APP depletion alters selective pre- and post-synaptic proteins

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ABSTRACT

The normal role of Alzheimer's disease (AD)-linked amyloid precursor protein (APP) in the brain remains incompletely understood. Previous studies have reported that lack of APP has detrimental effects on spines and electrophysiological parameters. APP has been described to be important in synaptic pruning during development. The effect of APP knockout on mature synapses is complicated by this role in development. We previously reported on differential changes in synaptic proteins and receptors in APP mutant AD transgenic compared to wild-type neurons, which revealed selective decreases in levels of pre- and post-synaptic proteins, including of surface glutamate receptors. In the present study, we undertook a similar analysis of synaptic composition but now in APP knockout compared to wild-type mouse neurons. Here we demonstrate alterations in levels of selective pre- and post-synaptic proteins and receptors in APP knockout compared to wild-type mouse primary neurons in culture and brains of mice in youth and adulthood. Remarkably, we demonstrate selective increases in levels of synaptic proteins, such as GluA1, in neurons with APP knockout and with RNAi knockdown, which tended to be opposite to the reductions seen in AD transgenic APP mutant compared to wild-type neurons. These data reinforce that APP is important for the normal composition of synapses.

1. Introduction

Despite nearly three decades since the formulation of the amyloid cascade hypothesis of Alzheimer's disease (AD) the normal role of the β-amyloid precursor protein (APP) and its various metabolites remain poorly understood. AD is increasingly viewed as a disease of synapses. Loss of the pre-synaptic protein synaptophysin has long been known to be a better brain correlate of cognitive decline in AD (Masliah et al., 1989; Terry et al., 1991) than the hallmark neuropathological lesions that characterize AD, amyloid plaques and neurofibrillary tangles, composed of β-amyloid (Aβ) and tau, respectively.

To study the role of APP, knockout mice were generated (Zheng et al., 1995), which were found to be smaller than their wild-type littermates, and it was subsequently reported that they had memory deficits (Seabrook et al., 1999; Senechal et al., 2008). Primary neurons from APP knockout (APP KO) mice were reported to have reduced dendritic arbors (Perez et al., 1997; Tyan et al., 2012) while some studies noted increased outgrowth and branching of axons (Young-Pearse et al., 2008; Billnitzer et al., 2013). Moreover, spine density was

described as decreased in APP KO compared to wild-type mouse brains (Lee et al., 2010; Tyan et al., 2012), and in vitro knockdown of APP was shown to reduce spine density in rat hippocampal neurons in culture (Lee et al., 2010). Seemingly contrary to these findings, it was reported that cultured hippocampal knockout neurons showed increased synaptophysin puncta and longer dendrites compared to their wild-type counterparts (Priller et al., 2006); a subsequent study by the same group further reported increased spine density in cortical layers 3 and 5 of 4–6 months old APP KO mouse brains (Bittner et al., 2009), a discrepancy reviewed by Jung & Herms (Jung and Herms, 2012).

We previously reported on differential alterations in synaptic composition in cultured neurons from Tg2576 AD transgenic compared to wild-type mice (Almeida et al., 2005), where decreased levels particularly in GluA1 and PSD-95 were noted in the AD transgenic neurons. Using a similar approach, we set out to investigate synaptic proteins in cultured neurons and brains from APP KO mice. Since APP has been reported to have important roles in neural development (Young-Pearse et al., 2007; Nicolas and Hassan, 2014), we further silenced APP in wild-type mouse neurons in culture following neurite outgrowth and

synapse formation. Here we provide evidence for alterations in levels of selective synaptic proteins, particularly in the AMPA receptor subunit GluA1, with lack of APP, that tended to show increased levels compared to the decreases in such synaptic proteins in AD transgenic neurons. Taken together our results underscore important roles for APP in normal synaptic composition.

2. Methods

2.1. Cloning

Viral vectors with short hairpin RNA (shRNA) targeting mouse APP (Young-Pearse et al., 2007) were created by restriction digestion and ligation into a modified pENTR plasmid(Campeau et al., 2009). Gateway recombination was used to transfer the shRNA and scrambled control RNA into a pLenti (Campeau et al., 2009) acceptor plasmid. Lentiviral vectors were prepared by a viral vector core facility in Lund University and titers were determined by immunofluorescence. Transduction of WT neurons was at 10 DIV and analysis at 14 DIV.

2.2. Antibodies

The following antibodies were employed: drebrin (Enzo, New York, USA, AB_1533466; Abcam, UK, AB_2230303), GluA1 (Millipore, Sweden, AB_2113602), NR1 (Upstate, New York, USA, AB_2112158), post-synaptic density-95 (PSD-95) (Millipore, Sweden AB 2092365), synaptophysin (Millipore, Sweden, AB_95187), 369 (C-terminal region of APP (Buxbaum et al., 1990)), Alpha Tubulin (Sigma-Aldrich. Sweden, AB_477593) and beta-actin (Sigma-Aldrich, Sweden, AB_476743). We applied secondary antibodies conjugated to Alexa-488, −546 and −647 (Thermo Fisher Scientific, Sweden) or HRP-conjugated (Biotechna, UK).

2.3. Cell culture

Primary neuronal cultures, as well as harvesting of brain tissue, were performed in accordance with the ethical guidelines and approved by the Ethical committee for the use of laboratory animals at Lund (M46-16) and Weill Cornell Medical College. Primary cortico-hippocampal neurons were harvested as described (Takahashi et al., 2004) from C57Bl/6J (Jackson Labs, Maine, USA, JAX 000664) and APP KO (Jackson Labs, Maine, USA, JAX 004133) mouse embryos at embryonic day 16 (E16). Primary neuronal cultures were maintained in Neurobasal medium with added glutamine, B27 and penicillin/streptomycin (Thermo Fisher Scientific, Sweden). Before plating the primary neurons, glass coverslips were coated with poly-D-lysine molecular weight > 300,000 (Sigma-Aldrich, Sweden) followed by rinses in autoclaved distilled water. Cell suspensions were plated in 10% FBS and 1% penicillin-streptomycin in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Sweden); after 3–5 h media were exchanged for complete Neurobasal medium. One embryo corresponds to one set of cultures. No clear differences in cell density or viability were noticed between WT and APP KO neurons at 12 and 19 DIV.

2.4. Viral vector transductions

Primary neuronal cultures were transduced at 10 DIV with lentiviral vectors containing either the shRNA targeting APP or a scrambled control at a multiplicity of infection of 5. Cells were maintained 4 days post-transduction and at 14 DIV either fixed for fluorescence microscopy or harvested for western blot analysis.

2.5. Mouse brain tissue

Equal amounts (25 mg) of brain tissue snap-frozen in liquid nitrogen derived from analogous cortical regions (frontal cortex) from brains of

male APP KO and wild-type mice both sacrificed (decapitation) at different ages (3 and 12 months) were assayed for determination of synaptic protein levels. Brain samples were lysed in radio-immunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Roche Diagnostics, Sweden).

2.6. Western blot

Lysates were prepared using modified RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 tablet per 50 ml complete protease inhibitor cocktail, and phosphatase inhibitor cocktail set II (Sigma-Aldrich, Sweden). Protein concentrations were determined by the Bradford technique (Bio-Rad, California, USA) or BCA protein assay kit (Thermo Scientific, Sweden), and equal amounts of protein from each sample were analyzed by NuPage 4–12% Bis-Tris SDS-PAGE (Thermo Fisher Scientific, Sweden), Tris-Glycine SDS-PAGE or 10–20% Tricine SDS- PAGE (Sigma-Aldrich, Sweden), followed by immunoblotting on PVDF membranes (Sigma-Aldrich, Sweden). Membranes were immunoblotted with primary antibodies and intensities were quantified using Scion Image software (NIH, RRID:SCR_008673) or Image Lab 5.2.1 (Bio-Rad, Sweden, RRID:SCR_014210). For full blots see Supplementary Information.

2.7. Immunofluorescence

After fixation cells were rinsed in PBS at room temperature (RT). Cells were then blocked in 0.1% Saponin (Sigma-Aldrich, Sweden), 1% bovine serum albumin (BSA; Sigma-Aldrich, Sweden), and 2% normal-goat serum (NGS; Thermo Fisher Scientific, Sweden) in PBS for 1 h at RT. Primary antibodies were diluted in 2% NGS in PBS and incubated overnight. Cells were then rinsed in PBS and incubated with the secondary antibodies in 2% NGS in PBS. Cells were rinsed in PBS and DAPI (Sigma-Aldrich, Sweden) was used as counterstain in primary neuronal cultures diluted at 1:2000. Cells were mounted on microscope slides with Slowfade gold antifade reagent (Thermo Fisher Scientific, Sweden). Fluorescence was analyzed with an inverted epifluorescence Olympus IX70 microscope, or a Leica SP5 or SP8 confocal microscope. Quantifications of fluorescence intensities and puncta densities were performed by a blinded experimenter using the integrated morphometric analysis feature in Metamorph (Molecular Devices, California, USA, SCR 002368). Thresholds were set so that only puncta with intensities ($2\times$) brighter than that of the neurite shafts were quantified; > 40 cells from 3 separate cultures were analyzed for each genotype. Analysis of spine density and length were performed manually by a blinded experimenter using ImageJ (SCR 003070). In all analyses blinding was performed before analysis by coding the names of the image files. Sholl analysis was performed using an ImageJ plugin (Ferreira et al., 2014), briefly, thresholded bitmap images were measured from soma to furthest dendrite in the MAP2 channel. Then concentric circles of gradually increasing (30 μm) radii were calculated, and the crossing points with the MAP2 channel automatically counted.

2.8. Experimental design and statistical analysis

All statistical analysis was performed using GraphPad Prism 7.2 (SCR 002798). Sample size is denoted as n = number of cells analyzed, and N = number of animals. Prior to hypothesis testing data was tested for normality with D'Agostino-Pearson omnibus K2 normality test for determination of statistically appropriate test. Larger sample size data sets were found to fit normal distribution and therefore parametric tests were used. For experiments where n < 8, data was assumed to fit a normal distribution without formal testing. All graphs are depicted as mean ± 95% confidence interval. For in between litter comparisons samples were normalized to the wild-type mean of that litter. Statistical comparisons were made using two-tailed unpaired *t*-tests for
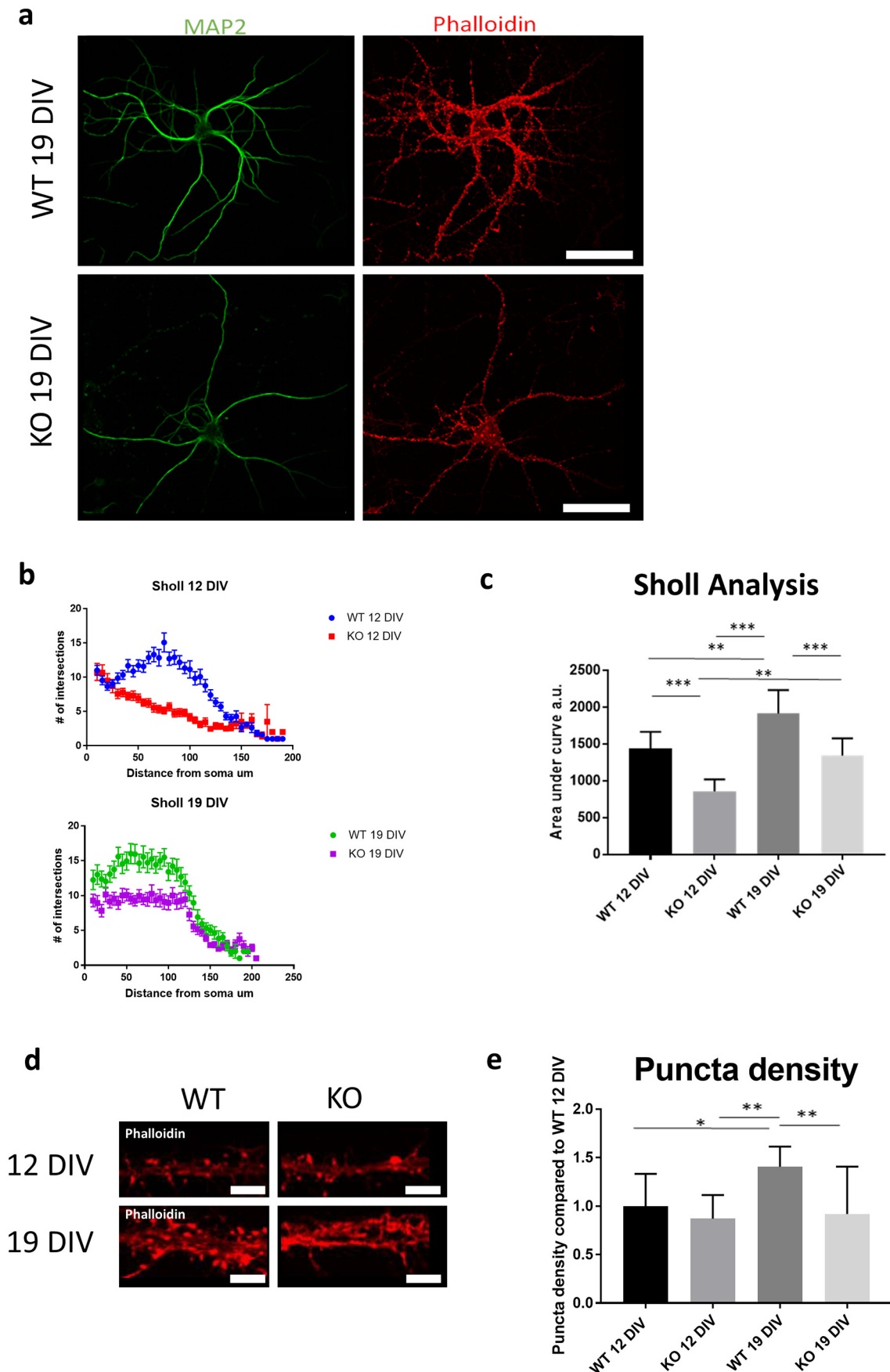
Fig. 1. APP knockout affects the morphology of neuronal cultures. (a) Representative images of MAP2 and phalloidin labeling in WT and APP KO cultures at 19 DIV. Scale bar = 50 μm. (b) Sholl analysis shows reduced dendritic branching in APP KO compared to WT neurons at 12 and 19 DIV. (c) Area under the curve for the Sholl analysis curves. (d) Representative micrograph of phalloidin labeling for spines at 12 and 19 DIV. (e) Phalloidin puncta density is decreased in 19 DIV APP KO compared to WT neurons; graph depicts mean ± 95% confidence interval with N = 3, n = 30 neurons, scale bar = 3 μm.
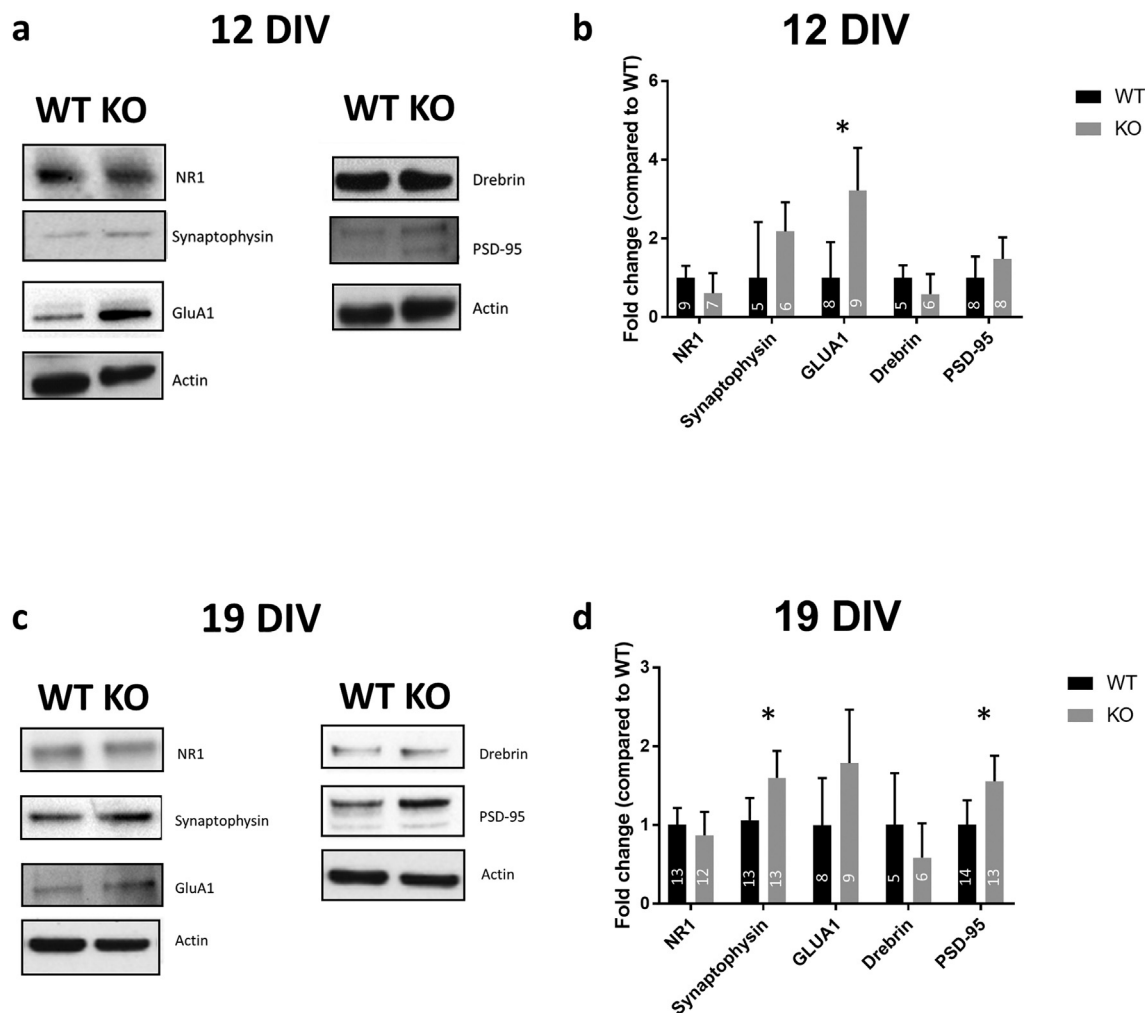
Fig. 2. Levels of selective pre- and post-synaptic proteins in APP KO compared to wild-type neurons in culture by western blot. (a) Representative Western blot of select synaptic proteins in 12 DIV WT and KO cultures. (b) Quantifications of (a), normalized to beta-actin. There was a significant increase in levels of the post-synaptic AMPA receptor subunit GluA1 (322 ± 66% increase, p = 0.0025, WT n = 8, KO n = 8) at 12 DIV. (c) Representative Western blot of select synaptic proteins in 19 DIV WT and KO cultures. (d) Quantifications of (c), normalized to beta-actin. Levels of post-synaptic PSD-95 (155% ± 14.9%, p = 0.013, WT n = 14, KO n = 14) and pre-synaptic synaptophysin (159% ± 15.7%, p = 0.014, WT n = 14, KO n = 14) were higher in the APP KO cultures at 19 DIV; graphs depict mean and 95% confidence intervals and n is indicated within each bar graph. Western blot scans are cropped; the bands analyzed were NR1 (130 kDa), synaptophysin (38 kDa), GluA1 (106 kDa), drebrin (120 kDa) and PSD-95 (95 kDa).

comparisons between two groups and paired t-tests for paired tests, and one way ANOVA followed by Tukey's test when comparing multiple groups. Statistical significance was placed at $p < 0.05$ denoted *, $p < 0.005$ denoted ** and $p < 0.001$ denoted *** unless otherwise stated.

3. Results

3.1. Neuronal morphology is altered in APP knockout neurons

It has previously been shown that APP KO affects neuronal morphology and function (Perez et al., 1997; Tyan et al., 2012). Since there are some discrepancies in the literature, we characterized our cortical cultures of APP KO and wild-type (WT) neurons at 12 and 19 days in vitro (DIV). Sholl analysis revealed that the APP KO neurons show reduced dendritic arbors compared to WT neurons both at 12 and 19 DIV (Fig. 1a,b and c). To investigate spine density, we labeled the neurons with phalloidin, which revealed reduced density of phalloidin puncta in APP knockout compared to WT neurons at 19 DIV (Fig. 1d and e).

3.2. APP knockout leads to increased levels of GluA1

To investigate the effects of APP KO on synaptic composition, APP KO or WT primary neurons cultured for 12 or 19 DIV were analyzed by western blot. We previously reported on decreases in selective synaptic proteins, including pre-synaptic synaptophysin and the post-synaptic AMPA receptor subunit GluA1 and PSD-95 in neurons of Tg2576 AD transgenic mice overexpressing the Swedish mutation of APP, compared to WT mice (Almeida et al., 2005). Remarkably, APP KO compared to WT neurons at 12 DIV showed a threefold increase in levels of GluA1 (322 ± 66% increase; p = 0.0025; n = 8; Fig. 2), while there were only non-significant trends for increases in the pre-synaptic protein synaptophysin and the post-synaptic protein PSD-95, and decreases in the NR1 subunit of the NMDA receptor. At 19 DIV there was a 1.5 fold increase in levels of PSD-95 (155% ± 14.9%, p = 0.013; n = 14) and a 1.6 fold increase in levels of synaptophysin (159% ± 15.7%, p = 0.014), as well as a non-significant change, albeit a trend for a 1.7 fold increase, in levels of GluA1 (178.7% ± 26.8%, p = 0.064; n = 14) in APP KO compared to WT neurons (Fig. 2).

To further examine the effects of APP knockout on synapse composition, the relative immuno-fluorescence of select synaptic proteins
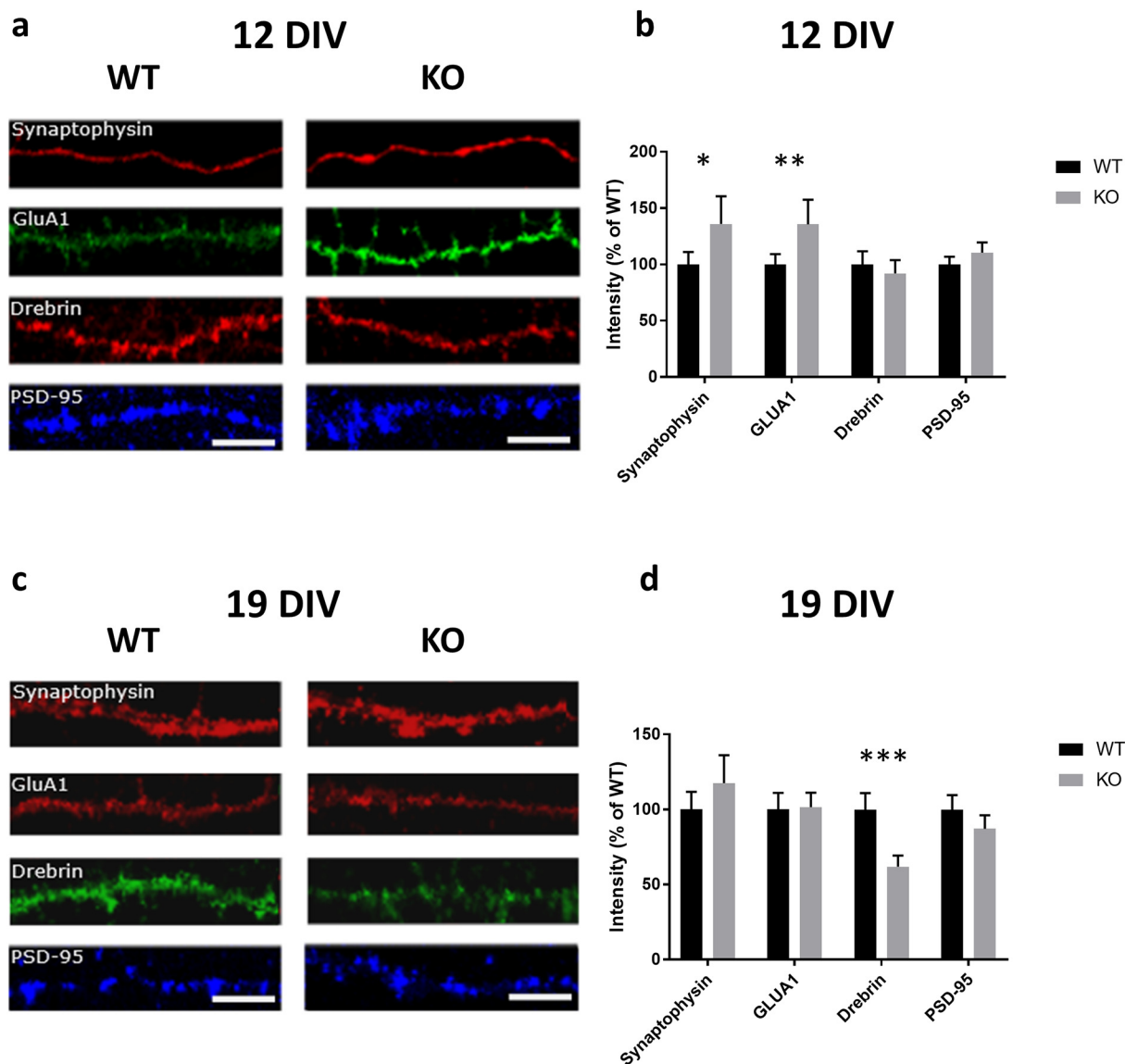
Fig. 3. Levels of selective pre- and post-synaptic proteins in APP KO compared to wild-type neurons in culture by immunofluorescence microscopy. Levels of pre-synaptic synaptophysin, and post-synaptic GluA1, drebrin and PSD-95 were quantified in wild-type and APP KO primary neurons. (a–b) At 12 DIV immuno-fluorescence puncta of synaptophysin and GluA1 were increased in APP KO compared to wild-type neurons. (c–d) However, at 19 DIV synaptophysin and GluA1 puncta were similar in intensity to wild-type neurons, while drebrin puncta intensities were significantly decreased (40% ± 4%, p < 0.001) in APP KO compared to wild-type neurons; scale bar = 5 μm, graphs represent mean ± 95% confidence intervals, n = 40 neurons.

was measured in neurites of APP KO and WT neurons in culture. At 12 DIV, APP KO compared to WT neurons showed increased labeling of the presynaptic protein synaptophysin (136.1% ± 13.3%, p = 0.0018). Further, there was increased labeling of the AMPA receptor subunit GluA1 (135.8% ± 11.74%, p = 0.003) in the APP KO neurons at 12 DIV (Fig. 3a and b). No statistically significant differences in the post-synaptic proteins PSD-95 or the actin binding protein drebrin were evident at 12 DIV, although there was a trend for increased PSD-95 labeling (p = 0.06). At 19 DIV, levels of drebrin were now markedly decreased by 40% ± 4% (p < 0.0001), while the other synaptic proteins were all comparable to WT levels, although rather than the trend for an increase at 12 DIV, there was now a trend for a decrease in PSD-95 labeling in APP KO neurons, which however did not reach significance (p = 0.086; Fig. 3c and d).

To extend these findings from primary neurons in culture, we next examined synaptic proteins in brain homogenates of frontal cortex of APP KO compared to WT mice. At 3 months of age, western blotting of brain lysates showed an increase in levels of synaptophysin

(254 ± 14%; p < 0.0001) and GluA1 (187 ± 12%; p = 0.0001) in APP KO compared to WT mouse brains (Fig. 4a and b). However, at 12 months of age there were no longer statistical differences in levels of synaptophysin or GluA1, although there were now statistically significant increases in levels of PSD-95 (186 ± 19%; p = 0.0010) but decreases in levels of drebrin (47 ± 9%, p = 0.048) in APP KO brains (Fig. 4a and c).

Thus, our results both in APP KO primary neurons and brains support statistically significant increases in selective synaptic proteins. In particular, post-synaptic GluA1, known to decline in AD, was increased at early time points in culture (12 DIV) or early ages in brain (3 months) in APP KO mice, which however was not maintained at later time points.

3.3. Levels of GluA1 are increased in neurons with lentiviral knockdown of APP

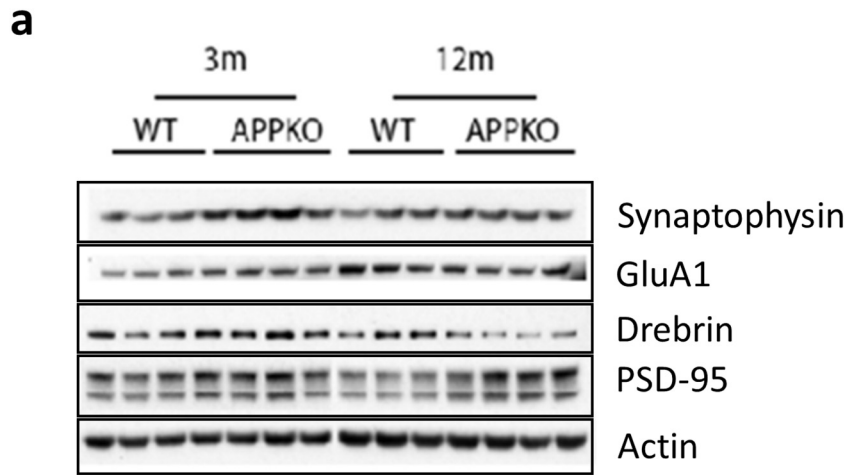Given the evidence for the involvement of APP in brain

a

Fig. 4. Levels of selective pre- and post-synaptic proteins in APP KO compared to wild-type mouse brains by western blot. (a) Western blots of 3 and 12 month-old WT and APP KO brain. An overall similar pattern of changes in selective pre- and post-synaptic proteins was seen in APP KO brains compared to neurons in culture. (b) Quantifications of 3 month-old mouse brains normalized to beta-actin; levels of pre-synaptic synaptophysin (254 ± 14%; p < 0.0001) and post-synaptic GluA1 (187 ± 12%; p = 0.0001) were significantly higher in APP KO (n = 4) compared to wild-type (n = 3) mouse brains at 3 months of age. (c) Quantifications of 12 month-old mouse brains normalized to beta-actin. While GluA1 and synaptophysin were not significantly altered at this age, there were increased levels of post-synaptic PSD-95 (186 ± 19%; p = 0.0010) and decreased levels of post-synaptic drebrin (47 ± 9%, p = 0.048) in 12 month-old APP KO mouse brains. Western blot scans are cropped; dotted line in (a) represents cut out molecular weight marker.

b

c

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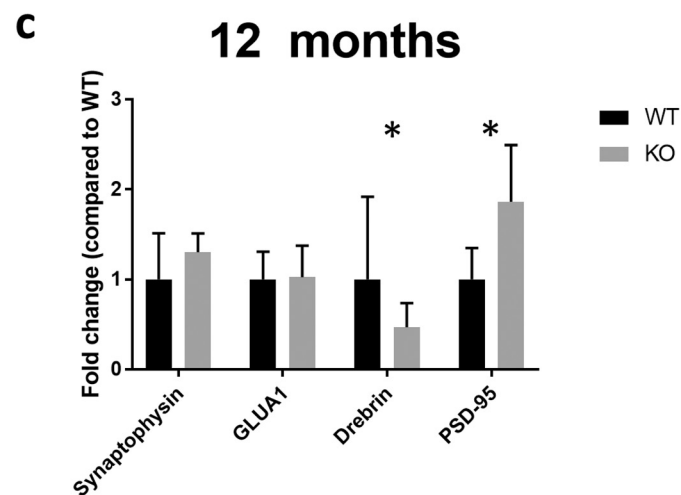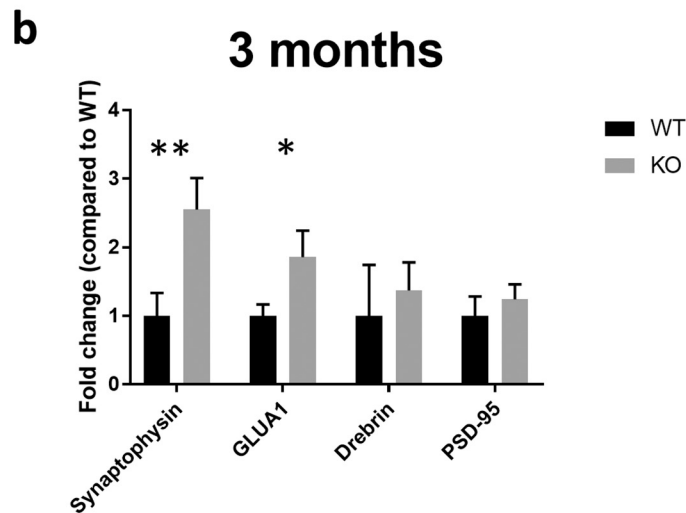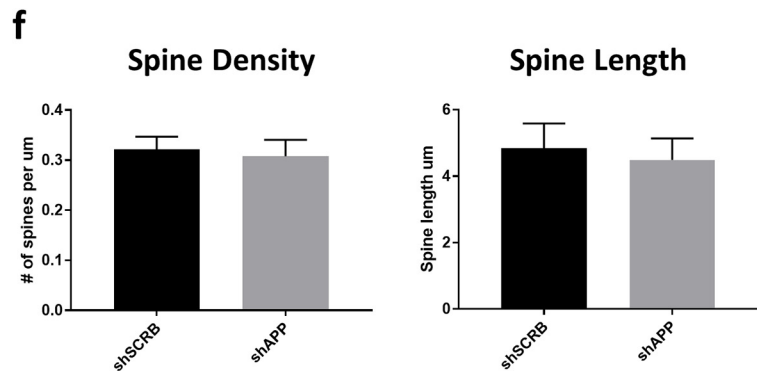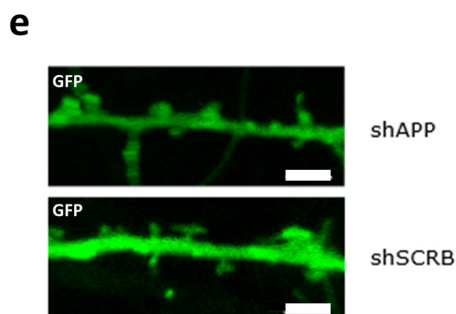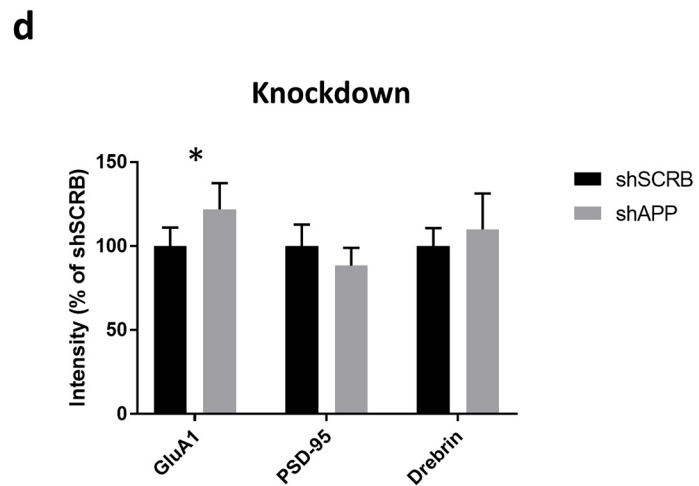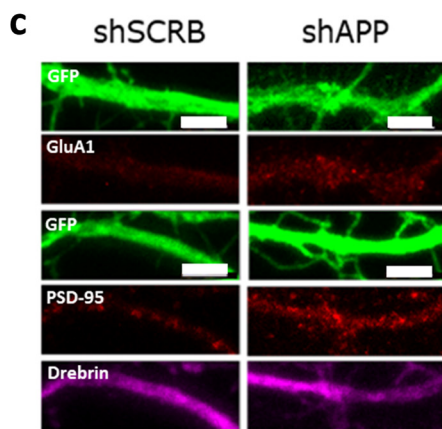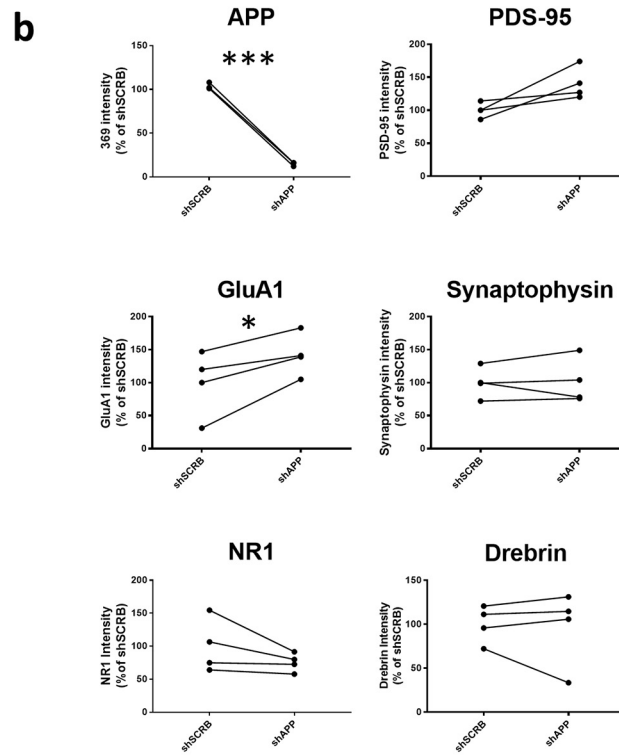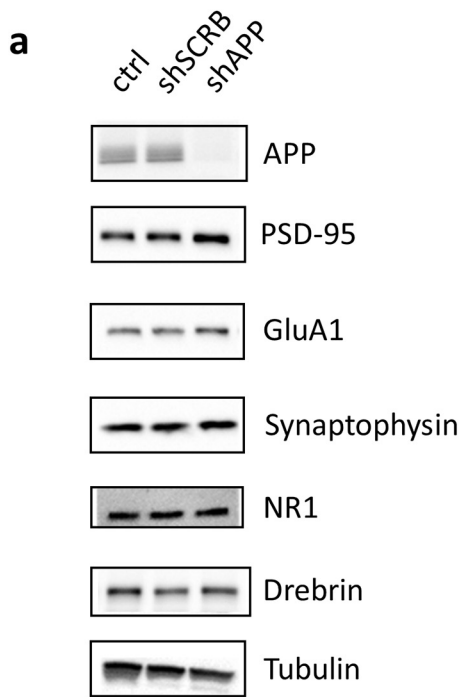
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Fig. 5. Levels of selected pre- and post-synaptic proteins with shRNA mediated knockdown of APP. (a) Levels of synaptic proteins were determined by western blot (normalized to tubulin) for 14 DIV cultures of WT neurons that were transduced with a lentiviral vector carrying shRNA against APP at 10 DIV; APP knockdown was verified by antibody 369 against the APP C-terminus on western blot (one- way ANOVA p < 0.001). (b) Levels of post-synaptic GluA1 (142% ± 22.43%, p = 0.0082, paired t-test) and PSD-95 (140% ± 23.9% p = 0.067, paired t-test, p = 0.0225, unpaired two-sample t-test) were increased with shAPP transduction; n = 4. Each dot in the graphs depicts one independent experiment. (c) Representative dendritic segments after transduction with scrambled (shSCRB) or shAPP lentiviral constructs. (d) Quantifications of immunofluorescence analysis. Levels of GluA1 increased with shAPP knockdown of APP (121% ± 7.8%; p = 0.023); scale bar = 3 μm, graphs show mean ± 95% confidence interval, n = 40 neurons. (e) Confocal images of representative dendritic spines. (f) Quantification of spine density and length; no significant differences between scrambled and shRNA APP knockdown was detected; graph depicts mean ± 95% confidence interval, scale bar = 3 μm. Western blot scans are cropped.

development, we next used RNAi to knock down APP in cultured wild-type neurons in order to investigate the role of APP after the formation of synapses. Typically a few days is needed for effective lentiviral RNAi knockdown in primary neurons, and to avoid too early knockdown, in which case synapse development would be affected, or too late knockdown, since transduction can be more toxic to more aged neurons in culture, we chose transduction at 10 DIV and analysis at 14 DIV. We verified the knockdown of APP using Western blot with a C-terminal APP antibody. Importantly there was no effect on APP levels with the scrambled control compared to non-transduced controls (Fig. 5a).

APP RNAi knockdown compared to the scrambled control RNAi at 10 DIV caused a significant increase in the levels of GluA1 (142% ± 22.43%, p = 0.0082) in cultured neurons when analyzed at 14 DIV by western blot (Fig. 5a and b). There was only a trend for increases in PSD-95 with APP knockdown (140% ± 23.9% p = 0.067), and levels of the pre-synaptic protein synaptophysin remained unchanged. Moreover, immunofluorescence analysis of dendritic segments also showed an increase in levels of GluA1 (121% ± 7.8%; p = 0.023), but not in levels of PSD-95 or drebrin (Fig. 5c and d). Lastly, we investigated whether spine density was altered with APP knockdown in wild-type neurons. However, we did not detect any significant differences in spine density or length between the APP knockdown and control neurons (Fig. 5e and f). Thus, knockdown of APP in wild-type mouse neurons when initiated at 10 DIV and analyzed at 14 DIV replicated the elevation in GluA1 seen in APP KO neurons, but not the alterations in spine density.

4. Discussion

Numerous lines of evidence support that Aβ can have detrimental effects on synapses. However, the precise mechanism(s) of APP/Aβ's involvement in AD remain to be determined. The following provide support for synapses as being early and critical sites of damage in AD: 1. APP is transported down axons and dendrites, and β- and γ–secretases localize to synapses (Koo et al., 1990; Tampellini et al., 2009; DeBoer et al., 2014; Lundgren et al., 2015; Schedin-Weiss et al., 2016); 2. Aβ is preferentially generated, secreted and degraded upon increased synaptic activity (Kamenetz et al., 2003; Cirrito et al., 2005; Tampellini et al., 2009); 3. APP mRNA is locally translated at synapses (Westmark and Malter, 2007); 4. Aβ accumulates and aggregates in synapses early in AD (Takahashi et al., 2002; Takahashi et al., 2004); 5. Extracellular Aβ binds to synapses (Lacor et al., 2004; Willen et al., 2017); 6. Aβ accumulation in synaptosomes correlates with dementia and can differentiate cognitively normal controls with high AD pathology from dementia cases (Bilousova et al., 2016); 7. APP has been shown to function as a trans-synaptic adhesion molecule (Wang et al., 2009; Stahl et al., 2014).

Here we provide evidence that APP and/or its proteolytic cleavage products are important for normal synaptic composition. We are not aware of a similar comparison of levels of selective pre- and post-synaptic proteins in APP KO primary neurons and brain combined with comparisons to APP knockdown. Overall the most consistent increase was in levels of the post-synaptic AMPA receptor subunit GluA1, which was increased in APP KO neurons and brain at early time points in primary neurons and young mouse brains, as well as with APP knockdown in wild-type neurons. Remarkably, GluA1 was also the most

consistently affected in our prior analysis of selective pre- and post-synaptic changes in AD transgenic compared to wild type mouse neurons (Almeida et al., 2005), although in contrast to the absence of APP, the AD mutation led to a decrease in levels of GluA1. GluA1 is particularly important for synaptic plasticity and learning and memory, and future work should focus on the precise mechanism(s) whereby APP and/or its cleavage products affect levels of this important post-synaptic AMPA receptor subunit.

Our analysis was not always consistent when it came to changes in some synaptic proteins in neurons with time in culture (12 versus 19 DIV) and compared to or between younger and older mouse brains (3 versus 12 months), or with APP knockdown compared to APP knockout. Moreover, findings on Western blot and immunofluorescence did not always overlap. It is important to note that Western blot assays total levels of a selective protein, while immunofluorescence of neurite segments provides more localized information on levels in neurites. The elevated levels on Western blot with unchanged or even reduced levels of a synaptic protein by immunofluorescence could, for example, reflect reduced axonal or dendritic transport to synapses and thus accumulation in neuron soma in APP KO compared to WT neurons.

Summarizing our results (see Fig. 6), we demonstrate that 1. APP knockout alters the morphology (dendritic branching and spines) of primary cortical neurons; 2. AMPA receptor subunit GluA1 is increased in cultured neurons at 12 DIV and brains of 3 months old APP KO mice; 3. Increased levels of GluA1 also with shRNA knockdown of APP supports an important role of APP in the composition of synapses rather than effects in APP KO mice being solely due to the role of APP in development, although we cannot fully exclude an effect on continued maturation between 10 DIV (RNAi transduction) and 14 DIV (analysis). 4. Lack of APP alters post-synaptic proteins in mature cultures and adult mice, at 19 DIV neurons and 12 month-old mice, respectively, as shown by increases in PSD-95 and decreases in drebrin.

Decreased phalloidin puncta and levels of drebrin were detected in APP KO compared to wild-type neurons at 19 DIV despite an increase in levels of the post-synaptic protein PSD-95. This reduction in phalloidin puncta indicates a reduction in spine density in 19 DIV APP KO neurons, an observation that fits with previous reports on altered neuronal morphology in APP KO neurons (Tyan et al., 2012; Weyer et al., 2014). Further, since the phalloidin puncta density is not decreased significantly at 12 DIV in the knockout compared to WT, but the 19 DIV WT neurons show a higher spine density than 12 DIV WT neurons, this could reflect decreased formation and/or increased removal of spines in APP KO neurons between 12 and 19 DIV. In fact, reduced spine turnover was shown in older APP KO mice, where environmental enrichment failed to increase spine density compared to wild-type mice (Zou et al., 2016). Neurons targeted by the APP shRNA vector, also showed PSD-95 increases, however, without a change in spine density. It is possible that changes in spine density might require more time than the APP knockdown used here, with shRNA transduction at 10 DIV and analysis at 14 DIV. Alternatively, PSD-95 increases have not been shown to increase spines (Cane et al., 2014), while drebrin is more closely correlated with spines (Koganezawa et al., 2017), and drebrin was decreased in the APP KO neurons at 19 DIV but not with APP knockdown (Fig. 6).

We detected increased levels of pre-synaptic synaptophysin at 3 months in the APP KO mice; this finding, together with the increased
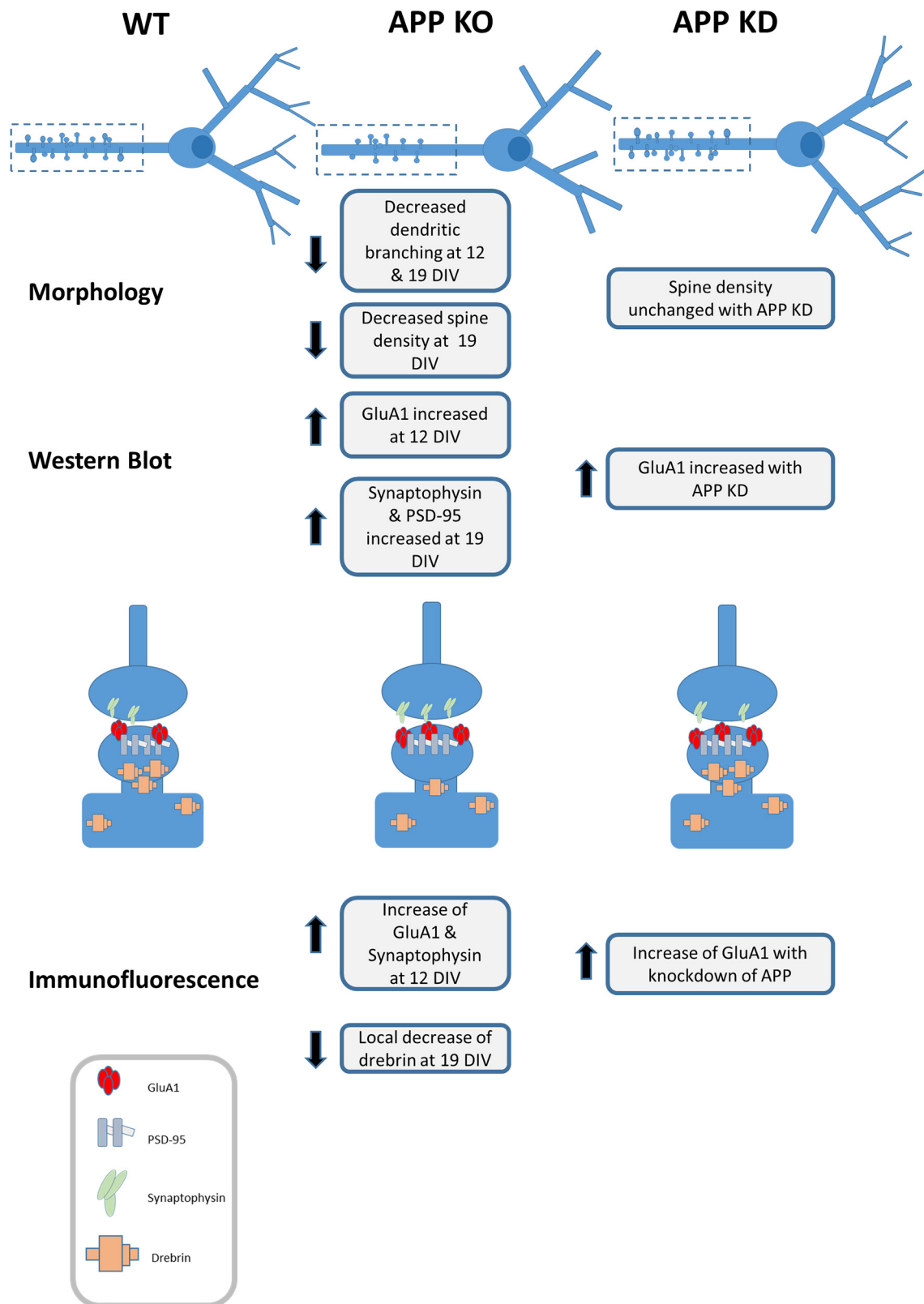
Fig. 6. Schematic summary. Comparisons of morphologies of dendrites to WT neurons revealed decreased dendritic branching (12 and 19 DIV) and decreased spines (19 DIV) in APP KO but not in APP KD neurons. Western blots revealed elevated GluA1 in APP KO (12 DIV) and APP KD compared to WT neurons, as well as elevated synaptophysin and PSD-95 in APP KO compared to WT neurons at 19 DIV. Immunofluorescence labeling revealed local increases of GluA1 in APP KO (12 DIV) and APP KD, and in synaptophysin in APP KO (12 DIV) neurons. A local decrease of drebrin labeling was evident in APP KO neurons (19 DIV).

synaptophysin immunofluorescence labeling in cultured APP KO neurons, fits with earlier reports showing increased synaptophysin labeling in hippocampi of p20 APP KO mice (Priller et al., 2006). The increased synaptophysin with APP KO could result from increased axonal branching and outgrowth during early development that has been described (Perez et al., 1997; Young-Pearse et al., 2008; Billnitzer et al., 2013). Moreover, APP was recently reported to act as a receptor for slit-1, further implicating APP in early axon development (Wang et al., 2017).

In conclusion, synapses appear to be key sites of APP biology and function, and it will be important to provide a clearer molecular understanding of the precise role(s) of APP and its cleavage products at synapses. Taken together, our results strengthen the important role of APP in synaptic composition.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2019.02.003.

Acknowledgements

Conflicts of interest

We have no conflicts of interest to declare.

Author contributions

The study was conceived by G.K.G. Biochemical assays were conducted by I.M, E.C-Z, K.W, M.F, and N.E. Cloning was performed by M.F. Microscopy was performed by I.M, E.C-Z, M.F and K.W. The manuscript was written by I.M and G.K.G, and edited by I.M., E.C-Z, M.F, K.W, S.F, L.T. and G.K.G.

References

Almeida, C.G., Tampellini, D., Takahashi, R.H., Greengard, P., Lin, M.T., Snyder, E.M., et al., 2005. Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. Neurobiol. Dis. 20 (2), 187–198. https://doi.org/10.1016/j.nbd.2005.02.008.

Billnitzer, A.J., Barskaya, I., Yin, C., Perez, R.G., 2013. APP independent and dependent effects on neurite outgrowth are modulated by the receptor associated protein (RAP). J. Neurochem. 124 (1), 123–132. https://doi.org/10.1111/jnc.12051.

Bilousova, T., Miller, C.A., Poon, W.W., Vinters, H.V., Corrada, M., Kawas, C., et al., 2016. Synaptic amyloid-beta oligomers precede p-tau and differentiate high pathology control cases. Am. J. Pathol. 186 (1), 185–198. https://doi.org/10.1016/j.ajpath.2015.09.018.

Bittner, T., Fuhrmann, M., Burgold, S., Jung, C.K., Volbracht, C., Steiner, H., et al., 2009. Gamma-secretase inhibition reduces spine density in vivo via an amyloid precursor protein-dependent pathway. J. Neurosci. 29 (33), 10405–10409. https://doi.org/10.1523/JNEUROSCI.2288-09.2009.

Buxbaum, J.D., Gandy, S.E., Cicchetti, P., Ehrlich, M.E., Czernik, A.J., Fracasso, R.P., et al., 1990. Processing of Alzheimer beta/A4 amyloid precursor protein: modulation by agents that regulate protein phosphorylation. Proc. Natl. Acad. Sci. U. S. A. 87 (15), 6003–6006.

Campeau, E., Ruhl, V.E., Rodier, F., Smith, C.L., Rahmberg, B.L., Fuss, J.O., et al., 2009. A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS One 4 (8), e6529. https://doi.org/10.1371/journal.pone.0006529.

Cane, M., Maco, B., Knott, G., Holtmaat, A., 2014. The relationship between PSD-95 clustering and spine stability in vivo. J. Neurosci. 34 (6), 2075. https://doi.org/10.1523/JNEUROSCI.3353-13.2014.

Cirrito, J.R., Yamada, K.A., Finn, M.B., Sloviter, R.S., Bales, K.R., May, P.C., et al., 2005. Synaptic activity regulates interstitial fluid amyloid-beta levels in vivo. Neuron 48 (6), 913–922. https://doi.org/10.1016/j.neuron.2005.10.028.

DeBoer, S.R., Dolios, G., Wang, R., Sisodia, S.S., 2014. Differential release of beta-amyloid from dendrite- versus axon-targeted APP. J. Neurosci. 34 (37), 12313–12327. https://doi.org/10.1523/jneurosci.2255-14.2014.

Ferreira, T.A., Blackman, A.V., Oyrer, J., Jayabal, S., Chung, A.J., Watt, A.J., et al., 2014. Neuronal morphometry directly from bitmap images. Nat. Methods 11, 982. https://doi.org/10.1038/nmeth.3125.

Jung, C.K.E., Herms, J., 2012. Role of APP for dendritic spine formation and stability. Exp. Brain Res. 217 (3), 463–470. https://doi.org/10.1007/s00221-011-2939-x.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., et al., 2003. APP

processing and synaptic function. Neuron 37 (6), 925–937.

Koganezawa, N., Hanamura, K., Sekino, Y., Shirao, T., 2017. The role of drebrin in dendritic spines. Mol. Cell. Neurosci. 84, 85–92. https://doi.org/10.1016/j.mcn.2017.01.004.

Koo, E.H., Sisodia, S.S., Archer, D.R., Martin, L.J., Weidemann, A., Beyreuther, K., et al., 1990. Precursor of amyloid protein in Alzheimer disease undergoes fast anterograde axonal transport. Proc. Natl. Acad. Sci. U. S. A. 87 (4), 1561–1565.

Lacor, P.N., Buniel, M.C., Chang, L., Fernandez, S.J., Gong, Y., Viola, K.L., et al., 2004. Synaptic targeting by Alzheimer's-related amyloid beta oligomers. J. Neurosci. 24 (45), 10191–10200. https://doi.org/10.1523/jneurosci.3432-04.2004.

Lee, K.J., Moussa, C.E., Lee, Y., Sung, Y., Howell, B.W., Turner, R.S., et al., 2010. Beta amyloid-independent role of amyloid precursor protein in generation and maintenance of dendritic spines. Neuroscience 169 (1), 344–356. https://doi.org/10.1016/j.neuroscience.2010.04.078.

Lundgren, J.L., Ahmed, S., Schedin-Weiss, S., Gouras, G.K., Winblad, B., Tjernberg, L.O., et al., 2015. ADAM10 and BACE1 are localized to synaptic vesicles. J. Neurochem. 135 (3), 606–615. https://doi.org/10.1111/jnc.13287.

Masliah, E., Terry, R.D., DeTeresa, R.M., Hansen, L.A., 1989. Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. Neurosci. Lett. 103 (2), 234–239.

Nicolas, M., Hassan, B.A., 2014. Amyloid precursor protein and neural development. Development 141 (13), 2543–2548. https://doi.org/10.1242/dev.108712.

Perez, R.G., Zheng, H., Van der Ploeg, L.H., Koo, E.H., 1997. The beta-amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity. J. Neurosci. 17 (24), 9407–9414.

Priller, C., Bauer, T., Mitteregger, G., Krebs, B., Kretzschmar, H.A., Herms, J., 2006. Synapse formation and function is modulated by the amyloid precursor protein. J. Neurosci. 26 (27), 7212–7221. https://doi.org/10.1523/JNEUROSCI.1450-06.2006.

Schedin-Weiss, S., Caesar, I., Winblad, B., Blom, H., Tjernberg, L.O., 2016. Super-resolution microscopy reveals gamma-secretase at both sides of the neuronal synapse. Acta Neuropathol. Commun. 4, 29. https://doi.org/10.1186/s40478-016-0296-5.

Seabrook, G.R., Smith, D.W., Bowery, B.J., Easter, A., Reynolds, T., Fitzjohn, S.M., et al., 1999. Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. Neuropharmacology 38 (3), 349–359.

Senechal, Y., Kelly, P.H., Dev, K.K., 2008. Amyloid precursor protein knockout mice show age-dependent deficits in passive avoidance learning. Behav. Brain Res. 186 (1), 126–132. https://doi.org/10.1016/j.bbr.2007.08.003.

Stahl, R., Schilling, S., Soba, P., Rupp, C., Hartmann, T., Wagner, K., et al., 2014. Shedding of APP limits its synaptogenic activity and cell adhesion properties. Front. Cell. Neurosci. 8, 410. https://doi.org/10.3389/fncel.2014.00410.

Takahashi, R.H., Milner, T.A., Li, F., Nam, E.E., Edgar, M.A., Yamaguchi, H., et al., 2002. Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am. J. Pathol. 161 (5), 1869–1879.

Takahashi, R.H., Almeida, C.G., Kearney, P.F., Yu, F., Lin, M.T., Milner, T.A., et al., 2004. Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. J. Neurosci. 24 (14), 3592–3599. https://doi.org/10.1523/jneurosci.5167-03.2004.

Tampellini, D., Rahman, N., Gallo, E.F., Huang, Z., Dumont, M., Capetillo-Zarate, E., et al., 2009. Synaptic activity reduces intraneuronal Abeta, promotes APP transport to synapses, and protects against Abeta-related synaptic alterations. J. Neurosci. 29 (31), 9704–9713. https://doi.org/10.1523/jneurosci.2292-09.2009.

Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill, R., et al., 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann. Neurol. 30 (4), 572–580. https://doi.org/10.1002/ana.410300410.

Tyan, S.H., Shih, A.Y., Walsh, J.J., Maruyama, H., Sarsoza, F., Ku, L., et al., 2012. Amyloid precursor protein (APP) regulates synaptic structure and function. Mol. Cell. Neurosci. 51 (1–2), 43–52. https://doi.org/10.1016/j.mcn.2012.07.009.

Wang, Z., Wang, B., Yang, L., Guo, Q., Aithmitti, N., Songyang, Z., et al., 2009. Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis. J. Neurosci. 29 (35), 10788–10801. https://doi.org/10.1523/jneurosci.2132-09.2009.

Wang, B., Li, H., Mutlu, S.A., Bowser, D.A., Moore, M.J., 2017. The amyloid precursor protein is a conserved receptor for slit to mediate axon guidance. 4 (3). https://doi.org/10.1523/eneuro.0185-17.2017.

Westmark, C.J., Malter, J.S., 2007. FMRP mediates mGluR5-dependent translation of amyloid precursor protein. PLoS Biol. 5 (3), e52. https://doi.org/10.1371/journal.pbio.0050052.

Weyer, S.W., Zagrebelsky, M., Herrmann, U., Hick, M., Ganss, L., Gobbert, J., et al., 2014. Comparative analysis of single and combined APP/APLP knockouts reveals reduced spine density in APP-KO mice that is prevented by APPsalpha expression. Acta Neuropathol. Commun. 2, 36. https://doi.org/10.1186/2051-5960-2-36.

Willen, K., Sroka, A., Takahashi, R.H., Gouras, G.K., 2017. Heterogeneous association of Alzheimer's disease-linked amyloid-beta and amyloid-beta protein precursor with synapses. J. Alzheimers Dis. 60 (2), 511–524. https://doi.org/10.3233/jad-170262.

Young-Pearse, T.L., Bai, J., Chang, R., Zheng, J.B., LoTurco, J.J., Selkoe, D.J., 2007. A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. J. Neurosci. 27 (52), 14459–14469. https://doi.org/10.1523/JNEUROSCI.4701-07.2007.

Young-Pearse, T.L., Chen, A.C., Chang, R., Marquez, C., Selkoe, D.J., 2008. Secreted APP regulates the function of full-length APP in neurite outgrowth through interaction with integrin beta1. Neural Dev. 3, 15. https://doi.org/10.1186/1749-8104-3-15.

Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J.S., Hopkins, R., Smith, D.W., et al., 1995. β-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81 (4), 525–531. https://doi.org/10.1016/0092-8674(95)90073-X.

Zou, C., Crux, S., Marinesco, S., Montagna, E., Sgobio, C., Shi, Y., et al., 2016. Amyloid precursor protein maintains constitutive and adaptive plasticity of dendritic spines in adult brain by regulating D-serine homeostasis. EMBO J. 35 (20), 2213–2222. https://doi.org/10.15252/embj.201694085.