AWARD NUMBER: W81XWH-16-1-0263

TITLE: Impaired mTOR Macroautophagy and Neurocognitive Deficits in Tuberous Sclerosis Complex

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This study is designed to identify mTOR-downstream molecules or pathways that account for synaptic and cognitive deficits in TSC, with the goal of identifying targets for more specific treatment. We had focused on macroautophagy (autophagy hereafter), a homeostatic catabolic degradation process downstream of mTOR, which is inhibited by hyperactive mTOR in Tsc1/2 deficient mouse brain. During the first project year, we had found significant cognitive impairment in Tsc2+/- mice and Atg7CKO autophagy deficient mice at the age of 3 months. These mice however did not show cognitive deficits at 1 month of age. Prior to the occurrence of cognitive impairment, Atg7CKO mice exhibited an increase in NMDA:AMPA ratio, increased frequency of miniature EPSCs and increased dendritic spine density, all indicating a blockade in postnatal synapse maturation. Atg7CKO mice moreover showed impaired CA3-CA1 long-term potentiation (LTP) and long term depression (LTD), both of which are well-known electrophysiological surrogates of hippocampus dependent learning and memory. Our findings therefore suggest that Autophagy is essential for synapse maturation and the development of normal synaptic plasticity and cognitive functions. We will continue to examine whether autophagy deficiency may underlie cognitive impairment in Tsc1/2 mutant mice during the next reporting period.
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1. INTRODUCTION:

Cognitive impairments, including long term and working memory deficits, are commonly neuropsychiatric features of a majority of TSC patients. Recent studies in Tsc1 or Tsc2 heterozygous mutant murine models suggest that disinhibited mTOR is sufficient to cause the cognitive impairments, which promoted the preclinical research to assess therapeutic effects of mTOR inhibitors on TSC related cognitive deficits. The effect of mTOR inhibitors is however transient and the efficacy may be limited by their side effects. Unraveling the downstream substrates of overactive mTOR will be critical for developing more targeted and effective therapies for the neurocognitive symptoms in TSC. This study is designed to identify mTOR-downstream molecules or pathways that account for synaptic and cognitive deficits in TSC, with the goal of identifying targets for more specific treatment while limiting side effects of mTOR inhibitors. We will focus on macroautophagy (autophagy hereafter), a homeostatic catabolic degradation process downstream of mTOR, which is inhibited by hyperactive mTOR in Tsc1/2 deficient mouse brain. Impaired mTOR-autophagy increases dendritic spine synapse density by suppressing postnatal synaptic pruning, a process necessary for the maturation of functional synaptic connections and neural circuits and required for multiple forms of learning and memory. We propose to study whether impaired autophagy may underlie cognitive impairments in TSC mice by disrupting synapse maturation.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Tuberous Sclerosis, cognitive impairment, autophagy, neuron, synapse maturation, mTOR

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Aim 1: To determine the role of impaired mTOR-autophagy in cognitive dysfunction in Tsc1 or Tsc2 (Tsc1/2) deficient mice (Time frame: months 1-24).

Aim 2: To identify synaptic mechanisms of impaired mTOR-autophagy for cognitive dysfunction in Tsc1/2 deficient mice (Time frame: months 1-24).

Aim 3: To identify molecular mechanisms of impaired autophagy for synaptic dysfunction in Tsc1/2 deficient mice (Time frame: months 13-36).

What was accomplished under these goals?

Task 1: Determine the role of impaired mTOR-autophagy in cognitive dysfunction in Tsc1 or Tsc2 (Tsc1/2) deficient mice

We had a delay with the start of this project due to the wait for ACURO animal use approval, and due to an accidental lost the Atg7flox/+;Camkcre+ breeding mouse line. We had thus spent 4 months to re-derived the female Atg7flox/+;Camkcre+ and then the female Tsc2+/- :Atg7flox/+;Camkcre+ breeders (2016.11-2017.02). We however obtained a cohort of Camkcre mediated Atg7 conditional knockout (Atg7CKO) and control mice, as well as a cohort of Tsc2+/-. 
control mice. These mice were grouped into two different age groups: 1-month old and 3-month old mice, and were tested for cognitive function at different ages. Our major findings are: 1) Atg7CKO mice exhibited hippocampus-related cognitive impairment at 3 months of age, indicated by impaired spatial learning (Fig. 1A) and memory (Fig. 1B) in a Morris water maze (MWM), impaired spatial learning in a 2-day radial arm water maze test (RAWA, Fig. 1C) and impaired contextual memory in fear

Figure 1. Cognitive deficits in 3-month old Atg7CKO mice. (A,B) Morris Water Maze test. (A) Escape latency to the hidden platform during learning sessions; (B) Spatial memory assessed in the probe trial for the percentage of time spent in the target quadrant (#4, indicated by the red square) relative to non-target quadrants (#1, #2, #3). (B) 2-day radial arm test. (C) Contextual and Cued fear conditioning test. Data are plotted as the mean ± SEM (Control, n = 19; Atg7CKO, n=14). Compared to controls, *, p<0.05; **, p<0.01, Student t test.

Figure 2. Cognitive deficits in 3-month old Tsc2+/- mice. (A,B) Morris Water Maze test. (A) Escape latency to the hidden platform during learning sessions; (B) Spatial memory assessed in the probe test. (C) Contextual and Cued fear conditioning test. Data are plotted as the mean ± SEM (Control, n = 10; Tsc2+/-, n=10). Compared to controls, *, p<0.05; **, p<0.01, Student t test.

Figure 3. Lack of cognitive impairment in 1-month old Tsc2+/- mice. (A,B) Morris Water Maze test. (A) Escape latency to the hidden platform during learning sessions; (B) Spatial memory assessed in the probe test. (C) Contextual and Cued fear conditioning test. Data are plotted as the mean ± SEM (Control, n = 10; Tsc2+/-, n=10).

Figure 4. Lack of cognitive impairment in 1-month old Atg7CKO mice. (A) Atg7CKO mice behaved normally in an open field test. (B,C) Morris Water Maze test. (A) Escape latency to the hidden platform during learning sessions; (B) Spatial memory assessed in the probe test. (D) Contextual and Cued fear conditioning test. Data are plotted as the mean ± SEM (Control, n = 17; Atg7CKO, n=14).
conditioning test (Fig. 1D); 2) Tsc2 +/- mice showed hippocampus-related memory at 3 months of age, manifested by a reduction in spatial memory in the MWM test and contextual memory in the fear conditioning test; 3) using different cohort of mice, we found that both Atg7CKO and Tsc2 +/- mice did not show cognitive impairment at the age of 1 month (Fig. 3, 4). A visible platform trial was administered after the water maze test. All control, Atg7CKO and Tsc2 +/- mice reached a visible platform in <1 min (p > 0.05), excluding the presence of sensory deficits that may prevent the animals from identifying visual cues. Swimming speed was not reduced in the mutants (not shown), suggesting that the impaired spatial reference memory is a result of defects in cognition but not motility or altered motivation. Both Tsc2 +/- and Atg7CKO mice behaved normally in the open field tests (Fig. 3A, data for Tsc2 +/- mice not shown).

Task 2: Identify synaptic mechanisms of impaired mTOR-autophagy for cognitive dysfunction in Tsc1/2 deficient mice (Time frame: months 1-24).

We have extended our preliminary study on the characterization of CA3-CA1 synapses in P35 Atg7CKO mice. We confirmed the lack of significant differences in resting membrane potentials, membrane resistance, and membrane capacitance between control and Atg7CKO mice (Fig. 5A). Spontaneous AMPA- and NMDA-mediated EPSCs was recorded at membrane voltages of -70 mV and +40 mV, respectively, and confirmed with either AMPA (CNQX) or NMDA (APV) receptor antagonists. NMDA:AMPA ratio was constructed by dividing the peak NMDA current (at +40 mV) by the peak AMPA current (at -70 mV) for each neuron. We found that in the Atg7CKO mice the NMDA current was significantly enhanced (Fig 5B), which resulted in an increase in NMDA:AMPA ratio (Fig 5B). To determine the average quantal AMPA response of CA1 synapses, we made recordings of miniature EPSCs (mEPSC) in the presence of 50 μM D-APV and 1 μM tetrodotoxin (TTX). The mean amplitude of mEPSC events was similar between control and Atg7CKO mice (control: 27.6 ± 1.80 pA, n = 7; Atg7CKO: 29.6 ± 2.05 pA, n = 7, p > 0.05, Kolmogorov–Smirnov (KS) two-sample test), suggesting that the average synaptic density of AMPA receptors, and thus the synaptic weight of CA1 synapses, remained intact in the Atg7CKO mice. The frequency of mEPSC was significantly higher in the Atg7CKO mice than in controls (Figure 5C, Control: 1.84 ± 0.35 Hz, n=7; Atg7CKO: 3.04± 0.35 Hz. p <0.05, KS test), indicating that the number of synaptic contacts or release probability may be substantially altered.

![Figure 5. Altered CA1 synaptic activity in P35 Atg7CKO mice. (A) Passive properties of neurons in control and Atg7CKO mice. Cell capacitance and resistance values were provided by Clampex 10 acquisition software. Resting potential was estimated from zero current potential -the potential imposed to the cell to zero the pipette offset after the membrane rupture. The mean values reported for all passive properties are not statistically different (student t test). (B) NMDA- and AMPA-mediated EPSC and NMDA:AMPA ratio (right) in control and Atg7CKO mice. *, compared to controls, p<0.05; (C) mEPSC amplitude and frequency in control and Atg7CKO mice. *, compared to controls, p<0.05.](image-url)
We examined dendritic tree complexity and dendritic spine density/morphology using DiOlistic labeling with a Helios Genegun system, as in Tang et al., Neuron 2014. Compared to controls, Atg7CKO CA1 neurons did not show changes in basal dendritic tree complexity (not shown), but a remarkable increase in dendritic spine density (Fig.6A). These data were consistent with our above findings of increase mEPSC frequency in the Atg7CKO mice. Altogether, our results support an increase in functional excitatory synapses in P35 Atg7CKO mice. Note that these mice do not develop cognitive impairment at this age when excitatory synapse is overproduced. It is likely that the increase in excitatory synapses due to autophagy deficiency may be the root of cognitive impairment that occurs in a later life. We will seek further evidence during the 2nd year of this award.

To examine changes in synaptic plasticity that may account for hippocampus dependent cognitive dysfunction, we have continued our previous study on CA3-CA1 long-term potentiation (LTP), a well-known electrophysiological surrogate of hippocampus dependent learning and memory. LTP was induced with a theta-burst stimulation protocol (4 pulses at 100 Hz, with the bursts repeated at 5 Hz and each tetanus including 3 ten-burst trains separated by 15 seconds). Baseline field excitatory post-synaptic potentials (fEPSP) was recorded every minute for 15 minutes at an intensity that evokes a response approximately 35–40% of the maximum evoked response. Responses were measured as fEPSP slopes, and were expressed as percentage of baseline. Compared to controls, Atg7CKO mice showed significantly impaired LTP induction (Fig 3B).

We next asked whether autophagy might play a role in the development of long term depression (LTD) triggered upon activation of group 1 metabotropic glutamate receptors (mGluR-LTD), which is thought to contribute to learning flexibility, and is deregulated in certain types of neurodevelopmental disorders of cognition. As the selective group 1 mGluR agonist 3,5-dihydroxyphenylglycine (DHPG) effectively induces mGluR, we characterized DHPG-induced mGluR-LTD in control and Atg7CKO mice. Strikingly, autophagy deficiency significantly impaired DHPG-induced mGluR-LTD at hippocampal CA1 synapses: exposure of hippocampal slices from control mice to DHPG at 50 μM for 10 min induced robust mGluR-LTD at CA1 synapses (Fig. 6C). However, in the Atg7CKO mice, the level of DHPG-induced mGluR-LTD was substantially attenuated (Fig. 6C).

These results confirmed a blunting of hippocampal synaptic plasticity in Atg7CKO mice that specifically lack autophagy in pyramidal neurons, suggesting that normal autophagy is required for the normal development of LTP and mGluR-LTD in the hippocampus.

**Aim 3: Identify molecular mechanisms of impaired autophagy for synaptic dysfunction in Tsc1/2 deficient mice** (Time frame: months 13-36).

It is well-established that specific inhibition of mTORC1 results in sharp translational down-regulation of a set of ribosomal proteins and translation factors through the 4EBP-EIF4E
signaling axis. This interaction is facilitated by 5'-terminal oligopyrimidine (5'-TOP) tracts, which are CT-rich motifs common to the 5'-UTRs of target mRNAs. Our pilot studies computed genome-wide translation efficiencies for the rapamycin treated and untreated control and Tsc2+/− mice and ranked all genes based on their translation efficiency fold-change. Unfortunately, our preliminary results failed to reveal altered translation of neuronal enriched genes in the Tsc2+/−; RiboTag mice (n=2), suggesting that the disrupted protein and synaptic protein homeostasis in the Tsc2+/− mice may be due to suppressed protein degradation, instead of exaggerated translation. When both wt and Tsc2+/− mice were treated with rapamyin, we found that genes containing the TOP-motif are significantly enriched (padj < 0.00001) among the genes that are translationally downregulated by rapamycin treatment (Fig. 7A). We next sought to determine whether there are cell type-specific alterations in translation efficiency in response to rapamycin treatment. Our initial hypothesis is that these would be largely restricted to neurons, since the Tsc1 deletion occurs only in Camk2A-expressing cells.

Figs. 2B-C showed GSEA for translation efficiency fold-change for neuron-specific and oligodendrocyte-specific genes, respectively. Surprisingly, while there are some neuron-specific genes that are translationally downregulated by rapamycin, the effects are much more global and dramatic among genes specific to myelinating oligodendrocytes (padj < 0.00001).

In the second year, we will repeat this measure by using more replicates. If it turns true that the Tsc2 heterozygous mutation does not interfere with the translational profile and that rapamycin exerts some effect on oligodendrocyte phenotype, we will have to modify our original research strategy on ribosome profiling analysis of Tsc1/2 deficient-Autophagy knockout mice, perhaps by profiling the proteome of total hippocampal lysates and excitatory synapses (see our plan for the next reporting period), so that we can isolate the contribution of neuronal autophagy specific effects on synapse/neuronal development.

We had also initiated the complicate mouse breeding for excitatory synapse specific proteomics. We had crossed Atg7flox/flox mice to PSD95flox/flox mice. The resulting Atg7flox/++;PSD95flox/+ mice were self-crossed to obtain Atg7flox/flox;PSD95flox/flox mice (November 2016- February 2017). We next crossed the Atgflox/flox;PSD95flox/flox mice to PSD95flox/flox;CamkCre+ mice to obtain female Atg7flox/++;PSD95flox/flox;Camkcre+ breeders (March 2017- May 2017). We are now in the process of crossing Atgfox/flox;PSD95flox/flox mice to the female Atg7flox/++;PSD95flox/flox;Camkcre+ mice (June 2017-present) to obtain Atg7CKO;PSD95flox/flox and PSD95flox/flox;CamKCre mice for both synapse proteomics or synaptic protein analysis during the next reporting period.

Figure 7 Translational profiling in rapamycin treated mice. (A) Genes translationally downregulated by rapamycin treatment; (B&C) Neuronal (B) and oligodendrocyte (C) specific genes that are translationally downregulated by rapamycin.
What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state “Nothing to Report.”

In the second year of the award, we will assess whether upregulated mTOR can disrupt cognitive functions through its effects on autophagy (Aim 1). Both Tsc1/2 and Atg7 knockout Tsc1/2 deficient mice will be treated with or without rapamycin (Rapa) to isolate the effect of autophagy from other downstream events of mTOR. We will measure learning and memory cognitive functions in mice using Morris water maze and fear conditioning tests. Behaviors will be compared among Tsc2 control, Tsc2+-, Tsc2+/-:Rapa, and Tsc2+/-:Atg7CKO:Rapa mice to isolate the role of mTOR-autophagy in cognitive dysfunction in Tsc2+/- mice.

We will examine whether impaired autophagy interferes with CA3-CA1 synapse maturation during the postnatal synapse development in the TSC1/2 deficient mice (Aim 2). Acute brain slices will be cut from the hippocampus of wt mice, rapamycin treated or untreated Tsc2+/-, Tsc2+/-:Atg7CKO, Tsc1mGFAPCreCKO, and Tsc1-Atg7mGFAPCreDKO mice at postnatal day P19-20 and P35 for the measure of basal synaptic transmission, AMPA:NMDA ratio, pair pulse facilitation. We will assess the morphological changes in dendritic spine synapses using Diolistics in all mouse lines treated with or without rapamycin.

For Aim 3, we had proposed to assess the differentially translated genes between rapamycin treated or untreated Tsc2+/-, Tsc2+/-:Atg7CKO, Tsc1mGFAPCreCKO and Tsc1-Atg7mGFAPCreDKO mice. As stated above, during next reporting period, we will first repeat our preliminary studies to confirm whether Tsc2+- mutation interferes with the translational program in vivo and whether rapamycin can be used to isolate the effect of neuronal autophagy from other downstream cellular and signaling events of mTOR. If this is the case, we will modify our original research strategy on ribosome profiling analysis of Tsc1/2 deficient-autophagy knockout double mutant mice, perhaps by profiling the proteome of total hippocampal lysates. In tandem with translational or proteome analysis, we will seek to characterize excitatory synapse molecular composition. We will purify flag-tagged synaptosomes specifically from excitatory neurons from Atg7 deficient mice and in Tsc2+/- mice, treated with or without rapamycin.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state “Nothing to Report.”
Nothing to report

**What was the impact on other disciplines?**
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

Nothing to report

**What was the impact on technology transfer?**
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

Nothing to report

**What was the impact on society beyond science and technology?**
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

Nothing to report

## 5. Changes/Problems:

The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

### Changes in approach and reasons for change
*Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

Nothing to report

### Actual or anticipated problems or delays and actions or plans to resolve them
*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

Due to the accidental loss of mouse breeders, the wait for ACURO approval for animal use, and delays in hiring a postdoc after February 2017, we had been lack of sufficient mice to complete all experiments planned in the first year.

We will complete these incomplete experiments during the 2nd year. To finish all experiments planned in years 1-2 during the next reporting period, we will increase Dr. Hongyu Li’s effort to 100% in the 2nd year Dr. Xiaoping Wu will start his electrophysiological recording in the 2nd year as all mice begin to be available. He will be assisted by a second postdoc who will join the lab in September 2017.

These changes will not have a significant impact on expenditures.

### Changes that had a significant impact on expenditures


Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

**Significant changes in use or care of human subjects**

Nothing to report

**Significant changes in use or care of vertebrate animals.**

Nothing to report

**Significant changes in use of biohazards and/or select agents**

Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

**Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

**Journal publications.**

Nothing to Report

**Books or other non-periodical, one-time publications.** Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Status: awaiting publication
Acknowledgement of federal support (Not Applicable).

Other publications, conference papers, and presentations.
Nothing to report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change.”

Name: Guomei Tang
Project Role: Principle investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-9479-5331
Nearest person month worked: 1.2 months
Contribution to Project: Dr. Tang supervised the project, performed work in mouse breeding, behavioral analysis, molecular biology/RNA analysis and histology.
**Funding Support:** Dr. Tang’s funding portfolio currently includes: NIMH K01 (K01MH096956); The Simons Foundation Autism Research Initiative (SFARI) Pilot award (SFARI 40220); DOD award W81XWH-16-1-0263 and DOD W81XWH-15-1-0112.

Name: Hongyu Li
Project Role: Postdoc
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1 month
Contribution to Project: Dr. Li assisted with mouse breeding and behaviors.
Funding Support: Dr. Li’s funding portfolio currently includes The Simons Foundation Autism Research Initiative (SFARI) Pilot award (SFARI 40220) and DOD W81XWH-16-1-0263.

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

**The PI has a previously active grant closed in June 2017:**

The Simons Foundation Autism Research Initiative (SFARI) Pilot award (#345915) Sulzer, PI; Tang, PI
Title: “Neuronal translation in Tsc2+/- and Fmr1−/− mutant ASD mouse models”

What other organizations were involved as partners?
*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**

**COLLABORATIVE AWARDS:** For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.
QUAD CHARTS:  If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Nothing to Report

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.