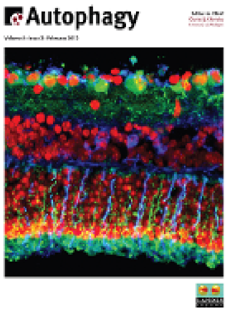

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Atg5 and Ambra1 differentially modulate neurogenesis in neural stem cells

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Keywords: autophagy, Ambra1, Atg5, neural progenitors, neurogenesis, neuritogenesis, differentiation

Abbreviations: Ambra1, activating molecule in Beclin 1-regulated autophagy; *Atg5*, autophagy-related gene 5; *Atg7*, autophagy-related gene 7; eOBSC, embryonic olfactory bulb stem cells; E, embryonic day; ER, endoplasmic reticulum; LC3, microtubule-associated protein 1 light chain 3; 3-MA, 3-methyl-adenine; MP, methylpyruvate; *Ngn*, Neurogenin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WM, wortmannin

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Neuroepithelial cells undergoing differentiation efficiently remodel their cytoskeleton and shape in an energy-consuming process. The capacity of autophagy to recycle cellular components and provide energy could fulfill these requirements, thus supporting differentiation. However, little is known regarding the role of basal autophagy in neural differentiation. Here we report an increase in the expression of the autophagy genes *Atg7, Becn1*, *Ambra1* and *LC3* in vivo in the mouse embryonic olfactory bulb (OB) during the initial period of neuronal differentiation at E15.5, along with a parallel increase in neuronal markers. In addition, we observed an increase in LC3 lipidation and autophagic flux during neuronal differentiation in cultured OB-derived stem/progenitor cells. Pharmacological inhibition of autophagy with 3-MA or wortmannin markedly decreased neurogenesis. These observations were supported by similar findings in two autophagy-deficient genetic models. In *Ambra1* loss-of-function homozygous mice (*gt/gt*) the expression of several neural markers was decreased in the OB at E13.5 in vivo. In vitro*, Ambra1* haploinsufficient cells developed as small neurospheres with an impaired capacity for neuronal generation. The addition of methylpyruvate during stem/progenitor cell differentiation in culture largely reversed the inhibition of neurogenesis induced by either 3-MA or *Ambra1* haploinsufficiency, suggesting that neural stem/progenitor cells activate autophagy to fulfill their high energy demands. Further supporting the role of autophagy for neuronal differentiation *Atg5*-null OB cells differentiating in culture displayed decreased TuJ1 levels and lower number of cells with neurites. These results reveal new roles for autophagy-related molecules Atg5 and Ambra1 during early neuronal differentiation of stem/progenitor cells.

e.

Introduction

In the developing nervous system, stem and progenitor cells undergo a complex program, which mediates differentiation into neurons, astrocytes and oligodendrocytes. This program not only controls phenotypic decisions but also determines cell survival or death at each stage.[1,2] The self-eating/autophagy process was first described in neurons, viewed by electron microscopy, over four decades ago.[3,4] It has taken many years to recognize macroautophagy (autophagy hereafter) as a cellular process implicated in multiple physiological and pathological situations (for reviews, see refs. 5–7) and to start unraveling autophagy's molecular regulation.[8] The first proteomic analysis of the basal autophagy interactions in human cells has recently revealed a network of 751 interactions among 409 candidate interacting proteins.[9] As such, the developmental regulation of these complex set of proteins involved in autophagy is poorly understood,[10] and little is known of the role of autophagy-related proteins in neural development.

The primary characteristic of the autophagic process is the recycling of cytosolic constituents, which are initially sequestered within double-membrane vesicles, known as autophagosomes, and later degraded through the lysosomal pathway.[11] In many cell types, autophagy plays a pro-survival role, protecting cells from starvation,[12] preventing the accumulation of protein aggregates and damaged organelles and supplying the cell with amino acids and energy.[13] A highly regulated interplay between autophagy, cell growth and cell death appears necessary for normal neural development in mammals.[14] The critical function of the autophagy regulator Ambra1 (activating molecule in Beclin 1-regulated

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autophagy) was demonstrated in *Ambra1^gt/gt^* mice, which exhibit severe neural tube defects and embryonic lethality associated with impaired autophagy.[15,16] *Ambra1^gt/gt^* mice also exhibit decreased expression of neurogenic genes such as *Ngn2,* accumulation of ubiquitinated proteins, unbalanced cell proliferation and excessive apoptotic cell death.[15] Thus in addition to its role in adaptive responses to nutrient deprivation or abnormal protein accumulation, autophagy appears to represent a key component of the developmental process.[16] In agreement, our recent findings[14,17] support a role of autophagy during cell death associated with central nervous system development, where it appears to be essential for cell engulfment. Earlier studies in nonmammalian systems anticipated the importance of autophagy in development and differentiation, with several developmental alterations described in autophagy mutants in yeast, plants, fungi, flies and worms.[18] Beclin 1 was the first Atg protein deleted in mammals, resulting in early embryonic lethality in mice at embryonic day (E)7.5, reduced embryo size and visceral endoderm malformations.[19]

The specific role of autophagy in neural differentiation is far from clear, and the impact of autophagy imbalance during a physiological stress, as occurs when stem/progenitor cells switch from a state of intense proliferation to neuronal differentiation, remains unknown. In contrast to the view of Ambra1 as a crucial protein for nervous system normal development, the autophagy protein Atg5 has not been considered essential in that process. This conclusion is based on the grossly normal central nervous system until birth in mice globally deficient for *Atg5*.[20] Moreover, in mice specifically deficient for *Atg5* in neural cells, conditional *Atg5^flox/flox^* through the use of a nestin-Cre recombinase, the neurodegenerative phenotype was not dramatic until 3 weeks of postnatal age.[21] In addition, these mice showed an accumulation of ubiquitin-positive inclusion bodies in neurons of the olfactory bulb prenatally (among many other regions). In another study, the conditional Atg5 deletion in Purkinje-cell induced axonal swelling around the terminal observed after 4 weeks of age, leading to neuronal death.[22]

Stem/progenitor cells from the mouse embryo olfactory bulb (eOBSC) are a well-characterized tool for the study of neuronal differentiation. These cells allow the production of a large number of neurons over multiple passages under defined conditions in primary culture.[23-25] Due to their synchronous differentiation in the absence of mitogenic factors, eOBSC provide a useful model for the study of neurogenesis and its relationship with essential cellular processes such as autophagy.

Ambra1 is expressed at very high levels in the OB of adult mice,[15] perhaps in association to the continuous neurogenesis which occurs in this region throughout the life of the organism.[26,27] During development, from E13.5 to 15.5, the germinal zone surrounding the OB ventricle consists of a layer of neuroepithelial cells. One day later, at E16.5, a cell layer formed by the projecting mitral neurons is evident in OB sections.[23] The neurogenic program is thus highly active in OB neural stem and precursor cells from E13.5–15.5 in vivo. In agreement, expression of the neurogenic genes *NeuroD1* and *Ngn1*, a proneural transcription factor, is detected in the OB at E13.5.[28]

As autophagy is a potential source of energy during the cellular restructuring associated with neuronal differentiation and neurite outgrowth, we investigated whether regulated basal autophagy occurs during OB development in vivo. In wild-type mouse OB, we observed a progressive increase in the expression of the autophagy genes *Atg7, Becn1, LC3* and *Ambra1*, during the differentiation of mitral neurons in vivo. In agreement, an increase in eOBSC autophagic activity was observed under differentiation conditions in culture. Inhibition of autophagy caused a significant decrease in the number of neurons generated and in the number of neurites exhibited by each neuron. In addition, *Ambra1* functional deficiency prevented the generation of neurospheres in culture, while *Ambra1* haploinsufficency resulted in the generation of smaller neurospheres and fewer differentiated neurons. Importantly, these changes were reversed, to a large extent, by the metabolic substrate methylpyruvate. Moreover, *Atg5*-null eOBSCs also manifested reduced neuronal differentiation in vitro. Together, these pharmacological and genetic ablation data define new roles for autophagy-related molecules in the early phase of neuronal differentiation.

Results

Basal autophagy is increased in the OB in vivo and in OBSC during early neuronal differentiation in vitro. Neural stem and progenitor cells in the germinal layer of the OB give rise to projecting neurons, the mitral cells. In mice, this early phase of neurogenesis, which occurs between E12 and E15[29] is triggered by the combined action of several transcription factors, including the proneural factors Neurogenin1 (*Ngn1*), *Ngn2* and *NeuroD*.[28] We hypothesized that the autophagy machinery may be active during this early period of neuronal differentiation. We thus analyzed the gene expression patterns of several autophagy genes in mouse OB at different stages in vivo by semi-quantitative and real-time quantitative RT-PCR (**Fig. 1A**). *Ngn1, NeuroD* and the neuronal marker *β-III-Tubulin* were increased during neuronal differentiation (**Fig. 1A**). *Atg7, Becn1, LC3* and *Ambra1* were all expressed

Figure 1 (See opposite page). Autophagy is increased during neural differentiation in vivo and in vitro. (A) Quantitative (bar graphs, the value at E13.5 is arbitrarily set as 1), and semiquantitative (gel insets) RT-PCR from pooled OBs from mouse littermates at the indicated embryonic ages. The displayed genes by RT-qPCR show significant statistical differences, $p < 0.05$. (B) E13.5 eOBSC were cultured as neurospheres and differentiation induced by mitogen deprivation. At the indicated times, cells were collected and protein was extracted for immunoblot with β-III-Tubulin (identified by TuJ1 antibody). Gel is representative out of five experiments with cells cultured for 3 h, 24 h and 72 h. GAPDH was used as a loading control. The TuJ1/GAPDH ratio is shown (mean ± SEM of five experiments), $p < 0.05$. (C) Representative fields of cells at 24 h and 72 h. Cells are stained for $TuJ1^+$ or $MAP2ab^+$ cells (red) and DAPI to visualize nuclei (blue). Scale bar = 100 μm. (D) E13.5 eOBSC were cultured in differentiation conditions and collected at different times of culture in the presence or in absence of ammonium chloride and leupeptin during the last 3 h of culture. LC3-II* displays a less exposed film. The LC3-II/GAPDH ratio is shown (mean ± SEM of five experiments, $p < 0.05$). (E) Representative fields of cells at 24 h and 72 h. We can observe $TuJ1^+$ cells in red and LC3 puncta in green. The nuclei are stained with DAPI (blue). Arrows mark cells with increased LC3 immunostaining. Scale bar = 100 μm.

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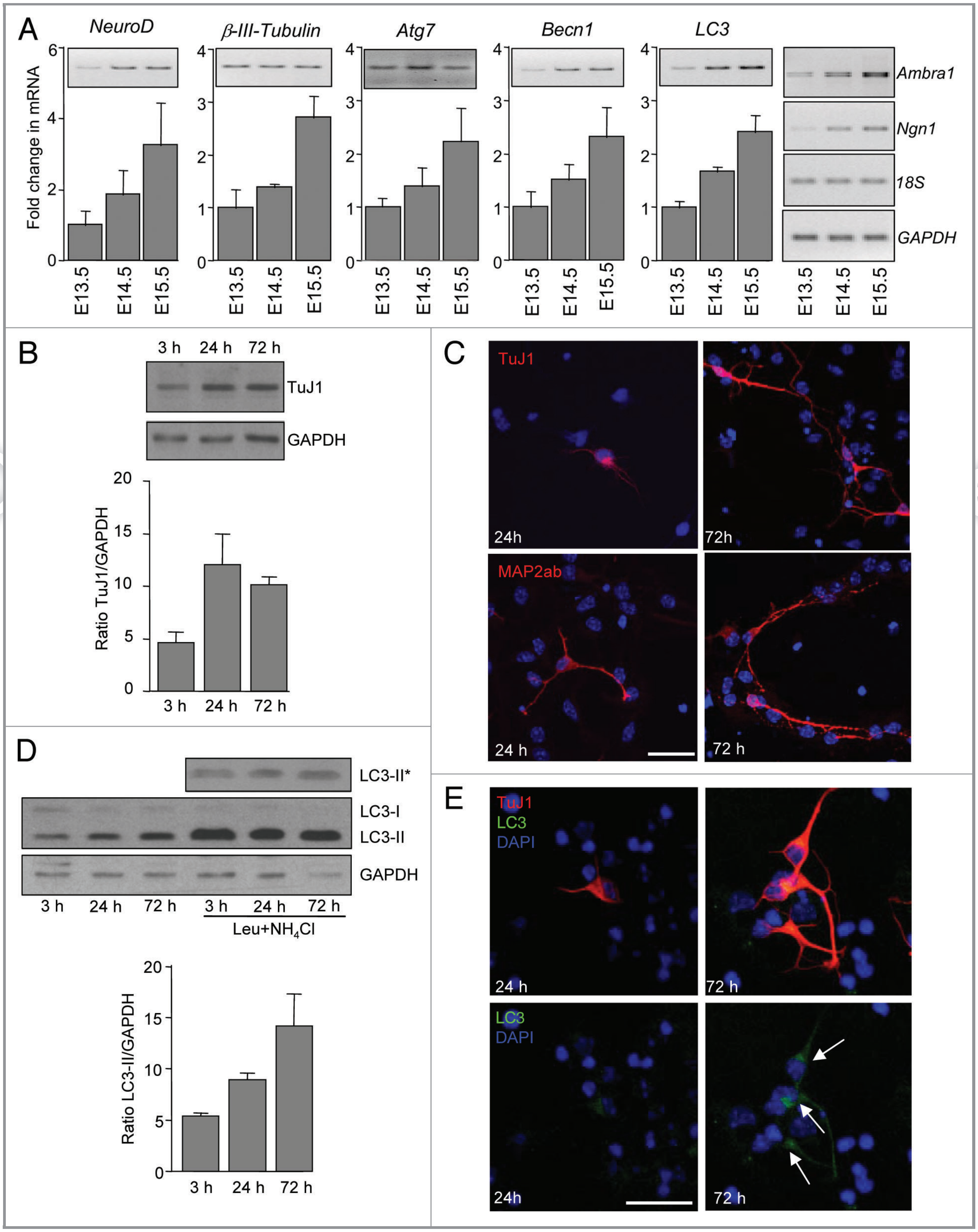

Figure 1. For figure legend, see page 188.

at E13.5 in the OB, and increased progressively up to E15.5. As the cellular composition of the OB is heterogeneous, and we specifically sought to characterize newborn neurons, we next established eOBSC cultures in which the transition from proliferative to differentiated cells can be easily monitored in short-term cell culture following the withdrawal of mitogenic factors.[23] eOBSC grown as neurospheres and allowed to differentiate for 72 h exhibited a marked increase in the levels of β-III-Tubulin, visualized by TuJ1 antibody in western blot (**Fig. 1B**) and immunofluorescence (**Fig. 1C**) and labeling with the neuronal marker MAP2ab (**Fig. 1C**). Concomitant to cell differentiation we observed an increase in the lipidated form of LC3 and increased autophagic flux (**Fig. 1D**). In addition, undifferentiated cells expressed low levels of LC3 by immunofluorescence while differentiated neurons, labeled with the neuronal marker TuJ1, manifested LC3 positive puncta (**Fig. 1E**). Together, these data show an increase in autophagy coincident with early neuronal differentiation.

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Autophagy inhibition decreases neuronal differentiation. eOBSC allowed to differentiate in culture generated a small number of TuJ1-positive ($TuJ1^+$) neurons after 16 h, which after 72 h had increased to account for almost 50% of total cells (**Fig. 2A**). To determine whether autophagy induction was essential for neuronal differentiation, we performed short-term incubations with 3-MA, which blocks autophagy through the inhibition of class III PtdIns3-kinase.[30] Cells were incubated for 3 h with 3-MA, rinsed and allowed to differentiate in fresh 3-MA free culture medium for up to 72 h. As shown in **Figure 2B and C**, 3 h incubation with 3-MA reduced LC3-II levels. However, as expected, this effect was transient and no differences in the lipidated form of LC3 were observed at later time-points. More importantly, the initial blockade of autophagy with 3-MA led to a significant and sustained decrease in the number of differentiated $TuJ1^+$ cells (**Fig. 2A and D**). These results were confirmed by incubation with wortmannin,[30] a more specific and sustained inhibitor of autophagy (**Fig. 2E**). Moreover, sustained inhibition with wortmannin for 72 h decreased the number of $TuJ1^+$ cells with neurites (**Fig. 2F**). The discrepancy between the level of TuJ1 protein in western blot, similar between control and 3-MA treated cells at 72 h, and the decreased number of differentiated cells, $TuJ1^+$, is probably due to differences in epitope exposure by the two techniques and the specific identification of strongly labeled cells by immunocytochemistry. Altogether these data show that pharmacological blockade of autophagy using two different inhibitors reduced neuronal differentiation.

Normal functional levels of Ambra1 are critical for the generation of neurospheres and neurons. The autophagy regulator Ambra1 is required for normal development of the central nervous system, and its absence in the *Ambra1*$^{gt/gt}$ mouse results in embryonic lethality at E14.5.[15] In OB extracts from E13.5 *Ambra1*$^{gt/gt}$ mice, we observed statistically significant reduced expression of *NeuroD* and *β-III-Tubulin* by RT-qPCR compared with wild type littermates (**Fig. 3A**). A tendency to decreased expression of *LC3*, *Atg7* and *Becn1* was also observed although it did not reach statistical significance (data not shown). In order to confirm that autophagy is required for neuronal differentiation, we quantified neurosphere formation and neuronal differentiation in eOBSC from *Ambra1* mutant mice. Homozygous *Ambra1*$^{gt/gt}$ eOBSC were incapable of generating neurospheres. In contrast, haploinsufficient *Ambra1*$^{+/gt}$ cells formed neurospheres, though of a smaller size than their *Ambra1*$^{+/+}$ counterparts (**Fig. 3B and C**). None of the neurospheres from *Ambra1*$^{+/gt}$ cultures exhibited a diameter over 175 μm, whereas roughly 10% of those from *Ambra1*$^{+/+}$ cultures were of this size diameter or greater (**Fig. 3C**). When proliferating *Ambra1*$^{+/gt}$ eOBSC were allowed to differentiate in culture *Ambra1*$^{+/gt}$ cells showed decreased levels of LC3-II in comparison to wild-type littermates (**Fig. 3D**) and less autophagic flux at 24 h (**Fig. 3D and E**) indicating a reduction in autophagy in *Ambra1*$^{+/gt}$ cells. Importantly, *Ambra1*$^{+/gt}$ eOBSC displayed fewer differentiated neurons (detected using two different neuronal markers) at 72 h as compared with *Ambra1*$^{+/+}$cultures (**Fig. 4A–C**). These data demonstrate that functional autophagy is required for eOBSC neuronal differentiation in vitro and in vivo.

Methylpyruvate restores neuronal differentiation in Ambra1$^{+/gt}$ eOBSC and after 3-MA treatment. We recently demonstrated that autophagy inhibition with 3-MA reduces ATP levels during retinal neurogenesis, an effect reversed by methylpyruvate (MP), a permeable analog for the citric acid cycle.[17] To determine whether the requirement of autophagy in stem/progenitor cell differentiation is dependent on energy status, we incubated eOBSC with 3-MA for 3 h, after which the cell culture medium was supplemented with MP during cell differentiation. Interestingly, the impairment of eOBSC neuronal differentiation by 3-MA was largely reversed in the MP-treated cultures (**Fig. 5A**). No morphological differences were detected between $TuJ1^+$ neurons in the control and 3-MA+MP groups (**Fig. 5B**). Thus in autophagy-deficient cultures, restoration of normal ATP availability by the addition of MP appears essential for eOBSC neuronal differentiation.

We next studied in greater detail the morphology of neurons generated after 72 h in culture in both control and impaired autophagic conditions. The progressive differentiation of eOBSC was quantified according to the presence and number of primary and secondary neurites on individual cells. 3-MA decreased the proportion of $TuJ1^+$ cells with primary neurites by 50%, an effect that was attenuated by the addition of MP (**Fig. 5C**). The appearance of secondary neurites, a hallmark of more advanced maturation, was highly sensitive to impaired autophagy, with 3-MA treatment decreasing the number of secondary neurites by over 90% compared with control cells. Restoration of energy balance by the addition of MP partially reversed this effect, resulting in the generation of an intermediate number of cells with secondary neurites (**Fig. 5D**). More importantly, the addition of MP also completely rescued the neuronal differentiation capacity of *Ambra1*$^{+/gt}$ eOBSC after 72 h (**Fig. 6A and B**). Together, we show that inhibition of autophagy impairs neuronal differentiation. This effect is reversed by supplying cell cultures with a permeable substrate for the citric acid cycle, suggesting that autophagy represents an essential source of energy during early neuronal differentiation.

eOBSCs from Atg5$^{-/-}$ embryos show decreased neuronal differentiation in vitro. To further confirm that autophagy is

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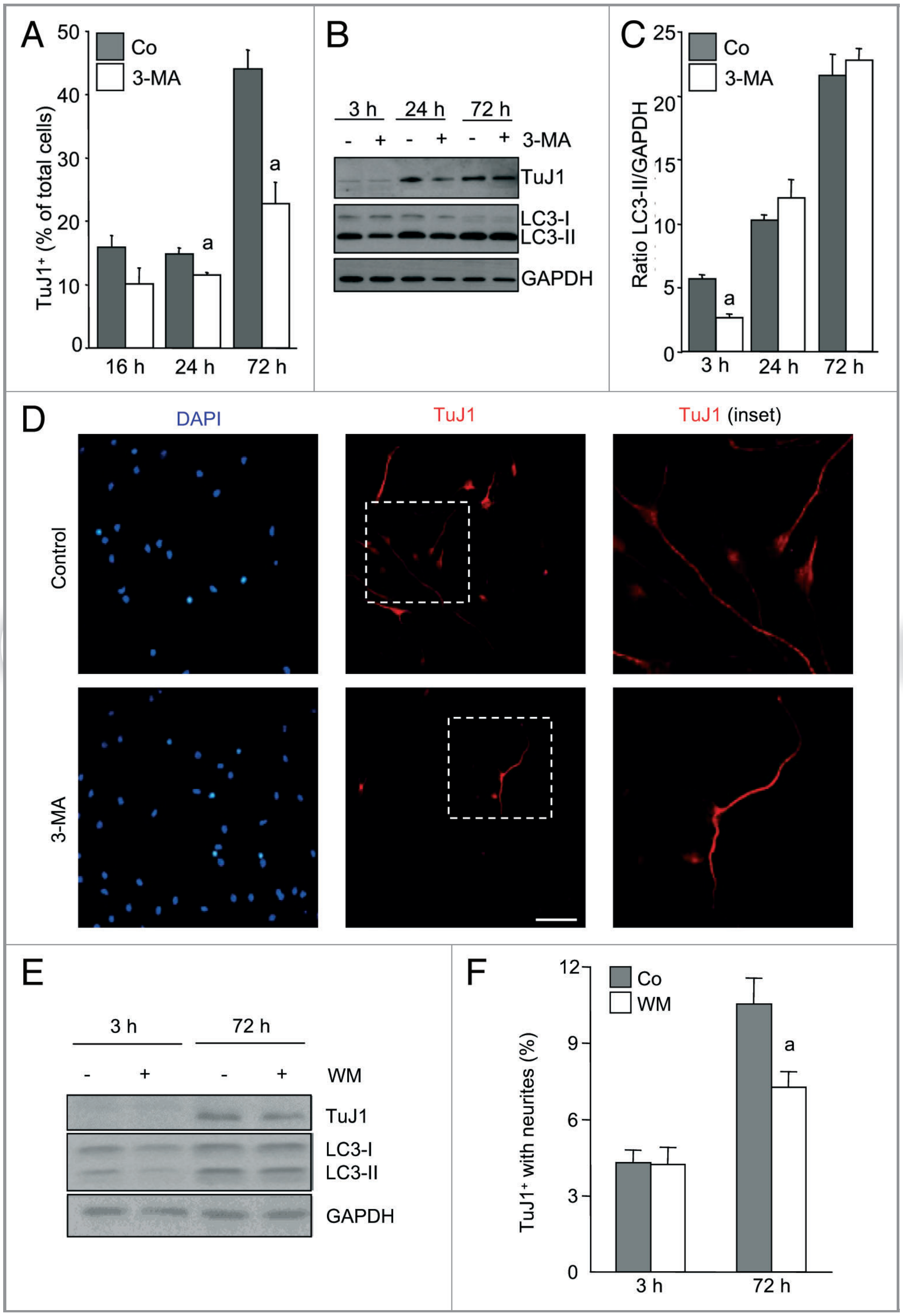

Figure 2. Autophagy inhibition attenuates the generation of neurons from eOBSC. E13.5 mouse eOBSC were allowed to differentiate by deprivation of mitogens. Three hours after plating, 3-MA (10 mM) was added for 3 h. After washing, the culture was maintained for 72 h under standard cell differentiation conditions. (A) Cells were then collected for immunostaining against β-III-Tubulin (identified by TuJ1 antibody) and DAPI. Percentage of TuJ1$^+$ differentiated neurons at the time-points indicated in control (solid bars) and 3-MA treated (open bars) cells. Results represent the mean ± SEM from five experiments, with each culture performed in triplicate. (a) $p < 0.05$ *vs* corresponding time control. (B) Immunoblot of TuJ1 and LC3 representative of three experiments from cells cultured for 3 h, 24 h and 72 h after 3-MA treatment (for 3 h) GAPDH was used as a loading control. (C) Densitometric analysis of LC3-II/GAPDH ratio (mean ± SEM) of three experiments with duplicate samples). (a) $p < 0.05$ *vs* corresponding time control. (D) Representative fields of control and 3-MA treated cells at 72 h. Nuclei are stained with DAPI (blue) and the morphology of TuJ1$^+$ cells (red) is shown at lower (middle panels) and higher (right panels) magnifications (the enlarged field is indicated by dashed lines in the middle panels). Scale bar = 100 μm. (E) Immunoblot of LC3 and β-III-Tubulin expression in cells cultured for 3 h and 72 h after 3 h wortmannin treatment. GAPDH was used as a loading control. (F) Percentage of TuJ1$^+$ cells with neurites at the time-points indicated in control cultures (solid bars) and cultures treated with wortmannin during the whole incubation period (open bars). Results represent the mean ± SEM from three experiments. (a) $p < 0.05$ *vs* corresponding time control.

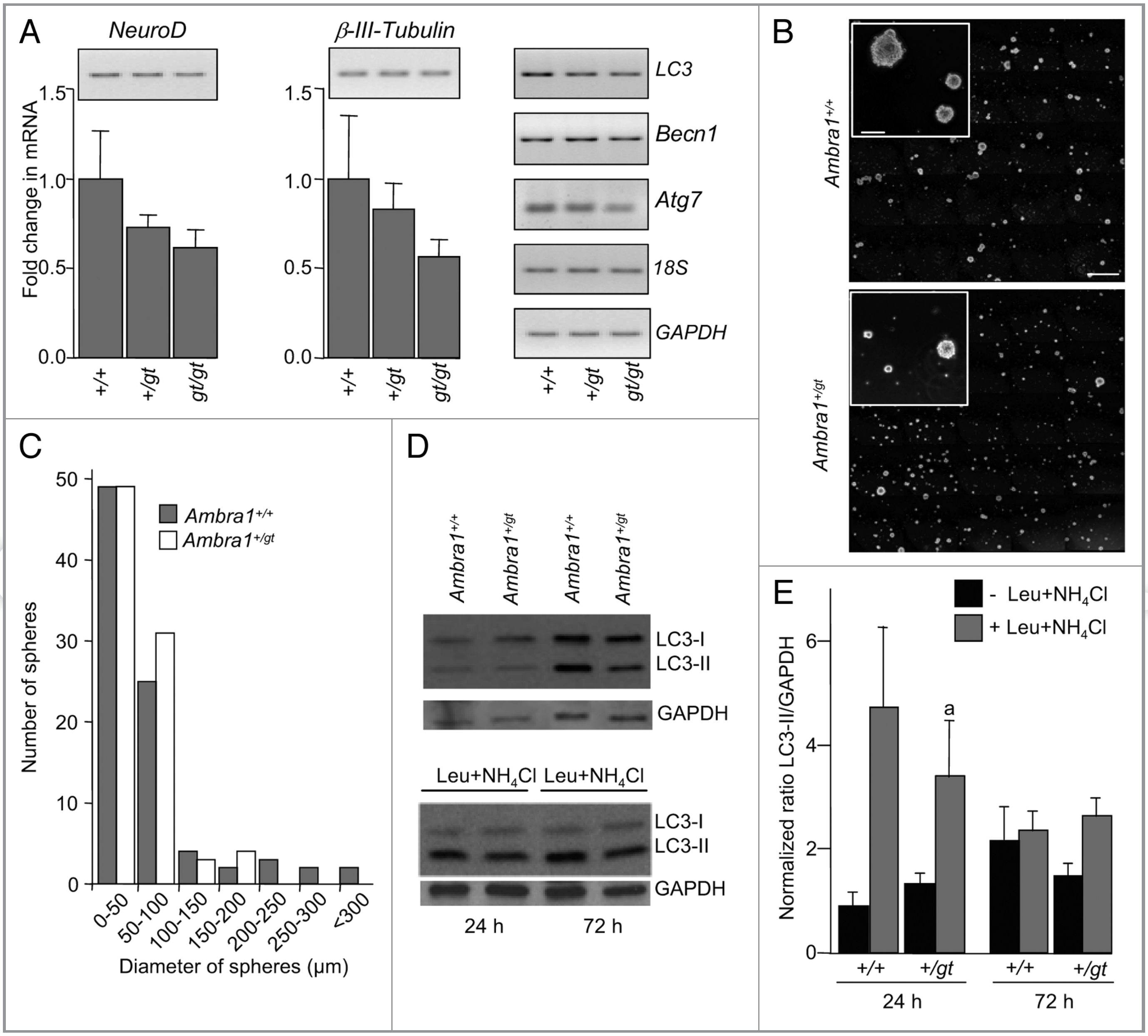

Figure 3. *Ambra1* mutant mice have deficits in gene and protein expression in the OB in vivo and decreased ability to generate neurospheres from eOBSC in vitro. (A) Quantitative (graphs) and semiquantitative (gels) RT-PCR was performed with total RNA isolated from OBs pooled from *Ambra1*$^{+/+}$, *Ambra1*$^{+/gt}$ and *Ambra1*$^{gt/gt}$ mice littermates at E13.5. Neural genes (*NeuroD, β-III-Tubulin*) and autophagy related genes (*Atg7, Becn1, LC3*) were analyzed in parallel with loading controls (*18S, GAPDH*) by semi-quantitative RT-PCR and by RT-qPCR. Statistical analysis of RT-qPCR show a significant difference in *Ambra1*$^{+/+}$ *vs. Ambra1*$^{gt/gt}$ in neural genes ($p < 0.05$). (B) eOBSC were grown from E13.5 embryos derived from the crossing of *Ambra1* haploinsufficient mice. *Ambra1*$^{gt/gt}$ cells failed to generate neurospheres. Representative fields of *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ neurospheres in culture after 48 h under proliferative conditions viewed under phase contrast optical microscopy; the inset shows a higher magnification. Scale bars = 1 mm in main photo and 100 µm in the inset. (C) Number of neurospheres with the diameter indicated in *Ambra1*$^{+/+}$ (solid bars) and *Ambra1*$^{+/gt}$ (open bars) derived cultures. eOBSC were derived from two separate litters, two independent experiments performed in triplicate, and the results pooled. Differences in neurosphere mean size were analyzed with a Poisson distribution with a χ^2 of 135.5, $p < 0.001$. (D) E13.5 *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ mice eOBSC were cultured in differentiation conditions without mitogens for 24 h or 72 h in the presence or absence of ammonium chloride and leupeptin during the last three hours of culture. Then the protein was extracted and analyzed by immunoblot for LC3. A decrease in the lipidated form of LC3 is observed in *Ambra1*$^{+/gt}$ cells in basal conditions and less autophagic flux is evident at 24 h in a representative blot of three independent experiments. We used GAPDH as a loading control. (E) The ratio of LC3-II/GAPDH is shown as mean ± SEM from three independent experiments, with *Ambra1*$^{+/+}$cells at 24 h considered arbitrarily as 1. (a) $p < 0.05$ *vs Ambra1*$^{+/+}$.

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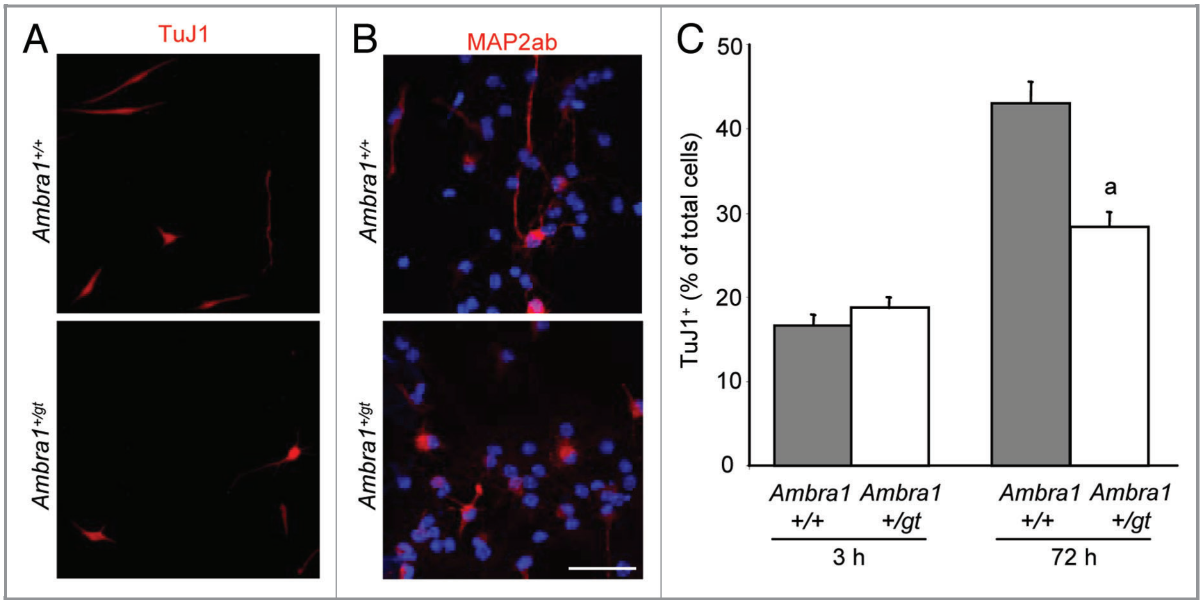

Figure 4. *Ambra1* haploinsufficiency results in decreased neuronal differentiation. *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ eOBSC were grown as neurospheres and were plated in differentiation conditions and cultured for up to 72 h. Cells were collected at 3 h and 72 h and fixed. (A) Representative fields of *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ neurons stained with TuJ1 or (B) MAP2ab antibodies (red) at 72 h. Nuclei are stained with DAPI (blue). Scale bar = 100 μm. (C) The percentage of TuJ1^{+} differentiated neurons is shown for *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ cultures at the indicated time-points. Results represent the mean ± SEM from two independent experiments from two litters, with each culture performed in triplicate. (a) $p < 0.05$ *vs* control.

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required for neuronal cell differentiation we isolated eOBSCs from *Atg5*$^{-/-}$ and *Atg5*$^{+/+}$ embryos at E13.5 and allowed them to differentiate in vitro by growth factor withdrawal. As it is shown in **Figure 7A**, LC3-II levels were undetected in the knockout cells indicating that autophagy is reduced at 72 h. Importantly, TuJ1 levels by western blot were also reduced at this time-point (**Fig. 7A and B**). Moreover, immunofluorescence analyses followed by quantitation also demonstrated that *Atg5*$^{-/-}$ cells cultures displayed reduced numbers of TuJ1-positive cells with neurites in comparison to wild-type littermates (**Fig. 7C and D**).

In conclusion, by using two pharmacological approaches and cells from two autophagy-deficient animal models we demonstrated for the first time that proper autophagy, particularly normal levels of Ambra1 and Atg5, are needed for progenitor stem cells differentiation into neurons in vivo and in vitro.

Discussion

Unraveling the mechanisms underlying stem cell transition from pluripotency to the differentiated state is essential for their future use in regenerative medicine. Using the mouse OB during neurogenesis and eOBSC cultures as in vivo and in vitro model systems, we described the induction of autophagy in parallel with neuronal differentiation. Both pharmacological and genetic disruption of the autophagic machinery impaired the differentiation process, decreasing the number of newborn neurons and hindering their maturation in culture. The rescue of the differentiation program by methylpyruvate addition strongly suggests that autophagy provides the high levels of energy required for the transition from proliferative precursor to postmitotic neuron.

During nervous system development, molecular signals including transcriptional factors and epigenetic changes instruct progenitor cells to generate different types of neuronal and glial cells.[31] A critical period of neurogenesis in the OB projecting neurons, the mitral cells, occurs between E13.5 and E15.5 in the mouse. We observed a progressive upregulation of the autophagy genes *Atg7*, *Becn1, Ambra1* and *LC3* throughout this period, along with a parallel upregulation of the neurogenic markers *Ngn1, NeuroD* and *β-III-Tubulin*. This strongly suggests that autophagy participates in the neuronal differentiation program, at the very least playing a permissive role in this process. A similar function was recently been proposed in human keratinocytes, in which autophagy constitutes an early signaling process required for keratinocyte commitment to the differentiation pathway.[32]

While the participation of autophagy in the transition from proliferation to differentiation during development is now well documented,[33] appropriate primary cell systems are needed to characterize the precise role of autophagy regulators. The availability of a well-defined synchronous stem/progenitor cell culture system, derived from the mouse embryonic olfactory bulb, has allowed us to demonstrate the involvement of autophagy in early phases of neuronal differentiation. Activation of autophagy, as determined by LC3 lipidation, occurred in parallel with an increase in the expression of the neuronal marker β-III-Tubulin, whereas autophagy inhibition with 3-MA reduced the number of neurons by 50% after 72 h in culture. A fully functional autophagic response appears to be crucial during the initial stages of differentiation in culture, as inhibition of autophagy with 3-MA or wortmannin for just 3 h had a profound effect on the final number of neurons and their capacity to differentiate. This

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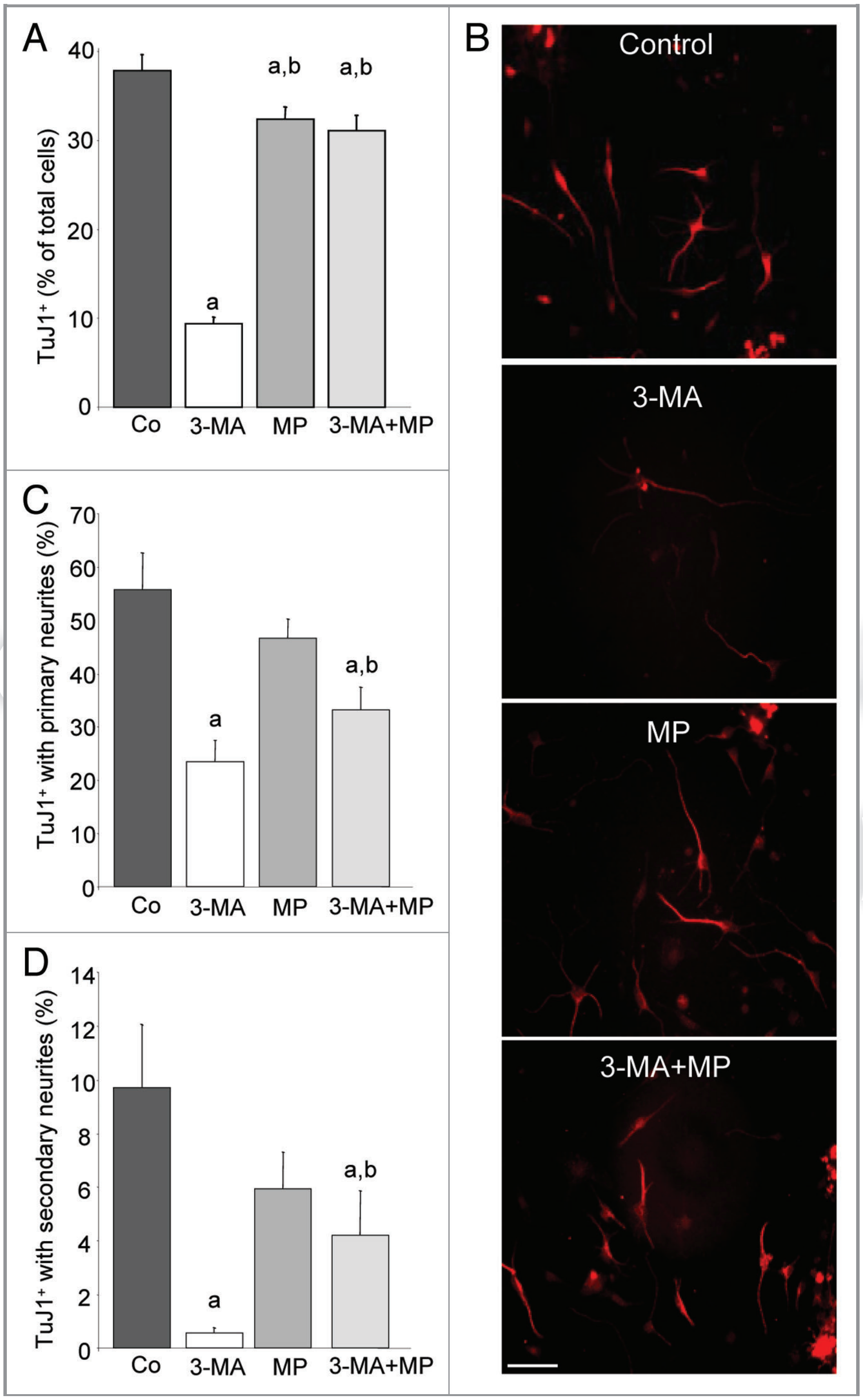

Figure 5. Neurite outgrowth is impaired by 3-MA treatment and partially restored by MP. eOBSC were cultured as described in **Figure 2** and stained with TuJ1 antibody. Neurites were classified as primary (predominant processes emerging directly from the cell body) or secondary (processes emerging from a primary neurite). (A) Percentage of total $TuJ1^+$ neurons in control culture, and following treatment with 3-MA, MP, or 3-MA and MP in combination (3-MA + MP, 10 mM each). (B) Representative fields of $TuJ1^+$ neurons after 72 h in culture under control conditions or following 3-MA ± MP treatments. Scale bar = 100 μm. (C) Percentage of $TuJ1^+$ neurons displaying at least one primary neurite in the same cultures as in (A). (D) Percentage of $TuJ1^+$ neurons displaying secondary neurites in the same cultures as A. Results represent the mean ± SEM of three experiments performed in triplicate. (a) $p < 0.05$ *vs* control, (b) $p < 0.05$ *vs* 3-MA-treated culture.

hypothesis is further supported by the fact that autophagic flux is reduced at 24 h in *Ambra1*$^{+/gt}$ with an effect in neuritogenesis at 72 h. The involvement of autophagy proteins in early OB differentiation was further supported by the observed decrease in *NeuroD* and *β-III-Tubulin* at E13.5 in mice with functional deletion of both *Ambra1* alleles. A tendency to decreased *Atg7, Becn1* and *LC3* expression was also observed at this time-point, just before embryonic lethality occurs. The requirement of autophagic processes for neuronal differentiation was confirmed in *Ambra1* haploinsufficient mice, in which the neuronal number was halved as compared with controls. The striking inability of *Ambra1*$^{gt/gt}$ to form neurospheres may also implicate autophagy in the proliferative state,[15] or in the maintenance of pluripotency of stem/precursor cells, an issue which merits further research.

Morevoer, the observation of reduced neuronal differentiation in the *Atg5*-null eOBSCs further supports our hypothesis that autophagy is essential for this process. Besides the role of Atg5 demonstrated recently in the survival of adult neurons under stress conditions,[34] autophagy may also be essential to generate the vestibular ganglion cells during inner ear development (manuscript in preparation, laboratory of I. Varela-Nieto, IIB, CSIC). In comparison with *Ambra1*, the phenotype of *Atg5* null cells was milder, pointing out to the existence of compensatory effects in the Atg5 cells, or reflecting additional effects of Ambra1 independent of its role in autophagy, and/or the requirement of Atg5 and Ambra1 in slightly different moments of the process of differentiation from a neuroblast to a neuron.

Autophagy is an energy-providing mechanism, which recycles cellular organelles and intracellular constituents to produce energy and amino acids.[13] In line with this view, the present study describes a primary role of autophagy in neurogenesis. Addition of MP, which acts as an energy supplier, partially restored neuronal generation following pharmacological inhibition of autophagy, and fully restored it in the *Ambra1* haploinsufficiency model. These observations thus point to energy supply as the primary function of autophagy in the context of neuronal differentiation. This proposal is supported by our previous studies in the developing retina, which demonstrated an essential role for

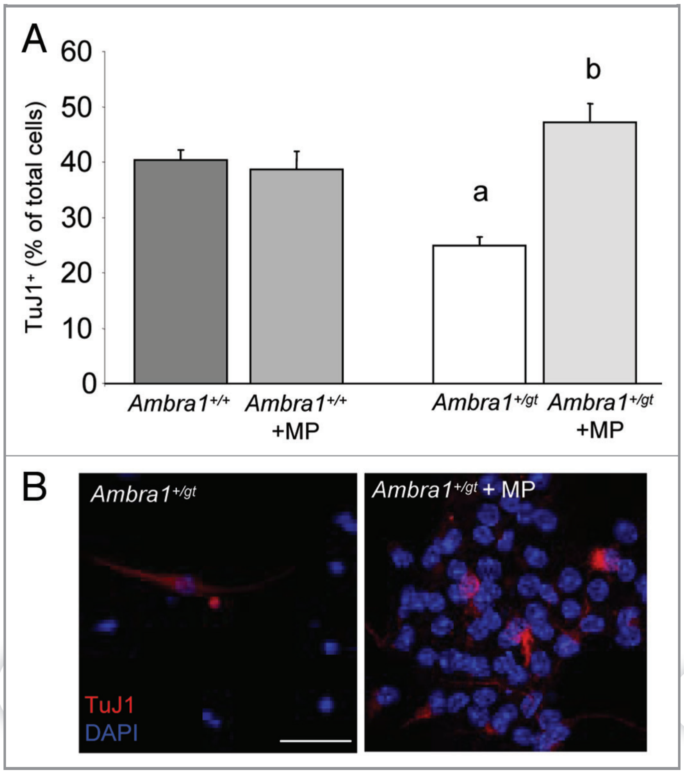

Figure 6. Neuronal differentiation is impaired in *Ambra1* haploinsufficient mice and restored by MP. eOBSC were grown from pooled E13.5 embryos derived from the crossing of *Ambra1* haploinsufficient mice. Cultures from *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ were grown initially as neurospheres and after at least three passages, cells were plated in differentiation conditions and cultured for up to 72 h, in the presence or absence of MP (10 mM). Cells were collected and fixed, and differentiation monitored. (A) Percentage of TuJ1^{+} differentiated neurons at 72 h in *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ cultures treated as indicated. Results represent the mean ± SEM from two independent experiments from two litters, with each culture performed in triplicate. (a) $p < 0.05$ *vs Ambra1*$^{+/+}$, (b) $p < 0.05$ *vs Ambra1*$^{+/gt}$ without MP. (B) Representative fields of *Ambra1*$^{+/+}$ and *Ambra1*$^{+/gt}$ + MP cells at 72 h. Nuclei are stained with DAPI (blue) and neurons labeled with TuJ1 (red). Scale bar = 100 µm.

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autophagy in cell corpse clearance during naturally occurring cell death associated with neurogenesis.[17,35] In this context, autophagy maintains the ATP levels necessary for the exposure of engulfment signals,[14] a phenomenon also observed in embryoid body cavitation.[36] A fully functioning autophagy response is essential to maintain energy levels in several other situations, such as starvation, when the induction of autophagy helps maintain cell viability in vitro[12] or amino acid concentrations in neonates before nursing.[20,37] Autophagy is also essential for preimplantation embryonic development, as oocytes from *Atg5* knockout mice fail to develop beyond the four- and eight-cell stages, and the resultant autophagy-null embryos exhibit decreased rates of protein synthesis.[38] Energy and amino acids generated by autophagy are thus essential for tissue homeostasis at different stages during the life cycle of vertebrates, including early embryogenesis, neural development, birth, the postnatal stage and adult starvation. Further studies are needed to unravel the hierarchy between energy requirements, mTOR pathway and autophagy during neuronal differentiation.

Neuritogenesis was also impaired after autophagy inhibition, and partially restored by the addition of MP. Although additional studies are needed to determine whether this effect is independent of autophagy inhibition, or a direct consequence of impaired differentiation, some observations point toward specific effects of starvation-independent autophagy. For example, previous findings in healthy neurons[39] and the reported requirement of Atg7 for maintenance of axonal homeostasis.[40] Decreased activity of Ulk1 (the mouse ortholog of yeast *Atg1*) also prevents neurite outgrowth in vitro and in vivo, and results in decreased expression of neuron-specific β-III-Tubulin.[41] In the hybrid NG108-15 cell line, blockade of autophagy with 3-MA, or by silencing *Becn1* or *Atg5*, prevents cAMP-induced differentiation.[42] Other autophagy genes are also essential for cellular remodeling. Differentiation of primary mouse embryonic fibroblasts into adipocytes is blocked in cells derived from *Atg5*-null mice, suggesting that autophagy facilitates the reshaping of the cytoplasm necessary for adipocyte differentiation.[43] Autophagy can also mediate the specific elimination of mitochondria, ER, peroxisomes and ribosomes.[44,45]

The cell-autonomous basal autophagy described in the present study was developmentally regulated, as it increased in parallel with neuronal differentiation, both in vivo and in cultured eOBSC. We thus propose that autophagy is a major contributor to the intensive cell-remodeling process that occurs during early neuronal differentiation. This is supported by the demonstrated requirement of Ambra1 and Atg5 for normal neurogenesis in vitro and in vivo. Our findings underscore the importance of fully elucidating the interplay between autophagy machinery and the cell differentiation process to identify molecular targets for future neural stem-cell based therapies.

Materials and Methods

Olfactory bulbs and quantitative and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Animals were housed, cared and euthanized in accordance with European Union guidelines and experiments were approved by the CIB ethics committee for animal experimentation. After removing the brain from the skull, both OBs were dissected and pooled according to mouse strain (C57BL/6J or *Ambra1*) and developmental day (13.5, 14.5 and 15.5; the day on which vaginal plug was detected was considered E0.5). C57BL/6J mice were obtained from the Jackson Laboratory, and *Ambra1* mutant mice were generated in the laboratory of F. Cecconi.[15] *Atg5* knockout animals were kindly provided by N. Mizushima.[20] Total RNA was extracted from tissue using Trizol (Invitrogen, 15596-018) and reverse transcription performed on 1 µg of total RNA using Oligo(dT)18-20 (Invitrogen, 18418-020) and Superscript III enzyme (Invitrogen, 18080-44). Quantitative real-time PCR was performed with a TaqMan Universal PCR Master mix using probes from Universal Probe Library Set in a 7900 HT-Fast real time PCR System (Roche Applied Biosystems). Each value was adjusted by using 18S RNA levels as a reference. For semi-quantitative RT-PCR all genes were processed in parallel and

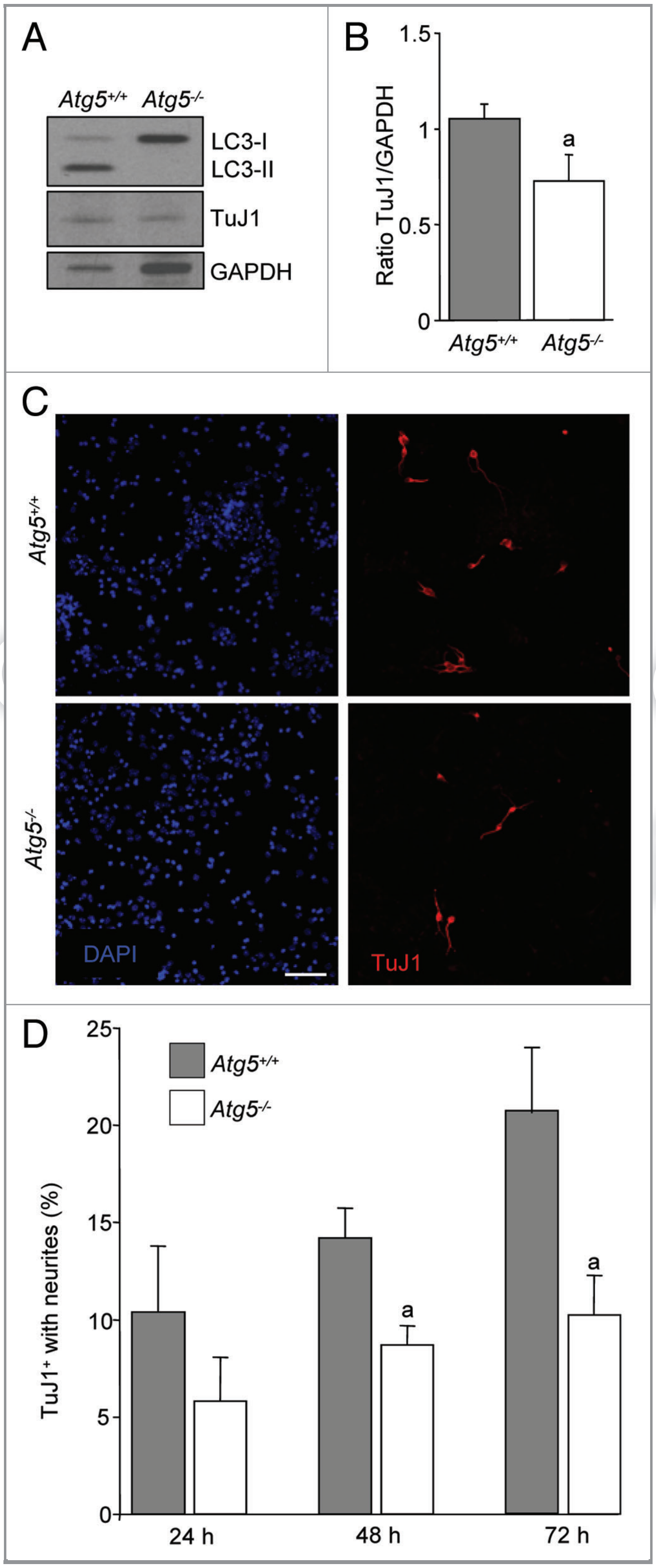

Figure 7. *Atg5 null eOBSC* display decreased neuronal differentiation. $Atg5^{+/+}$ and $Atg5^{-/-}$ eOBSC were grown initially as neurospheres and after at least three passages, were plated in differentiation conditions without mitogens and cultured for up to 72 h. (A) Immunoblot of differentiated cells after 72 h of culture in differentiation conditions. In $Atg5^{-/-}$ cells there is an absence of LC3-II form, and a decrease of TuJ1 protein expression. (B) Densitometric analysis of TuJ1/GAPDH ratio (mean ± SEM of three experiments), (a) $p < 0.05$. (C) Representative fields of $Atg5^{+/+}$ and $Atg5^{-/-}$ neurons stained with TuJ1 (in red) and DAPI staining for nuclei (blue). (D) Percentage of $TuJ1^{+}$ cells with neurites at different time-points of culture, in $Atg5^{+/+}$ and $Atg5^{-/-}$ cells. Results represent the mean ± SEM from two independent experiments from three litters, with each culture performed in triplicate. Scale bar = 100 µm. (a) $p < 0.05$ *vs* $Atg5^{+/+}$.

the same parameters for image analysis were applied. The primers used are listed in **Table 1**.

Genotyping of *Ambra1* and *Atg5* mutant embryos. *Ambra1* and *Atg5* E13.5 embryos were genotyped by processing tail bud tissue. In *Ambra1* embryos RNA was isolated as described above and PCR was performed using two pairs of primers: 5'-AACGCATTTATACCCAGTCCA-3' (primer A) and 5'-ACCATAACGTATCGGCCCATC-3' (primer B), mapping upstream and downstream of the gene-trap insertion site, respectively; and primer A together with 5'-CCCAGTCACG-ACGTTGTAAAA-3' (primer C), the latter mapping onto the lacZ reporter sequence. In *Atg5* embryos DNA was isolated with proteinase K (0.6 mg/ml, Sigma, P2308) in high salt buffer, and PCR was performed using 3 primers: 5'-ACAACGTCGAGCACAGCTGCGCAAGG-3', (primer A) 5'-GAATATGAAGGCACACCCCTGAAATG-3' (primer B) 5'-GTACTGCATAATGGTTTAACTCTTGC-3' (primer C).

Neural stem/progenitor cell cultures. Neural/progenitor stem cells were prepared from the OB of E13.5 mouse embryos, as previously described.[23] The OB was dissected and mechanically disaggregated. Cells were resuspended in DMEM/F12/N2 medium, consisting of Dulbecco's modified Eagle's medium (DMEM/F12, GIBCO, 42400-028), with N2 supplement containing insulin (10 µg/ml, Sigma, I1507), apotransferrin (Sigma, T2252), putrescine (Sigma, P5780), progesterone (Sigma, P6149) and sodium selenite (Sigma, S5261). Cells were then plated onto uncoated tissue culture dishes at a density of 3.5×10^4 cells/cm^2 and incubated at 37°C in 5% CO_2. FGF-2 (Preprotech, 100-18B) and EGF (Preprotech, AF-100-15) (20 ng/ml each) were added daily to expand the proliferating precursor cell population up until the first passage, after which they were added only on days of passage (every 3–4 d).

Proliferative eOBSC and neurosphere scoring. Cultured $Ambra1^{+/gt}$ eOBSC were plated at a density of 6×10^3 cells/cm^2 under proliferation conditions (DMEMF12/N2 with mitogens). To quantify the number of neurospheres, a total of five random fields per well were photographed, using the 10 × objective of a phase contrast microscope (Zeiss Axioplan). Neurosphere diameter was measured using LAS AF Leica Software. Only neurospheres with diameters of over 20 µm were scored. Cultures from different passages and litters were analyzed.

Differentiating cultures and treatments with 3-MA, wortmannin and methylpyruvate. To induce cell differentiation, neurospheres from cultures of less than 20 passages were plated at a density of 10^5 cells/cm^2 on coverslips coated with 15 mg/ml polyornitin (Sigma, P4957) and 1 mg/ml fibronectin (GIBCO, 33010-018) under differentiation conditions (DMEM/F12/N2

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Table 1. Sequence of the primers used in quantitative and semi-quantitative RT-PCR

		Quantitative PCR	Semi-Quantitative PCR
Gene		Primer	Primer
Ambra1	F	5′GAGCACCCAATTTACCCAGA3′	5′AACGCATTTATACCCAGTCCA3′
	R	5′GATCATCCTCTGGGCGTAGTA3′	5′ACCATAACGTATCGGCCCATC3′
Atg7	F	5′CCGGTGGCTTCCTACTGTTA3′	5′CCGGTGGCTTCCTACTGTTA3′
	R	5′AAGGCAGCGTTGATGACC3′	5′AAGGCAGCGTTGATGACC3′
Beclin1	F	5′CAGGCGAAACCAGGAGAG3′	5′GCTCCATTACTTACCACAGC3′
	R	5′CGAGTTTCAATAAATGGCTCCT3′	5′CTAGGATCTCCAAACAGCGT3′
LC3 A	F	5′CATGAGCGAGTTGGTCAAGA3′	5′TGATCATCGAGCGCTACAAG3′
	R	5′CCATGCTGTCTGGTTA3′	5′ACCATGTAGAGGAATCCGTC3′
Neuro D	F	5′GCTCCAGGGTTATGAGATCG3′	5′GCTCCAGGGTTATGAGATCG3′
	R	5′CTCTGCATTCATGGCTTCAA3′	5′CTCTGCATTCATGGCTTCAA3′
Ngn1	F	5′CGATCCCCTTTTCTCCTTTC3′	5′CGATCCCCTTTTCTCCTTTC3′
	R	5′TGCAGCAACCTAACAAGTGG3′	5′TGCAGCAACCTAACAAGTGG3′
β-III-Tubulin	F	5′GCGCATCAGCGTATACTACAA3′	5′GCGCCTTTGGACAGGTATTC3′
	R	5′CATGGTTCCAGGTTCCAAGT3′	5′GGGGAGGACATCTAGGACTG3′
18S	F	5′TGCGAGTACTCAACACCAACA3′	5′TGCGAGTACTCAACACCAACA3′
	R	5′TTCCTCAACACCACATGAGC3′	5′TTCCTCAACACCACATGAGC3′
GAPDH	F	5′AGCTTGTCATCAACGGGAAG3′	5′AGCTTGTCATCAACGGGAAG3′
	R	5′TTTGATGTTAGTGGGGTCTCG3′	5′TTTGATGTTAGTGGGGTCTCG3′

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without mitogens) and incubated for 3 h. To analyze autophagic flux during cell differentiation we cultivated cells during the last 3 h of culture in the presence of ammonium chloride (20 mM, Sigma, A9434) and leupeptin hemisulfate (0.1 mM, Fisher Scientific, BP2662). To study the effect of autophagy inhibition on neural differentiation, 3-MA (10 mM, Sigma, M9281) or wortmannin (100 nM, Calbiochem, 681675) was added to the culture medium, alone, or in combination with methylpyruvate (10 mM, Sigma, 37117), for 3 h. The medium was then replaced with fresh medium and the cells cultured up until 72 h, in the presence or absence of MP, or with wortmannin. The culture medium was supplemented with sodium bicarbonate (1.5 mg/ml, Sigma, S7561) to maintain the pH balance in the presence of MP, and replaced every 24 h. Cells were then fixed for immunostaining, or lysed for immunoblot analysis.

Immunostaining of cultured cells and neurite scoring. Cells cultured for the indicated periods of time were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 min. Cells were incubated overnight at 4°C with primary antibodies against: β-III-Tubulin (TuJ1, rabbit polyclonal, 1:1000; Covance, MRB-435P), LC3 (1:100, MBL, M152-3) or MAP2ab (1:200, Sigma, M1406) followed by incubation with Texas Red secondary antibody (1:1000; Molecular Probes, T2767) or with Alexa 488 antibody (1:250, Molecular Probes, A1101). Controls were performed to confirm primary and secondary antibody specificity. Cultures were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, D9542) to visualize nuclei. To determine the number of $TuJ1^+$ cells, a total of 10 random fields per coverslip were analyzed using a 40 or 63× objectives under a fluorescence filter. Neurites were classified as primary (predominant processes emerging directly from the cell body) or secondary (processes emerging from a primary neurite).

Immunoblotting. Cells harvested at the indicated time-points were incubated in lysis buffer [50 mM TRIS-HCl pH 7.4, 300 mM NaCl, 0.01% Triton X-100, 1 mM EDTA, 1 mM Orthovanadate (Sigma, S6508), 25 mM NaF (Sigma, S6521), 4 mM sodium pyrophosphate (Sigma, S9515) and an entire mini EDTA-free protease inhibitor tablet (Roche Diagnostics, 11836170001)] for 20 min at 4°C. Cell lysates were centrifuged at 20,000 g at 4°C for 15 min, and the supernatant collected and stored at -20°C for further analysis by immunoblotting. Protein extracts (35 µg, quantitation performed by BCA method, Thermo Scientific, 23227) were fractionated by electrophoresis on 15% polyacrilamide gels and transferred to PVDF membranes (Whatman Protran, 10401396). Membranes were treated with 5% nonfat dry milk, 0.05% Tween 20 in PBS for 2 h at room temperature (RT), and incubated overnight at 4°C with primary antibodies: β-III-Tubulin (TuJ1, mouse monoclonal 1:500, Covance, MMS-435P), LC3 (1:2000, MBL, PM036), and GAPDH (1:5000, Abcam, ab8245). After washing, membranes were incubated for 2 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies and subsequently with enhanced chemiluminescence (ECL) reagent (Pierce, 34080). The optical density of specific bands was measured by densitometry using ImageJ software. Protein levels were normalized relative to those of GAPDH.

Statistical analysis. Results are expressed as the mean ± SEM of the number of experiments indicated in the figure legends. Statistical analyses were performed using ANOVAs. To understand which treatments were different, we used individual contrasts. Differences in the mean size of neurospheres in **Figure 3C** were analyzed using a

Poisson distribution. The level of significance was set at $p < 0.05$ (two-tailed).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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