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The sensing of essential amino acid deficiency in the anterior piriform cortex, that requires the uncharged tRNA/GCN2 pathway, is sensitive to wortmannin but not rapamycin

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ABSTRACT

Animals detect and reject their first essential/indispensable amino acid (IAA) deficient meal within 20 min; this IAA sensing requires an intact anterior piriform cortex (APC). In the biochemical responses to IAA deficiency in the APC we have shown that: uncharged tRNA is the primary sensor; IAA transport is increased; and signaling, including the extracellular-regulated kinase (ERK1/2), is activated. The mammalian target of rapamycin (mTOR) is a potential AA sensor and is regulated by AA transport. Previously, the inhibitors, rapamycin for mTOR, wortmannin for phosphoinositide 3 kinase (PI3K) and PD98059 for ERK, each blocked the upregulation of the System A transporter in IAA depleted APC neurons. Here we injected these same inhibitors into the APC and measured intake of an IAA deficient diet. Rapamycin had no effect on the rejection of the IAA deficient diet, but wortmannin increased ERK activation and intake of the deficient diet before 40 min and PD98059 acted after 40 min to increase the second meal. While the specific wortmannin target involved in blocking the behavioral response remains unclear, we conclude that mTOR is dispensable for sensing IAA deficiency in the APC, and that ERK is associated with the secondary learned responses to IAA deficient diets.

1. Introduction

Nearly half of the amino acids (AA) present in protein cannot be synthesized or stored by metazoans; these are the essential, or dietary indispensable amino acids (IAA), which must be obtained by diet selection for survival. In the classical behavioral test for IAA deficiency, animals detect and reject a diet lacking an IAA (Harper et al., 1970); they do so within 20 min of the onset of feeding (Gietzen et al., 2007; Koehnle et al., 2003; Koehnle et al., 2004b). This adaptive response leads to selection of food containing the limiting IAA. Lesion studies showed that an intact anterior piriform cortex (APC) is essential for the detection of IAA deficiency in vivo (Leung and Rogers, 1971). The APC is now accepted as the behaviorally relevant chemosensor for IAA depletion, projecting to neural circuits that control feeding (Beverly et al., 1990; Gietzen et al., 1998; Hao et al., 2005). Concentrations of the limiting IAA are decreased in the APC shortly after animals begin eating the deficient diet (Koehnle et al., 2004a) validating this in vivo model of IAA depletion. Replacing 1–2 nmol of the limiting IAA stereotaxically into the rat APC (Beverly et al., 1990; Koehnle et al., 2004a) abolishes the behavioral rejection of the deficient diet. Nanomole injections into the APC using L-IAA alcohols, which competitively inhibit tRNA acylation, mimic IAA deficiency in terms of both the rejection behavior and the biochemical responses (Hao et al., 2005). Moreover, general control non-derepressing kinase 2 (GCN2)-deficient animals fail to reject an IAA deficient diet Hao (Hao et al., 2005; Maurin et al., 2005). Thus, the earliest detection of IAA limitation in the APC is via the GCN2 system, which is activated by uncharged tRNA; this results in phosphorylation of eukaryotic initiation factor 2α (eIF2α) (Gietzen et al., 2004) and inhibition of global protein synthesis at the initiation of translation, reviewed in (Wek et al., 2006).

The output neurons of the highly chemosensitive (Ekstrand et al., 2001) APC are the glutamatergic (Jung et al., 1990) pyramidal cells of layer II, which receive inhibitory input from several neurotransmitter

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systems in a well-studied recurrent excitatory circuitry (Ekstrand et al., 2001). These are the primary cells activated in the response to IAA deficiency; their signaling results in the behavioral rejection of a deficient diet (Gietzen et al., 2007). In these APC pyramidal cells, P-eIF2α is co-localized with extracellular signal-regulated protein kinase (P-ERK1/2) (Sharp et al., 2006), apparently as a secondary signal.

Other putative nutrient sensors that have not yet been investigated in the APC include the mammalian target of rapamycin (mTOR), a receptor tyrosine kinase linked to the phosphoinositide 3-kinase (PI3K) pathway (Gough, 2009). (Nicklin et al. (2009) showed recently that mTOR is regulated by AA transport in which glutamine has an important role. The adaptive upregulation of the AA System A transporter in the APC requires at least one phosphorylation event that can be blocked by rapamycin (Rap), wortmannin (Wort), or the ERK inhibitor, PD98059 (Blais et al., 2003). Consistent with this observation, the selective system A transporter substrate, alpha amino butyric acid, is strongly affected by glutamine in APC neurons (Blais et al., 2003). In light of these findings, we looked for a role for mTOR in the responses to IAA deficiency in the APC, separately or in cooperation with other signaling systems, such as GCN2, ERK, or Wort substrates including mTOR and the PI3Ks.

There are two protein complexes formed by mTOR: mTOR complex 1 (mTORC1) is the Rap-sensitive target (Oshiro et al., 2004); mTORC2 is insensitive to Rap (Han et al., 2007), but is affected by Wort at appropriate doses (Han et al., 2007; Hara et al., 1998; Vanhaesebroeck et al., 2001). In animals, mTOR is responsive to AA supply (Wang et al., 2008) and a variety of other metabolic signals (Corradetti and Guan, 2006). Branched-chain AAs (BCAA), particularly leucine, activate an mTORC1 signaling pathway in several different tissues (Anthony et al., 2004; Lynch et al., 2002) including the hypothalamus (Cota et al., 2006). Yet, the responses of mTOR to changes in IAA availability are variable (Pham et al., 2000). In neurons, glutamatergic activity activates the mTORC1 system as well as ERK (Lenz and Avruch, 2005). As noted above, we have seen P-ERK in IAA deficiency (Sharp et al., 2002), but whether mTOR responds to IAA deficiency in the APC has not been determined.

The specific inhibitor of mTORC1, Rap (Anthony et al., 2004; Hara et al., 1998), binds to the tacrolimus (FK506) binding protein, FKBP12 and the resulting complex inhibits the function of mTOR by dissociation of an essential peptide component, raptor, from the mTORC1 complex (Oshiro et al., 2004). It has been suggested that IAA withdrawal and Rap affect overlapping but distinct sets of signaling elements (Peng et al., 2002). Among the downstream effectors of mTORC1, ribosomal protein S6 kinase 1(S6K1) and the eIF4E binding protein (4EBP1) are the best characterized; when phosphorylated, they serve as readouts of increased mTORC1 activity (Hara et al., 1998). Wortmannin is a fungal metabolite that inhibits mTOR, but its selectivity depends on the dose used.

There are reports that the mTORC2 complex is not affected by IAA depletion, e.g., (Jacinto et al., 2006). Others suggest that mTORC2 multimerization may be sensitive to IAA sufficiency (Takahara et al., 2006), and indeed, mTORC2 controls the actin cytoskeleton in a nutrient-dependent fashion (Loewith et al., 2002). In addition mTORC2 serves as the long-sought phosphoinositide-dependent kinase D2 (PDK2), which phosphorylates Akt/ Protein Kinase B (PKB) on S473, so P-Akt(Ser473) serves as a readout for mTORC2 activity (Sarbassov et al., 2005). Thus mTORC2 may have effects via Akt/PKB as well as more recently identified targets (Alessi et al., 2009).

To study the two mTOR complexes we first used, as behavioral outcomes, the rats' feeding responses to IAA deficient diets after injection of Rap or Wort into the APC. Biochemically we made immunoblot analyses of phosphorylated substrates in APC tissue after the Rap and Wort treatments. We looked at the specific biochemical readouts for mTORC1, the phosphorylation of S6K1, and for mTORC2, P-Akt(Ser 473), in the APC after feeding control or IAA deficient diets.

The results show that both mTORC1 and mTORC2 are dispensable for detection of IAA deficiency in the APC. However, the feeding responses of rats to IAA deficiency were sensitive to Wort injection into the APC, which could be due to an effect of human vacuolar protein sorting kinase 34 (hVps34), a Class III PI3K that has been implicated in IAA sensing (Gulati et al., 2008). The ERK inhibitor, PD98059, increased later intake of the deficient diet (the second meal after >40 min delay), consistent with the timing of an effect on the well known conditioned taste aversion to IAA deficient diets (Simson and Booth, 1973).

2. Methods

2.1. Animals and diets

Male Sprague–Dawley rats, 180–200 g (Harlan, Indianapolis, IN), were housed in individual cages at 22 ± 1 °C with a 12 h light: dark cycle. The vivarium and animal protocols were approved by the local Animal Care and Use Committee and were in accordance with the US National Institutes of Health Guidelines. The diets, containing free L-AAs (Ajinomoto, Teaneck, NJ) as the sole protein source, have been described in detail previously (Koehnle et al., 2003). The basal diet (BAS) contained ~75% of the rats' protein requirement with 50% of their requirement for the limiting IAA and met all other known nutrient requirements of rats. For a threonine-devoid (TBD) diet, threonine was deleted from BAS and the difference was adjusted isocalorically with carbohydrate. For an imbalanced diet, the limiting IAA was kept at the same level as in the basal diet, while the other IAAs were increased as described (Koehnle et al., 2003).

Rats were prefed BAS for 7–10 days before each experiment. In this well-established nutritional model the BAS pre-feeding regimen causes a modest depletion of labile protein and reduces the concentration of the limiting IAA in plasma and brain (Peng et al., 1972), thus allowing rapid recognition of the acute IAA-devoid (or imbalanced) diet-induced deficiency in vivo (Koehnle et al., 2004a; Russell et al., 2003).

2.2. Food intake measurements

On each of the baseline and experimental days, the rats were deprived of food for the last 3 h of the light period in a 12 h light/dark cycle. At the onset of the dark period, a pre-weighed food cup containing BAS or the test diet was placed in each cage. Food intake was measured as the difference in the weight of the food cup (corrected for spillage) at 20 min and 40 min, plus 1, 3, 6, and 21 h in the first Rap studies. The average of 3 consecutive days of BAS intake, measured immediately prior to the experimental day, was used to establish baseline food intake.

2.3. Groups and experimental design

Rats were randomly assigned by baseline food intake to groups such that the experimental groups had equal baseline food intake. There were 6–10 rats per group at the beginning of each study. After it was determined that the drugs did not affect BAS diet intake (see below), the groups were given Sal (vehicle) or drug injections and both groups also were given the IAA-devoid diet. At the completion of the 20 min feeding studies, and for an additional 10 Rap-injected animals, brains were taken for Western blot experiments, as described below.

For the first Rap study 8 rats/gp were fed BAS, given injections of either vehicle (Sal) or Rap into the APC, and food intake was measured at 20, 40, 60 min, 3 h and 21 h. Then 9 rats/gp were given injections as above and TBD diet intake was measured at the same intervals. There were 6, 7 or 10 rats/gp in the remaining 3 studies. Because branched-chain amino acids (BCAA) are important in mTOR signaling, we tried

an isoleucine (Ile) basal prefeed and the Ile devoid diet (Dev) in the protocol described above. The intake of Ile Dev was measured at 20 and 40 min and 3 h. In all, we used 48 rats in the Sal groups and 58 rats in the Rap groups.

For the Wort studies 56 rats were used: 29 Sal and 27 Wort. Exceptions to the general experimental design: the first study used a crossover design such that in the first trial, groups were Sal/BAS vs Wort/BAS and in the second trial they were reversed so that Sal/Bas from trial 1 became Wort/TBD and Wort/BAS became Sal/TBD. The next 3 studies followed the basic study design, but the last one included an additional Bas/Sal group.

In the PD98059 study, as noted above, the rats were housed in modules with computerized measurement of their feeding behavior; 12 rats were used, 6/gp. In this study the threonine imbalanced diet was used and meal patterns were recorded by computer, as described (Koehnle et al., 2004b; Russell et al., 2003). After elimination of 1 rat for misplaced cannulas and 1 for food intake 2 SD from the group mean, there were 5 rats in each group.

2.4. Procedures

2.4.1. Surgeries

After 7 days adaptation to the vivarium, rats were anesthetized with a ketamine cocktail (0.1 ml/100 g of body weight, i.p.) as used previously (Washburn et al., 1994). Bilateral 24-gauge stainless steel cannulae were directed to stereotaxic coordinates 3 mm above the injection site in the APC (2.0 mm anterior to bregma, 3.7 mm lateral to midline, and 6.5 mm ventral to the surface of the dura mater), as done routinely in our laboratory (Beverly et al., 1990). The rats were allowed several days for recovery, during which their body weights and food intakes were monitored to ensure that these conditions returned to pre-surgical levels, prior to the basal pre-feeding period.

2.4.2. Injections and drugs

Each animal was injected once, bilaterally, on the experimental day, as in IAA repletion studies (Beverly et al., 1990). Injections were made 1 h before the diets were introduced. Rats received bilateral 0.5 μl injections of either vehicle or the appropriate drug (Rap, Wort, or PD98059 [Sigma, St Louis, MO], each at 0.05 nmol/side [total dose, 0.1 nmol/rat] in saline) at a constant rate of 0.1 μl/min, using micro-injection pumps (Bioanalytical Systems, West Lafayette, IN). At the end of each study, cannula placements were verified by injecting India ink and histological evaluation. Data from animals with misplaced cannulae, or that had not eaten at least 0.8 g of the test diet were eliminated prior to analysis of the data set.

As noted above, Rap is a selective inhibitor of mTORC1 (Hara et al., 1998). At nanomolar doses Wort can serve as a selective inhibitor for the PI3Kinases, as well as polo-like kinases (Seeburg et al., 2005). In selected cells, at higher (μM) doses than were used in the present studies, Wort also blocks the activation of ERK and other kinases. Yet, the nM doses of Wort that we used clearly did not block activation of ERK (indeed, P-ERK1/2 levels were increased, see Results), showing that our doses were low enough to be selective (Vanhaesebroeck et al., 2001). PD98059 inhibits the upstream mitogen-activated protein kinase kinase (MEK) selective for ERK1/2 (Alessi et al., 1995). Each of these inhibitors, at in vitro doses (100nM) comparable to the injected doses used here, blocked the activation of system A amino acid transporter by IAA deficiency in primary cultures of APC neurons (Blais et al., 2003).

2.4.3. Immunoblotting

At the appropriate time after introduction of the experimental diets, rats were decapitated, brains removed and APC slices were made. Transverse sections of frozen brain were cut ~1.9–3.9 mm rostral to Bregma. The APC was dissected as described (Gietzen et al., 2004; Sharp et al., 2006), and divided into an area medial to the lateral olfactory tract (APC_{lot}) and a more ventro-rostral segment (APC_{vr}) (Ekstrand et al., 2001; Sharp et al., 2006). For immunoblot analysis, either tissues from 5–7 rats were pooled, or bilateral APCs from individual rats were used. Protease inhibitors and two phosphatase inhibitor cocktails (Catalogue Nos. p8340, p2850 [Cocktail 1], and p5726 [Cocktail 2], respectively; Sigma) were added to the extraction buffer, each at a 1:100 dilution. Cocktail 1 inhibits the L-isozymes of alkaline phosphatases and serine/threonine protein phosphatases (PP) such as PP1 and PP2A. Cocktail 2 inhibits the acid and alkaline phosphatases and tyrosine protein phosphatases (www.sigmaaldrich.com).

Brain tissues were prepared for Western blotting as described previously (Gietzen et al., 2004; Sharp et al., 2006; Sharp et al., 2004). The primary antibodies used (all from Cell Signaling [Danvers MA] except as noted) were: phosphorylated (P-) calcium calmodulin protein kinase II (P-CaMKII) phosphorylated on Thr286 (Promega, Madison, WI), diluted 1:200; P-$p^{44/42}$MAP Kinase on Thr202/Tyr204 (P-ERK1/2), diluted 1:1000; P-eIF2α on Ser51, diluted 1:500; P-4E-Binding Protein 1 (P-4EBP1) on Thr37 and/or Thr46, diluted 1:1000; P-$p70^{s6k}$ (P-S6K1) on Thr389, diluted 1:1000; and P-Akt(Ser473), diluted 1:2000 (in 5% bovine serum albumin rather than milk). In some cases the phosphorylated protein was normalized to its non-phosphorylated form, determined by stripping and re-probing the same membrane. In other cases, the protein values were normalized either to the saline control or to the density of the total protein in each lane as determined by protein staining using Coomassie Blue (Sigma). In the figures, separate gel segments are bordered by black bands. Within a black banded section of a figure, adjustments for brightness or contrast were made uniformly.

2.5. Statistics

For comparisons of data from more than 2 groups we used ANOVA with Fischer's least significant differences test for post-hoc comparison among group means. For 2 groups the Student's *t* test was used. Where appropriate, repeated measures analysis (e.g., food intake over several time points) or paired *t* tests (for control vs devoid treatment in hemi-sections of the same brain slice) were used. Values are given as means ± SEM. Significance was assumed at $P<0.05$.

3. Results

3.1. Rapamycin injections into the APC, behavior and mTORC1

Bilateral Rap administration into the APC did not affect the ability of the rats to recognize the TBD diet, as the saline (Sal) and Rap-injected groups ate almost exactly the same amount of diet throughout to 21 h; data for the first 20 and 40 min are given in Fig. 1A. As expected (Koehnle et al., 2004b; Russell et al., 2003), Sal-injected rats consumed significantly less of the TBD diet than the control, BAS, during each measurement period after 20 min (Sal/TBD vs Sal/BAS: $P<0.05$). Rapamycin injection into the APC did not affect rats' consumption of BAS, compared to the Sal control group, measured at any time point from 20 min to 21 h after presentation of the diet (pre-planned comparisons: Rap/BAS vs Sal/BAS and Rap/TBD vs Sal/TBD: $P>0.05$ not significant [NS], Fig. 1A). Rapamycin administration also failed to affect the rats' recognition of an Ile Dev diet; Rap-injected animals ate similar small amounts of the Ile Dev diet as did the Sal controls ($n=9$/group, Rap/Ile Dev vs Sal/Ile Dev: $P=$NS).

To validate the effectiveness and dose of the Rap as used in these studies, animals were given injections as above and immunoblots were made of APC tissue taken at 20 min after introduction of BAS. (Diets were placed in the cages 1 h after the injections were completed, so the tissues were taken 1 h 20 min after the injections.) In these immunoblots, the readouts for effective Rap treatment, P-S6K1 and P-4EBP1, (Hara et al., 1998) were tested. Importantly, the Rap-injected rats had a

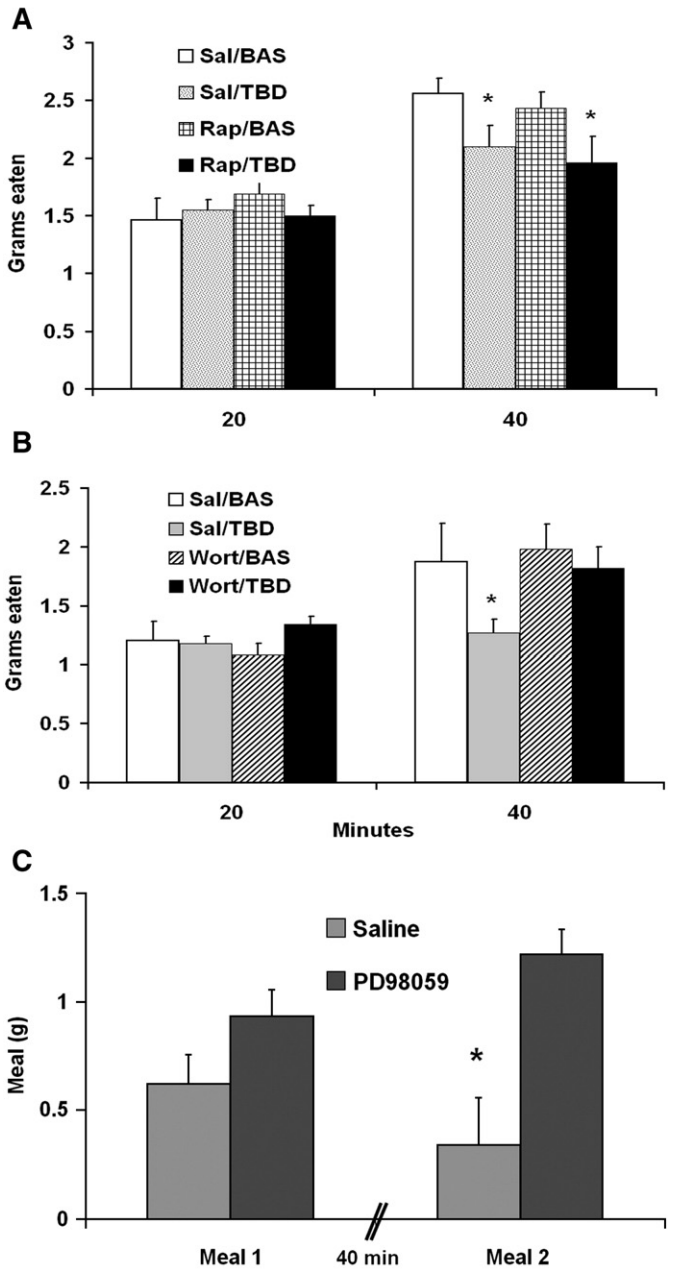

Fig. 1. Food intake (in g eaten) of basal or IAA depleting diets introduced 1 h after injections into the APC. (A) Effects of rapamycin (Rap) or saline (Sal) pre-injections, administered into the APC 1 h prior to feeding, on intake of the basal diet (BAS) or threonine-basal devoid diet (TBD). Food intake measurements were made at 20 min and 40 min after introduction of the test diet. Values represented by bars and error markers are group means and SEM. At 40 min, both Rap and Sal groups ate significantly less TBD than their respective BAS controls (Stars "*" indicate $P<0.05$ for BAS vs TBD) indicating that Rap did not block the usual rejection of the IAA–devoid diet (Sal/TBD vs Rap/TBD, $P=$NS). (B) Effects of wortmannin (Wort) or saline (Sal) pre-injections into the APC on food intake of basal (BAS) or threonine-basal devoid (TBD) diets at 20 and 40 min after onset of feeding. There was no effect of Wort on intake of the basal diet (Sal/BAS vs Wort/BAS, $P=$NS), but the Wort injected rats had increased their intake of the devoid diet (TBD) by 40 min, compared with the saline-treated group, for which devoid diet intake was significantly less (*$=P<0.05$ for Sal/TBD vs Wort/TBD). These results show that Wort injections into the APC blocked the rejection of the IAA-devoid diet before 40 min. (C) Effects of the ERK1/2 inhibitor, PD98059, or saline injections into the APC on meal size of threonine imbalanced diet in the first and second meals. There was no significant effect on the first meal size (Meal 1, Saline vs PD98059, $P=$NS) or on the intermeal interval, but PD98059 increased the size of meal 2 (Saline vs PD98059, right, $P=0.023$).

significant reduction of P-S6K1(Thr389) in APC tissue, both APC_{lot} and APC_{vr}, (Fig. 2A and B, top), and a directional (albeit not significant) reduction in the phosphorylation of 4EBP1 at Thr37/Thr46 (Fig. 2A and B). Therefore, the absence of an effect of Rap on the feeding response to IAA deficiency was not due to the effectiveness or dose of the drug.

Rapamycin treatment caused a slight decrease in P-ERK1/2 in the APC_{vr}, but not the APC_{lot}, compared to Sal-treated rats, and had no significant effect on P-CaMKII or P-eIF2 (both: $P=$NS; Fig. 2A and B) after 20 min of eating BAS. In a separate study using tissue taken 40 min after onset of a BAS meal (Nemanic et al., 2003), APCs from animals treated similarly with Rap had decreased phosphorylation of S6K1, ERK, and CaMKII (each $P<0.05$), suggesting a continuing effect on S6K1 but not 4EBP1 along with decreases of ERK and CaMKII later, at 40 min. It is important that Rap administration did not affect the activity of the eIF2α kinase, GCN2, as measured by P-eIF2α, at either 20 or 40 min, in any of our trials.

3.2. Diet effects

The effects of an IAA deficient diet include increased P-eIF2α, P-ERK1/2, and P-CaMKII in the APC at 20 min after introduction of these diets, as reported previously (Gietzen et al., 2004; Sharp et al., 2006; Sharp et al., 2004). These results were confirmed here and extended to show an unexpected increase in P-S6K1 in the IAA depleted APC tissue (all, $P<0.05$, Fig. 3).

If mTORC2 were to be activated by AA and inhibited by IAA depletion we reasoned that the phosphorylation of its readout, P-Akt (S473), should be decreased in the IAA deficient APC tissue. Importantly, P-Akt(S473) was clearly not decreased, but if anything slightly increased, in APC tissue after 40 min eating the devoid diet (D) vs the corrected diet (C; $P=$NS; Fig. 3A and B).

3.3. Wortmannin in the APC

Wortmannin had no effect on intake of BAS ($P=$NS, Fig. 1B). However, the Wort-injected animals did not reject the TBD diet, eating

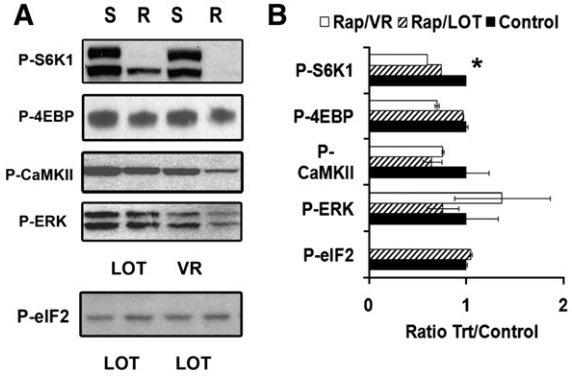

Fig. 2. Effects of rapamycin injection on proteins in the APC. (A) Phospho-proteins in the APC after saline (S) or rapamycin (R) injection into the APC. Rats were fed the basal diet, food deprived for the last 3 h of the light period, and injected into the APC with Rap 1 h prior to the dark period and introduction of the basal diet meal. Samples were taken 20 min after the onset of the meal. LOT, tissue taken adjacent to the lateral olfactory tract in the APC, VR, collected from the ventro-rostral area of the APC as described in (Sharp et al., 2006). Proteins are listed at the left of the figure: for phosphorylated (P-)$p70^{s6k}$(P-S6K1) both 70 and 85 bands are seen, P-4E binding protein 1 (P-4EBP), P-calcium calmodulin dependent protein kinase II (P-CaMKII), P-mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (P-ERK), showing both 42 and 44 bands, and P-eIF2α, shown in duplicates of the LOT. (B) Means and standard errors for immunoband density expressed as the ratio of treatment group/control. Rap/LOT = Rap injection/LOT tissue and Rap/VR = Rap injection/VR tissue. Densities were normalized to the band proteins as measured by densitometry of the blot stained with Coomassie Blue. Data shown are ratios of the treatment (Trt)/saline (Control) across 3–5 separate studies, each with tissue from 2–4 rats/group; *Indicates significant difference from the respective saline control for P-S6K1 in Rap/LOT and Rap/VR, both less than control, $P<0.05$.

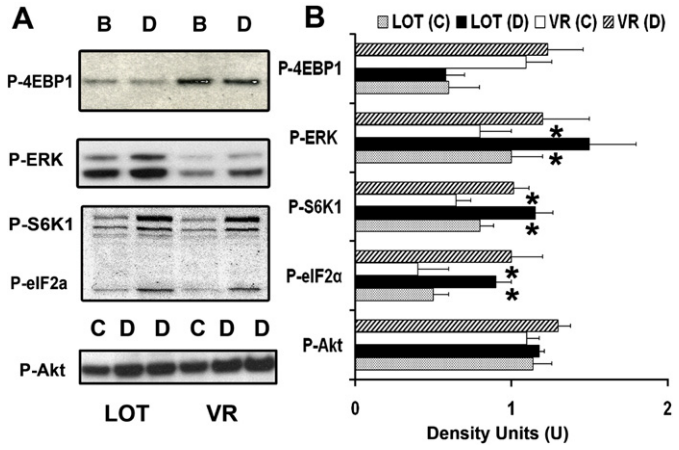

Fig. 3. Diet effects on proteins in the APC. (A) Immunoblot segments prepared from pooled APC tissue taken after (non-injected) animals had eaten either basal (B) or threonine-basal devoid diet (D) for 20 min. LOT: APC_{lot}, VR: APC_{vr}, anatomical locations of tissue samples, as described in Fig. 1 and in the methods. P-4EBP1 showed no change between diet treatments. The increases in P-ERK1/2 and P-eIF2α, after IAA-devoid diet ingestion, replicated our previous results (Sharp et al., 2006). P-S6K1 was increased in the devoid diet tissues. For the P-Akt study, rats were fed either a control (complete [C]) diet or a threonine-devoid (D) diet for 40 min; the bands represent pooled bilateral APClot or APCvr sections from individual rats. (B) Protein density values for P-4EBP1, P-ERK1/2, P-S6K1, P-eIF2α and P-Akt(S473), normalized to total band proteins within that gel, and averaged over 3–6 studies each having 3–4 replicates from pooled tissue samples, except for P-Akt, as noted above. C = control basal or corrected diet, D = Devoid diet. There was no effect of the devoid diet on P-4EPB1 or P-Akt. After 20 min eating the threonine-devoid diet, P-S6K1, P-ERK and P-eIF2α were increased in both areas of the APC; the star " *" indicates significance at $P<0.05$ for control (C) vs devoid (D).

as much as the controls ate of BAS; the usual rejection of the devoid diet was seen only in the saline-injected rats at 40 min ($P<0.05$) (compare Sal/TBD vs Wort/TBD in Fig. 1B).

Wortmannin pretreatment increased the phosphorylation of ERK1/2 in the APC rats fed the TBD diet. This significant increase, in addition to the already-elevated levels due to the IAA-devoid diet, was seen for P-ERK1/2 after Wort injection ($P<0.025$, Fig. 4A and B). There was no effect of Wort on P-S6K1, P-CaMKII or P-eIF2α in these Western blots ($P=$ NS).

3.4. ERK inhibition with PD98059 increased intake of the second IAA-devoid meal

Neither the size of meal 1, nor total food intake before 40 min, differed between drug and saline-injected animals. Following the first meal, similar near-40 min intermeal intervals were seen (PD98059 group: 38.4 ± 19.3 min vs saline group: 42.0 ± 12.4 min; means ± SE; $P=$ NS). Thereafter, the size of the second meal was significantly larger in the PD98059-treated group (Fig. 1C; $P<0.02$), while the saline-treated group ate very little.

4. Discussion

Nutrient sensing is of great current interest and has wide metabolic and clinical implications. Among the amino acid sensing systems, the uncharged tRNA-GCN2-eIF2α pathway in the APC is necessary for animals to sense and reject IAA deficient diets (Hao et al., 2005), but it remains unclear whether this system is fully sufficient for such sensing. Two additional putative AA sensors were studied here, mTOR (Hara et al., 1998) and hVps34 (Gulati et al., 2008).

The major findings of this work are that: 1) mTOR is not involved in the sensing of IAA deficiency by the behaviorally relevant APC; 2) Wortmannin blocked the rejection of the IAA deficient diet, possibly implicating hVps34; and 3) The extracellular-regulated kinases, ERK1/2, appear to be associated with secondary responses known to include learned aversions. A schematic model for the findings reported here is given in Fig. 5.

4.1. Mammalian TOR complexes, mTORC1 and mTORC2

If mTORC1 provides the signal of IAA deficiency, Rap should mimic IAA deficiency and decrease BAS food intake; it did not. Conversely, if a drug inhibits the sensor for IAA deficiency, the animals treated with that drug (in this case, Rap) should fail to recognize the deficient diet, and continue eating it at control levels of feeding. Although the dose and bioavailability of Rap were validated by decreased P-S6K1 in control APC tissue (Fig. 2), the feeding responses to both TBD and Ile Dev diets were unaffected by Rap injections into the APC. Rapamycin also had no effect on the other proteins (Fig. 2) that have previously been associated with IAA depletion in the APC (Gietzen et al., 2004;

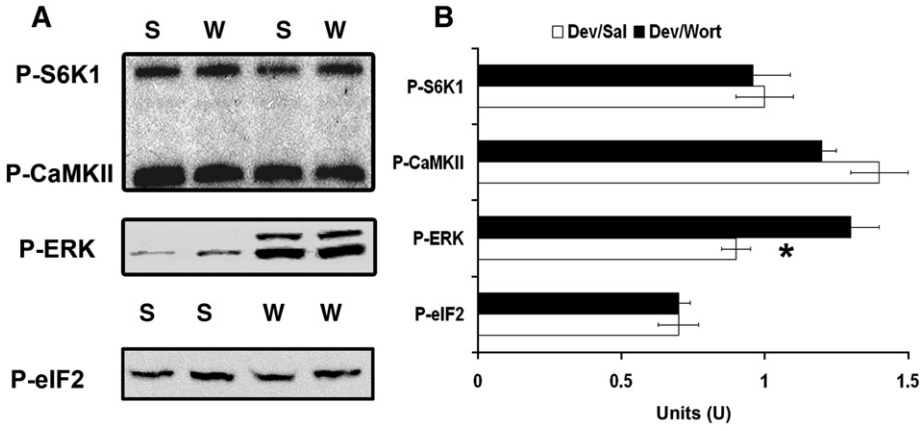

Fig. 4. Effects of wortmannin injection on proteins in the APC of devoid-fed rats. (A) Immunoblot segments showing proteins in the APC after saline or Wort pre-injections into the APC 1 h prior to feeding, and 20 min ingesting the TBD diet. As for Fig. 2, proteins are listed at the left of the blot segments. Treatments are listed across the top: S indicates saline injection and W indicates Wort injection into the APC. For the blot segment illustrating P-S6K1 and P-CaMKII, the first S and W lanes on the left are for the APC_{lot} and lanes on the right are for the APC_{vr}, as in Fig. 2. For both P-ERK and P-eIF2α, the left 2 lanes are duplicates of APC samples from the saline-injected rats and the right 2 lanes are duplicates of whole APC samples from Wort-injected rats. (B) Values for normalized immunoblot densities after Wort injections and the devoid (Dev) diet. The treatments were saline (Sal) or Wort injection into APCs of animals fed the IAA-devoid diet as described in the methods. There was a significant increase in P-ERK after Wort treatment (Dev/Sal vs Dev/Wort, $P<0.025$); there were no other significant differences for the other proteins due to Wort in these studies. Values are means and SE for Density Units (U) for 2–5 studies per protein, each with duplicates of 6–7 pooled APC tissues.

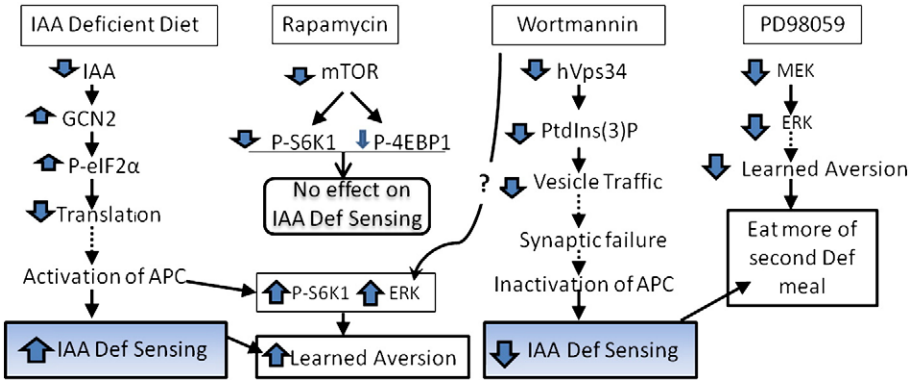

Fig. 5. Model of pathways for the effects described in the text. The diagram shows effects of an IAA deficient diet via the GCN2 pathway (left column), Rap injections (second column), Wort injections (third column), and PD98059 (right column). Symbols: short, wide filled arrows show the direction of an effect; the small filled arrow at P-4EBP1 indicates a directional but non-significant effect. Arrows with dotted lines indicate hypothesized effects. The solid arrow with "?" indicates an unexplained effect (for increased ERK after Wort). Effects previously known or shown in the present study are indicated by solid arrows. Abbreviations are the same as used in the text. Null effects for P-proteins are not included in the figure for clarity; several such findings are relevant to our conclusions and may be seen in Figs. 2–4.

Sharp et al., 2002; Sharp et al., 2006; Sharp et al., 2004) and Fig. 3. Therefore the sensing of IAA deficiency by the APC is not sensitive to Rap. Moreover, Wort had no effect on S6K1, the mTORC1 readout, so the Wort effect on feeding is not likely due to mTORC1. Taken together, these results rule out a role for mTORC1 in the APC's sensing of IAA deficiency.

MammalianTORC2 is Rap-insensitive, at least in the short period of 20–40 min (Sarbassov et al., 2006) within which the rodent rejects an IAA deficient diet. Thus, the failure of Rap to affect feeding in our rats does not rule out mTORC2, nor does the feeding response to Wort necessarily support such a conclusion. However, the selective indicator of mTORC2 activity, P-Akt(S473), was unchanged after feeding an IAA deficient diet, showing that mTORC2 was not responsive to IAA deficiency in the APC. Thus, we have ruled out mTORC2 as well, consistent with the findings of others, e.g., (Jacinto et al., 2006).

4.2. Rapamycin and its biochemical readout, ribosomal S6 kinase (S6K1)

Decreased phosphorylation of S6K1 has become the expected result of both Rap treatment and AA depletion (Anthony et al., 2004; Hara et al., 1998). As noted, we did see the expected decrease of P-S6K1 in the APCs from Rap/BAS animals. However, P-S6K1 was increased in the APC after the TBD meal, rather than decreased, as it should have been if the TBD diet and Rap had similar effects. This unexpected increase in P-S6K1 in the APC after eating a TBD diet could result from a relative increase in non-limiting AAs (Blais et al., 2003), such as the BCAAs, which would signal mTORC1 to phosphorylate S6K1 (Hara et al., 1998; Xu et al., 1998). Yet when we tested this idea in APC, the BCAAs were not consistently increased at 30 min after introduction of the IAA deficient diet (Koehnle et al., 2004a), eliminating this possibility. As an alternative explanation, the increased P-S6K1 might be secondary to the stimulation of p90 ribosomal S6 kinase (RSK) by ERK (Tsokas et al., 2007). Although Wort increased ERK, it had no effect on S6K1, so our evidence does not favor this pathway, either. It remains possible that the increased P-S6K1 could be due to the known glutamatergic activity of the APC pyramidal cells, as glutamate has been shown to increase both P-S6K1 and ERK in fetal neurons (Lenz and Avruch, 2005).

Gulati et al. (2008) found that P-S6K1 was increased by addition of AAs, particularly leucine, in a calcium-dependent system associated with hVps34, which can lead to increased P-S6K1 via mTORC1. They showed that Wort blocked the P-S6K1 increase that was due either to release of calcium from intracellular stores or the increased AAs or both (Gulati et al., 2008). For this mechanism to serve our model, P-S6K1 should have been inhibited by Wort, but it was not, nor did Wort affect the downstream increases in levels of P-CaMKII, an indicator of increased intracellular Ca^{2+} (Sharp et al., 2004). Thus, it appears that the effects of Wort on feeding in this study were not due to calcium or P-CaMKII. Still, a role for hVps34 itself is consistent with our findings (see below).

4.3. Wortmannin: effects on behavior and ERK

Because Wort had no effect on intake of the control diet, BAS, it was not toxic to the animals, and did not mimic the effects of IAA deficiency. Rather, the continued ingestion of the TBD diet between 20 and 40 min by the Wort injected rats indicates that Wort in the APC blocked the usual behavioral response to IAA deficiency, i.e., Wort blocked rejection of the deficient diet.

In the immunoblots made after Wort treatment in TBD-fed animals, the absence of increases in P-eIF2α, P-S6K1, P-4EBP1 and P-CaMKII might be due to a ceiling effect from the TBD-stimulations, which could blunt further increases. Still, we should have been able to see any decreases occurring after the Wort injections, which were expected, if the behavioral effects of Wort in blocking rejection of the TBD diet were due to phosphorylation of S6K1, CaMKII, or eIF2. Recently Kilberg's group (Thiaville et al., 2008) showed that ERK is required for phosphorylation of eIF2α in human cancer cells, but this required a period of hours, not the minutes associated with a sensory function. In contrast, in the present study, Wort had no effect on the increased P-eIF2α seen in the APC 20 min after introduction of the TBD diet, as shown in Fig. 4. Thus, the present effects of Wort appear to be distinct from the GCN2 system as well.

The sole effect on phosphorylated proteins in the Wort/TBD groups was a significant increase in P-ERK1/2 (Fig. 4). Because P-ERK1/2 is increased in the APC after a TBD diet, secondary to and co-localized with P-eIF2α in the same neurons (Sharp et al., 2006), these results implicate ERK1/2 in the later neural signaling, secondary to detection of the IAA depletion (Sharp et al., 2006). Therefore, it was important to see if inhibition of ERK would affect intake of an IAA depleting diet. In this study, food intake was monitored by on-line computerized data collection, and subjected to meal-pattern analysis (Koehnle et al., 2004b; Russell et al., 2003).

4.4. Extracellular-regulated protein kinase 1/2 (ERK1/2)

The feeding responses after inhibition of ERK by injection of PD98059, as seen in Fig. 1C, show that the P-ERKs1/2 are associated with the learned aversion seen in the second meal, rather than the earlier sensory function. Increased P-ERK due to generalized neuronal activity in piriform cortex pyramidal neurons has been associated with learning, independent of IAA deficiency, (Cohen-Matsliah et al.,

2007). Because Wort treatment caused an increase, above the already-elevated levels of phosphorylated ERK1/2 in the APC due to IAA depletion, there must be at least 2 separate pathways involved. This is consistent with our postsynaptic observations in the APC (Sharp et al., 2006) where clearly the neurons are activated in IAA deficiency, and with the effect of PD98059 to increase intake of the second IAA deficient meal after 40 min, when learned aversion to the IAA deficient diet is seen (Simson and Booth, 1973). Also consistent with our findings, inhibition of ERK did not affect mTORC1 in a conditioned taste aversion learning study using the gustatory cortex, where Rap attenuated taste learning (Belelovsky et al., 2009). The effects of PI3K on ERK include both activation and inhibition; the inhibitory effect is via Akt in glial cells (Mograbi et al., 2001), which could explain how Wort increased ERK, but the precise mechanism of this finding is still to be determined.

4.5. Wortmannin and phosphatidylinositol 3 kinases (PI3Ks)

The dose of Wort that we used should be selective for the 3 classes of PI3Ks. The Class I and II PI3Ks are reported not to be involved in nutrient sensing in differentiated cells (Tassa et al., 2003), and the time course of the polo-like kinases (>1 h) is too long for a sensory function (Seeburg et al., 2005), so these potential Wort targets will not be considered further here.

The sole Class III PI3K, hVps34, is sensitive to our doses of Wort and acts on a single substrate, phosphatidylinositol, to yield phosphatidylinositol 3 phosphate (PtdIns(3)P) (Backer, 2008; Vanhaesebroeck et al., 2001). Its many actions include nutrient signaling (Gulati et al., 2008), growth (Vanhaesebroeck et al., 2001) and regulation of protein trafficking (Backer, 2008; Gulati et al., 2008). In cultured cells rapid (min) hVps34 activation, upstream of mTOR, correlates positively to AA increases and negatively to leucine depletion (Gulati et al., 2008). Conversely, others have shown that activation of hVps34 by depletion of leucine (or total AA) is independent of mTOR (Tassa et al., 2003). These opposing results may be explained by differing responses in different cell types (Pham et al., 2000) or different responses based on the state of differentiation of the cells (Tassa et al., 2003). Based on the time course of the responses and the low nM dose used here, we suggest that the results of Wort to block the rejection of an IAA deficient diet that we saw could involve hVps34.

4.6. Effects of the class III PI3K, hVps34

The known effects of hVps34 include autophagy and intracellular protein trafficking (Backer, 2008; Gulati et al., 2008). Because autophagy occurs over days (Gulati et al., 2008) or hours (Tassa et al., 2003), rather than the minutes required for sensing IAA deficiency (Koehnle et al., 2003; Koehnle et al., 2004b), the trafficking of synaptic proteins may more likely play a role in the neuronal synapses of the APC, precisely as suggested for the "signalosome" that responds to AA excess in Gulati's model (2008). If Wort inhibits hVps34, and thus PtdIns(3)P, this could hamper synaptic functioning in the APC. This would serve as a chemical lesion, simulating the electrolytic lesions that originally identified the APC as the chemosensor for IAA deficiency (Leung and Rogers, 1971). Although suggestive and consistent with the available data, a clear demonstration of a role for hVps34/PtdIns(3)P in sensing IAA deficiency in the APC will require further study.

5. Conclusions

The present results show that mTOR is clearly dispensable in the brain's chemosensor for the sensing of IAA deficiency, either in its mTORC1 or mTORC2 complex. The mechanism of the Wort effect on IAA deficient diet intake remains to be solved, but it does not appear to involve either GCN2 or mTOR. Based on the timing of the feeding behavior, the downstream effects of ERK likely are associated with the subsequent learned aversion to the diet. This would be consistent with immunohistochemical results in the APC (Sharp et al., 2002; Sharp et al., 2006) and the behavioral results using the MEK inhibitor, PD98059 as seen in Fig. 1C.

Because an intact APC is required for the sensing of IAA deficiency (Leung and Rogers, 1971), it is tempting to suggest that both the GCN2 target, eIF2α and a Wort target, such as hVps34, could be important in this sensory role of the APC. In this scenario, both eIF2α for continued protein translation and hVps34 for synaptic vesicle trafficking would be necessary. Absent these essential elements, the neuronal circuitry of the APC might be unable to function properly, and animals would fail to reject the IAA deficient diet.

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