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mAChR-dependent decrease in proteasome activity in the gustatory cortex is necessary for novel taste learning

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ABSTRACT

Regulation of protein degradation via the ubiquitin proteasome system is crucial for normal learning and synaptic plasticity processes. While some studies reveal that increased proteasome degradation is necessary for different types of learning, others suggest the proteasome to be a negative regulator of plasticity. We aim to understand the molecular and cellular processes taking place in the gustatory cortex (GC), which underlie appetitive and aversive forms of taste learning. Previously, we have shown that N-methyl D-aspartic acid receptor (NMDAR)-dependent upregulation of proteasome activity 4 h after novel taste learning is necessary for the association of novel taste with malaise and formation of conditioned taste aversion (CTA). Here, we first identify a correlative increase in proteasome activity in the GC immediately after novel taste learning and study the upstream and downstream effectors of this modulated proteasome activity. Interestingly, proteasome-mediated degradation was reduced in the GC, 20 min after novel taste consumption in a muscarinic acetylcholine receptor (mAChR)-dependent and NMDAR-independent manner. This reduction in protein degradation led to an increased amount of p70 S6 kinase (p70S6k), which was abolished in the presence of mAChR antagonist scopolamine. Infusion of lactacystin, a proteasome inhibitor, to the GC precluded the amnestic effect of scopolamine. This study shows for the first time that following novel taste learning there is a cortical, mAChR-dependent reduced proteasome activity that enables the memory of taste familiarity. Moreover, inhibition of degradation in the GC attenuates novel taste learning and of p70 S6 kinase correlative increased expression. These results shed light on the complex regulation of protein synthesis and degradation machineries in the cortex following novel taste experience.

1. Introduction

Protein degradation regulation plays a complex role in learning and synaptic plasticity. For example, proteasome inhibitors impair late phase long term potentiation (l-LTP) (Cai, Frey, Sanna, & Behnisch, 2010). However, when they are given concomitantly with protein synthesis inhibitors, l-LTP is rescued (Fonseca, Vabulas, Hartl, Bonhoeffer, & Nagerl, 2006). This intricate effect of proteasome inhibitors is explained partially by their potential to increase the expression levels of both positive and negative regulators of plasticity (Dong, Bach, Haynes, & Hegde, 2014; Dong, Upadhya, Ding, Smith, & Hegde, 2008).

Proteasome regulation affects not only LTP but also long term depression (LTD). For example, metabotropic glutamate receptor (mGluR)-dependent LTD was shown to be enhanced in the presence of proteasome inhibitors, unlike N-methyl-D-aspartic acid receptor (NMDAR)-dependent LTD, that relies on protein ubiquitination, but not proteasomal degradation (Citri, Soler-Llavina, Bhattacharyya, & Malenka, 2009). Furthermore, early LTD is reinforced into protein synthesis-dependent late LTD by application of proteasome inhibitors (Li, Korte, & Sajikumar, 2015). Thus, under certain circumstances, proteasomal degradation is a negative regulator of both LTP and LTD forms of synaptic plasticity. At the structural level, proteasomes are recruited to synaptic spines (Bingol & Schuman, 2006), and can be activated by CaMKII phosphorylation of proteasome subunit Rpt 6 (Djakovic, Schwarz, Barylko, DeMartino, & Patrick, 2009; Djakovic et al., 2012).

On the behavioral level, numerous experiments have tested the effect of local proteasome inhibition in different brain regions and reported no effect or memory impairment (Figueiredo et al., 2015; Jarome, Werner, Kwapis, & Helmstetter, 2011; Lee et al., 2008; Lopez-salon et al., 2001; Massaly, Francès, & Moulédous, 2014; Merlo & Romano, 2007; Reis, Jarome, & Helmstetter, 2013; Ren et al., 2013; Rodriguez-Ortiz, Balderas, Saucedo-Alquicira, Cruz-Castaneda, & Bermudez-Rattoni, 2011; Rosenberg, Elkobi,

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Dieterich, & Rosenblum, 2016). At the same time, proteasome inhibition rescues the amnestic effect induced by protein synthesis inhibition. This was ascribed to the assumption that a different effective dosage is required to impair or rescue memory consolidation or reconsolidation (Lee et al., 2008; Sol Fustiñana, Federman, Freudenthal, & Romano, 2014).

This paradox outcome of protein degradation can be possibly reconciled by the context-specific effect of the manipulation; while downregulation of protein degradation can induce a burst of protein synthesis by increasing the amount of plasticity-related proteins, it can also increase the amount of negative regulators, thereby impeding protein synthesis (Dong et al., 2014). Timing is also critical; protein synthesis elicited by learning or by stimulation has a time window in which it may be sensitive to inhibition (Alberini, 2008; Artinian et al., 2008; Merhav & Rosenblum, 2008). In our previous study, focusing on the taste system, we found that proteasome activity is upregulated in the gustatory cortex (GC) 4 h following novel taste consumption. Using a low dose of proteasome inhibitor lactacystin, which was effective *in vivo* for approximately 2 h, we showed that this increase in proteasome activity is necessary for conditioned taste aversion (CTA) (Rosenberg et al., 2016).

Importantly, memory of CTA is protein synthesis dependent and the translation machinery is highly regulated in the GC following novel taste learning (Belelovsky, Elkobi, Kaphzan, Nairn, & Rosenblum, 2005; Belelovsky, Kaphzan, Elkobi, & Rosenblum, 2009; Elkobi, Ehrlich, Belelovsky, Barki-Harrington, & Rosenblum, 2008; Gildish et al., 2012; Heise et al., 2016; Ounallah-Saad, Sharma, Edry, & Rosenblum, 2014; Rosenblum, Meiri, & Dudai, 1993; Stern, Chinnakkaruppan, David, Sonenberg, & Rosenblum, 2013; Taha, Gildish, Gal-Ben-Ari, & Rosenblum, 2013).

Here, we first tested the hypothesis that proteasome activity is differentially modulated in time following novel taste learning. Later, we identified the upstream neurotransmitter affecting proteasome activity and tested causality between the different molecular correlates using pharmacological tools.

2. Materials and methods

2.1. Subjects

Adult male Wistar rats weighing 200–250 g (Harlan, Jerusalem, Israel) were maintained on a 12-h light/dark cycle. All procedures were performed in strict accordance with the University of Haifa regulations and the US National Institutes of Health guidelines (NIH publication number 8023).

2.2. Micro-surgery and micro-infusions

Micro-infusions into the GC were performed via chronically implanted cannula. Rats were anesthetized with Equithesin (0.3 mL/100 g) (2.12% w/v $MgSO_4$, 10% v/v ethanol, 39.1% v/v 1,2,-propranolol, 0.98% w/v sodium pentobarbital, and 4.2% w/v chloral hydrate), restrained in a stereotactic apparatus (Stoelting, USA), and implanted bilaterally with a 10 mm guide stainless steel cannula (23 gauge) aimed at the rat gustatory cortex (anteroposterior, +1.2 mm relative to bregma; lateral, ±5.5 mm; ventral, −5.5 mm; Paxinos & Watson, 2005). The cannula were positioned in place with acrylic dental cement and secured by two skull screws. A stylus was placed in the guide cannula to prevent clogging. Following the microsurgery, animals were injected i.m. with 0.2 mL amoxycilin and 0.2 mL dypiron to ease their pain, and were allowed to recuperate for one week.

For micro-infusion, the stylus was removed from the guide cannula, and a 28 gauge injection cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The injection cannula was connected via PE20 tubing to a Hamilton micro-syringe driven by a micro-infusion pump (Harvard PHD 2000). Micro-infusion was performed bilaterally in a volume of 1 μL per hemisphere delivered over 1 min. The injection cannula was left in position before withdrawal for an additional 1 min to minimize dragging of the injected fluid along the injection tract.

2.3. Pharmacology

For the behavioral set of experiments, the rats were bilaterally injected either with lactacystin (10 μM, Enzo life science, Farmingdale, NY, USA) or with vehicle (aCSF containing 124 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM Dglucose, and 2.4 mM $CaCl_2$, and brought to a concentration of 10 μM) 4 h before consumption. Rats were also injected systemically with scopolamine (0.05 mg/kg) or saline (0.9% NaCl) 60 min before novel taste consumption.

For the biochemical experiments, the rats were bilaterally injected with NMDAR blocker (2R)-amino-5-phosphonopentanoate (50 μM; APV, Sigma-Aldrich, Israel) 20 min before incidental taste learning. The rats were decapitated 20 or 120 min after incidental taste learning, and insular cortices were dissected, snap frozen and stored at −80 °C until further use 24 h later (as described in Rosenberg et al., 2016).

2.4. Behavior

2.4.1. Incidental taste learning

Two groups of rats were water deprived for 24 h, and then pretrained for 3 days to get their daily water ration once a day for 20 min from two pipettes, each containing 10 mL of water. On the fourth day, the experimental group was exposed to an unfamiliar (novel) taste (saccharin 0.1%) for 20 min, while the control group was exposed to water for the same time period. The rats were sacrificed either 20 or 120 min following consumption, and their GC was removed, snap frozen, and stored at −80 until further use (Berman, Hazvi, Rosenblum, Seger, & Dudai, 1998).

2.4.2. Attenuation of neophobia (AN)

These experiments were performed as previously described (Rosenberg et al., 2016). Rats were separated into individual housing cages and subjected to a 3-day water restriction training session, in which once a day for 20 min they were offered 20 mL of water from two pipettes, each containing 10 mL. On the fourth day (novel) the rats received water and saccharin (0.1%) as a multiple-choice test and consumption volumes were recorded. After two successive days of water restriction training, the rats were tested in a multiple-choice test again (familiar). The behavioral data are presented in terms of preference index, expressed as a percentage, [mL saccharin/(mL water + mL saccharin)] × 100, in which the quantities are those consumed during each test. AN is presented as significant increase in preference during the familiar session compared to the novel session.

2.5. Biochemical procedures

2.5.1. Measuring chymotrypsin-like protein degradation

Brain samples were homogenized in 20 mM Tris, 0.32 M sucrose, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, and 0.2% Triton X-100. Total lysate was sampled for SDS PAGE and western blotting and the rest of the sample was centrifuged for 15 min at 15,000*g*. Protein amount was quantified using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Life Technologies, Thermo Scientific, NY) and equal amounts of protein were added to a buffer containing 50 mM Tris, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.1 mM