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RESEARCH ARTICLE

Oral and pre-absorptive sensing of amino acids relates to hypothalamic control of food intake in rainbow trout

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ABSTRACT

To assess the putative role of taste and pre-absorptive sensing of amino acids in food intake control in fish, we carried out an oral administration with L-leucine, L-valine, L-proline or L-glutamic acid in rainbow trout (*Oncorhynchus mykiss*). Treatment with proline significantly reduced voluntary food intake at 2 h and 3 h after oral administration, while glutamic acid showed a less pronounced satiating effect at 3 h. The mRNA expression of taste receptor subunits *tas1r1*, *tas1r2a*, *tas1r2b* and *tas1r3* was measured in the epithelium overlying the bony basihyal of the fish (analogous to the tetrapod tongue) at 10, 20 or 30 min following treatment. No significant changes were observed, except for a *tas1r* down-regulation by valine at 30 min. Of the downstream taste signalling genes that were analysed in parallel, *plcb2* and possibly *trpm5* (non-significant trend) were down-regulated 20 min after proline and glutamic acid treatment. The signal originated in the oropharyngeal and/or gastric cavity presumably relays to the brain as changes in genes involved in the regulation of food intake occurred in hypothalamus 10–30 min after oral treatment with amino acids. In particular, proline induced changes consistent with an increased anorexigenic potential in the hypothalamus. We have therefore demonstrated, for the first time in fish, that the peripheral (pre-absorptive) detection of an amino acid (L-proline), presumably by taste-related mechanisms, elicits a satiety signal that in hypothalamus is translated into changes in cellular signalling and neuropeptides regulating food intake, ultimately resulting in decreased food intake.

KEY WORDS: Taste receptor, L-Proline, Fish, Hypothalamus, Oral epithelium, Satiety signals, Taste

INTRODUCTION

Satiation and satiety, defined as the processes that bring a meal to an end or that delay the start of the following meal until hunger returns, respectively, are determined through sensory, cognitive, post-ingestive and post-absorptive signals acting from peripheral organs to the brain (Morell and Fiszman, 2017). In mammals, protein is widely recognized as the dietary nutrient providing the highest satiating effect (Morell and Fiszman, 2017). Its satiating power is thought to vary depending on the type of protein, i.e. on its specific amino acid composition (Veldhorst et al., 2009), although contradictory results have been reported (Lang et al., 1998). In other vertebrates, available evidence is scarce. Many species of fish are carnivorous, having high dietary protein requirements (Tacon and Cowey, 1985) and being strongly dependent on dietary amino acids for functioning. However, the impact of protein and amino acids on their satiety and hunger mechanisms is relatively unknown. The few available studies in fish regarding this issue focused on post-absorptive signalling (Conde-Sieira and Soengas, 2017; Delgado et al., 2017; Soengas et al., 2018). In previous studies in rainbow trout, we administered L-leucine, L-valine and L-proline, either intracerebroventricularly (i.c.v.; Comesaña et al., 2018a) or intraperitoneally (i.p.; Comesaña et al., 2018b). We observed changes in food intake as well as in hypothalamic amino acid-sensing systems and neuropeptides that control feed intake. We determined that similar central amino acid-sensing systems operate in fish as in mammals, coupled to the expression of neuropeptides, although with some specificities depending on the particular amino acid. Thus, for instance, circulating leucine clearly has an anorexigenic effect, being similarly involved in the homeostatic control of food intake in fish as in mammals. Valine, contrary to mammals, was also involved in the regulation of food intake, although with different effects (orexigenic or anorexigenic) depending on the site of administration (i.c.v. or i.p.). Furthermore, we also observed that the homeostatic regulation of food intake appears to be independent of the palatability of these amino acids (Comesaña et al., 2018b).

Most studies addressing pre-absorptive (peripheral) satiety signals in mammals have focused on those originating in the gut; few studies have tried to elucidate the role of signals from the oral cavity (Berthoud, 2008; Fromentin et al., 2012). Nutrients from ingested food, especially glucose and amino acids, interact with specific receptors to produce the sense of taste (Fromentin et al., 2012; Han et al., 2019). Gustatory cells in taste buds of the oral cavity transduce taste stimuli into electrochemical signals and transmit them through afferent nerve fibers to the fish brain with many similarities to mammals in terms of morphology, innervation and central neural circuitry (reviewed in Okada, 2015; Kasumyan, 2019). This early detection of nutrients in taste buds allows prediction of the nutritional value of food before its ingestion, therefore enabling a judicious selection of food as well as anticipating an imminent increase in systemic nutrient availability before its digestion and assimilation (Efeyan et al., 2015). In mammals, the cellular mechanisms of the signalling pathway of taste receptors are relatively well known. The main receptors responsible for the detection of glucose and amino acids in mammals belong to the taste receptor type 1 (T1R) family, which has three members that associate in heterodimers to form the sweet (T1R2–T1R3) and umami (T1R1–T1R3) receptors (Chandrashekar et al., 2006). Downstream G-proteins and secondary messengers are involved in the process of taste transduction. The first step is the dissociation of the G-protein heterodimer containing α-gustducin and βγ-gustducin where both subunits are capable of independently

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initiating different downstream signalling cascades. One cascade operates via phospholipase C (PLC) to inositol trisphosphate (IP_3) production and release of intracellular Ca^{2+} stores, and another via adenylyl cyclase (AC) to cAMP production, which inhibits basolateral K^+ channels through phosphorylation by cAMP-activated protein kinase A (PKA). Both pathways eventually lead to taste receptor cell membrane depolarization and neurotransmitter release (Margolskee, 2002). Available evidence suggests conserved taste signalling pathways across vertebrates (Behrens and Meyerhof, 2016), with most of the molecules being shared between mammals and teleost fish including T1rs, Plcb2 and Trpm5 (Ishimaru et al., 2005; Yasuoka et al., 2004; Oike et al., 2007; Yoshida et al., 2007). However, in fish, unlike mammals, amino acids are detected by both T1R1–T1R3 and T1R2–T1R3 (Oike et al., 2007). Gustducin is apparently not present in fish and its function must be covered by other G-proteins such as Gnai1 (Ohmoto et al., 2011).

In the present study, we explored whether specific amino acids in the oral cavity are detected by oral taste receptors, which could act as a pre-absorptive satiety signal communicating with the hypothalamus to regulate food intake. In order to ensure that the action of the amino acids was pre-absorptive, we performed preliminary experiments (not shown) using a physiological dye. We determined that within the established sampling time some dye could have reached the stomach, but none would have reached the intestine. We first assessed changes in rainbow trout food intake after oral administration of different amino acids such as leucine, valine, proline and glutamic acid. We selected leucine, valine and proline based on previous studies showing their impact in food intake regulation through post-absorptive amino acid-sensing systems in the same species (Comesaña et al., 2018a,b). We additionally tested the response to glutamic acid as this amino acid is considered the most important signal in umami taste in mammals. Fish do not have an umami taste given that the umami receptor homologue is a broad amino acid receptor (Oike et al., 2007), but glutamic acid could still be an important signal of availability of dietary proteins considering that it is the most abundant amino acid in almost all proteins (Fromentin et al., 2012; Torii et al., 2013). In a second study, we evaluated the mRNA abundance of different genes related to the mammalian taste signalling pathway in rainbow trout within the epithelium overlying the bony basihyal of the fish. Although not a true vertebrate tongue, we will refer to that structure as analogous to a 'tongue'. In hypothalamus we assessed the mRNA abundance of neuropeptides and transcription factors, and levels and phosphorylation status of proteins involved in food intake control at various pre-absorptive times following oral administration of the same amino acids.

MATERIALS AND METHODS

Fish

Rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), were obtained from a local fish farm (Piscifactoría de la Calle, A Estrada, Spain) and maintained under laboratory conditions at the Universidade de Vigo for 1 month in 100-litre tanks equipped with filters and aerators, with a 12 h light:12 h dark photoperiod (lights on at 08:00 h, lights off at 20:00 h), in dechlorinated tap water at 15°C. Fish were fed once daily (09:00 h) to satiety with a commercial diet (Dibaq-Diproteg SA, Fuentepelayo, Spain) containing 48% crude protein, 14% carbohydrates, 25% crude fat, 11.5% ash and 20.2 MJ kg^{-1} of feed. The experiments described comply with the guidelines of the European Union Council (2010/63/UE) and of the Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Experimental design

Following a 1-month acclimation period, a total of 70 fish were randomly assigned to eight 100-litre experimental tanks. Five tanks were used for the first set of experiments (food intake assessment) and three tanks were used for the second set of experiments. In the second set of experiments, fish were fasted for 24 h before treatment to ensure that basal levels of main hormones involved in metabolic control were achieved (Moon, 1998; Mommsen et al., 1999; Caruso and Sheridan, 2011). On the day of the experiment, fish were lightly anaesthetized with 2-phenoxyethanol (0.02% v/v; Sigma, St Louis, MO, USA) and weighed. Fish received 1 ml 100 g^{-1} oral administration of water alone (control) or containing 40 µmol ml^{-1} L-leucine, L-valine, L-proline or L-glutamic acid (all from Sigma). The dose was calculated from the amount of leucine ingested per day by a trout fed a standard commercial diet (Wacyk et al., 2012). For oral administration, a syringe without a needle was used to slowly dispense the solution onto the surface of the 'tongue', allowing the treatment to be exposed in the mouth for 30 s.

In the first set of experiments, for the assessment of food intake, we placed fish (mass 70.9±2.7 g) in each of five tanks. Food intake was registered for three consecutive days before initiating treatments in each tank. Evaluation of food intake was then done 1, 2 and 3 h after oral administration of water alone (control) or containing L-leucine, L-valine, L-proline or L-glutamic acid. After feeding to satiety (when fish do not eat any more despite food being available in the water), uneaten food and feed waste remaining at the bottom of the conical tanks were withdrawn, dried and weighed, and this value was used to calculate the amount of food consumed by all fish in each tank, as the difference from the feed offered (Polakof et al., 2008a,b). The evaluation of each time point was repeated three times on different days, with each time point also being assessed on different days, alternating days of oral administration with days of resting without administration. This resulted in N=9 tests per treatment. Repetitions were done randomly, changing the tank assigned for each treatment in the previous administration. This set of experiments lasted 20 days (3 days of assessment of basal levels, 9 days of food intake measurement and 8 days of resting).

In a second set of experiments the fish (mass 80.7±3.3 g; N=40) were orally administered with water alone (control, N=8 fish) or containing L-leucine (N=8 fish), L-valine (N=8 fish), L-proline (N=8 fish) or L-glutamic acid (N=8 fish), as described above. After 10 min, fish were lightly anaesthetized with 2-phenoxyethanol (0.02% v/v). Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, immediately deproteinized (using 0.6 mol l^{-1} perchloric acid) and neutralized (using 1 mol l^{-1} potassium bicarbonate) before freezing on dry ice and storing at −80°C until further assay. Fish were then killed by decapitation, and the hypothalamus and 'tongues' (apical part) were dissected, snap-frozen and stored at −80°C. Six fish per group were used for the assessment of mRNA levels by reverse transcription-quantitative PCR (RT-qPCR) and changes in the phosphorylation status of proteins by western blot. The same experimental procedure was carried out 20 and 30 min post-administration. For this set of experiments, we used three tanks with 40 fish each, and one tank was used for each sampling time (10, 20 and 30 min). Fish were anaesthetized and administered the treatment in batches of four, being kept individually until euthanasia in order to accurately sample at the post-treatment time.

Assessment of metabolite levels

Levels of glucose and lactic acid in plasma were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain). Total α-amino acids were assessed colorimetrically using the nynhydrin method (Moore, 1968) with alanine as standard.

Analysis of mRNA abundance by RT-qPCR

Total RNA of hypothalamus and 'tongue' samples was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA) and subsequently treated with RQ1-DNAse (Promega, Madison, WI, USA). Total RNA (2 µg) was reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) in a reaction volume of 20 µl. Gene expression levels were determined by RT-qPCR using the iCycler iQ (Bio-Rad, Hercules, CA, USA). Analyses were performed on 1 µl cDNA (previously diluted 1:2) using Maxima SYBR Green qPCR Master Mix (Life Technologies), in a total PCR reaction volume of 15 µl, containing 50 nmol l^{-1} of each primer. We assessed mRNA abundance of transcripts related to: (1) food intake control in hypothalamus, including the transcription factors brain homeobox transcription factor (*bsx*), cAMP response-element-binding protein (*creb1*), forkhead box O1 (*foxO1*), and the neuropeptides agouti-related protein 1 (*agrp1*), neuropeptide Y (*npy*), pro-opio melanocortin a1 (*pomca1*), and cocaine- and amphetamine-related transcript (*cartpt*); (2) hypothalamic integrative sensors such as mechanistic target of rapamycin (*mtor*), and protein kinase AMP-activated catalytic subunits α1 and α2 (*prkaa1* and *prkaa2*); and (3) taste signalling genes in 'tongue' such as taste receptor type 1 member 1, member 2a, member 2b and member 3 (*tas1r1*, *tas1r2a*, *tas1r2b* and *tas1r3*), guanine nucleotide-binding protein G subunit α-1 (*gnai1*), phospholipase C β2 (*plcb2*), inositol 1,4,5-trisphosphate receptor type 3 (*itpr3*) and transient receptor potential cation channel subfamily M member 5 (*trpm5*). Most transcripts were measured using previously described primers in the same species (Comesaña et al., 2018b; Conde-Sieira et al., 2018; Polakof et al., 2011; Polakof and Soengas, 2013), with the exception of *tas1r1*, *tas1r2a*, *tas1r2b*, *tas1r3*, *plcb2*, *itpr3* and *trpm5*. For these transcripts, new primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-.4.0/primer3/) from sequences available in GenBank (Table 1). A fragment of each sequence containing the amplicon was amplified by conventional PCR and run on a 1.2% agarose gel. The corresponding bands were cut from the gel, purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced in an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA) in Servicio de Determinación Estructural, Proteómica y Genómica (CACTI, Universidade de Vigo). Forward and reverse primers used for each gene expression assay are shown in Table 1. Thermal cycling was initiated with incubation at 95°C for 90 s using hot-start iTaq DNA polymerase activation followed by 40 cycles, each one consisting of heating at 95°C for 20 s, and specific annealing and extension temperatures (1) for 20 s. Following the final PCR cycle, melting curves were systematically performed and monitored (temperature gradient at 0.5°C s^{-1} from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without RNA were run in each qPCR assay as negative controls. Relative expression of the target transcripts in hypothalamus was calculated using *actb* (β-actin) and *eef1a1* (elongation factor 1α) as reference genes, which were stably expressed in this experiment, following the Pfaffl method (2001). However, in 'tongue', Keratin 8b (*krt8b*) was additionally evaluated as a reference gene that it is specifically expressed in the epithelia of taste buds in mammals, although this has not been specifically demonstrated in fish (Markl and Franke, 1988; Venkatesan et al., 2016; Takai et al., 2019), and therefore we cannot discard the possibility that *krt8b* may not be present in fish taste buds. The aim was to ensure that differences in expression are not caused by a different number of taste buds being coincidentally

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by qPCR

	Forward primer	Reverse primer	Annealing temperature (°C)	Database	Accession no.
actb	GATGGGCCAGAAAGACAGCTA	TCGTCCCAGTTGGTGACGAT	59	GenBank	NM_ 001124235.1
agrp1	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60	GenBank	CR376289
bsx	CATCCAGAGTTACCCGGCAAG	TTTTCACCTGGGTTTCCGAGA	60	GenBank	MG310161
cartpt	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60	GenBank	NM_001124627
creb1	CGGATACCAGTTGGAGGAGGA	AGCAGCAGCACTCGTTTAGGC	60	GenBank	MG310160
eef1a1	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59	GenBank	AF498320
foxO1	AACTCCCACAGCCACAGCAAT	CGATGTCCTGTTCCAGGAAGG	60	GenBank	MG310159
gnai1	GCAAGACGTGCTGAGG	CGGTGACTCCCTCAAA	60	GenBank	XM_021601007
itpr3	GCAGGGGACCTGGACTATCCT	TCATGGGGCACACTTTGAAGA	60	GenBank	XM_021616029.1
krt8b	TGGCTACTCCAGTGGTTTCG	CCGCTACCGGAGCTGTAGTT	60	GenBank	X92522.1
mtor	ATGGTTCGATCACTGGTCATCA	TCCACTCTTGCCACAGAGAC	60	GenBank	EU179853
npy	CTCGTCTGGACCTTTATATGC	GTTCATCATATCTGGACTGTG	58	GenBank	NM_001124266
plcb2	GGATTGCTGGAAGGGAAAACC	CGGGGTACTGTGACGTCTTGA	60	GenBank	XM_021584705.1
pomca1	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60	TIGR	TC86162
prkaa1	ATCTTCTTCACGCCCCAGTA	GGGAGCTCATCTTTGAACCA	60	GenBank	HQ403672.1
prkaa2	GGGCTACCATTAAAGACATTAGGG	ACTCGGTGCTCTCAAACTTG	58	GenBank	HQ403673.1
tas1r1	GTTGTGTTCTCCAGCAAAAGC	TCTGTCCCTATCCACACCTTG	60	GenBank	XM_021614415.1
tas1r2a	ATAGTTTTTGCCGGGCAGAGC	CCTGCAATCCACACTTTGCTG	59	GenBank	MT240253
tas1r2b	GATGAGTGGGCCAGGAATGG	CCTCCCACCGGCTGACTTTA	59	GenBank	MT547708
tas1r3	GCCCTGTGGAGCCCATCTTA	CCACACAGTAGGTCAGGGTGGA	60	GenBank	XM_021569423.1
trpm5	GCCAGAGTCAGGAAGCTCAGG	TGTGGCTCTTAGCGATGTCCA	60	GenBank	XM_021569424.1

actb, β-actin; *agrp1*, agouti-related protein; *bsx*, brain homeobox transcription factor; *cartpt*, cocaine- and amphetamine-related transcript; *creb1*, cAMP response element binding protein; *eef1a*1, elongation factor 1α; *foxO1*, forkhead box O1; *gnai1*, guanine nucleotide-binding protein G subunit α1; *itpr3*, inositol 1,4,5-trisphosphate receptor type 3; *krt8b*, keratin8b; *mtor*, mechanistic target of rapamycin; *npy*, neuropeptide Y; *plcb2*, phospholipase C β2; *pomca1*, pro-opio melanocortin A1; *prkaa1*, protein kinase AMP-activated catalytic subunit α1; *prkaa2*, protein kinase AMP-activated catalytic subunit α2; *tas1r1*, taste receptor type 1 member 1; *tas1r2a*, taste receptor type 1 member 2 form a; *tas1r2b*, taste receptor type 1 member 2 form b; *tas1r3*, taste receptor type 1 member 3; *trpm5*, transient receptor potential cation channel subfamily M member 5.

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included in each sample. The expression of *krt8b* in this experiment was compared with that of *actb* and *eef1a1*, and a similar pattern of raw data (that is, non-normalized) was observed. Furthermore, geNorm analysis (Vandesompele et al., 2002) identified both *krt8b* and *eef1a1* as the most stable genes across treatments and time points, and the BestKeeper algorithm (Pfaffl et al., 2004) highlighted that the combination of these two genes was the optimal strategy for normalization. Therefore, both *krt8b* and *eef1a1* were chosen for normalization in 'tongue'.

Western blot analysis

Protein was isolated from the phenol-ethanol phase saved during RNA extraction using Trizol reagent (Life Technologies). The concentration of protein in each sample was determined using the Bradford assay with bovine serum albumin as standard. Protein lysates (20 µg) were used for western blotting using appropriate antibodies from Cell Signaling Technology (Leiden, The Netherlands): 1:500 Anti-phospho AMPKα (Thr172, reference no. 2531), 1:500 anti-AMPKα (reference no. 2532), 1:250 anti-phospho-FoxO1 (Thr24, reference no. 9464), 1:250 anti-FoxO1 (reference no. 9454), 1:500 anti-phospho-mTOR (Ser2448, reference no. 5536) and 1:1000 anti-β-tubulin (reference no. 2146); or Sigma: 1:250 anti m-TOR (reference no. T2949). All these antibodies cross-react successfully with the proteins of interest in rainbow trout (Sánchez-Gurmaches et al., 2010; Velasco et al., 2016). After washing, membranes were incubated with an IgG-HRP secondary antibody (reference no. 2015718; Abcam, Cambridge, UK) and bands were quantified by Image Lab software version 5.2.1 (Bio-Rad; https://www.bio-rad.com/en-uk/product/image-lab-software) in a ChemiDoc Touch Imaging system (Bio-Rad). Bands were assessed by LC-MS/MS at CACTI (Universidade de Vigo), and then compared with available sequences using Uniprot software (https://www.uniprot.org/align/).

Statistics

Comparisons among groups were carried out with one-way ANOVA followed by Dunnett's test using the SigmaStat (Systat Software, San José, CA, USA) statistical package (https://systatsoftware.com/products/sigmastat/). Shapiro–Wilk and Levene's tests were used to confirm normality and homoscedasticity of the data, respectively. When necessary, data were transformed to logarithmic or square root scale to fulfil the conditions of normality and homoscedasticity. Differences were considered statistically significant at $P<0.05$.

RESULTS

Food intake was significantly lower after oral administration with proline compared with the control at 2 h and 3 h, and lower after oral administration with glutamic acid compared with the control at 3 h (Fig. 1A). When the average food intake was considered (Fig. 1B), proline treatment significantly decreased intake relative to the control.

In the second set of experiments, from parameters assessed in plasma only, lactic acid was significantly reduced relative to the control 30 min after leucine treatment (Fig. 2F).

The analysis of mRNA abundance of taste receptors in the oral epithelium, in a region corresponding to the tetrapod 'tongue' (Fig. 3), showed that *tas1r1* decreased significantly compared with the control 30 min after valine treatment (Fig. 3C). No significant changes occurred in *tas1r2a*, *tas1r2b* and *tas1r3* mRNA abundance compared with the control at the time points assessed. Regarding the taste-transduction genes (Fig. 4), mRNA abundance of *plcb2* significantly decreased compared with the control after proline or glutamic acid treatment at 20 min (Fig. 4E). No significant changes occurred for *gnai1*, *itpr3* and *trpm5* compared with the control,

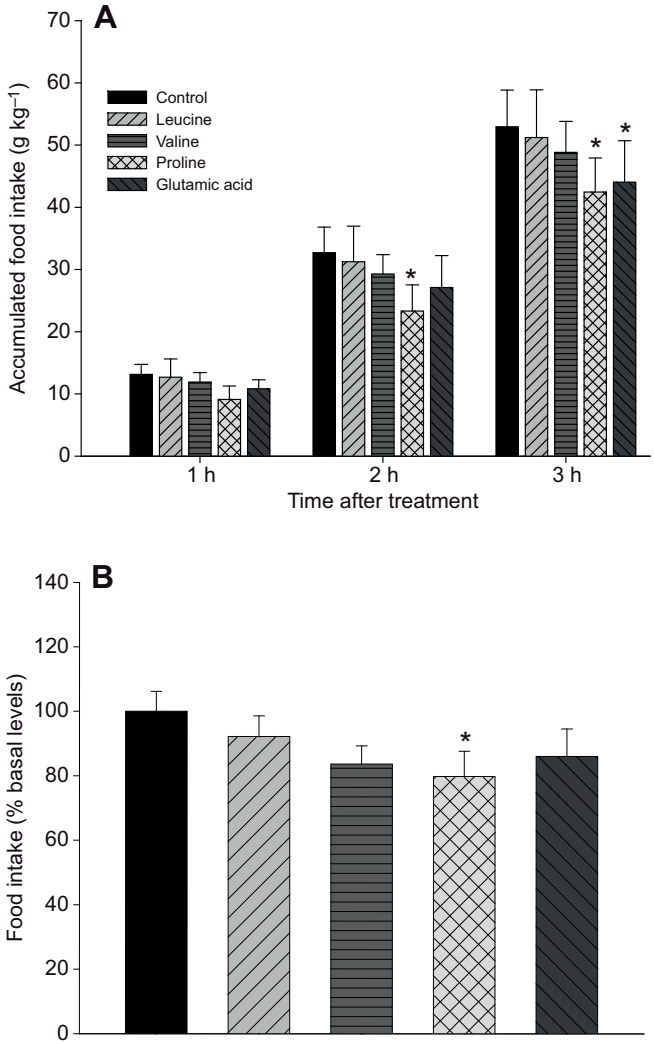

Fig. 1. Accumulated and average food intake recorded in rainbow trout. Food intake was measured at 1, 2 and 3 h after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. (A) Accumulated food intake is displayed as mean±s.e.m. mass of food ingested per body mass, from three different experiments at each time in which 10 fish were used per group in each tank. (B) Average food intake is displayed as mean±s.e.m. of the percentage of food ingested with respect to baseline levels (calculated as the average of food intake in the 3 days prior to the experiment), from three different experiments at each time in which 10 fish were used per group in each tank, resulting in $N=9$ for the averaged food intake. *Significant differences ($P<0.05$) compared with the control group (Dunnett's test).

although *trpm5* showed a similar trend (non-significant, $P=0.08$) to *plcb2* at 20 min.

Proline treatment induced a significant increase in mRNA abundance of *agrp1* (Fig. 5C), *cartpt* (Fig. 5I) and *pomca1* (Fig. 5L) 30 min post-administration, and a decrease of *npy* 10 min after treatment (Fig. 5D), in comparison with the control group. Valine oral administration increased the expression of *pomca1* 20 min after treatment (Fig. 5K).

Leucine oral administration decreased mRNA abundance of *bsx* 10 min after treatment compared with the control (Fig. 6A). No significant changes occurred in mRNA abundance of *creb1* compared with the control. Most significant changes, relative to the control, were observed in mRNA abundance of *foxO1*, which

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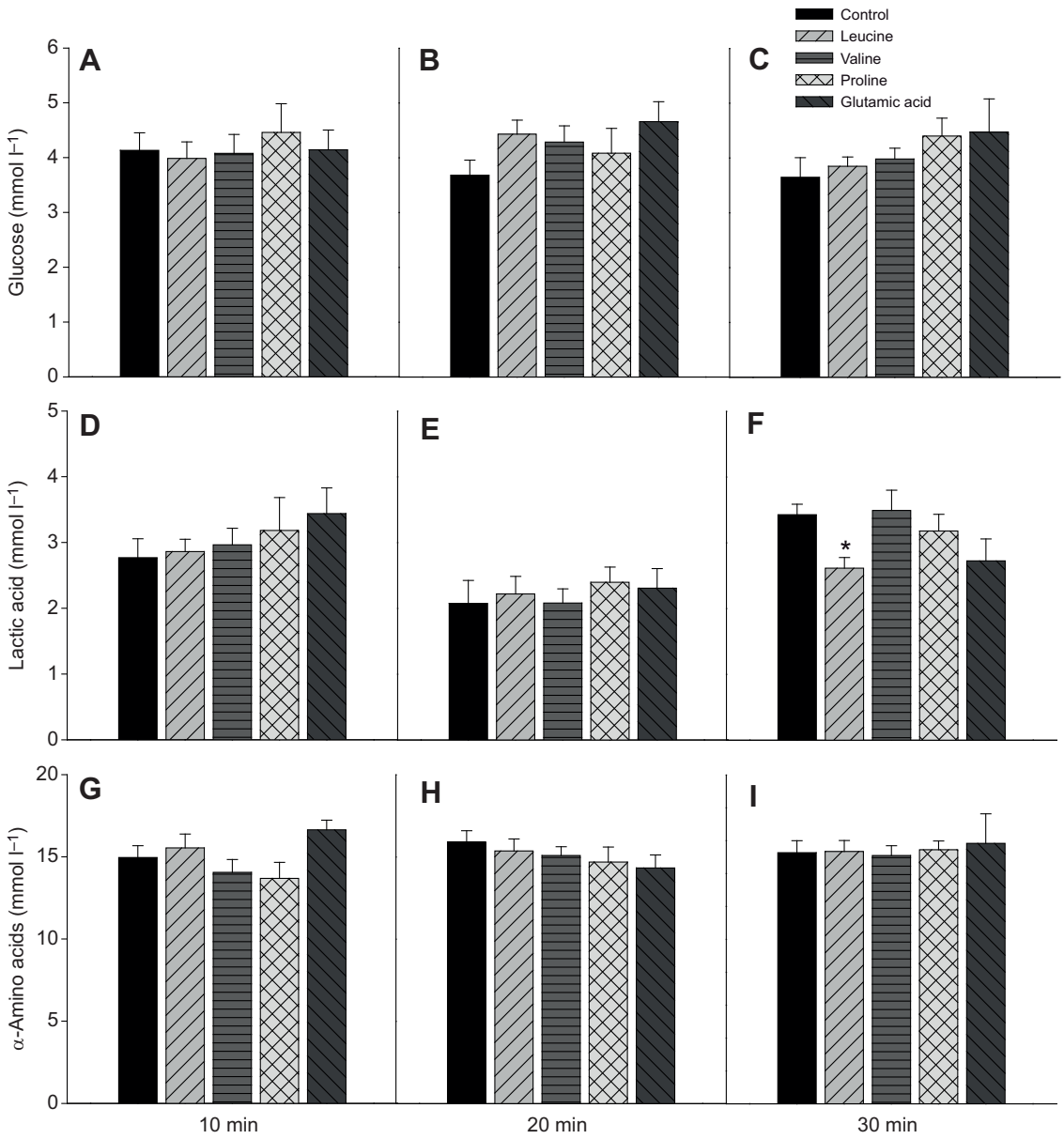

Fig. 2. Plasma levels of glucose, lactic acid and α-amino acids. Levels of glucose (A–C), lactic acid (D–F) and α-amino acids (G–I) were measured in rainbow trout 10 (A,D,G), 20 (B,E,H) and 30 min (C,F,I) after oral administration of 1 ml 100 g^{-1} body mass of water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of *N*=8 fish per treatment. *Significant differences (*P*<0.05) compared with the control group (Dunnett's test).

increased at 20 min after oral administration of glutamic acid (Fig. 6H) and 30 min after oral administration of valine and proline (Fig. 6I).

Valine, proline and glutamic acid induced an increase in the expression of *mtor* 30 min after oral administration, compared with the control group (Fig. 7C). Proline additionally increased the expression of *prkaa2* 30 min after treatment (Fig. 7I).

Finally, no significant changes were observed in the abundance of protein and phosphorylation status of FoxO1, Ampkα and mTor (Fig. 8).

DISCUSSION

Oral and pre-absorptive effects of different amino acids on food intake

Mammalian studies have shown that satiety and satiation can be modified by sensory exposure to food. Furthermore, satiety is usually greater when a rich food is consumed orally, and proportional to oral exposure time (i.e. solid foods induce stronger appetite-suppressing responses than similar liquid foods), than when infused into the gastrointestinal tract (Morell and Fiszman, 2017). However, these studies relate to complex foods rather than single amino acids. Many fish, in contrast to mammals, do not keep the grasped food item in the oral cavity for a long time, do not masticate food, and digestive enzymes are not produced in the oral cavity. Nevertheless, even if very briefly, evaluation of the sensorial and nutritional properties of grasped food clearly occurs in the oral cavity and determines the fishes' ingestive behaviour. After taking food into the mouth, fish either swallow or reject it by spitting it out (Jobling et al., 2012; Kasumyan, 2019). Amino acids are potent taste stimuli, and are the taste compounds that have received by far the most attention in behavioural and electrophysiological studies in fish (Hara et al., 1994; Kasumyan and Døving, 2003).

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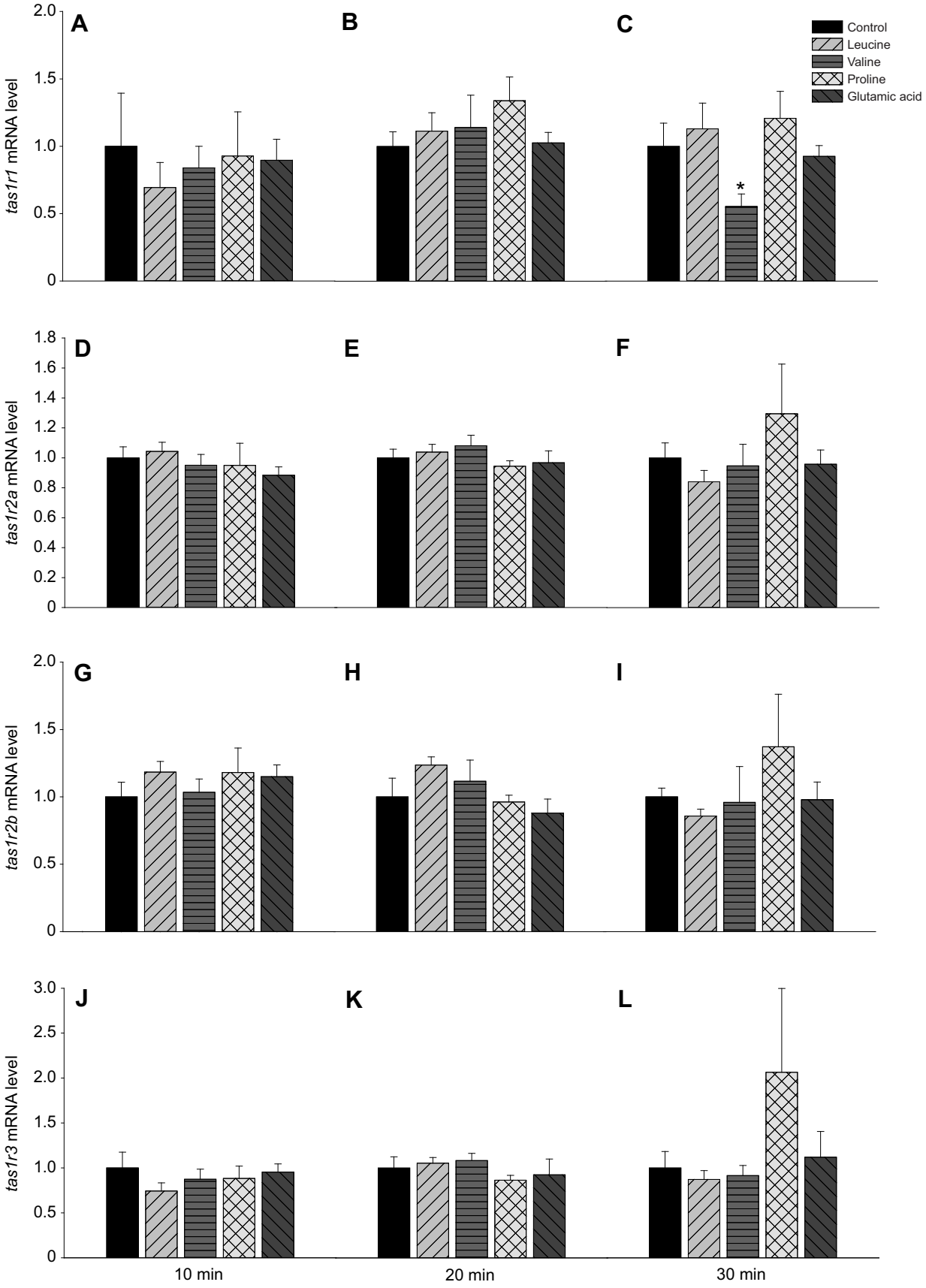

Fig. 3. mRNA abundance of T1R family taste receptors. mRNA levels of *tas1r1* (A–C), *tas1r2a* (D–F), *tas1r2b* (G–I) and *tas1r3* (J–L) in 'tongue' of rainbow trout 10 (A,D,G,J), 20 (B,E,H,K) and 30 min (C,F,I,L) after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of N=6 fish per treatment. Gene expression results are relative to control and are normalized by *krt8b* and *eef1a1* expression. *Significant differences (P<0.05) compared with the control group (Dunnett's test).

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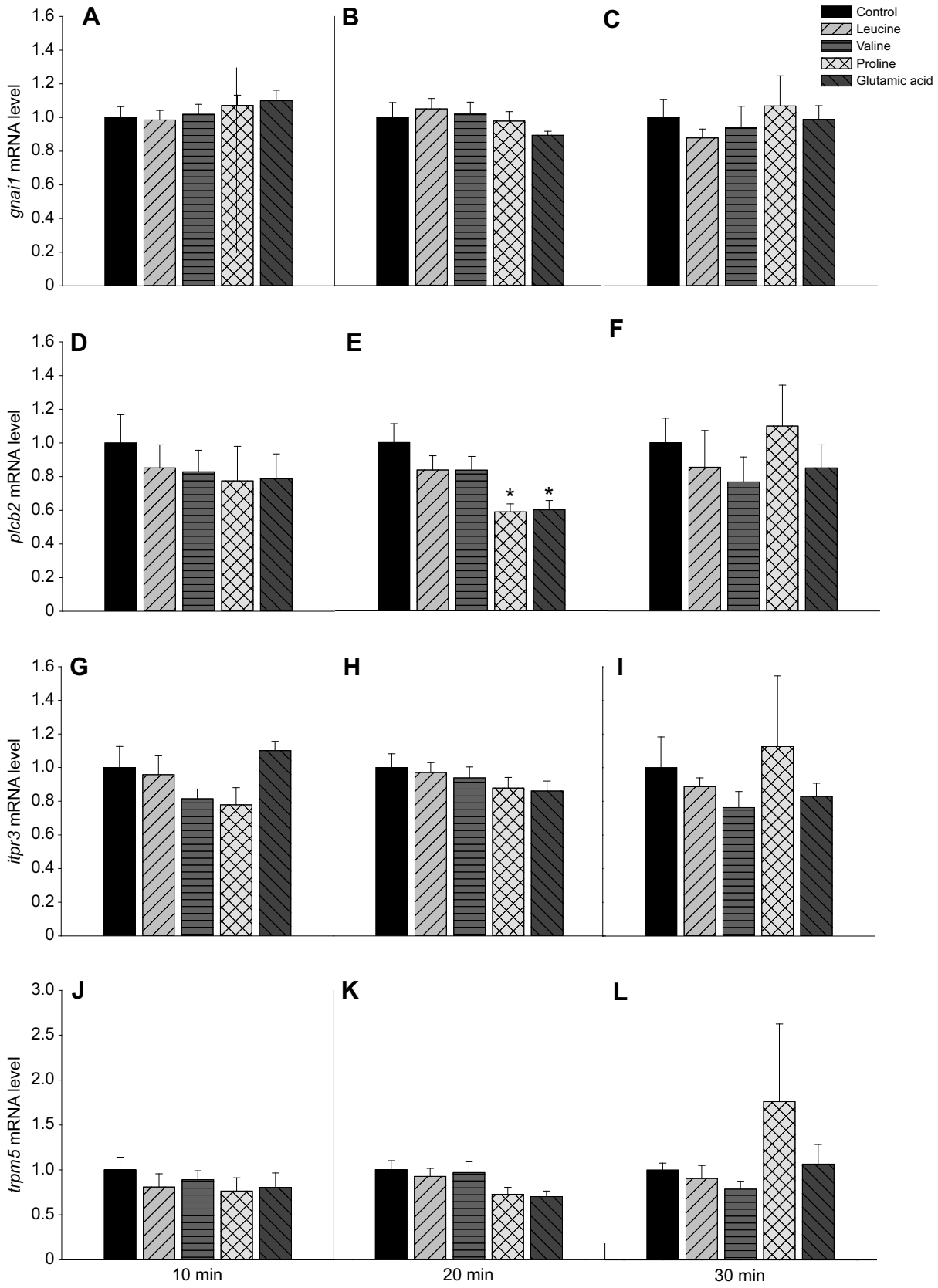

Fig. 4. mRNA abundance of components of downstream signalling cascade from taste receptors. mRNA levels of *gnai1* (A–C), *plcb2* (D–F), *itpr3* (G–I) and *trpm5* (J–L) in 'tongue' of rainbow trout 10 (A,D,G,J), 20 (B,E,H,K) and 30 min (C,F,I,L) after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of N=6 fish per treatment. Gene expression results are relative to group and are normalized by *krt8b* and *eef1a1* expression. *Significant differences (P<0.05) compared with the control group (Dunnett's test).

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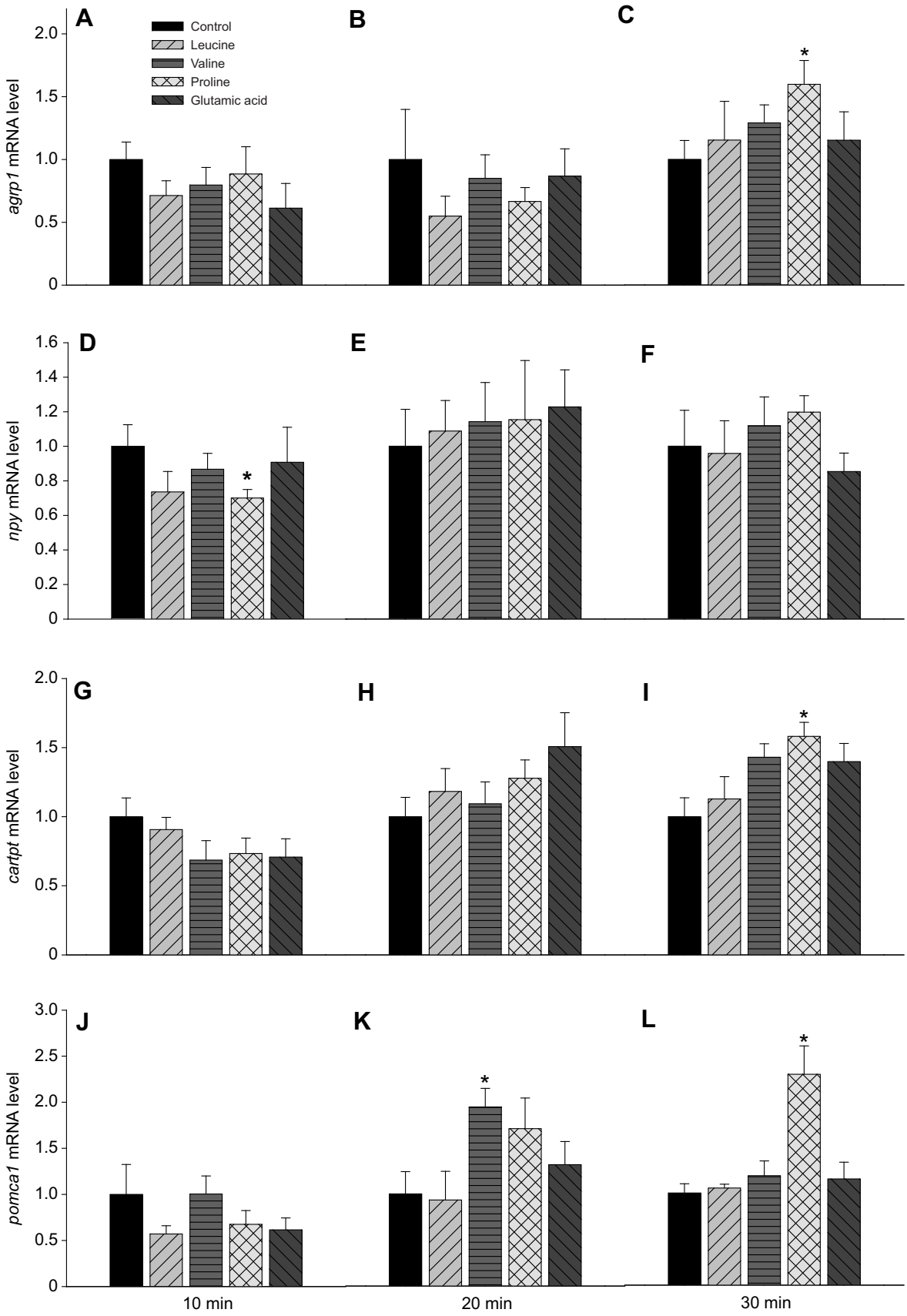

Fig. 5. mRNA abundance of hypothalamic neuropeptides. mRNA levels of *agrp1* (A–C), *npy* (D–F), *cartpt* (G–I) and *pomca1* (J–L) in hypothalamus of rainbow trout 10 (A,D,G,J), 20 (B,E,H,K) and 30 min (C,F,I,L) after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of N=6 fish per treatment. Gene expression results are relative to control and are normalized by *actb* and *eef1a1* expression. *Significant differences (P<0.05) compared with the control group (Dunnett's test).

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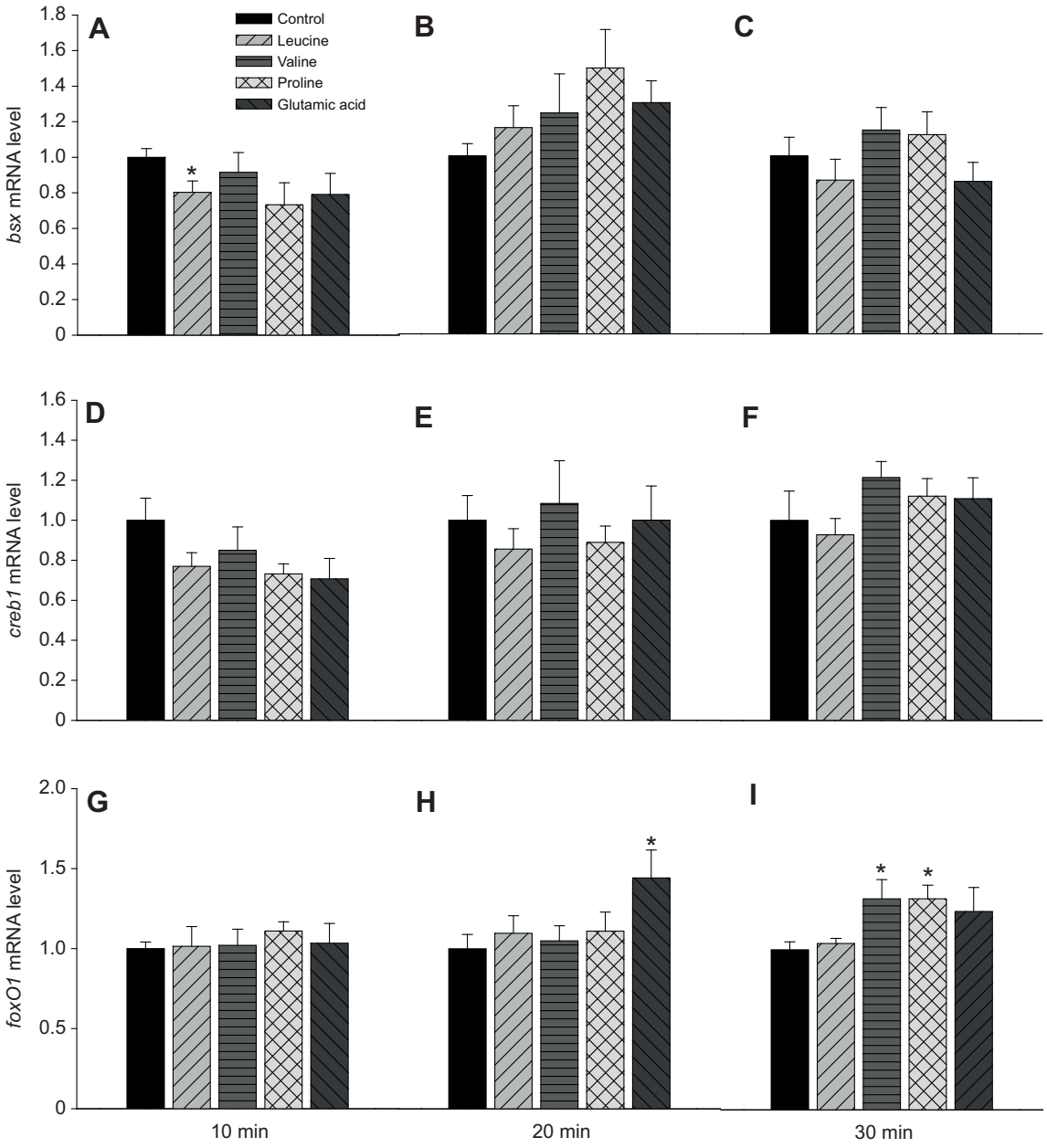

Fig. 6. mRNA abundance of hypothalamic transcription factors. mRNA levels of *bsx* (A–C), *creb1* (D–F) and *foxO1* (G–I) in hypothalamus of rainbow trout 10 (A,D,G), 20 (B,E,H) and 30 min (C,F,I) after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of *N*=6 fish per treatment. Gene expression results are relative to control and are normalized by *actb* and *eef1a1* expression. *Significant differences (P<0.05) compared with the control group (Dunnett's test).

In a first experiment, we tested the feeding response to a single oral administration of different amino acids. Proline reduced food intake compared with the control (approximately 20% on average) significantly at 2 h and 3 h following oral treatment. Of the other amino acids assessed, only glutamic acid displayed a significant decrease in food intake 3 h after treatment. The fact that proline in a previous study (Comesaña et al., 2018b) did not affect food intake 6 h after i.p. administration suggests that it might have a different effect when it is sensed peripherally (pre-absorptive). Similarly, the fact that leucine administered orally did not affect food intake, contrasting with its strong anorexigenic effect after both i.c.v. and i.p. administration, indicates that the response measured here reflects only pre-absorptive stimuli.

The striking differences in the food intake response to both leucine and proline depending on its pre-absorptive or post-absorptive signalling are probably quite logical if we consider some key particularities of these amino acids. These differences could link to a particular physiological relevance as protein or amino acid availability signals at different moments of meal ingestion, digestive and metabolic processing. Leucine is an essential branched chain amino acid. In fish, just as in mammals, essential amino acids show the highest correlation between dietary and postprandial circulating free amino acid composition, in contrast to non-essential amino acids, which are more rapidly metabolized or converted to other metabolites (Jürss and Bastrop, 1995). In addition, branched chain amino acids show the highest changes in plasma amino acid pool in periods of fasting as well as after feeding (Navarro et al., 1997),

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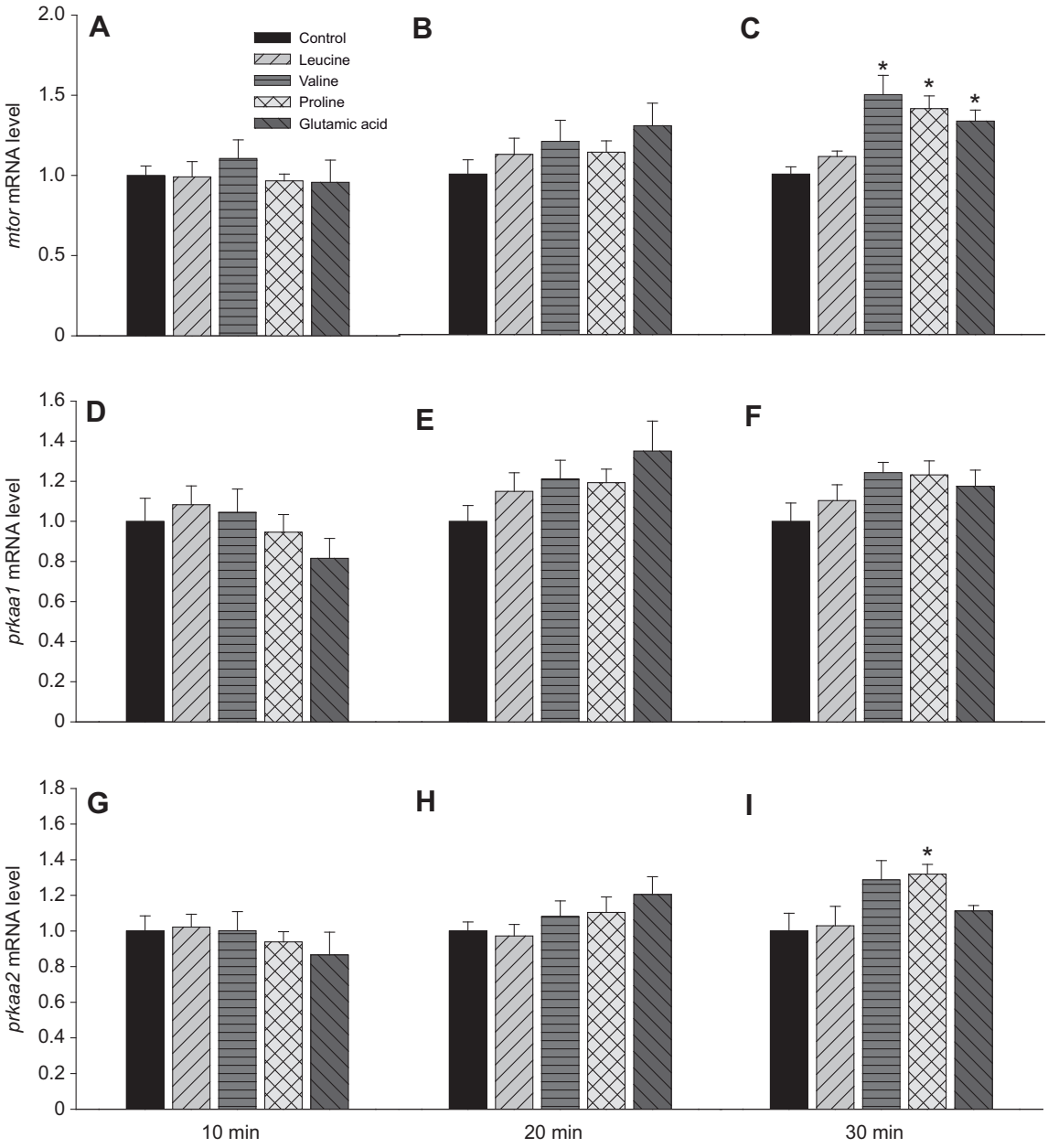

Fig. 7. mRNA abundance of hypothalamic parameters related to integrative sensors mTOR and AMPK. mRNA levels of *mtor* (A–C), *prkaa1* (D–F) and *prkaa2* (G–I) in hypothalamus of rainbow trout 10 (A,D,G), 20 (B,E,H) and 30 min (C,F,I) after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of *N*=6 fish per treatment. Gene expression results are relative to control and are normalized by *actb* and *eef1a1* expression. *Significant differences (P<0.05) compared with the control group (Dunnett's test).

making them ideal homeostatic sensors of endogenous amino acid availability. Proline is an imino amino acid with diverse and important roles in cell metabolism and physiology that can be synthesized endogenously from L-glutamic acid. However, in fish it is considered a conditionally essential amino acid as rates of endogenous synthesis are inadequate during early life stages, and possibly also in adults (Li et al., 2009; Wu et al., 2011). In addition, the requirement of proline for whole-body protein synthesis is the greatest of all amino acids on a per-gram basis (Wu et al., 2011). Finally, proline typically has a high palatability in carnivorous fishes (Li et al., 2009; Morais, 2017), including rainbow trout (Jones, 1989), which could relate to its value in identifying 'good protein'-rich food sources.

A stronger effect of proline compared with the other tested amino acids is consistent with classical electrophysiological studies in rainbow trout, where proline was by far the most effective amino acid stimulating both the facial and glossopharyngeal nerves innervating taste buds in the extraoral surface, rostral oral cavity (palate) and posterior part of the oral cavity (Marui et al., 1983; Kohbara and Caprio, 2001). Furthermore, this imino acid was one of the most potent stimulators of facial nerve responses in zebrafish (Oike et al., 2007). The electrophysiological gustatory response in trout as well as in several other salmonids has a limited range, responding to a small number of amino acids. This is in contrast to other fish species with a much broader spectrum of gustatory activation (Hara et al., 1994; Yamashita et al., 2006). Therefore, care

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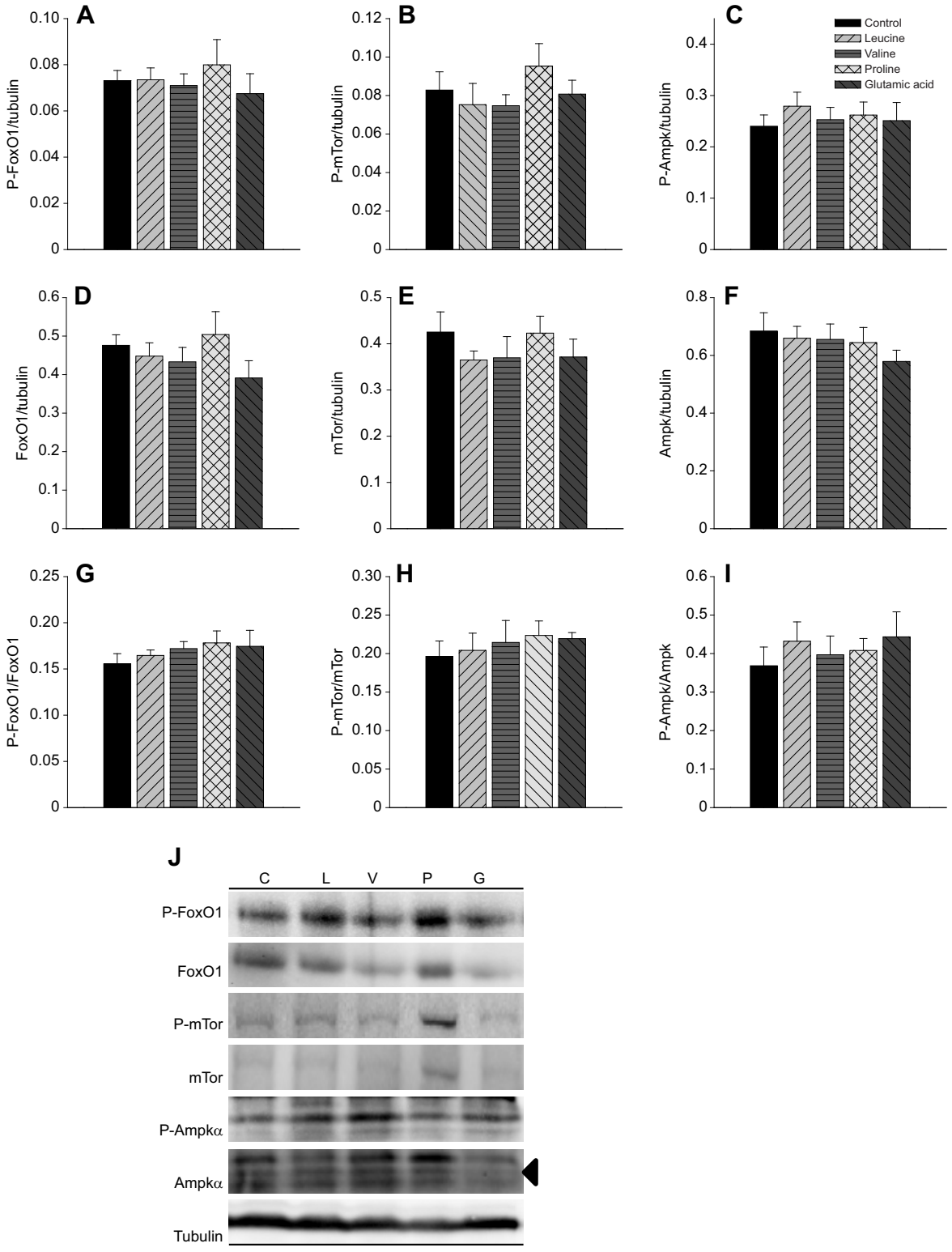

Fig. 8. Western blot analysis of hypothalamic protein abundance and phosphorylation status. FoxO1 (A,D,G), mTor (B,E,H) and Ampkα (C,F,I) were assessed in hypothalamus of rainbow trout 30 min after oral administration of 1 ml 100 g^{-1} body mass of distilled water alone (control) or containing 40 µmol ml^{-1} of L-leucine, L-valine, L-proline or L-glutamic acid. Each value is the mean±s.e.m. of *N*=6 fish per treatment. (J) Total protein (20 µg) was loaded onto the gel per lane. Western blots were performed on six individual samples per treatment, and a representative blot per treatment is shown here (C, control; L, leucine; V, valine; P, proline; G, glutamic acid).

should be taken in extrapolating the results from this study to other non-salmonid fish species, where a higher oral taste responsiveness to different types of amino acids is more likely.

However, there is clear evidence in fish that different amino acids can have different activation thresholds of not only taste nerves (Marui et al., 1983; Kohbara and Caprio, 2001) but also taste

receptors (Oike et al., 2007). In the present study all amino acids were examined at isomolar concentrations. Therefore, we cannot disregard the possibility that different results might have been obtained if the amino acids were tested at different concentrations. In fact, these results indicating a satiating effect of proline administered orally contrast with our previous study when both proline and particularly leucine showed a significantly higher voluntary consumption compared with the control when offered at 0.1 mol l^{-1} in agar pellets (Comesaña et al., 2018b). Hence, the lower concentration (0.04 mol l^{-1}) in the present study could be at least partly responsible for the differences, as the chemosensory response of gustatory nerve fibers in trout in response to proline and leucine has been shown to differ depending on concentration (D-Pro, L-Leu, L-Phe, Gly, L-Arg and L-Trp were also found to be stimulatory, but only at the highest concentration tested: 0.01 mol l^{-1}; Kohbara and Caprio, 2001). Additionally, we cannot disregard a potential effect of olfactory receptors modulating the feeding response in the previous study, where amino acids could have easily leached from the agar pellets.

Finally, of the other tested amino acids only L-glutamic acid showed a slight satiating effect, which was only significant at 3 h after treatment, but not when the mean food intake of the whole period was considered. Glutamic acid, as the most abundant amino acid in proteins, is the most ubiquitous signal for the presence of dietary protein (Fromentin et al., 2012; Torii et al., 2013). Fish literature describes this amino acid as being a poorly effective palatability stimulus (Kasumyan and Døving, 2003; Kasumyan, 2019), as well as a weak activator of the T1R1–T1R3 taste receptor or facial nerves in fish species (Oike et al., 2007), including trout (Marui et al., 1983; Kohbara and Caprio, 2001). Most mammalian studies addressing pre-absorptive umami signalling have been performed after intragastric infusion of amino acids. In these studies, glutamic acid, as well as arginine and lysine, have been shown to inhibit food intake in rats 1 h after their intragastric administration (Jordi et al., 2013). In addition, Kondoh et al. (2009) observed that vagal gastric afferent fibers of rat stomach responded only to glutamic acid from all of the 20 amino acids tested. They also demonstrated that the response was mediated in the lumen rather than systemically given that intravenous administration of glutamic acid did not have an effect. This could be expected, considering that intestinal mucosal cells metabolize virtually all enteral glutamic acid during absorption, which therefore does not reach the blood circulation at noticeable levels after food ingestion (Kondoh et al., 2009). In the present study it is also possible that the administrated glutamic acid could have exerted some satiating effect through the activation of vagal gastric afferent fibers, as at least some of the administrated solution would have reached the stomach; further studies are needed to establish this. Therefore, the results from this study support the existing notion that glutamic acid has a less relevant role in peripheral (pre-absorptive) protein taste sensing in fish than in mammals, but some effect, even if weaker than L-proline, cannot be disregarded.

Transcriptional response of *tas1r* receptors and taste-signalling genes in oral epithelia

Many teleosts, including rainbow trout, have a highly developed gustatory system. Moreover, fish have the greatest amount of taste buds compared with other vertebrates, located in the body surface or specialised appendages, in gill rakers or the oral cavity (reviewed by Morais, 2017; Kasumyan, 2019). Within the oropharyngeal cavity, taste buds are found throughout the oral epithelia but a tendency for a decrease in their density in the rostrocaudal direction has been reported (Marui et al., 1983; Devitsina and Golovkina, 2011). This distribution makes the apical area analogous to the tetrapod tongue a good area to analyse taste receptors.

Different molecular studies have characterized the teleost T1R family of taste receptors (Ishimaru et al., 2005). In fish both heterodimers, T1R1–T1R3 and T1R2–T1R3, respond to amino acids (Oike et al., 2007), unlike mammals, where T1R2–T1R3 responds specifically to sweet substances (Chandrashekar et al., 2006). Furthermore, T1R2–T1R3 has a wider response profile, being activated by a far broader range of amino acids (Oike et al., 2007). Changes in the expression of these receptors in response to specific nutrients has been evaluated in fish brain (Otero-Rodiño et al., 2015; Comesaña et al., 2018b), intestine (Polakof and Soengas, 2013; Cai et al., 2018), liver and Brockman bodies (Otero-Rodiño et al., 2016), as part of mechanisms of amino acid and glucose sensing (Efeyan et al., 2015; Conde-Sieira and Soengas, 2017). However, their transcriptional regulation by potential ligands has not been assessed in an oral tissue until now. With the exception of *tas1r1* down-regulation 30 min after valine treatment, our results did not show a significant change in expression in any of the taste receptor subunits assessed in response to the oral presence of amino acids. There is no evidence that the activation of these receptors is associated with transcriptional responses, and the present results suggest that this is not the case. However, changes observed in downstream taste transduction molecules as well as in central mechanisms controlling food intake in the hypothalamus, as discussed next, suggest that these changes occur quickly and over a short time span. Hence, it cannot be completely disregarded that such a response, if present, could have been missed.

Multiple mammalian G-proteins are implicated in taste transduction of sweet, umami and bitter tastes, functioning as heterodimers containing a G_{α} subunit and a $G_{\beta\gamma}$ dimer. When taste receptors are activated, G_{α} dissociates from $G_{\beta\gamma}$ and both subunits are capable of initiating different downstream signalling cascades. In one of these cascades, G-proteins then activate phospholipase C β2 (PLCβ2) to generate diacylglycerol and inositol trisphosphate. The interaction of inositol with its receptor in the endoplasmic reticulum triggers the release of intracellular calcium stores and the subsequent opening of the TRPM5 channel, resulting in cell depolarization (Behrens and Meyerhof, 2016; Hisatsune et al., 2007). In fish, current evidence suggests that most of the taste signalling effectors are conserved between mammals and teleost fish, including Plcb2 and Trpm5 (Ishimaru et al., 2005; Yasuoka et al., 2004; Oike et al., 2007; Yoshida et al., 2007) and are similarly involved in taste discrimination (Aihara et al., 2008).

In the present study, a clear effect of amino acid oral treatment was observed in *plcb2* expression, which was significantly down-regulated 20 min after proline and glutamic acid administration, relative to the control. Furthermore, although not significant (P=0.08), a similar trend was observed in *trpm5* expression. These results suggest a potential role for *plcb2*, and possibly also for *trpm5*, in the transduction of taste signals from specific amino acids. High expression of *plcb2* is found in a subset of cells from taste buds not only in mammals but also in fish (Asano-Miyoshi et al., 2000; Yasuoka et al., 2004), where it also co-localizes with *trpm5* (Yoshida et al., 2007). PLCβ2 co-expresses with both T1R and T2R (bitter) families of taste receptors, although in a different subset of cells, and has been implicated in the transduction of preferable (T1R-related) and aversive (T2R-related) tastes in fish, similar to mammals (Aihara et al., 2008). No changes were measured in the other investigated taste-signalling genes; however, similar to *tas1r* genes, we cannot exclude that these could be regulated via other

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(non-transcriptional) mechanisms and/or that changes occurred more quickly than that which could be accurately assessed in the present study.

It is especially relevant that the only two amino acids that had a significant effect on *plcb2* expression were also those that had an effect on food intake, which was particularly marked for proline. It remains to be determined how the detection of amino acids at the oral level is transmitted to the brain and can subsequently affect food intake, but given the present results we suggest that taste signalling through *plcb2* and possibly *trpm5*, putatively through the activation of T1R receptors co-expressed in the same taste receptor cells, might be at least partly involved. However, other families of taste receptors or alternative taste signalling pathways may also play a role. These possibilities are very likely considering that in mammals, other families of receptors such as the metabotropic glutamic acid receptors (mGluRs) and the calcium-sensing receptor (CaR) have also been implicated in umami or amino acid taste signalling through a TRPM5-independent pathway (Conigrave et al., 2002; Yasumatsu et al., 2012).

Pre-absorptive effects of amino acids on hypothalamic mechanisms of food intake regulation

In order to assess the effects of amino acids when sensed peripherally on the feeding response, we examined changes in different hypothalamic systems involved in the control of food intake after oral administration of different amino acids. The study was carried out over a short time scale (assessed by a preliminary study) ensuring that the signal is derived mostly from the oropharyngeal cavity and stomach. It is very unlikely that at 30 min the amino acids could have reached the upper intestine and entered circulation by this time, as also established by the absence of relevant changes in metabolite levels in plasma.

The initial phase assessed in this study should correspond to the cephalic phase response (CPR), which refers to rapid physiological responses initiated by sensory exposure to food. The sensory information is conveyed via afferent pathways to the brain, which in turn elicits autonomic efferent responses through vagal nerves (Han et al., 2019). Each nutrient can have a different effect. Glucose, for instance, induces an insulin response, whereas amino acids trigger a pancreatic response (Mattes et al., 2019). Therefore, in the course of the 30 min periods that were evaluated, the hypothalamic responses could result from the integration of different stimuli relayed by sensory neurons associated with oropharyngeal taste receptor cells (through facial or glossopharyngeal nerves), through gastric vagal afferent fibers, via humoral pathways (release of peptides by enteroendocrine cells in the stomach), or even by a combination of these pathways.

Remarkably, the strong satiating effect of proline, associated with a significant down-regulation of *plcb2*, was generally correlated with changes in hypothalamic mRNA abundance of neuropeptides involved in the control of food intake. These include the rise in levels of anorexigens *cartpt* and *pomca1* and the decrease in levels of the orexigen *npy*, although the increase observed in the level of *agrp1* was inconsistent. The capacity of oral proline treatment to induce changes in food intake through hypothalamic regulation mechanisms is also supported by the finding of changes in mRNA abundance of integrative sensors (*mtor* and *prkaa2*) and transcription factors (*foxO1*). This is in agreement with changes in these parameters in the hypothalamus of rainbow trout observed under similar anorectic conditions (Velasco et al., 2017; Otero-Rodiño et al., 2017; Conde-Sieira et al., 2018).

Interestingly, the hypothalamic mRNA abundance of several integrative sensors and transcription factors also changed after oral treatment with the other amino acids assessed such as leucine (*bsx*), valine (*foxO1* and *mtor*) and glutamic acid (*mtor* and *foxO1*). These results suggest that some intracellular signalling pathways in hypothalamus were affected by the oral administration of leucine, valine or glutamic acid. As previously discussed, the different amino acids were evaluated at isomolar concentrations, and different activation thresholds are likely for both taste receptors and facial or glossopharyngeal nerve responses. Hence, there is the possibility that amino acids other than proline would require a higher concentration to elicit an effect.

Changes in hypothalamic integrative sensors and transcription factors occurred only at the mRNA level without changes in protein expression, which probably require a longer time to become evident.

A critical aspect of this study was the time of sampling, considering the expected fast central response to oral or gastrointestinal taste signalling. For instance, areas of the rat brain related to food intake control responded very quickly to an intragastric infusion of glutamic acid, with a peak at 10–12 min (Kondoh et al., 2009). In fish, a delay might be predicted considering that physiological processes, including those related to food intake and metabolism, are slower than in mammals due to their ectothermic nature (Rønnestad et al., 2017; Soengas et al., 2018). In the present study, the effect in taste signalling at the oral level was seen at 20 min after treatment, whereas central effects occurred 10–30 min (but mostly at 30 min) after treatment. Such extremely quick responses do not enable us to distinguish the contribution of different pathways based on temporal patterns of expression. Hence, future physiological studies where some of these pathways are selectively inhibited would be highly desirable to better understand the peripheral effects of amino acids and how the signal is conveyed to the brain. However, this study is an important first step towards establishing pre-absorptive effects of different amino acids, which have been shown to be different from their post-absorptive or metabolic effects (after i.p. administration) in the same fish species.

Conclusions

The present study provides new information regarding the pre-absorptive regulation of food intake by amino acids in rainbow trout. Both proline and glutamic acid were sensed in taste receptor cells in taste buds of the oral epithelia, in an area corresponding to the tetrapod tongue. These effects are indicated by changes in the expression of *plcb2*, and likely also *trpm5* (albeit non-significantly), 20 min after oral administration. Furthermore, both amino acids significantly reduced subsequent food intake (2–3 h after oral treatment), although the effect was markedly stronger for proline. Nevertheless, only proline significantly affected hypothalamic gene expression in a manner consistent with an increased anorexigenic potential. Interestingly, proline, an imino acid eliciting the strongest electrophysiological gustatory response in trout, is a strong activator of fish T1Rs, and typically has high palatability in carnivorous fish species, including trout. It is possible that proline and the other tested amino acids, in particular glutamic acid, might also have affected other pathways of the cephalic phase response which were not evaluated in this study, including the activation of sensory neurons by other families of taste receptors, the activation of gastric vagal afferent fibers, or even humoral pathways. Furthermore, different results could have been obtained at different test concentrations, as amino acids can have different activation

thresholds of T1R receptors and taste nerves. However, this study presents the first evidence that pre-absorptive sensing of specific amino acids, at least partly mediated by oral taste signalling, has the potential to affect food intake in teleost fishes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.L.S., S.M.; Formal analysis: S.C., M.C.-S., C.V.; Investigation: S.C., M.C.-S., C.V.; Data curation: S.C., J.L.S., S.M.; Writing - original draft: S.C., J.L.S., S.M.; Writing - review & editing: S.C., M.C.-S., J.L.S., S.M.; Visualization: J.L.S.; Supervision: J.L.S., S.M.; Project administration: J.L.S., S.M.; Funding acquisition: J.L.S., S.M.

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