Expression of human amyloid precursor protein in *Drosophila melanogaster* nerve cells causes a decrease in presynaptic gene mRNA levels

D.I. Rodin[1], A.L. Schwarzman[1,2] and S.V. Sarantseva[1]

[1]Molecular and Radiation Biophysics Division,
National Research Centre "Kurchatov Institute" B.P. Konstantinov Petersburg
Nuclear Physics Institute, Gatchina, Russia
[2]Molecular Genetics Department,
Institute of Experimental Medicine of The North-West Branch of the Russian
Academy of Medical Sciences, Saint-Petersburg, Russia

Corresponding author: D.I. Rodin
E-mail: rodin.dmitry@icloud.com

ABSTRACT. Amyloid precursor protein (APP) is a key player in Alzheimer's disease. The proteolytic cleavage of APP results in various short peptide fragments including the toxic amyloid-beta peptide, which is a main component of senile plaques. However, the functions of APP and its processed fragments are not yet well understood. Here, using real-time polymerase chain reaction, we demonstrate that exogenous expression of APP, its mutant form APP-Swedish, or two truncated forms in *Drosophila melanogaster* causes a significant ($P \leq 0.05$) drop in the mRNA levels of the presynaptic proteins synaptotagmin-1 and neuronal synaptobrevin. The results obtained from this study suggest a potential role of APP or its fragments in the regulation of synaptic gene transcription.

Key words: Alzheimer's disease; *Drosophila*; Synaptic protein; Amyloid precursor protein

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is characterized by loss of cognitive and functional abilities. One of the main pathomorphological markers of AD is extracellular senile plaques that generally consist of the amyloid-beta peptide (Aβ). To date there are several hypotheses to explain the development of AD but the most accepted is the amyloid cascade hypothesis (Haass and Selkoe, 2007; Walsh and Selkoe, 2007; Zhang et al., 2014).

According to this hypothesis, production of toxic Aβ oligomers causes neurodegeneration and the development of AD. The primary evidence in support of this hypothesis is that familial forms of AD are caused by mutations in several genes that encode proteins involved in the generation of Aβ, including the *APP* gene, and the presenilin 1 (*PS1)* and 2 (*PS2*) genes (Hardy and Selkoe, 2002; Haass and Selkoe, 2007). Aβ oligomers are considered to be the main agents in the development of synapse dysfunction in the case of familial forms of AD (Walsh et al., 2002; Walsh and Selkoe, 2004). In contrast, an alternative hypothesis suggests that disruption of the normal cellular functions of APP is the main cause of synapse dysfunction in AD (Sarantseva et al., 2009; Nizzari et al., 2012).

It has been demonstrated that early memory and cognitive function impairment during the progression of AD strongly correlate with synapse dysfunction (Terry et al., 1991), accompanied by a considerable reduction of the levels of certain presynaptic proteins such as synaptophysin and synaptotagmin in the cortex and hippocampus of patients. Synapse dysfunction occurs well before Aβ accumulation (Masliah and Mallory, 2001), but this effect has not yet been successfully explained.

In our previous study, we showed that hyper-expression of human *APP* in *Drosophila melanogaster* nerve cells led to neurodegeneration in fly brain, as well as loss of memory and learning ability, which are considered to be the processes observed in AD pathogenesis. Consequently, we assumed that hyper-expression of *APP* in the nerve cells of transgenic flies led to neuropathological processes specific for AD. In the transgenic flies, we observed synapse disruption characterized by a reduced level of the proteins synaptotagmin 1 (syt1) and neuronal synaptobrevin (n-syb) in the mushroom body brain area of *Drosophila* (Sarantseva et al., 2009). However, the mechanism that was responsible for the synaptic protein level drop was not clear.

In this study, we show that hyper-expression of the *APP* gene and its fragments affect transcription of the presynaptic genes *syt1* and *n-syb* and that the decrease in presynaptic proteins is preceded by a drop in the corresponding gene expression levels.

MATERIAL AND METHODS

Drosophila strains used for experiments

Drosophila strains used in this study included *UAS-APP* (henceforth referred to as *APP*) carrying the human *APP* gene, *UAS-APP-Swedish* (*APP-Sw*) carrying the human *APP* gene with a mutation that leads to the familial form of AD, *UAS-APPΔNT* (*APPΔNT*) and *UAS-APPΔCT* (*APPΔCT*), carrying the truncated forms of APP695, and *UAS-BACE* (BACE), carrying the human beta-secretase gene. All *APP* strains were obtained from the *Drosophila* Bloomington Stock Center (Indiana University, Bloomington, IN, USA); *UAS-BACE* was kindly provided by R. Reifegerste. *APP*, its forms, and *BACE* were expressed in *Drosophila*

neurons using the tissue-specific transcription driver *elav-GAL4c155* (Indiana University, Bloomington, IN, USA).

Flies were kept on standard yeast medium at a temperature of 25°C and a photoperiod of 12 h.

RNA extraction and reverse transcription

For each experiment, 40 flies were frozen in liquid nitrogen and decapitated. Heads were homogenized and RNA was extracted using a ZR Tissue & Insect RNA MicroPrep™ kit (Zymo Research, Irvine, CA, USA). During extraction, RNA samples were treated with DNase to reduce the amount of genomic DNA. RNA extraction was followed by reverse transcription with Oligo(dT) 18 Primers and RevertAid M-MuLV Reverse Transcriptase from the RevertAid™ First-Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA).

Real-time polymerase chain reaction (PCR)

The mRNA level for the *syt1* and *n-syb* genes were estimated by TaqMan real-time PCR with ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Waltham, MA, USA). The *RP-49* and *GAPDH2* genes were used as the reference genes. Primer and probe sequences (Bigle, Russia) used for PCR were as follows: *syt1*: fwd 5'-CCT GGT CAG CGT TGA AGG A-3', rev 5'-GCA GCG AGA AGC AGA TAT CT-3', probe 5'-FAM-AGG GCG GAC AGG AAA-RTQ1-3'; *n-syb*: fwd 5'-GGC GGC GTG TAA GCA ATC-3', n-syb rev 5'-CCC GCT GAA GGA GCA CAC TA-3', probe 5'-FAM-CGC TGC CAG GAC GAA AGT TTC TCG A-RTQ1-3'; *PSN*: fwd 5'-TGG ACT GCC TGG GCT GTA-3', rev 5'-TCC TCT TGG CGA AAG GAC A-3', probe 5'-FAM-CTG CCA TTT CTA TTT GGG ATC TT-RTQ1-3'; *RP49*: fwd 5'-AGC ACT TCA TCC GCC ACC-3', rev 5'-CGA CGC ACT CTG TTG TCG-3', probe 5'-R6G-CTA AGC TGT CGC ACA AAT GGC G-BHQ1-3'; and *GAPDH2*: fwd 5'-TGG CCA AGG TGA TCA ACG ACA A-3', rev 5'-ACA ACT TGC CGG AAG GTC CAT-3', probe 5'-R6G-TGA TGA CCA CCG TTC ATG CCA CCA CCG CT-BHQ1-3'. Denaturation at 95°C for 10 min was followed by 50 amplification cycles (melting at 95°C for 30 s; annealing at 60°C for 30 s; synthesis at 72°C for 30 s). The mRNA levels of *syt1* and *n-syb* were normalized to mRNA levels of *GAPDH2* or *RP-49*. The relative amount of *syt1* and *n-syb* mRNA was estimated using relative standard curve method according to protocol provided by Applied Biosystems. Standard curves were built for genes of interest as well as for the reference genes. Every sample was tested in triplicate.

Statistical analysis

Statistical analysis was performed using the KyPlot software (KyensLab Inc., Tokyo, Japan). One-way ANOVA was followed by planned multiple comparisons between relevant groups with the Tukey-Kramer test.

RESULTS

We studied the effect of expression of *APP*, its truncated forms, and *APP-Sw* in nerve cells of *Drosophila* on the mRNA levels of the presynaptic proteins syt1 and n-syb.

Aβ production is a result of APP cleavage by β- and γ-secretases. *Drosophila* has no or minimal activity of β-secretase and the *Drosophila* homologue of the human *APP* gene (*APPL*) does not have the Aβ sequence (Luo et al., 1990; Fossgreen et al., 1998; Greeve et al., 2004). This allows us to analyze the effects of *APP* expression in *Drosophila* independently from Aβ effects.

Levels of *syt1* and *n-syb* mRNA were analyzed in 2- and 30-day-old flies of the following genotypes: *elav/+* (control), *elav*; *APP*, *elav*; *APP-Sw*, *elav*; *APPΔCT*, *elav*; *APPΔNT*, *elav*; *APP/BACE*, and *elav*; *BACE*; *APP-Sw*. The results were normalized relatively to the control genotype, *elav*;+.

The mRNA levels of *syt1* and *n-syb*, normalized to the reference genes *RP-49* and *GAPDH2* in flies aged 2 and 30 days, are presented in Figures 1 and 2, respectively.

According to the data, the relative mRNA levels of *syt1* and *n-syb* were decreased even during the first days of life in all transgenic flies analyzed. The expression level of *syt1* in 30-day-old flies was significantly reduced ($P \leq 0.05$) compared to control flies of the same age, with the exception of the single genotype, *APPΔCT*, where statistical significance was not obtained, although a similar tendency toward reduction of expression level was observed. The expression level of *n-syb* in 30-day-old flies was reduced in flies expressing *APP* and *APP-Sw.*

Furthermore, in order to demonstrate an absence of an *APP* hyper-expression effect on the overall profile of organismal gene expression, we analyzed the expression level of *Drosophila psn*. The mRNA levels of *psn* in flies aged 2 and 30 days, normalized to the reference genes *RP-49* and *GAPDH2*, are presented in Figure 3. No reduction of *psn* mRNA level was observed in any of the genotypes analyzed. In contrast, we observed increased transcriptional activity of *psn* in 30-day-old flies.
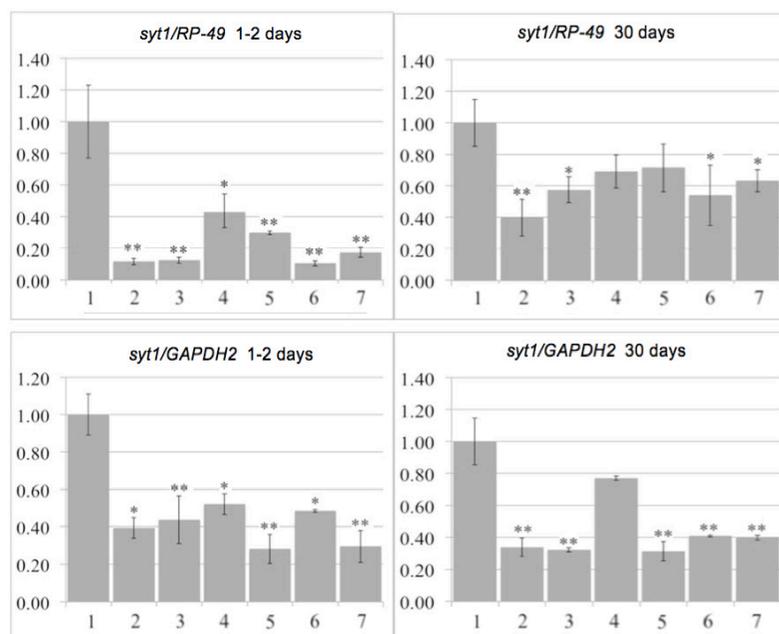
Figure 1. Relative mRNA expression levels of *syt1* in 2- or 30-day-old flies. Genotypes: 1) elav (control); 2) elav; APP; 3) elav; APP-Sw; 4) elav; APPΔCT; 5) elav; APPΔNT; 6) elav; APP/BACE; 7) elav; BACE; APP-Sw. mRNA levels of *syt1* were normalized to the reference genes *RP-49* (top) and *GAPDH2* (bottom). *P ≤ 0.05, **P ≤ 0.001.
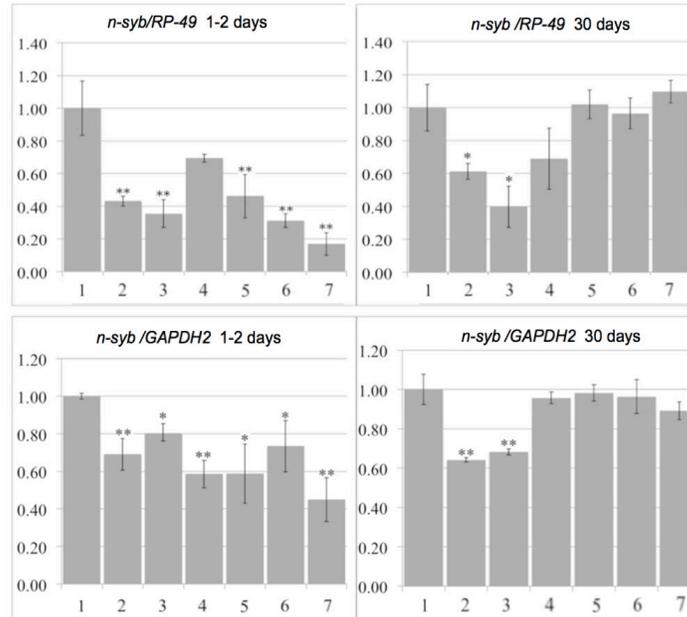
Figure 2. Relative mRNA expression levels of *n-syb* in 2- or 30-day-old flies. Genotypes: 1) elav (control); 2) elav; APP; 3) elav; APP-Sw; 4) elav; APPΔCT; 5) elav; APPΔNT; 6) elav; APP/BACE; 7) elav; BACE; APP-Sw. mRNA levels of *n-syb* were normalized to the reference genes *RP-49* (top) and *GAPDH2* (bottom). *P ≤ 0.05, **P ≤ 0.001.
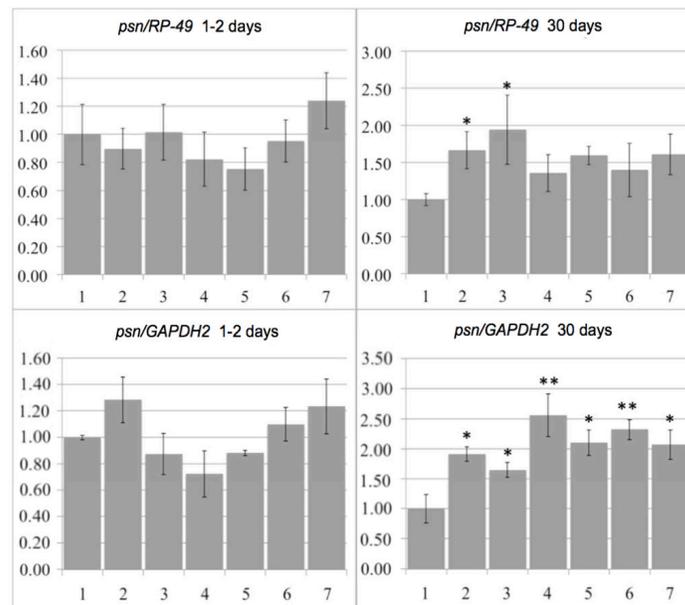
Figure 3. Relative mRNA expression level of *psn* in 2- or 30-day-old flies. Genotypes: 1) elav (control); 2) elav; APP; 3) elav; APP-Sw; 4) elav; APPΔCT; 5) elav; APPΔNT; 6) elav; APP/BACE; 7) elav; BACE; APP-Sw. mRNA levels of *psn* were normalized to the reference genes *RP-49* (top) and *GAPDH2* (bottom). *P ≤ 0.05, **P ≤ 0.001.

DISCUSSION

Many studies show that early memory and cognitive function impairment in patients with Alzheimer's disease are followed by a considerable reduction of presynaptic protein levels in the brain (Masliah and Terry, 1993; Davidsson and Blennow, 1998). The level of synaptotagmin was considerably reduced in the areas responsible for memory formation and consolidation: hippocampus and entorhinal area of cortex, while at the same time the decrease in synaptobrevin level was independent from brain area (Sze et al., 2000). Similar results were observed in transgenic mice with hyper-expression of human *APP* (Chapman et al., 1999; Mucke et al., 2000). For example, a study of synaptic density in the brain of mice with hyper-expression of *APP-Sw* showed a significant reduction of the level of syt protein in the cortex and hippocampus (Wang et al, 2012). Unfortunately, these effects have not yet been successfully and clearly interpreted. Transcript analysis carried out on the brains of patients with sporadic AD (average age of 82.7 years) using Affymetrix HuFL chips showed a reduced level of mRNA transcripts in most synaptic protein groups (Yao et al., 2003). On the other hand, experiments on transgenic mice (22 months old) showed a wide spectrum of synaptic protein levels (Yao et al., 2003). In addition, significant reductions of protein expression levels of VAMP1 and VAMP2 (coding 2 isoforms of synaptobrevin) and of syt (Mufson et al., 2002) were registered in the brains of patients with AD (Bossers et al., 2009). It should be noted that in the experiments described above there were no data for the early stages of AD, which could potentially explain the inability of these studies to distinguish a primary effect of impaired synaptogenesis from other effects of the neuropathological process.

D. melanogaster, a genetically well studied model organism, has been widely used as an experimental model for studying mechanisms underlying neurodegenerative diseases including AD (Finelli et al., 2004; Greeve et al., 2004; Bilen and Bonini, 2005). It has been shown that the genes responsible for formation of the γ-secretase complex are highly conserved between *Drosophila* and mammals; accordingly, *Drosophila* has an active γ-secretase complex (Fossgreen et al., 1998). However, the *Drosophila* homolog of human β-secretase (dBACE) does not cleave APP at the β-site, and therefore dBACE cleavage of *APP* does not lead to Aβ formation in flies (Greeve et al., 2004). In addition, whereas *Drosophila* has a homolog of human APP (APPL), the Aβ sequence is not conserved in APPL (Rosen et al., 1989). However, it has been shown that expression of human *APP* in combination with the human β-secretase gene in *Drosophila* leads to Aβ deposition and associated pathologies (Greeve et al., 2004).

Modeling AD in *Drosophila* allows estimation of the very early changes in synaptic protein levels and of alterations in cognitive function. In our previous studies, we demonstrated that hyper-expression of *APP* and *APP-Sw* together with *BACE* co-expression caused a significant decrease in syt1 and n-syb protein levels in the antennal lobes and mushroom body, which are *Drosophila* brain structures responsible for memory formation. The decrease in protein levels correlated with disruption of cognitive function (odor recognition) (Sarantseva et al., 2009).

In our current research, we studied the effects of *APP* expression on the mRNA levels of synaptic proteins in transgenic *Drosophila*, which allowed for the discrimination of the effects of exogenous APP and Aβ. We demonstrated that *APP* hyper-expression changed the mRNA levels of *syt1* and *n-syb*. The data obtained in our experiments showed significant reduction of *syt1* mRNA levels in 2-day-old flies in all genotypes tested, consequent to expression of *APP* and its variant forms as well as with co-expression of *APP* and *BACE*, which leads to Aβ production. Similar results were observed in 30-day-old flies for *syt1*, with the exception of

flies that expressed the shortened forms of *APP*: *APPΔCT* and *APPΔNT*. However, in 30-day-old flies the mRNA level of *n-syb* was reduced only in flies with full-length *APP* expression (*APP* and *APP-Sw*). As previously described, the use of transgenic *Drosophila* allowed us to distinguish the effects of *APP* expression from those of Aβ production. The results obtained in our experiments showed that the reduced mRNA levels of *syt1* and *n-syb* were caused by *APP* expression independently from Aβ secretion. Although our study was carried out on transgenic flies, other studies have demonstrated that *APP* gene duplication caused AD development in five families with autosomal dominant early-onset Alzheimer disease (Rovelet-Lecrux et al., 2006).

We assume that not only Aβ but also other fragments derived after APP processing might play an important role in the change of presynaptic gene expression levels. For example, it has been shown that the C-terminal fragment of APP-AICD, a 6-kDa protein cleaved from APP by γ-secretase, can penetrate the nucleus and bind with different intracellular adoptive proteins, influencing many cellular processes including gene expression, synaptic plasticity, and memory formation (Cao and Südhof, 2001; Gao and Pimplikar, 2001; Kim et al., 2003; Muller et al., 2007). On the other hand, APP itself is also able to participate in transcriptional regulation. Neuronal *APP* hyper-expression has been shown to reduce the mRNA levels of proteins involved in cholesterol metabolism: SREBP, HMGCR, and cholesterol 24-hydroxy-lase) (Pierrot et al., 2013).

In conclusion, transcriptional disturbance of the genes encoding synaptic proteins might underlie the alterations of synaptic plasticity in patients with AD and can be recognized as one of the early events of the disease. *APP* expression changes or *APP* mutations might lead to reduction of the mRNA levels of synaptic proteins and thereby cause synaptic pathology in patients with AD.

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