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NBAD-hydrolase processing in brain and epidermis of *Drosophila melanogaster*.

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In recent years, research on N-β-alanyl-derivative metabolism in insects has shed new light on its physiological relevance. While NBAD (N-β-alanyldopamine), the first conjugate studied in this metabolism, was originally described as the main sclerotization precursor of insect brown cuticles (Hopkins and Kramer, 1992), new roles have been proposed in neural tissue (Pérez *et al*., 2004, 2010, 2011; Schachter *et al*., 2007). Another studied N-β-alanyl-derivative is carcinine (N-β-alanylhistamine, NBAHA), which has been suggested as of physiological importance for the visual system (Borycz *et al.*, 2002; True *et al.*, 2005; Wagner *et al.,* 2007). Both NBAHA and NBAD have been proposed as shuttle/recycling agents of histamine (HA) and dopamine (DA), between glial and neuronal cells (Borycz *et al.*, 2002; True *et al.*, 2005; Pérez *et al.*, 2010).

NBAD and NBAHA are both synthesized by the same enzyme: NBAD-synthase, also known as Ebony protein in *Drosophila melanogaster*. This enzyme shows rather wide substrate specificity, since tyramine, octopamine, norepinephrine, tyrosine, and serotonin can also be conjugated to β-alanine (Pérez *et al*., 1997, 2002, 2004, 2010; Richardt *et al*., 2003; Schachter *et al*., 2007).

The hydrolysis of these conjugates is catalyzed by NBAD-hydrolase (also known as Tan), which is encoded in *D. melanogaster* by the gene *tan* (Wright, 1987; True *et al*., 2005) and has recently been partially characterized in *C. capitata* and *D. melanogaster* (Badaracco *et al*., 2009; Aust *et al*., 2010; Pérez *et al*., 2011). As the synthase, it shows a wide substrate specificity, since it hydrolyses at least NBAD, NBAHA, and NBANE (Wright, 1987; True *et al*., 2005; Pérez *et al*., 2011). The study of NBAD-hydrolase has revealed a constitutive expression in neural tissue and epidermis throughout the *Drosophila* life cycle (True *et al*., 2005; Badaracco *et al.*, 2009; Pérez *et al*., 2011).

By expression in *E. coli*, Tan was described as a homo-dimeric protein with subunits of around 30 and 15 kDa apparent molecular weight (aMW). Apparently, these subunits arise from self-processing of a precursor polypeptide of around 45 kDa (Wagner *et al*., 2007; Aust *et al*., 2010). A Gly-Cys motif, at position 121, was crucial for this self-processing, and the Tan[1] mutant protein, with

an Arg for Pro mutation (at position 217), showed no processing in *E. coli*. We decided to study this enzyme and its expression further in the t^1 mutant.

To our knowledge, this is the first *Drosophila*-expressed study of this protein. Moreover, it is the first study suggesting different tissue-specific expression/processing of the Tan protein.

Materials and Methods

D. melanogaster were reared in commercial fly medium: Formula 4.24 Instant Drosophila Medium (Carolina Biological Supply). Both wild type Canton S (CS) and *tan*[1] (t^1) mutant strains were from the Bloomington Stock Center. Flies of 1-2 weeks were anesthetized with ice, decapitated under binocular magnifying glass, and the bodies frozen in liquid N_2. Brains were dissected (n = 100 each experiment) by separating both the eyes and lamina and stripping away as many tracheas as possible. The resulting brains and "head carcasses + eyes" were immediately frozen in N2 and stored at -80ºC. All dissections were carried out in Petri-dishes on top of ice, at 4ºC and with pre-chilled buffer. We have recently shown that homogenates from beheaded bodies show similar levels of hydrolase enzymatic activity as carcasses (which had been stripped of internal organs), whereas the activity in internal organs was negligible (Pérez *et al.*, 2011). Thus, we considered decapitated bodies as epidermis-tissue material (in contrast to the brain/neural tissue material). Protein extracts were prepared by homogenizing the tissues in 70 mM Tris/HCl pH 7.5 buffer, containing 10% Glycerol, 20 mM EDTA-Na$_2$ pH 8, 0.01 mM Pepstatin A, 1 mM PMSF and saturated with phenylthiourea. These homogenates were cleared by centrifuging ($20000 \times g$, 15 min, at 4ºC) and the supernatants used for western blots. Protein concentration was from 1 mg/ml to 10 mg/ml (Bradford method). Protein extracts were boiled in Laemmli buffer, loaded (50 μg) in a 12.5% polyacrylamide gel (Laemmli, 1970) and separated in a minigel apparatus (BioRad). Protein transfer was done by electroblotting onto PVDF membrane (Termo Scientific) at 400 mA for 1 hour 30 minutes, using Towbin transfer buffer (192 mM Glycine, 25 mM Tris/HCl pH 8, 20% methanol). Western blots were performed as described in Wittkopp *et al.* (2002). The membranes were temporarily stained in 0.02% Ponceau S (to confirm even protein loading and running), washed until all the Ponceau was removed, blocked with 3% non fat milk in phosphate buffered saline, 0.15% Tween 20 (PBST) for 1h at room temperature. Membranes were then incubated with primary antibody (1:500 rat anti-Tan, in PBST 3% non-fat milk) overnight at 4°C and washed (15, 20, and 25 minutes) in PBST. Finally, membranes were incubated with goat anti-rat horseradish peroxidase (Jackson ImmunoResearch) secondary antibodies (1:1000) in PBST containing 3% non fat milk for 2 hours at room temperature, washed in PBST (10, 20, and 30 minutes), and developed with ECL (G&E, Healthcare). The affinity-purified Tan antiserum was a generous gift by Dr. B. Hovemann (Rhur University, Bochum), the description of which is found in Wagner *et al.* (2007) and a personal communication. Rat pre-immune serum was used as a control (not shown). ImageJ was used to measure Rfs by measuring the peak value of intensity for each band and to measure the total intensity for each band. Relative intensity distribution was then calculated by dividing the intensity from each line by the total intensity of all the peaks with aMW of 51 kDa or less (aMWs are calculated to the nearest 0.5 kDa).

Results and Discussion

Wild-type bodies showed three immunoreactive bands of aMW 43 kDa, 28.5 kDa, and 14.5 kDa species. The latter seem to correspond to the previously described 30 and 15 kDa processing subunits of *E. coli*-expressed Tan protein (Aust *et al.*, 2010). The t^1 mutant carries a point mutation

that probably changes the spatial structure of the protein, which could explain its inability to hydrolyze NBAD and has been proposed to inhibit processing (Aust *et al.*, 2010; Pérez *et al.*, 2011). It shows a new, heavier, peptide of 51 kDa aMW, the 43 kDa band, and the 14.5 kDa aMW band and no trace of the 28.5 kDa subunit.   This shows the first difference observed with the data from expression in *E. coli*: Both the novel 51 kDa peptide and the 14.5 kDa band were previously not described for the Tan[1] protein mutation. This is important to point out since it shows a specialized processing of the Tan protein. We have no further information about the 51 kDa peptide, which is almost 8 kDa heavier than the expected full-lengh peptide (43.7 kDa). We hypothesize that this could be a post-translational modification (*e.g.*, Ubiquitination), possibly targeting the non-functional protein for degradation. More research is needed to clarify this hypothesis. With regards to the 14.5 kDa peptide, we have no current explanation apart from the possibility that it might be a degradation product or an experimental artifact.
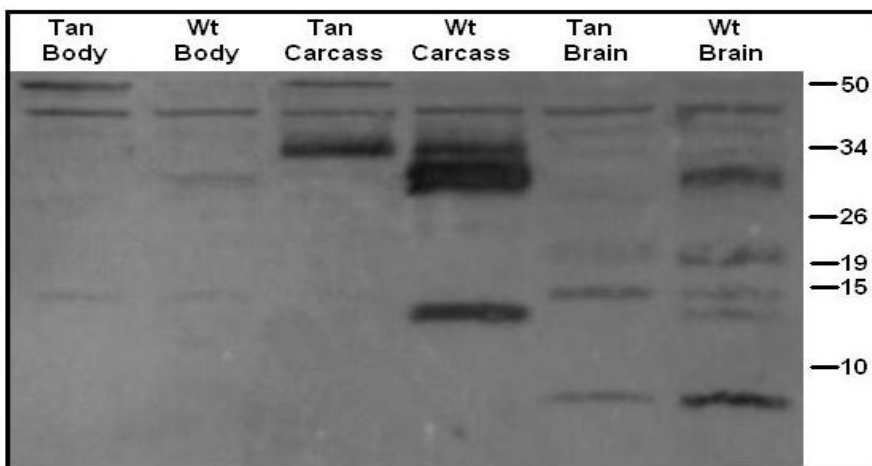

Figure 1. From left to right, the first two lanes show the homogenates from beheaded bodies, then head carcasses (lanes 3 and 4) and dissected brain ganglia (lanes 5 and 6) from mutant t^1 and wt, respectively. Numbers on the right side indicate molecular weight markers.

The analysis of the wt "head carcasses" shows a novel, more complex processing profile. In addition to the 43 and 28.5 kDa aMW bands (the latter of much higher intensity than in bodies), a new 34.5 kDa band was visualized, and the 28.5 kDa band showed a "double" appearance. This can be due to physiological processing but also to artifacts provoked by the unspecific proteases eventually activated during dissection, in spite of the protease-inhibitor cocktail present. Surprisingly, a band of 13 kDa aMW, which was clearly distinguished from the 14.5 aMW band, was recorded, suggesting further processing. This might also be interpreted as an artifact. The difference in processing between bodies and heads might eventually be sustained by observation of the head carcass t^1 profile (lane 3) since, in addition to the pre-precursor (51 kDa) and the precursor (43 kDa) proteins present in body extracts, one of the novel bands (34 kDa) present in wt heads was also detected, whereas the canonic 28.5 kDa product and the novel 13 kDa peptide were absent. Were these peptides the product of degradation by unspecific proteases, it is probable that they would be present in wt and mutants alike.

Finally, analysis of wt brains (lane 6) showed, in addition to the canonical 43, 28.5, and 14.5 KDa bands, a new band of aMW 18 kDa and a faint band of the 13 kDa species as well as a small (less than 10 kDa aMW) peptide. Again, these "new" peptides can be the result of physiological processing (or post-translational modifications) of the Tan peptide as well as unspecific degradation. The t^1 brain showed the 43 kDa and the 14.5 kDa bands as well as the unusual (albeit very faint) 18 kDa peptide and maintains the mutant characteristic of the absence of a 28 kDa peptide as well as the 13 kDa band present in the wt head. Noteworthy is the absence of the 51 kDa band observed in other tissues and the strong presence of the 14.5 kDa peptide.

In order to visualize the relative amount of peptide distributed among the different processing intermediates and post-translational modifications, we graphed the percentage of the total intensity for each peptide species. Both graphs show a shift in the distribution from heavier (*i.e.*, non-processed) proteins, in body tissue, towards lighter (processed) peptides in head and neural tissue. This, together with the almost uniform expression of the 43 kDa peptide in all tissues of both wt and mutant, suggests an important post-translational control of Tan protein processing and, thereby, activity.
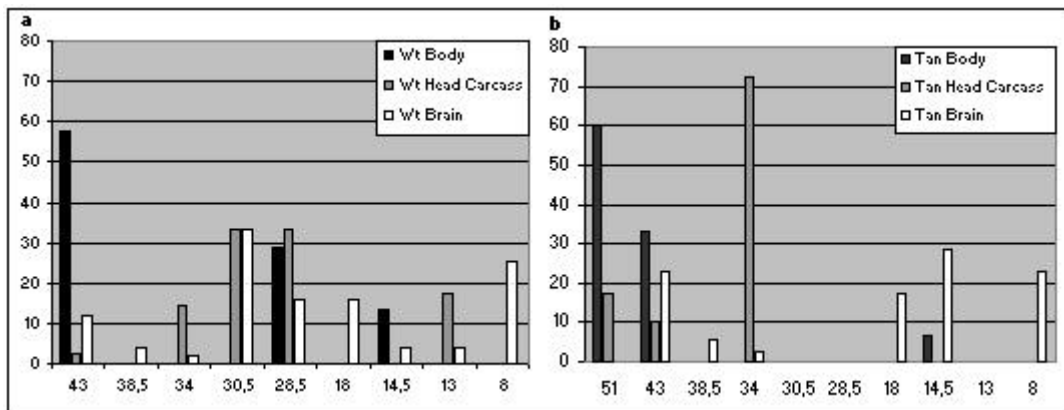

Figure 2. Relative intensity distribution of protein species for wt (a) and tan[1] (b).

The overall picture indicates that a previously overlooked processing of the Tan protein seems to occur, mainly demonstrated by the wide variety of novel peptide species present in head carcasses and brain tissue. In addition, the Tan[1] protein, which was previously suggested to be unable to produce the 28.5 (30) and 14.5 (15) kDa subunits, seems able to be at least partially processed to the 14.5 subunit. The complete absence of the other subunit might be the result of rapid degradation, since, theoretically, the production of the 14.5 kDa "half" should be accompanied by the complementary 28.5 kDa peptide. The anomalous "extra" weight of the Tan[1] protein is also a mystery. Ubiquitination as a consequence of its inactivating mutation seems plausible but does not explain the absence of this protein in brain tissue.

Further studies will be required to pinpoint exactly which of the peptides reported here are degradation artifacts, which are part of a *bona fide* physiological processing of the Tan protein, and which are post translational modifications. Apart from the proposed ubiquitination, the unusually high amount of phosphorilable residues (67, more than 17%) may provide an alternate explanation for the high variety of peptides, also adding to the hypothesis of a post-translational control of the protein's activity.

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Preliminary results of a forward genetic screen for X chromosomal dominant modifiers of *Drosophila melanogaster dfmr1*.

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Introduction

Fragile X syndrome is a neuro-developmental disease in humans. It is caused by a mutation in the gene *Fmr1,* which expands abnormally a CGG-repeat in its promoter region, thus leading to a subsequent hypermethylation and transcriptional inactivation of the gene (Sutcliffe *et al.*, 1992). *Fmr1* encodes the fragile X mental retardation protein (FMRP).

The most important clinical symptoms of the disease include mental retardation, sleep disturbances, autism, and impaired motor coordination. They reflect the key role of FMRP in the brain, where it is predominantly expressed (Devys *et al.*, 1993).

The lack of this protein is accompanied by defects in synaptic maturation and morphology, synaptic connectivity disturbances, and dysfunction throughout the nervous system (reviewed in Tessier and Broadie, 2009; Pfeiffer and Huber, 2009; Gatto and Broadie, 2011).

Research on animal models confirmed the main characteristics of fragile X syndrome – neuronal defects, synaptic abnormalities in synaptic development and function, circadian rhythms disturbances, impaired long term plasticity, abnormal mGluR signaling, and learning and memory deficits (reviewed in Bassell and Warren, 2008; Gatto and Broadie, 2009; Mercaldo *et al.*, 2009; Pfeiffer and Huber, 2009).

Drosophila studies have shown that dFMRP functions in axon growth, path finding, and activity dependent pruning and refinement of synaptic elaborations(Dockendorff *et al.*, 2002; Morales *et al.*, 2002; Michel *et al.*, 2004; Pan *et al.*, 2004; Tessier and Broadie, 2008).

FMRP is a selective mRNA-binding protein (with two KH domains and an the RGG box), which is a negative regulator of protein synthesis of its mRNA targets at synapses (Laggerbauer *et al.*, 2001; Li *et al.*, 2001; Sung *et al.*, 2003; Zalfa *et al.*, 2003; Qin *et al.*, 2005; Antar *et al.*, 2006; Price *et al.*, 2006). Accumulating evidence shows a role of FMRP in mRNA transport (Dictenberg *et al.*, 2008; Estes *et al.*, 2008) and in the regulation of mRNA stability (Zalfa *et al.*,2007; Gantois *et al.*, 2006; Miyashiro *et al.*, 2003; D'Hulst *et al.*, 2006; De Rubeis and Bagni, 2010).

Numerous candidate m-RNA targets, interacting with FMRP, were obtained by different approaches, though only a few of them have been validated *in vivo* (see the reviews: Zalfa and Bagni, 2004; Bassell and Warren, 2008; Callan and Zarnescu, 2011).

In order to exercise its multiple functions and to participate in different processes from the nucleus to the synapses, FMRP is thought to shuttle between nucleus and cytoplasm and to form different protein complexes. Models were created, suggesting that FMRP might take part in DNA