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Research Paper

sAPPβ and sAPPα increase structural complexity and E/I input ratio in primary hippocampal neurons and alter Ca^{2+} homeostasis and CREB1-signaling

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ABSTRACT

One major pathophysiological hallmark of Alzheimer's disease (AD) is senile plaques composed of amyloid β (Aβ). In the amyloidogenic pathway, cleavage of the amyloid precursor protein (APP) is shifted towards Aβ production and soluble APPβ (sAPPβ) levels. Aβ is known to impair synaptic function; however, much less is known about the physiological functions of sAPPβ. The neurotrophic properties of sAPPα, derived from the non-amyloidogenic pathway of APP cleavage, are well-established, whereas only a few, conflicting studies on sAPPβ exist. The intracellular pathways of sAPPβ are largely unknown. Since sAPPβ is generated alongside Aβ by β-secretase (BACE1) cleavage, we tested the hypothesis that sAPPβ effects differ from sAPPα effects as a neurotrophic factor. We therefore performed a head-to-head comparison of both mammalian recombinant peptides in developing primary hippocampal neurons (PHN). We found that sAPPα significantly increases axon length ($p = 0.0002$) and that both sAPPα and sAPPβ increase neurite number ($p < 0.0001$) of PHN at 7 days in culture (DIV7) but not at DIV4. Moreover, both sAPPα- and sAPPβ-treated neurons showed a higher neuritic complexity in Sholl analysis. The number of glutamatergic synapses ($p < 0.0001$), as well as layer thickness of postsynaptic densities (PSDs), were significantly increased, and GABAergic synapses decreased upon sAPP overexpression in PHN. Furthermore, we showed that sAPPα enhances ERK and CREB1 phosphorylation upon glutamate stimulation at DIV7, but not DIV4 or DIV14. These neurotrophic effects are further associated with increased glutamate sensitivity and CREB1-signaling. Finally, we found that sAPPα levels are significantly reduced in brain homogenates of AD patients compared to control subjects. Taken together, our data indicate critical stage-dependent roles of sAPPs in the developing glutamatergic system *in vitro*, which might help to understand deleterious consequences of altered APP shedding in AD patients, beyond Aβ pathophysiology.

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; ADAM, A Disintegrin And Metalloproteinase (α-secretase); APP, amyloid precursor protein; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BACE, β-site of APP cleavage enzyme (β-secretase); BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CREB, cAMP-response element binding protein; CSF, cerebrospinal fluid; DIV, days *in vitro*; EGFP, enhanced green fluorescent protein; GABA, γ-aminobutyric acid; HRP, horse-radish peroxidase; LOAD, late-onset Alzheimer's disease; LSM, laser-scanning-microscope; LTP, long-term potentiation; NMDA-R, *N*-methyl-D-aspartate receptor; PCR, polymerase chain reaction; PHN, primary hippocampal neuron; PSD, postsynaptic density; sAPP, soluble amyloid precursor protein; TEM, transmission electron microscopy

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1. Introduction

Besides being a central molecule in Alzheimer's disease (AD) pathophysiology, amyloid precursor protein (APP) and its cleavage products are known to be major players in establishing and maintaining neuronal architecture (Nicolas and Hassan, 2014). Depending on whether α- or β-secretase (BACE1) cleaves first, subsequent APP cleavage by γ-secretase fosters or precludes the generation of the neurotoxic amyloid β (Aβ) fragment (Prox et al., 2012). Alongside failure of Aβ clearance mechanisms and missense mutations in presenilin 1 or 2 genes, a shift in proteolytic APP processing towards BACE1 cleavage in AD patients that leads to gradually rising Aβ42 levels in the brain and therefore causing neuronal death is a well described hypothesis (Selkoe and Hardy, 2016). Besides the increased Aβ42 and soluble APPβ (sAPPβ) production, less sAPPα is also generated.

In contrast to the deleterious effects of Aβ on neurons, the soluble APPα cleavage product (sAPPα) is known to have neurotrophic or synaptic plasticity enhancing characteristics, as shown by increase of long-term potentiation (LTP) (Taylor et al., 2008). sAPPα, but also sAPPβ levels seem to be decreased in cerebrospinal fluid (CSF) of AD patients (Colciaghi et al., 2004; Hock et al., 1998; Lannfelt et al., 1995). BACE1 inhibition, which is a potential disease-modifying therapy for AD, leads to decreased sAPPβ levels in the CSF of treated subjects (Kennedy et al., 2016). Interestingly, a missense mutation within the gene encoding for the major α-secretase, ADAM10, resulting in attenuated activity has been recently associated with late-onset AD (LOAD) (Suh et al., 2013). Both findings can therefore lead to an imbalance of soluble APP cleavage products in the central nervous system (CNS). Until now, it has not been known how APP influences neural development and adult brain function or whether loss of these functions can account for AD pathophysiology. Hence, deciphering the physiological functions of both sAPP forms is of crucial importance.

In neuronal cell culture, it has been shown that sAPPα interacts with the p75 neurotrophin receptor to stimulate neurite outgrowth (Hasebe et al., 2013). Furthermore, sAPPα and sAPPβ decrease cell adhesion and thereby decrease dendrite outgrowth and increase axon outgrowth (Chasseigneaux et al., 2011). Neuroprotective effects are also described for sAPPα as it could be shown that it protects hippocampal neurons against Aβ induced oxidative injury (Goodman and Mattson, 1994) and proteasomal stress (Copanaki et al., 2010). Moreover, sAPPα has been shown to extenuate established synaptic and cognitive deficits in the APP/PS1ΔE9 AD mouse model (Fol et al., 2015). Another study generated a sAPPα-knock-in mouse to show that sAPPα is sufficient to rescue the abnormalities of APP-deficient mice, including reductions in brain weight and the impairment in spatial learning and LTP (Ring et al., 2007). Conversely, there is one study published demonstrating that cleavage of sAPPβ under trophic-factor deprivation generates a cleavage product that is able to induce death receptor 6 signaling and thereby lead to cellular self-destruction (Nikolaev et al., 2009).

Generally, effects of sAPPα on neurons are well established, whereas much less is known about sAPPβ function (Chasseigneaux and Allinquant, 2012). In addition, the intracellular pathways mediating the sAPP functions are also largely unknown. Since sAPPβ derives from amyloidogenic APP cleavage, we hypothesize that sAPPβ opposes neurotrophic sAPPα effects. Therefore, to further evaluate the roles of both sAPPs in neuronal development, we performed in this study a head-to-head comparison in developing primary hippocampal neurons at different time stages.

2. Materials and methods

2.1. Synthesis of recombinant sAPP peptides

sAPPα and sAPPβ gene fragments were amplified by PCR with human APP695 cDNA as template. sAPPs were then N-terminally tagged with the APP secretory sequence, 8× histidine tag, and EGFP. Constructs were subcloned in pcDNA™5/FRT (Invitrogen, Carlsbad, CA, USA) vector by *Nhe*I and *Apa*I restriction sites. The same construct (empty EGFP vector), without sAPP sequence (APP secretory sequence, 8× histidine tag, and EGFP), was used as a control peptide. For purification of sAPP and control proteins, Flp-In™HEK293 cells were stably transfected (Flp-In™ system, Invitrogen, Carlsbad, CA, USA) with sAPPα, sAPPβ and the EGFP construct. Ni-NTA charged agarose beads (Qiagen Hilden, Germany, #30210) were added to the supernatant of the cultivated cells containing the his-tagged sAPP/EGFP fusion proteins. After overnight binding, beads were washed three times with low imidazol buffer (40 mM imidazol) and sAPP/EGFP fusion proteins were subsequently eluted in high imidazol buffer (150 mM imidazol). For buffer exchange and protein concentration eluates were pooled and concentrated *via* 100 kDa cut-off columns (Millipore Darmstadt, Germany, #UFC810024). Proteins were recovered in PBS and analyzed qualitatively and quantitatively *via* SDS-PAGE, Western blot, BCA assay, and SimplyBlue™ SafeStain (Invitrogen, Carlsbad, CA, USA) (Fig. S1).

2.2. Lentiviral plasmid design and vector production

To generate infectious lentiviral particles, sequences of sAPPα and sAPPβ were cloned into pUltra Hot vector. sAPPα and sAPPβ gene fragments were amplified by PCR with the above-mentioned sAPPα/β pcDNA™5/FRT as template and inserted into pUltra Hot vector by *Age*I and *Nhe*I restriction sites. The expressed proteins were N-terminal fusion proteins with mCherry. pUltra Hot is a lentiviral vector backbone for bi-cistronic expression of the gene of interest and the fluorescent reporter mCherry under the control of a human ubiquitin promoter. pUltra Hot was a gift from Malcolm Moore (Addgene plasmid # 24130) and served as a control plasmid to induce viral stress on neurons. psPAX2 is a packaging plasmid encoding HIV-1 gag/pol sequences under the control of a SV40 promoter. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260). pMD2.G is an envelope-expressing plasmid encoding for VSV-G glycoprotein under the control of a CMV promoter. pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259). For the virus production, LentiX 293 T cells (Takara Clontech, Mountain View, CA, USA) were co-transfected (calcium phosphate transfection method) with pUltra Hot, psPAX2 and pMD2.G. Six hours after transfection, the medium was changed to remove transfection reagent in the conditioned medium to which the virus is secreted. 48 h after transfection conditioned medium was collected and filtered using a 0.2 µm sterile filter (Sarstedt, Nuembrecht, Germany). Conditioned medium was transferred to 38.5 ml Beckman Ultra-Clear™ tubes containing 3 ml 20% sucrose and spinned for 2.5 h at 4 °C and 24,000 rpm in a Beckman SW32Ti swinging bucket rotor. Supernatant was discarded and virus was resuspended in DPBS, aliquoted and stored at −20 °C until use.

2.3. Preparation of primary hippocampal neurons

Primary hippocampal neurons were prepared from brains of C57BL/6 embryonic mice (E18), as described previously (Kaech and Banker, 2006). Briefly, hippocampi were dissected from embryonic brains, the meninges were removed and the cells were dissociated by trypsinization (0.25%) for 20 min at 37 °C. The dissociated cells were resuspended in serum-free neurobasal medium (Gibco®, Invitrogen, Germany) supplemented with 10% B27 (Gibco®, Invitrogen, Germany) and 0.5 mM L-glutamine and seeded into poly-L-lysine-coated culture dishes at a density of 2.2×10^4 cells per cm^2 for immunocytochemistry (grown on coverslips) and 6.6×10^4 cells per cm^2 for cell lysis. After 45 min, medium was replaced completely by the same medium, to reduce astroglial growth. Cells were maintained at 37 °C in the presence of 5% CO_2 and 10% O_2 in a humidified incubator.