulum through its eponymous receptors (RyRs). Ryanodine (1 μ M) showed a directional decrease of P-eIF2 α ($F_{1,18}=0.39$, $P=NS$), and at 5 μ M ryanodine provided a significant blockade of the Dev-induced increase in P-eIF2 α expression ($F_{1,8}=8.326$, $P=0.02$), Figure 4a. There was no effect of ryanodine in the Cont medium ($F_{1,12}=0.13$, $P=NS$). P-CaMKII: In contrast to the results for P-eIF2 α , there was a directional decrease, but not a significant effect, of ryanodine on P-CaMKII (1 μ M: $F_{1,6}=1.76$, $P=NS$ or 5 μ M: $F_{1,12}=0.37$, $P=NS$, or Cont: $F_{1,18}=2.46$, $P=NS$), Figure 4b. This is again consistent with intracellular activity of P-eIF2 α , as appropriate for a cytoplasmic protein.

Electrophysiology: Ryanodine (50 μ M) in the Dev slice medium caused a clear reduction of the EPSP amplitude to Cont levels, Figure 4c.

InsP₃ channel: U73122, a mixed agonist of PLC [24,25] was used to probe the InsP₃ Ca^{2+} -release channel on the ER (10 or 20 μ M). In each of 4 studies

there was an early decline in the EPSP ($R^2=0.82$, slope=-0.181), followed by a steep increase ($R^2=0.75$, slope=0.0165; $Z>3.5$, $P<0.01$), consistent with early inhibitory activity followed by stimulation. This is consistent with action as a mixed antagonist/agonist of PLC, but will require further study.

Mitochondria: The blockers, Ruthenium red and FCCP, both failed to have any effect; in normalized units for Ruthenium red: P-eIF2 α (Dev: 0.67 ± 0.07 vs Dev+ Ruth Red: 0.65 ± 0.07 , $P=0.22$) or P-CaMKII (Dev: 0.98 ± 0.06 vs Dev+Ruth Red: 0.87 ± 0.08 , $P=0.53$, both NS). With FCCP (10 nM) Dev, 0.47 ± 0.04 vs Dev+FCCP 0.50 ± 0.04 for P-eIF2 α and Dev, 0.70 ± 0.07 vs Dev+FCCP, 0.64 ± 0.06 for P-CaMKII, $P=NS$. Therefore, the mitochondria are unlikely to be the source of $[Ca^{2+}]_i$ in this model.

Gene chip analysis: We updated and re-analyzed data obtained earlier from APC taken after feeding BAS or Dev diet for 2 hr, allowing time for transcription. Methods and portions of these data have been published in a report on GABA receptors [20]. Among 112 significantly altered transcripts, those associated with Ca^{2+} showed a P value of 0.007; those associated with intracellular trafficking (suggested to be involved by Gulati et al. [15]) were significant at $P=0.002$. CaN A β (Ppp3cb, -2.0 fold), along with the β subunit of CaMKII (Camk2b, -5.6), and the Ca^{2+} -regulated G β protein, (RAS, Gnb1, [26] +8.6 and +5.4), were among the significant findings for Ca^{2+} -associated transcripts.

Discussion

Taken together, biochemical (Western blot), electrophysiological (EPSP amplitude), and transcript-array results provide clear support for a Ca^{2+} requirement in regulating both the phosphorylation of eIF2 α and CaMKII, as well as the activation of the APC by IAA depletion. When we chelated $[Ca^{2+}]_i$, blocked its entry into the cell, or blocked its release from the ER via ryanodine, these phospho-protein levels, or the EPSP, or both, were reduced. Moreover, consistent with these suggestions, Ca^{2+} related transcripts were significantly affected in APC tissue from Dev fed rats.

An important finding from this work is that the kinase

activity of GCN2 did not depend on Ca^{2+} , showing that the similarly reduced P-eIF2 α levels in both BAP+Cont and BAP+Dev conditions must be due to dephosphorylation. Because P-eIF2 α was reduced in the presence of BAP, we conclude that this reduction implicates Ca^{2+} -dependent phosphatase activity. The phosphatase that acts selectively on eIF2 α phosphorylated by GCN2, is protein phosphatase 1 (PP1) with a regulatory (inhibitory) subunit, PPP1R15b, the constitutive repressor of eIF2 α phosphorylation (CReP) [27,28]. The constitutive feature of CReP is important, because the entire biochemical and behavioral sequence, including deficient diet rejection occurs in 20 min. The two other regulatory subunits of PP1 are growth arrest and DNA damage-inducible protein/MyD116 (GADD34/ PPP1R15a) and the viral ICP34.5 [29]; both require about 2 hr for activation [30]; such timing rules them out as the PP1 subunit in the present model.

The role of Ca^{2+} : A precedent for a [Ca^{2+}]_i effect on downstream components, including CaM [13] the RAS pathway [26] and calcineurin (CaN) is shown in brain ischemia [31]. CaN is dependent on CaM, which is clearly seen in layer II of the APC [32], reviewed in Sharp et al. [13]; CaM makes up 1% of total forebrain protein [33]. CaN (PP2B) is the only known protein serine/threonine phosphatase that is reported to be directly regulated by Ca^{2+} /CaM [34]; importantly CaN is the only Ca^{2+} -regulated phosphatase in brain [35]. CaN is found in both presynaptic [36] and postsynaptic [37] neurons and accounts for another 1% of total brain protein [38]. CaN has catalytic (A) and regulatory (B) subunits; in situ hybridization clearly shows high levels of both CaNA α and β in the very cell body layer of APC that is involved in IAA sensing, see Figure 4C in Takaishi et al. [32]. CaN also acts with PP1's regulatory subunits, which in the case of GCN2-phosphorylated eIF2 α would be CReP [28].

Conclusions

The role of the IAA-depletion/uncharged tRNA/GCN2/P-eIF2 α pathway in the APC's responses to IAA depletion is established [10]. With P-eIF2 α blocking the initiation of protein synthesis, inhibitory features in the

APC circuit are lost and the APC is activated [11]. As prolonged activation of the APC can lead to seizures, restoration of protein synthesis via control of P-eIF2 α is vital [39]. We suggest that PP1/CReP complexed with Ca^{2+} /CaM-dependent CaN, participates in the regulation of P-eIF2 α in the APC. Moreover, this important regulator of eIF2 phosphorylation, CReP, which is conserved in drosophila [28], has here been confirmed in the APC of the mammalian brain.

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Author Contributions

CMR-I did the immunohistochemistry and analyzed the Western blot data; JBR and SH did the electrophysiology; JBR analyzed the EPSP data and prepared those figures. CMR-I and SH prepared the genechip materials. MD contributed to analysis of the genechip data. DWG did the statistical analysis, assembled the figures and prepared the final drafts. All the authors contributed to the study design and the writing and editing of the manuscript.

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