INTRODUCTION

As final differentiated cells, mature neurons lose the ability to generate new neurons, contributing to the lack of a cure for neurodegeneration. Fortunately, a group of neural stem cells (NSCs) are preserved in the dentate gyrus of the hippocampus to generate new neurons in the adult mammal [1, 2].

Lipopolysaccharide-induced Autophagy Increases SOX2-positive Astrocytes While Decreasing Neuronal Differentiation in the Adult Hippocampus

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Inflammation alters the neural stem cell (NSC) lineage from neuronal to astrogliogenesis. However, the underlying mechanism is elusive. Autophagy contributes to the decline in adult hippocampal neurogenesis under E. coli lipopolysaccharide (LPS) stimulation. SRY-box transcription Factor 2 (SOX2) is critical for NSC self-renewal and proliferation. In this study, we investigated the role of SOX2 in induced autophagy and hippocampal adult neurogenesis under LPS stimulation. LPS (5 ng/100 g/hour for 7 days) was intraperitoneally infused into male Sprague–Dawley rats (8 weeks old) to induce mild systemic inflammation. Beclin 1 and autophagy protein 12 (Atg12) were significantly upregulated concurrent with decreased numbers of Ki67- and doublecortin (DCX)-positive cells in the dentate gyrus. Synchronously, the levels of phospho(p)-mTOR, the p-mTOR/mTOR ratio, p-P85s6k, and the p-P85s6k/P85s6k ratio were suppressed. In contrast, SOX2 expression was increased. The fluorescence micrographs indicated that the colocalization of Beclin 1 and SOX2 was increased in the subgranular zone (SGZ) of the dentate gyrus. Moreover, increased S100β-positive astrocytes were colocalized with SOX2 in the SGZ. Intracerebroventricular infusion of 3-methyladenine (an autophagy inhibitor) effectively prevented the increases in Beclin 1, Atg12, and SOX2. The SOX2+–Beclin 1+ and SOX2+–S100β+ cells were reduced. The levels of p-mTOR and p-P85s6k were enhanced. Most importantly, the number of DCX-positive cells was preserved. Altogether, these data suggest that LPS induced autophagy to inactivate the mTOR/P85s6k pathway, resulting in a decline in neural differentiation. SOX2 was upregulated to facilitate the NSC lineage, while the autophagy milieu could switch the SOX2-induced NSC lineage from neurogenesis to astrogliogenesis.

Key words: Autophagy, SOX2, mTOR signaling, Adult hippocampal neurogenesis, Lipopolysaccharide

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formation from neuronal stem cells (NSCs) to neural progenitor cells (NPCs) required for neural differentiation and maturation in adulthood is known as adult neurogenesis [3]. These newborn neurons integrate into the existing circuit of the hippocampus to maintain and improve learning and memory function [4-11]. Along the birth of new neurons, NSCs also produce astrocytes [12, 13]. Inflammation alters NSC differentiation and leads to a shift in lineage from neuronal to astrogliogenesis [14, 15], resulting in decreased newborn neurons. However, the underlying mechanisms are inconclusive.

Systemic administration of lipopolysaccharide (LPS) induces autophagy [16] and impairs NSC proliferation and differentiation in the adult hippocampus [2, 17-19]. Autophagy, which progresses the degradation of unwanted cell components, is initiated by the nucleation and elongation of phagophores. The formation of the autophagosomal membrane is mediated by the Class III PI3K complex, Beclin 1 with a multiprotein complex, while autophagy protein 12 (Atg12) conjugates to Atg5 for the formation of phagophores for further protein engulfment or organelle degradation [20]. Although autophagy-mediated astrogliogenesis in adult hippocampal neural stem cells has been documented [21], the underlying mechanism is still inconclusive.

In contrast to autophagy, accumulating evidence suggests that mTOR/s6k signaling governs protein synthesis to activate cell growth and division [22, 23]. After activation, mTOR activates s6k by phosphorylation to support neuronal differentiation [24, 25]. To date, the following two major isoforms of s6k have been identified: P70s6k, which is the cytoplasmic isoform, and P85s6k, which is the nuclear isoform [26]. Whether the wax and wane of autophagy and mTOR signaling occur in the hippocampus under LPS is unclear.

SOX2 is a transcription factor crucial for promoting the self-renewal and proliferation of NSCs [27]. Recently, SOX2-positive cells colocalized with autophagosomes were detected in the dentate gyrus of animals under stress [28]. Notably, SOX2 is widely expressed in hippocampal astrocytes [12]. These lines of evidence indicate the possibility that SOX2 might mediate LPS-induced autophagy to reduce neuron differentiation by inducing astrocyte production. Nonetheless, the interplay among SOX2 in LPS-induced autophagy, mTOR/s6k signaling, and adult hippocampal neurogenesis is unclear.

Peripheral or central LPS administration is a well-accepted model used to investigate the stress-associated decline in adult neurogenesis. Sustained, lower grade LPS administration is related to a mild level of systemic inflammation, such as microbiota dysbiosis [29, 30]. In this study, we examined the contribution of SOX2 and autophagy to the decline in adult neurogenesis in the hippocampus by using a rodent model of sustained, low-level *E. coli* lipopolysaccharide (LPS) peritoneal infusion (IP) mimicking microbiota dysbiosis-associated LPS increments [29, 30]. 3-Methyladenine (3MA, a common inhibitor of autophagy) or vehicle was intracerebroventricularly (icv) infused for seven days. The protein expression and distribution of the autophagy factors mTOR signaling, SOX2, cell proliferation, and neuronal differentiation in the dentate gyrus of the hippocampus were detected to identify the roles of autophagy and SOX2 in LPS-decreased adult neurogenesis.

**MATERIALS AND METHODS**

Male, 7-week-old Sprague–Dawley rats (SD from the Experimental Animal Center, National Science Council, Taiwan) were used in this study and allowed to acclimatize in a temperature- (22 ± 1 °C) and light- (12:12 light-dark cycle, light on from 08:00) controlled animal room for one week before the experiments. All experiments were carried out in accordance with the guidelines for animal experimentation endorsed by the Institutional Animal Care and Use Committee (IACUC) of Kaohsiung Chang Gung Memorial Hospital. The animals consumed regular chow *ad libitum* as the only food source. Their body weight and food and liquid intake per cage were measured and recorded each week. *Escherichia coli* LPS was intraperitoneally infused, and 3-methyladenine (3MA) was intracerebroventricularly infused (icv) by an osmotic minipump for 7 days. After deep anesthetization and transcardial perfusion with sterile saline, the forebrain was sampled for further study. A schematic depicting the study design is shown in Fig. 1.

**Implantation of osmotic minipump of lipopolysaccharide**

Regarding the intraperitoneal infusion (IP), systemic inflammation was induced by continuous intraperitoneal infusion via an osmotic minipump of LPS (5 ng·100 g·hour⁻¹ for 7 days; Sigma–Aldrich, St. Louis, MO, USA). On the day of implantation, animals were anesthetized with sodium pentobarbital (50 mg/kg, IP), and...
osmotic minipumps (ALZET® Micro-Osmotic Pump Model 1007D; Durect, Cupertino, CA, USA) were placed in the peritoneal cavity. Control animals received saline-filled osmotic minipumps, and the sham-operated animals underwent identical surgical procedures with the implantation of a vehicle-filled osmotic minipump. Following implantation, the abdominal muscles were closed in layers, and body temperature was maintained at 37°C with a heating pad until the animals recovered from anesthesia.

**Intracerebroventricular infusion**

Regarding the animals that received additional central infusion of the test agents, rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow were introduced for anesthesia. Then, the rats were placed in a standard stereotaxic device equipped with a gas anesthesia nose cover to maintain anesthesia throughout the surgery with 2% isoflurane and 600 ml/min oxygen flow. A drug-filled osmotic pump was surgically implanted with an ALZET Brain Infusion probe (Alzet 1002) into the right lateral ventricle with the tip of the infusion probe at the following coordinates with reference to Bregma: anterior/posterior -1.4 mm; medial/lateral 1.8 mm; and dorsal/ventral -3.0 mm (Paxinos and Watson, 2013 [31]). Intracerebroventricular infusion (icv) of 3MA (5 mM, infusion rate: 0.5 µl/hour, ALZET® MICRO-OSMOTIC PUMP MODEL 1007D) was carried out for 7 days. An infusion of sterile saline (Sal) served as the volume and vehicle control. Only animals that showed progressive weight gain after the operation were used in the subsequent experiments.

**Immunofluorescence**

To identify the distribution of SOX2 and Beclin 1, the forebrains were sampled and postfixed in 4% paraformaldehyde for 72 hours at 4°C after transcardial perfusion. Furthermore, the forebrains were cryoprotected with 30% sucrose solution. Then, the forebrains were sliced with a freezing microtome at a thickness of 30 µm. The paraformaldehyde-fixed brain sections were stained with mouse anti-Ki67 (1:1,000, Abcam, CA) for newly proliferated cells and goat anti-doublecortin (DCX, 1:750; Abcam, CA) for immature neurons. Immunohistochemical staining was performed using a Vectorstain ABC system (Vector Laboratories, Burlingame, CA) and nickel-enhanced diaminobenzidine incubation. The images were acquired and processed under an Olympus light microscope (BX51, Tokyo, Japan).

**Cell counting**

The entire hippocampal dentate gyrus was sliced into an average of 193 coronal sections at a thickness of 30 µm. The numbers of Ki67- and DCX-positive cells, which were stained black, were counted in every 6th section. Only the Ki67-positive cells located in the subgranular zone of the dentate gyrus were counted as the targeted proliferation cells. The total number of labeled cells per section was determined and divided by the slide selection ratio to obtain the total number of labeled cells per dentate gyrus [19].

**Total protein extraction**

For the Western blot and ELISA analyses, the procedures were conducted as previously described [25, 26, 32]. In brief, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg; Sigma) and perfused with phosphate-buffered saline. The fresh brains were dissected, and tissue samples from the hippocampus were homogenized with a Dounce grinder and a tight pestle in ice-cold lysis buffer (15 mM HEPES, pH 7.2, 60 mM KCl, 10 mM NaCl, 15 mM MgCl₂, 250 mM sucrose, 1 mM EGTA, 5 mM EDTA, 1 mM PMSE, 2 mM NaF, and 4 mM Na₃VO₄). A mixture of leupeptin (8 µg/ml), aprotinin (10 µg/ml), phenylmethylsulfonyl
fluoride (20 µg/ml), and trypsin inhibitor (10 µg/ml) was included in the isolation buffer to prevent protein degradation. The homogenate was centrifuged at 13,500 rpm for 15 minutes, and the supernatant was collected for protein analysis. The concentration of the total protein extracted was estimated by the Bradford method with a protein assay kit (Bio-Rad, Hercules, CA).

**Western blot analysis**

The proteins of interest in the hippocampus were separated by using 10–12% SDS–PAGE. The samples from each group contained equivalent total protein concentrations. The proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P membrane; Millipore; Bedford, MA, USA). The membranes were probed with specific antibodies against Beclin-1 (1:1,000, Cell Signaling), Atg5 (1:1,000, Cell Signaling), Atg12 (1:1,000, Cell Signaling), SQSTM1 (1:1,000, Cell Signaling), LC3B (1:1,000, Sigma-Aldrich), mTOR (1:1,000, Cell Signaling), phospho(p)-mTOR (1:1,000, Sigma–Aldrich), P70s6k (1:1,000, Cell Signaling), p-P70s6k (1:1,000, Cell Signaling), P85s6k (1:1,000, Cell Signaling), and p-P85s6k (1:1,000, Cell Signaling). Then, the membranes were incubated with the appropriate horseradish peroxidase–conjugated secondary antibody. Specific antibody-antigen complexes were detected using an enhanced chemiluminescence Western blot detection system (Thermo Fisher Bioscience). The bands of the targeted proteins were densitometric quantified by ImageJ software (NIH, MD, USA), and the amounts of detected proteins are expressed as the ratio to β-actin protein.

**Statistical analysis**

All values are expressed as the mean±SEM. For the biochemical experiments involving multiple groups, one-way analysis of variance with repeated measures was used to assess the group means. This analysis was followed by a Tukey multiple range test as a post hoc assessment of the individual means. p<0.05 was considered statistically significant.

**RESULTS**

**The LPS-induced increase in Beclin 1 and Atg12 in the hippocampus was reversed by 3MA intracerebroventricular infusion**

LPS (5 ng/100 g •hour•1 for 7 days, 1P) was administered to 8-week-old male Sprague Dawley rats to induce sustained, low-grade systemic infection concurrent with 3MA (a Class III PI3K inhibitor) icv infusion for 7 days (Fig. 1). The hippocampus was harvested at the end of the experiment for Western blot analyses. The results indicated that the expression levels of Beclin 1 (Fig. 2A; an essential factor for the nucleation of autophagy [33]) and Atg12 (Fig. 2C; an ubiquitin-like protein that contributes to autophagy vesicle formation [20, 34]) in the hippocampus were significantly upregulated by LPS administration. As a key factor for autophagosome formation, SQSTM1 directly binds LC3 to facilitate protein degradation by autophagy [35]. The results of the Western blot analyses further indicated that the expression levels of SQSTM1 (Fig. 2D), LC3B-I (Fig. 2E), and LC3B-II (Fig. 2F) were significantly enhanced in the LPS group. The 3MA treatment effectively abrogated these increases in Beclin 1 (Fig. 2A), Atg12 (Fig. 2C), SQSTM1 (Fig. 2D), and LC3B-II (Fig. 2F), while the increase in LC3B-I (Fig. 2E) was not reduced by 3MA. On the other hand, Atg5 did not significantly differ between groups (Fig. 2B). These results indicate that peritoneal LPS administration triggered the upregulation of hippocampal autophagy in seven days.

**3MA prevented LPS-decreased cell proliferation and neuronal differentiation in the dentate gyrus**

The cell counts of Ki67 (a marker of dividing cells)-positive cells and doublecortin (DCX, a marker of immature newborn neurons)-positive cells in the subgranular zone of the dentate gyrus were counted on Day 7 after LPS infusion. The results indicate that the numbers of dividing cells (Fig. 3A) and newborn neurons (Fig. 3B) were significantly reduced in the LPS group compared with those in the control group (Sal).

In the 3MA treatment group (L+3MA), the number of DCX-positive cells in the dentate gyrus was effectively maintained at the control level compared with that in the LPS group (Fig. 3B). On the other hand, 3MA had no significant effect on preventing the loss of Ki67-positive cells compared with the LPS group (Fig. 3A). These results suggest that LPS might decrease cell proliferation and differentiation in the hippocampus through different pathways. In particular, autophagy might mediate the decline in neuronal differentiation instead of cell proliferation.

**3MA prevented LPS-downregulated mTOR signaling in the hippocampus**

The mammalian target of rapamycin (mTOR), a central cell-growth regulator, inhibits autophagy [36]. To examine whether mTOR signaling was suppressed concurrent with LPS-enhanced autophagy, mTOR signaling was detected by Western blot analysis. The results indicated that the levels of total mTOR showed a decreasing trend in the LPS group, although no statistically significant difference was detected (Fig. 4A). On the other hand, expression of phospho(p)-mTOR (Fig. 4B) was significantly decreased.
Autophagy-lifted SOX2 Reduces Neurogenesis

in the LPS group. To further determine whether the decrease in p-mTOR was a result of mTOR reduction or inactivation of mTOR, the ratio of p-mTOR/mTOR was further evaluated. The results indicated that p-mTOR/mTOR was significantly reduced in the LPS group (Fig. 4C), suggesting inactivation of mTOR in the hippocampus under LPS administration. The reduced p-mTOR and p-mTOR/mTOR ratios imply a decrease in mTOR activation in the hippocampus by LPS stimulation. The 3MA icv infusion effectively prevented the reduced p-mTOR and p-mTOR/mTOR ratio.

Activated mTOR phosphorylates s6k, the downstream signal for protein synthesis, to support neuronal differentiation [24, 25]. There are two major isoforms of s6k as follows: P70s6k, which is the cytoplasmic isoform, and P85s6k, which is the nuclear isoform [26]. The results of the Western blot analysis indicated that the phosphorylation of the nuclear isoform P85s6k (Fig. 5A) and the ratio of p-P85s6k/P85s6k (Fig. 5C) were significantly downregulated in the LPS group, while the total P85s6k expression (Fig. 5B) showed a decreasing trend in the LPS group. However, no significant alterations were detected in p-P70s6k (Fig. 5D), P70s6k (cytoplasmic isoform; Fig. 5E), or the ratio of p-P70s6k/P70s6k (Fig. 5F). 3MA effectively prevented the reduction in P85s6k at both the expression and phosphorylation levels. These results further suggest that mTOR/P85s6k inactivation might lead to LPS-suppressed adult neurogenesis in the hippocampus.

3MA prevented the LPS-enhanced SOX2 in the hippocampus

SOX2 is a critical transcription factor in stem cells and plays an important role in the self-renewal and proliferation of NSCs [18] in the subgranular zone (SGZ) of the adult dentate gyrus. Immunofluorescence and Western blot analyses were conducted to detect the distribution and expression of SOX2, respectively. The immunofluorescence results (Fig. 6A) indicated that SOX2 (green) was detectable in the vehicle control group (Sal) and that the SOX2 signal was not restricted to the SGZ. In the LPS group, signal density and distribution of SOX2 were largely increased, particularly in the SGZ. To determine whether autophagy contributes to LPS-induced SOX2 upregulation, 3MA administration was synchronized with LPS infusion. Consistent with the immunofluorescence evidence, the protein expression of SOX2 was significantly upregulated in the hippocampus by LPS, which was prevented by 3MA treatment (Fig. 6B). These results indicate that 3MA effectively

**Fig. 2.** The increased protein expression of autophagy signaling in the hippocampus induced by LPS administration was prevented by icv infusion of 3MA. Representative gels (inset) and densitometric analysis of the Western blot results showing changes in the expression of (A) Beclin 1, (B) Atg5, (C) Atg12, (D) SQSTM1, (E) LC3B-I, and (F) LC3B-II. Values are the mean±SEM, n=10 to 12 animals per experimental group. *p<0.05, **p<0.01, ***p<0.001 in the post hoc Tukey’s multiple range tests. Sal: vehicle infusion, LPS: LPS (IP) with vehicle (icv), LPS+3MA: LPS (IP) infusion with 3MA (icv).
prevented LPS-induced SOX2 expression in the hippocampus.

**The LPS-altered SOX2'-Beclin 1' cells in the dentate gyrus were prevented by 3MA administration**

Based on the above results, we further investigated whether autophagy-associated SOX2 upregulation is an intracellular or intercellular effect by using double and triple immunofluorescence. The results from NeuN (a marker of mature neurons)-Beclin 1 immunofluorescence indicate that in the Sal group, some punctate signals of Beclin 1 (green) were located in the cytosol of neurons (red) in the granular layer of the dentate gyrus (Fig. 7A). The punctate signals of Beclin 1 in the granular layer were largely increased by LPS. The immunofluorescence of SOX2 (red)-Beclin 1 (green)-DAPI (blue) further indicated that rare SOX2'-Beclin 1' cells were detected in the SGZ of the dentate gyrus in the Sal group (Fig. 7B). Notably, the number of SOX2'-Beclin 1' cells (in-
Autophagy-lifted SOX2 Reduces Neurogenesis

The decreased protein expression of mTOR signaling in the hippocampus of animals treated with LPS was prevented by icv infusion of 3MA. Representative gels (inset) and densitometric analysis of the Western blot results showing changes in the expression of (A) mTOR, (B) phospho(p)-mTOR and the (C) p-mTOR/mTOR ratio. Values are the mean±SEM, n=10 to 12 animals per experimental group. *p<0.05, *** p< 0.001 in the post hoc Tukey’s multiple range tests. Sal: saline infusion, LPS: LPS IP infusion with saline (icv), LPS+3MA: LPS (IP) with 3MA (icv).

Suppression of s6k in the hippocampus of animals treated with LPS peritoneal infusion was prevented by icv infusion of 3MA. The expression of (A) p-P85s6k (85 kDa; phosphorylated nuclear isoform), (B) p85s6k (nuclear isoform), (C) the p-p85s6k/p85s6k ratio, (D) p-P70s6k (70 kDa; phosphorylated cytoplasmic isoform), (E) p70s6k (cytoplasmic isoform), and (F) the p-p70s6k/p70s6k ratio in the hippocampus. Values are the mean±SEM, n=10 to 12 animals per experimental group. **p<0.01 in the post hoc Tukey’s multiple range tests. Sal: saline infusion, LPS: LPS (IP) with saline (icv) infusion, LPS+3MA: LPS (IP) with 3MA (icv).

The LPS-altered SOX2⁺⁻p-mTOR⁺ cells in the dentate gyrus were prevented by 3MA administration

Furthermore, NeuN (red)-p-mTOR (green) double labeling was
conducted. The results indicated that p-mTOR signals (green) were located in the cytosol of neurons (red) in the granular layer of the dentate gyrus in the Sal group (Fig. 8A). The punctate signals of p-mTOR in the granular layer were largely decreased by LPS. The images from SOX2 (red)-p-mTOR (green)-DAPI (blue) triple staining further indicated that SOX2\(^*\)-p-mTOR\(^*\) cells (indicated by the yellow arrowhead in the SOX2\(^*\)-p-mTOR\(^*\) merged micrograph) were primarily detected in the SGZ of the dentate gyrus in the Sal group (Fig. 8B). The number of SOX2\(^*\)-p-mTOR\(^*\) cells was decreased in the LPS group (Fig. 8B). In the LPS+3MA group, the p-mTOR (Fig. 8A) and SOX2\(^*\)-p-mTOR\(^*\) (Fig. 8B) signals were maintained at the same levels as those in the Sal group. The quantification data of fluorescent signals further demonstrated these image observations (Table 2).

**The LPS-altered SOX2\(^*\)-p-s6k\(^*\) cells in the dentate gyrus were prevented by 3MA administration**

In addition, NeuN (red)-p-s6k (green) double labeling was conducted. Similarly, the results indicate that p-s6k signals (green) were located in the cytosol of neurons (red) in the granular layer of the dentate gyrus in the Sal group (Fig. 9A). The punctate signals of p-s6k in the granular layer were largely decreased by LPS. The images from SOX2 (red)-p-s6k (green)-DAPI (blue) triple staining further indicated that SOX2\(^*\)-p-s6k\(^*\) cells (indicated by the yellow arrowhead in the SOX2\(^*\)-p-s6k\(^*\) merged micrograph) were primarily detected in the SGZ of the dentate gyrus in the Sal group (Fig. 9B). The number of SOX2\(^*\)-p-s6k\(^*\) cells was decreased in the LPS group (Fig. 9B). In the LPS+3MA group, the p-s6k (Fig. 9A) and SOX2\(^*\)-p-s6k\(^*\) (Fig. 9B) signals were maintained at the same levels as those in the Sal group. These observations were further demonstrated by the quantification data of fluorescent signals.

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**Fig. 6.** The LPS-induced increase in SOX2 expression in the hippocampus was reversed by icv infusion of 3MA. (A) Representative immunofluorescence images showing the signals of SOX2 (green) in the hippocampus in the Sal, LPS, and LPS+3MA groups. (B) Representative gels (inset) and densitometric analysis of the Western blot results showing changes in the expression of SOX2. Values are the mean±SEM, n=10-12 animals per experimental group. *p<0.05 in the post hoc Tukey’s multiple range tests. SOX2: SRY-Box Transcription Factor 2. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine. Scale bar: 20 µm.
Fig. 7. The Beclin 1 signal was primarily in hippocampal neurons, and some SOX2+ cells with Beclin 1+ signals were in the dentate gyrus. Representative immunofluorescence images showing (A) NeuN+ (red)-Beclin 1+ (green), and (B) SOX2+ (red)-Beclin 1+ (green)-DAPI (blue) in the hippocampus in the Sal, LPS, or LPS+3MA groups. DAPI was used to identify the nuclei. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine. SOX2: SRY-box Transcription Factor 2. Scale bar: 20 µm. Yellow arrowhead: double-labeled cells.
SOX2

renewal and differentiation to support the existing neural circuits. Autophagy might trigger SOX2 upregulation to increase NSC self-renewal and differentiation in the adult hippocampus by

stimulation (such as microbiota dysbiosis) could enhance the autophagy level in the hippocampus within one week. The reduction of astrocytes at the mature stage [39]. To investigate whether LPS-induced SOX2-positive cells are prone to astrocytic differentiation under LPS, SOX2 (green) and S100β (red) double labeling was conducted. In the control group, SOX2-S100β was rarely detected under LPS, SOX2 (green) and S100β (red) double labeling was conducted. In the control group, SOX2-S100β was rarely detected (Fig. 10). Concurrent with the increased SOX2-positive cells in the dentate gyrus, the S100β signal was enhanced in the LPS group. Most importantly, the SOX2 signal was largely colocalized with the S100β signal (indicated by the yellow arrow). In the LPS+3MA group, less SOX2-S100β colocalized signal was detected in the hippocampus.

3MA prevented LPS-enhanced astrocytic SOX2 expression in the hippocampus

SOX2-positive NSCs are capable of differentiating into neurons or astrocytes [37, 38]. A decrease in neuronal differentiation might be a result of increased astrocytic differentiation. S100β is a marker of astrocytes at the mature stage [39]. To investigate whether LPS-induced SOX2-positive cells are prone to astrocytic differentiation under LPS, SOX2 (green) and S100β (red) double labeling was conducted. In the control group, SOX2-S100β was rarely detected (Fig. 10). Concurrent with the increased SOX2-positive cells in the dentate gyrus, the S100β signal was enhanced in the LPS group. Most importantly, the SOX2 signal was largely colocalized with the S100β signal (indicated by the yellow arrow). In the LPS+3MA group, less SOX2-S100β colocalized signal was detected in the hippocampus.

DISCUSSION

In this study, we demonstrated that the induced neuronal upregulation of Beclin 1, Atg12, SQSTM1, and LC3B-II concurrent with the downregulation of mTOR/s6k signaling contributed to an increase in SOX2-positive cells with a reduction in cell proliferation and neuronal differentiation in the adult hippocampus by sustained, low-grade peripheral LPS infusion. Intriguingly, Beclin 1-/-SOX2+ cells were increased, while p-mTOR-/-SOX2- and s6k-/-SOX2+ cells were reduced by LPS administration. Notably, a higher proportion of SOX2 signal was colocalized with S100β+ astrocytes in the LPS group than in the Sal group. 3MA effectively prevented the aforementioned changes. These results suggest that neuronal autophagy might trigger SOX2 upregulation to increase NSC self-renewal and differentiation to support the existing neural circuits. Unfortunately, the autophagy-associated microenvironment might lead SOX2+ cells to undergo astrogliogenesis instead of neurogenesis.

Autophagy plays dual roles in regulating NSC fates under physiological and pathological conditions [28, 40]. For instance, autophagy is involved in cell proliferation, differentiation, and maturation during neuron development. Similarly, our data further indicate that autophagy was detectable in the adult hippocampus in the control group (Sal) in this study. In contrast, the sustained activation of autophagy leads to cell death [41]. It has been reported that LPS suppresses adult neurogenesis [20] by inhibiting NSC proliferation, neural differentiation, and newborn neuron survival [30, 32-38], which have been strongly linked to the status of neuroinflammation. Our previous study demonstrated that sustained, low-grade peripheral LPS stimulation triggers neuroinflammation [26], mimicking a mild level of systemic infection. In support of previous studies [42], we demonstrated that sustained, low-grade peripheral LPS stimulation suppressed cell proliferation and neuronal differentiation in the SGZ of the dentate gyrus. 3MA prevented LPS-reduced neural differentiation and had limited capacity to prevent the suppressed cell proliferation. This evidence implies that LPS-induced autophagy might predispose to a reduction in neural differentiation in the adult hippocampus.

Table 1. Fluorescence intensity of SOX2, Beclin 1, and colocalized SOX2-Beclin 1 in the Sal, LPS and LPS+3MA groups

| Fluorescence | Sal | LPS | LPS+3MA |
|-------------|-----|-----|---------|
| SOX2 Area   | 4.889±0.21     | 7.474±5.36      | 4.778±4.70      |
| Raw intensity (RawIntDen) | 786.51±0.470    | 1.147±0.7553     | 876.75±4.7054    |
| Beclin 1 Area | 2.387±0.64     | 12.50±2.385     | 7.93±3.012       |
| Raw intensity (RawIntDen) | 491.33±9.51     | 2.267±0.49052    | 608.74±8.219862  |
| SOX2+Beclin 1 Area | 705±0.68 | 3.73±4.1999     | 229±8.7995     |
| Raw intensity (RawIntDen) | 142.61±8.554   | 426.16±6.31198   | 45.81±18.1267   |
| Values are the mean±SEM, n=3 animals per experimental group. "p<0.05, "p<0.01 vs. Sal group and "p<0.05, "p<0.01 vs. LPS group in the post hoc Tukey's multiple range tests. SOX2: SRY-Box Transcription Factor 2. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine.

(Table 3). This evidence suggests that LPS-altered autophagy and mTOR signaling primarily occurred in the granular layer of the dentate gyrus and that autophagy-associated SOX2 upregulation was mostly an intercellular effect with some intercellular effect.

(3MA). This evidence suggests that LPS-altered autophagy and mTOR signaling primarily occurred in the granular layer of the dentate gyrus and that autophagy-associated SOX2 upregulation was mostly an intercellular effect with some intercellular effect.
Fig. 8. The phospho(p)-mTOR signal was primarily in hippocampal neurons, and some SOX2+ cells with p-mTOR+ signals were in the dentate gyrus. Representative immunofluorescence images showing (A) NeuN+ (red)-p-mTOR+ (green), and (B) SOX2+ (red)-p-mTOR+ (green)-DAPI (blue) in the hippocampus in the Sal, LPS, or LPS+3MA groups. DAPI was used to identify the nuclei. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine. p-mTOR: phospho-mTOR. SOX2: SRY-box Transcription Factor 2. Scale bar: 20 µm. Yellow arrowhead: double-labeled cells.
and prevent impairment in neural differentiation. However, the inhibition of autophagy had no significant effect on maintaining the numbers of Ki67-positive cells in the SGZ. Our previous studies demonstrated that neuroinflammation is induced by systemic inflammation in this animal model [25, 26, 32]. Neuroinflammation sharply decreases adult hippocampal neurogenesis via activation of microglia [2]. It is well accepted that cytokines trigger Ki67-positive cell loss [43]. Therefore, it is possible that the irreversible cell proliferation in this study may be due to the sustained inflammatory microenvironment.

Notably, the wax and wane of autophagy and mTOR are crucial for the maintenance of neurogenesis. mTOR in the neural stem cell niche positively regulates downstream phospho(p)-s6k-induced protein synthesis to facilitate neuronal differentiation by the phosphorylation cascade [44]. It is conceivable that the inhibition of mTOR signaling facilitates the execution of autophagy to negatively control neurogenesis [45]. Consistent with previous studies [44, 45], our immunofluorescence data indicate that both the p-mTOR, p-s6k, and Beclin 1 signals were primarily located in the granular layer of the dentate gyrus instead of the SGZ. Combined with the semiquantification data by Western blot analysis and the distribution evidence by immunofluorescent staining, we found that the p-mTOR/p-s6k signal was higher with a low Beclin 1 signal in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal [22, 48]. Whether the activation of TSC1 and TSC2 mediates the LPS-associated inhibition of mTOR activity in the hippocampus requires further study.

Table 2. Fluorescence intensity of SOX2, p-mTOR, and colocalized SOX2-p-mTOR in the Sal, LPS, and LPS+3MA groups

| Fluorescence | Sal | LPS | LPS+3MA |
|--------------|-----|-----|---------|
| SOX2 Area    | 4,700±423.3 | 7,077±337.5*** | 3,874±678.7** |
| Raw intensity (RawIntDen) | 1,604±69.04 | 2,452±86.026* | 791,341±136,149** |
| p-mTOR Area  | 9,095±639.1 | 2,893±310.8 | 13,125±1,981*** |
| Raw intensity (RawIntDen) | 1,961±182,704 | 636,602±120,655* | 2,69×10^4±422,897*** |
| SOX2+p-mTOR Area | 2,839±55.19 | 556±18.61*** | 1,724±279.1*** |
| Raw intensity (RawIntDen) | 266,093±8,018 | 54,912±645.2*** | 153,426±21,742*** |

Values are the mean±SEM, n=3 animals per experimental group. *p<0.05, **p<0.01, ***p<0.001 vs. Sal group and "p<0.01, ""p<0.001 vs. LPS group in the post hoc Tukey’s multiple range tests. p-mTOR: phospho-mTOR, SOX2: SRY-Box Transcription Factor 2. Sal: saline infusion, LPS: peritoneal infection (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine.

SOX2 promotes the self-renewal and proliferation of NSCs [27]. Similarly, our data further indicate that the SOX2 signal primarily accumulates in the SGZ, although it was not restricted to this area in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal in the control group (Sal). Furthermore, we found that in the SGZ, some p-mTOR puncta (cytosol) were close to the SOX2 signal in the control group (Sal).
Autophagy-lifted SOX2 Reduces Neurogenesis

**Fig. 9.** The p-s6k signal was primarily in hippocampal neurons, and some SOX2⁺-p-s6k⁺ cells were in the dentate gyrus. Representative immunofluorescence images showing (A) SOX2⁺ (red)-p-s6k⁺ (green) and (B) SOX2⁺-p-s6k⁺ (green) in the hippocampus in the Sal, LPS, or LPS+3MA groups. DAPI (blue) was used to identify the nuclei. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine. SOX2: SRY-box Transcription Factor 2. Scale bar: 20 µm. Yellow arrowhead: double-labeled cells.
munofluorescence further indicates that the LPS-enhanced SOX2 signal located in astrocytes in the SGZ was increased. These lines of evidence imply that the decreased neural differentiation might be a result of increased astrocyte differentiation from SOX2-positive cells. LPS-enhanced autophagy might contribute to a switch in differentiation. Microenvironment changes, for example, LPS-reduced BDNF [49], might play roles in the waning of mTOR signaling [50] and neural differentiation [51]. Nonetheless, the interaction between neurons in the granular layer and SOX2+ cells in the neural stem cell niche under LPS-induced autophagy requires further investigation.

Altogether, the data from this study indicate that LPS-impaired adult neurogenesis was mediated by the upregulation of autophagy and the suppression of mTOR/P85s6k signaling. The increased number of SOX2-positive cells contributes to the increase in astrocytic differentiation. 3MA treatment prevented LPS-associated autophagy and the inactivation of mTOR to maintain neuronal differentiation in the adult hippocampus.

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### Table 3. Fluorescence intensity of SOX2, p-S6k, and colocalized SOX2-p-S6k in the Sal, LPS and LPS+3MA groups

| Fluorescence | Sal               | LPS               | LPS+3MA            |
|--------------|-------------------|-------------------|--------------------|
|              | Area              | 5.228±140.2       | 6.877±497.9*       | 4.141±307.3**      |
|              | Raw intensity (RawIntDen) | 1.051×10^6±22368 | 1.516×10^6±160.491* | 837.862±19.242**   |
| p-S6k        | Area              | 15.923±872.4      | 3.222±574.2***     | 9.445±837.7***     |
|              | Raw intensity (RawIntDen) | 3.153×10^6±134761 | 692.404±136992***  | 2.183×10^6±302.913*** |
| SOX2+p-S6k   | Area              | 2.887±211.0       | 465±777.8*         | 3.614±561.3***     |
|              | Raw intensity (RawIntDen) | 635.061±26.374   | 99.761±61.819*     | 585.605±162.954*   |

Values are the mean±SEM, n=3 animals per experimental group. *p<0.05, ***p<0.001 vs. Sal group and "p<0.05, ""p<0.01, """"p<0.001 vs. LPS group in the post hoc Tukey’s multiple range tests. SOX2: SRY-Box Transcription Factor 2. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine.

Fig. 10. The LPS-increased SOX2 signal colocalized with astrocytes in the hippocampus and was prevented by icv infusion of 3MA. Representative immunofluorescence images showing the SOX2 signal (green) colocalized with S100β in the hippocampus in the Sal, LPS, or LPS+3MA groups. DAPI (blue) was used to identify the nuclei. Sal: saline infusion, LPS: LPS peritoneal infusion (IP) with saline icv infusion, LPS+3MA: LPS (IP) with 3MA (icv). icv: intracerebroventricular infusion. 3MA: 3-Methyladenine. SOX2: SRY-Box Transcription Factor 2. Scale bar: 20 μm. Yellow arrowhead: SOX2-S100β double-labeled cells.
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ETHICS APPROVAL

All experiments were carried out in accordance with the guidelines for animal experimentation endorsed by the Institutional Animal Care and Use Committee (IACUC) of the Kaohsiung Chang Gung Memorial Hospital. IACUC number: 201506220.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed in this study are included in this article.

AUTHORS’ CONTRIBUTIONS

CWW and KLHW conceived and designed the study, analyzed, and interpreted the data, and wrote the manuscript. CWW, CYH, and ICC performed the animal experiments and tissue sampling. CWW, CYH, ICC, YCL, and CYW performed the molecular analyses, data acquisition and statistical analysis. CWW and KLHW critically revised the manuscript for important intellectual content. The authors read and approved the final version of this manuscript.

DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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www.enjournal.org 321
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