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BAG-1 is preferentially expressed in neuronal precursor cells of the adult mouse brain and regulates their proliferation in vitro

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ABSTRACT

BAG-1 protein has been well characterized as necessary for proper neuronal development. However, little is known about the function of BAG-1 in the adult brain. In this work, the expression and localization of BAG-1 in the mature mouse brain was studied. The levels of both BAG-1 isoforms decrease significantly in the brain during development. BAG-1 was found preferentially expressed in Neuronal Precursor Cells (NPCs) in the two major niches of neurogenesis. Lentiviral mediated overexpression of BAG-1 increased the proliferation rate of cultured NPCs. In addition, depletion of BAG-1 from NPCs induced a decrease in NPCs proliferation in the presence of a stress hormone, corticosterone. These data suggest a role for BAG-1 in mechanisms of neurogenesis in the adult mouse brain.
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1. Introduction

BAG-1 protein is a multifunctional protein that plays significant roles in regulation of apoptosis, gene transcription, and cellular differentiation. BAG-1 is expressed as three isoforms that all originate from the same messenger RNA by alternative start codons. Humans express BAG-1L, BAG-1M, and BAG-1S, while mice only express BAG-1L and BAG-1S [1]. In addition, BAG-1S and BAG-1L of mouse origin are slightly smaller than the human isoforms.

BAG-1 was first identified as a Bcl-2 binding partner with anti-apoptotic properties [2]. BAG-1 downregulates apoptosis through several mechanisms, and may play a role in progression of specific cancers [3]. Therefore, many reports have focused on changes in BAG-1 levels in cancerous tumors [4,5]. In parallel, BAG-1 has been identified as a marker of neuronal differentiation and development [6]. BAG-1 is highly expressed in developing neuronal tissue in the prenatal mouse. In addition, overexpression of BAG-1 in neurons can protect them from apoptosis following stress, such as glutamate toxicity [7]. Therefore, it has been suggested that BAG-1 may play an anti-apoptotic function in neurons, similar to its role in other tissues.

A central role for BAG-1 in the development of the nervous system was validated when a BAG-1 −/− mouse was engineered [8]. The mouse died a few days after birth and showed a 50% decrease in brain mass. The brain stained strongly for apoptotic markers, and the neurons failed to develop into fully mature neurons. A recent work has also determined that BAG-1 promotes axonal regeneration in injured retinal ganglion cells in vitro [9]. Therefore, BAG-1 is an important player in the generation and development of neurons in the central nervous system.

Despite the plethora of work on endogenous BAG-1 in the developing nervous system, there is little known about its role in the function of the adult nervous system. This study examined the expression and localization of BAG-1 in the adult mouse. A specific expression of BAG-1 was exhibited in proliferating neuronal precursor cells (NPCs) found in the subventricular zone and the dentate gyrus, as well as in migrating cells of the rostral migrating stream. The specific expression of BAG-1 in NPCs led us to examine its effect on these cells by lentiviral-mediated overexpression and siRNA. BAG-1 overexpression had a pro-proliferatory effect on NPCs in vitro. In addition, endogenous BAG-1 was found to be necessary to partially attenuate the anti-proliferatory effect of glucocorticoids on these cells. In conclusion, BAG-1 functions in the maintenance of NPCs in the mouse brain.

2. Materials and methods

2.1. Brain homogenation and immunoblotting

C57BL/6 mice were anesthetized (ketamine) and decapitated. Mice were provided by Harlan Laboratories and experiments were approved by the Institutional Animal Use and Care Committee

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(approval number 05811007-2). Brains were dissected and then homogenized in 10-times volume of Buffer H (20 mM HEPES, pH 7.4, 0.3 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) by 15 strokes in glass-teflon homogenizer. Equal amounts of protein were loaded on 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked in non-fat milk for 2 h, incubated with primary antibody overnight at 4 °C, washed and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Membranes were then developed with enhanced chemiluminescence. Primary antibodies used include anti-BAG-1 (1:1000; C-16, Santa Cruz Biotechnology), Tau-5 (1:10000), anti-Beta Actin (1:20000; Sigma), anti-pERK (1:20000; kindly provided by Rony Seger, Weizmann Institute of Science), anti-Hsp70 (1:1000; B-2, Santa Cruz Biotechnology).

2.2. Nuclear and cytoplasmic fractionation

Brain homogenate prepared as described above were centrifuged at 1000 × g for 10 min at 4 °C. Supernatant was collected as cytoplasmic fraction. Pellet was resuspended in 10 volumes homogenation buffer, and then centrifuged again at same conditions. Pellet was resuspended in homogenation buffer as nuclear fraction and nuclear membranes were disrupted by adding a final concentration of 0.1% SDS. Both cytoplasmic and nuclear protein concentrations were determined by Bradford, and equal amounts of protein were loaded onto SDS–PAGE gel.

2.3. Immunohistochemistry

Brains from seven week old C57BL/6 mice were fixed by intracardial perfusion, and stored in 1.25% paraformaldehyde and 30% sucrose at 4 °C until sectioning. For sectioning, brains were frozen, and sliced on a cryostat sliding microtome to 18 μm slices. Slices were treated with 2M HCl at 37 °C for 30 min, followed by a 10 min incubation in 50 mM borate buffer (pH 8.5). After blocking the slices in 20% donor horse serum for 2 h, the slices were incubated overnight in primary antibody in antibody buffer (2% donor horse serum, 0.2% Tween, in PBS). Primary antibodies include anti-Nestin (1:100; Chemicon), anti-BrdU (1:100; kindly provided by Dr. Raya Eilam, Weizmann Institute of Science), anti-Doublecortin (1:100; Dr. Raya Eilam), anti-BAG-1 (1:100; C-16, Santa Cruz Biotechnology), and anti-NeuN (1:200; Dr. Raya Eilam). Slices were then washed, reacted with dye-conjugated secondary antibody, and incubated with Hoechst. Slices were then mounted on coverslides with moviol glue. The slices were visualized by fluorescent microscopy using 488 nm laser excitations for fluorescein isothiocyanate and 545 nm for Cy3.

2.4. Proliferating neuronal precursor culture, and lysis

For each new primary culture, newborn C57BL/6 pups were decapitated and brains were collected in PBS. After brief disassociation by pipetting, brains were incubated for 5 min with trypsin at 37 °C. After brief centrifugation, pellet was resuspended in 1 ml Neurocult proliferation medium (NPM) (Including Neurocult basal medium and proliferation supplements; Stemcell Technologies, Canada), and disaggregated with vigorous pipetting until a single cell suspension was achieved. Cells were then plated in 25 ml of NPM supplemented with 20 ng/ml EGF. Cells were cultured for approximately 7 days. Medium containing floating neurospheres were collected and centrifuged. Neurospheres were resuspended in NPM, disaggregated with vigorous pipetting and replated for an additional 4 days. After another two passages, cells were incubated with virus concentration that had previously been determined to result in 90–100% cells infected (.25 μl virus/ml medium), as determined by GFP co-expression analysis. For lysis, floating neurospheres were pelleted by centrifugation at 400 rpm for 5 min. Cells were then incubated with lysis buffer (140 mM KCL, 3 mM $MgCl_2$, 1% Nonident P-40, 1% glycerol, 20 mM HEPES, pH 7.4, protease inhibitors as described above) for 10 min at 4 °C. Immunoblotting was performed as described above.

2.5. Immunocytochemistry

Neurospheres were collected from medium by centrifugation at 400 × g for 5 min. The neurospheres were then disassociated to a single cell suspension by vigorous pipetting and plated on 12 mm coverslides precoated with Poly-L-orthinine (50 microgram/ml). In some experiments, BrdU (1 μM) was added 4 h after plating. Sixteen hours following plating, cells were fixed with 4% paraformaldehyde in 4% sucrose for 20 min at room temperature. After permeabilization with 0.3% Triton X-100 for 3 min and blocking with 10% goat serum in PBS, the slides were incubated with primary antibodies overnight at room temperature. Primary antibodies include anti-Nestin (1:100; Chemicon), anti-BrdU (1:100; kindly provided by Dr. Raya Eilam, Weizmann Institute of Science), and anti-Doublecortin (1:100; Dr. Raya Eilam). Slides were then washed, reacted with a secondary antibody conjugated to CY3. The cells were then incubated with Hoechst solution for 3 min, and then mounted on slides. The slices were visualized by fluorescent microscopy using 488 nm laser excitations for GFP and 545 nm for Cy3.

2.6. Virus production and infection

Viruses were produced and packaged by transfection of viral producing plasmids in 293-HEK cells. The appropriate plasmids were kindly provided by Dr. Alon Chen (Weizmann Institute of Science, Israel). The pCSC expression plasmid containing cDNA overexpressing BAG-1 was co-transfected with pMDL, pRSV-Rev, and VSV-G plasmids in 12 confluent 150 mm tissue culture plates using the PEI protocol. The plates were washed the following day and then virus containing medium was collected the following 2 days. The viral particles were concentrated by centrifugation at 53000 × *g* for 2.5 h, and resuspended in Hank's Buffered Saline Solution and stored at −80 °C. Virus titer was determined by infection of 293 cells. For infections, viral particles were added to medium of proliferating neuronal precursors.

2.7. BrdU injections of mice

6 month old C57BL/6 mice were injected i.p. with 50 mg/kg BrdU every 12 h for 2 days, and then perfused the following day.

2.8. Plasmids

All lentiviral expression vectors were a kind gift from Dr. Alon Chen (Weizmann Institute of Science, Israel). BAG-1 was inserted into pCSC-SP lentiviral expression construct in the BamH1 site of the Multiple Cloning Site. pCSC-SP expression construct without BamH1 insert was used in control virus. For BAG-1 siRNA, the siRNA sequence was inserted into the p156RRL siRNA expression construct. The sequence used was 5′-GGGCAACTAGCCAAATGTC-3′ [8,10].

2.9. Statistics

Western blots were quantified by NIH imager software. Statistically significant differences between mean values were determined using unpaired Student's *t*-test. S.E. bars are provided. *P*

values and number of experiments used (n) are provided in each figure legend.

3. Results

To evaluate the levels of BAG-1 protein in the brain during mouse development and maturation, we analyzed brain homogenate using Western Blot. Protein levels of both BAG-1L (p50) and BAG-1S (p29) isoforms decreased during mice development (Fig. 1). However, the pattern of this decrease was different between the two isoforms. BAG-1L levels decreased significantly between E15 and 1 week postnatal, while BAG-1S levels declined suddenly between 1 and 7 weeks postnatal. In additional experiments, mice brains were fractionized into cytoplasmic and nuclear fractions using centrifugation. BAG-1S was the dominant isoform in both fractions at E15, while BAG-1L and BAG-1S levels were nearly equal in the nuclear fraction at stages of full development. However, both isoforms decreased in both fractions during mouse development. In conclusion, BAG-1 has a higher expression in the early development of the brain than in the adult brain.

To determine where BAG-1 is expressed in the adult mouse brain, sections were analyzed using immunohistochemistry. An antibody targeting BAG-1 revealed some low levels of ubiquitous staining in the hippocampus and cortex (data not shown). However, the most intense staining was visualized in the subventricular zone (SVZ), which borders the lateral ventricles (Fig. 2A and B). This zone is a niche for proliferating neuronal precursors, which subsequently migrate to the olfactory bulb and differentiate into fully functioning neurons of the olfactory system. Using BrdU incorporation as a marker for proliferating cells, we see that BAG-1 is highly expressed in the niche of cells that are proliferating (Fig. 2A). To verify that these cells are neuronal precursors, we stained with an antibody targeting Nestin, a cytosolic marker of NPCs. BAG-1 and Nestin were found in the same cell population (Fig. 2B). Analysis of the SVZ with confocal microscopy showed that BAG-1 is found in the nuclei of Nestin expressing cells (Fig. 2C). Therefore, BAG-1 is expressed in the nucleus of NPCs.

The one other site of NPCs in the brain, the dentate gyrus, was also analyzed for BAG-1 expression. Cells (84%) that stained positive for BrdU incorporation also stained positive for BAG-1 while only 27% of the BrdU negative cells stained positive for BAG-1 (Fig. 2D and E). Therefore, BAG-1 is found at relatively high levels in proliferating cells in both brain regions with proliferating neuronal precursors.

The abundant expression of BAG-1 among proliferating neuronal precursors was verified by Western blot analysis. Neurospheres, containing proliferating neuronal precursors, were cultured from newborn mice using standard protocols. Protein extract from the neurospheres were compared to extracts from total newborn mouse brain. The amounts of both BAG-1 isoforms were significantly higher in the cultured neuronal precursors than in the total brain extract (Fig. 2F). In addition, lysates from neurospheres were fractionated into nuclear and cytoplasmic fractions, and analyzed by Western blot. Both isoforms of BAG-1 were localized mainly in the nuclear fraction (Fig. 2G). These biochemical experiments corroborate well with the microscopy data to show that BAG-1 is highly expressed in neuronal precursor cells, and localizes mainly to the nucleus.

After initiating the process of differentiation to a neuronal phenotype, neuronal precursors from the SVZ migrate to the olfactory bulb through a defined pathway called the rostral migratory stream [11]. These cells, which do not yet have a fully neuronal

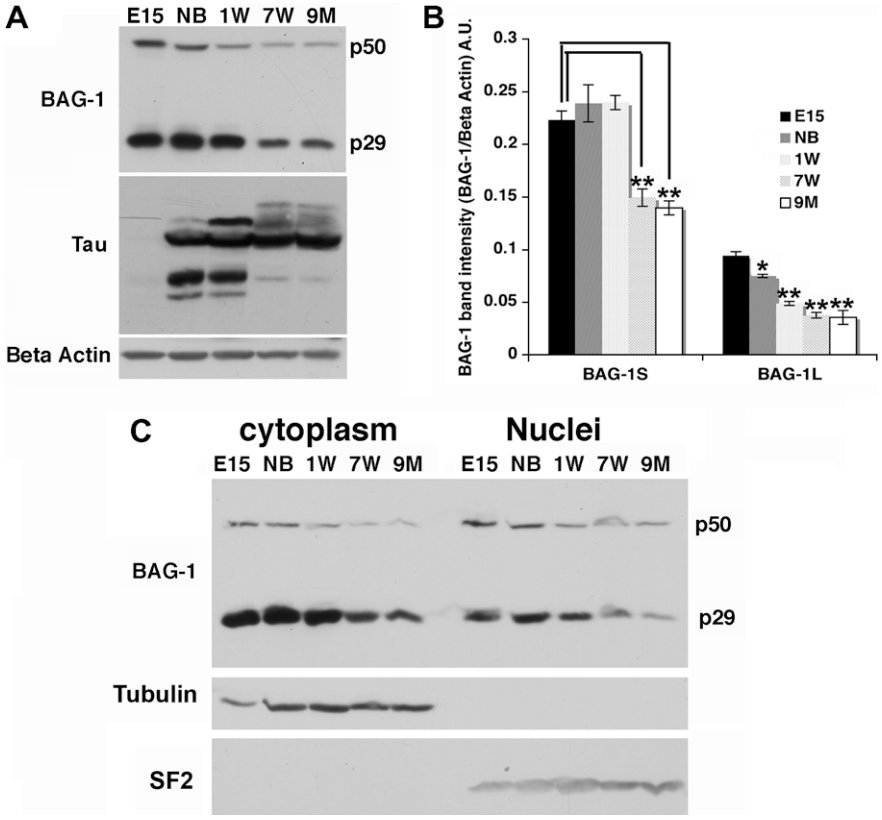

Fig. 1. BAG-1 expression during brain development. (A) BAG-1 levels in the brain were analyzed during mouse development by Western blot. Beta Actin levels are visualized to verify equal protein levels. Tau isoforms change according to stages in mouse development. (B) Quantification of the data from Fig. 1A using NIH imager. Results are represented as mean ± S.E. ($n = 3$; $^{*}P < 0.02$, $^{**}P < 0.01$). (C) Brains from mice at different developmental times were fractionated into nuclear and cytoplasmic fractions. Equal amounts of protein were loaded on SDS–PAGE gel and analyzed by Western blot with antibodies targeting BAG-1, splicing factor 2 (SF2; a nuclear marker), and tubulin (cytoplasmic marker).

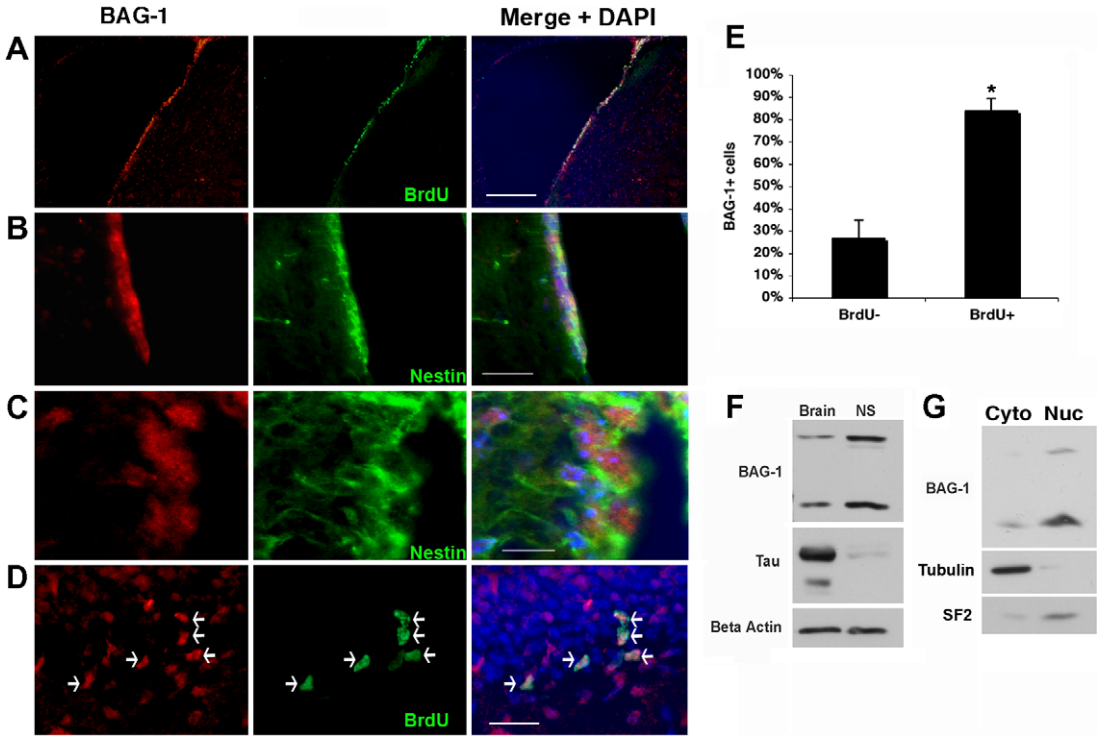

Fig. 2. BAG-1 expression in niches of neuronal precursor proliferation. (A–D) Mouse brain slices (18 μm) from BrdU injected mice were analyzed by immunohistochemistry with primary antibodies targeting BAG-1 and BrdU (A and D), or nestin (B andC).. The subventricular zone (A–C) and dentate gyrus (D) were examined by fluorescent microscopy. Scale bar sizes are 500 μm(A), 50 μm(B), 10 μm(C), and 50 μm(D). (E) Quantitative analysis of BrdU+ compared to BAG-1+ cells in the dentate gyrus. Results are represented as means ± S.E. ($^{*}P < 0.01$). Ten sections were analyzed from three mice for a total of 30 sections analyzed. All cells in the field were analyzed. (F) Total brain and NCS cultures (neurospheres) were analyzed by Western blot. BAG-1 levels are higher in neurosphere (labeled NS) lysate than in total brain homogenate. (G) Neurosphere lysate was separated into nuclear and cytoplasmic fractions, and analyzed by Western blot. Both BAG-1 isoforms are primarily in nuclear fraction.

phenotype, have distinct molecular markers, including doublecortin. We determined if migrating cells retain the high BAG-1 expression by co-immunohistochemistry. The cells migrating from the SVZ to the olfactory bulb retained high expression of BAG-1, as seen by costaining with antibodies targeting doublecortin and BAG-1 (Fig. 3A). Coronal sections of olfactory bulb tissue also revealed high levels of BAG-1 staining in migrating cells expressing doublecortin (Fig. 3B). Surrounding cells that no longer express doublecortin lost the BAG-1 expression. Confocal microscopy analysis shows that BAG-1 was usually visualized in the nuclei, while the doublecortin was in the cytoplasm (Fig. 3C). Staining of olfactory bulb for the neuronal marker NeuN also shows that NeuN expressing cells exhibit less BAG-1 staining (Fig. 3D). Therefore, BAG-1 expression was maintained through proliferatory and migratory stages, but then downregulated by full maturation.

To analyze the role of BAG-1 in proliferating cells, we engineered lentiviral vectors that express the cDNA for the human BAG-1 gene, as well as a vector that expresses siRNA that targets the BAG-1 gene. Viral particles containing these vectors, or a control vector, were infected into cultures of proliferating neuronal precursors. At least 90% of cells were infected as judged by co-expression of the marker protein GFP (not shown). Infection with viral particles expressing siRNA led to a decrease in approximately 80% of BAG-1 levels ($n = 4$, $P = 0.002163$). The siRNA targets all BAG-1 isoforms because they are translated from the same mRNA. Infection with viral particles overexpressing human BAG-1 led to an increase in BAG-1 levels. Extra bands seen on the Western blot analysis are due to the fact that human BAG-1L and BAG-1S run slightly higher on an SDS–PAGE gel, and human BAG-1M runs at the same weight as mouse BAG-1L [1] (Fig. 4A). After 3 days of incubation with the virus, a significantly higher amount of neurospheres were present in the cultures overexpressing BAG-1 (Fig. 4B). This suggests a higher rate of growth in precursors overexpressing BAG-1. However, downregulation of BAG-1 had no effect on neurosphere formation. To further study proliferation, we incubated infected cells with BrdU for 12 h, and detected BrdU incorporation with immunocytochemistry. A significantly higher percentage of BAG-1 overexpressing cells were labeled with BrdU compared to control and BAG-1 underexpressing cells (Fig. 4C and D). Nestin, a marker for NPCs, labeled all cells under all conditions, suggesting that manipulation of BAG-1 did not affect the cell's status (Fig. 4C). In addition, BAG-1 overexpression or downregulation didn't induce expression of the neuronal marker proteins doublecortin or tau (data not shown). Since BAG-1 may affect pERK activation under certain conditions, we checked pERK levels in infected cells. There were no changes in pERK levels, suggesting that activation of the pERK pathway is not responsible for the increase of NPC proliferation (Fig. 4A). These experiments indicate that BAG-1 can increase the proliferation rate of neuronal precursors, but basal levels may not be necessary to maintain the basal proliferation rate.

Neuronal precursor proliferation can be downregulated by stress signals, including glucocorticoids, such as corticosterone [12]. It has been shown that BAG-1 is a binding partner for glucocorticoid receptors, and can attenuate their activity [13]. Therefore, we tested if BAG-1 can regulate the effect of corticosterone on neuronal proliferation by treating viral infected cells with corticosterone. Corticosterone treatment reduced the number of neurospheres in culture by 18%. However, corticosterone together with siRNA led to a 33% decrease in total neurosphere number. In contrast, BAG-1 overexpression attenuated the effect of corticosterone on neurosphere formation (Fig. 5A). We also used the BrdU incorporation assay to explore the effect of BAG-1 and corticosterone on proliferation. Similarly, BAG-1 underexpression strength-

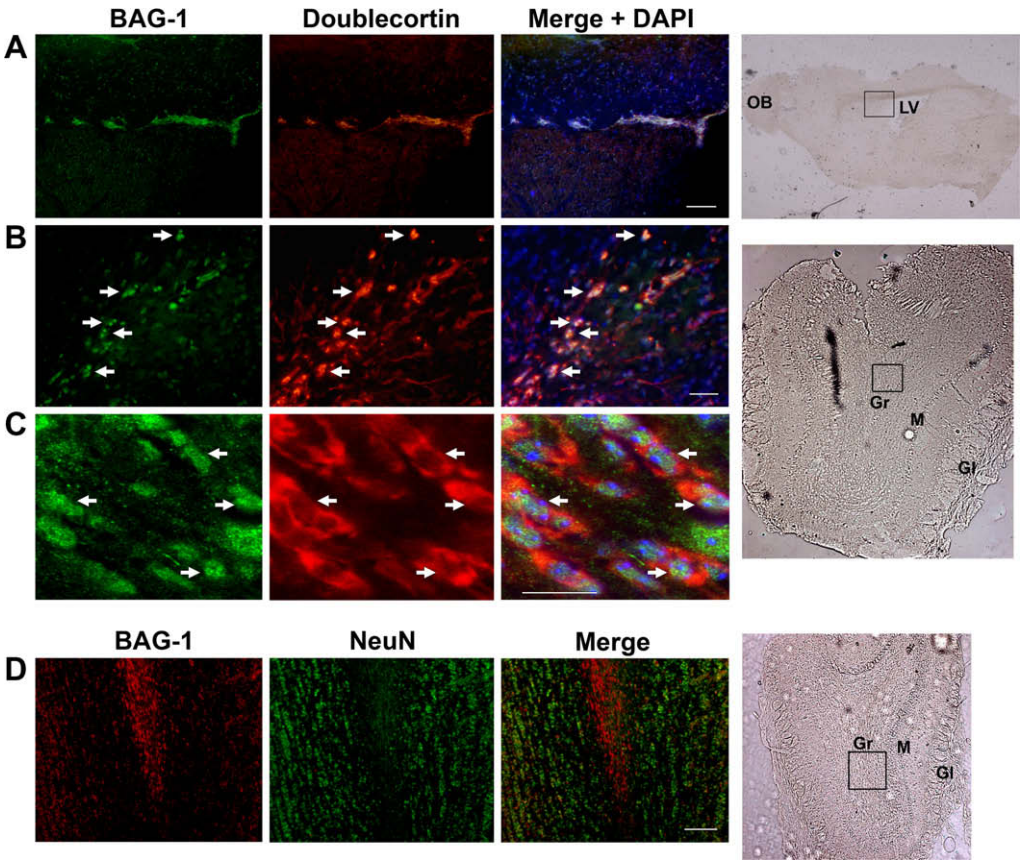

Fig. 3. BAG-1 expression in migrating neuronal precursors. (A) Sagittal slices of mouse brain were immunostained for BAG-1, and for doublecortin to visualize the migrating neuronal precursors in the rostral migratory stream. (B and C) Coronal section of mouse olfactory bulb was immunostained for BAG-1 and for doublecortin (DCX). White arrows show a portion of the cells containing both BAG-1 and doublecortin. BAG-1 is mainly nuclear. (D) Coronal section of mouse olfactory bulb immunostained for BAG-1 and for NeuN, a nuclear marker of mature neurons. Scale bar sizes are 250 μm (A), 50 μm (B) 10 μm (C) , and 100 μm (D). Phase contrast overview of the sections are provided for A, B, and D. Picture from C is taken from area adjacent to area visualized in B. LV, lateral ventricle, OB, olfactory bulb, Gl, glomeruli, M, mitral body layer, Gr, granule cell layer.

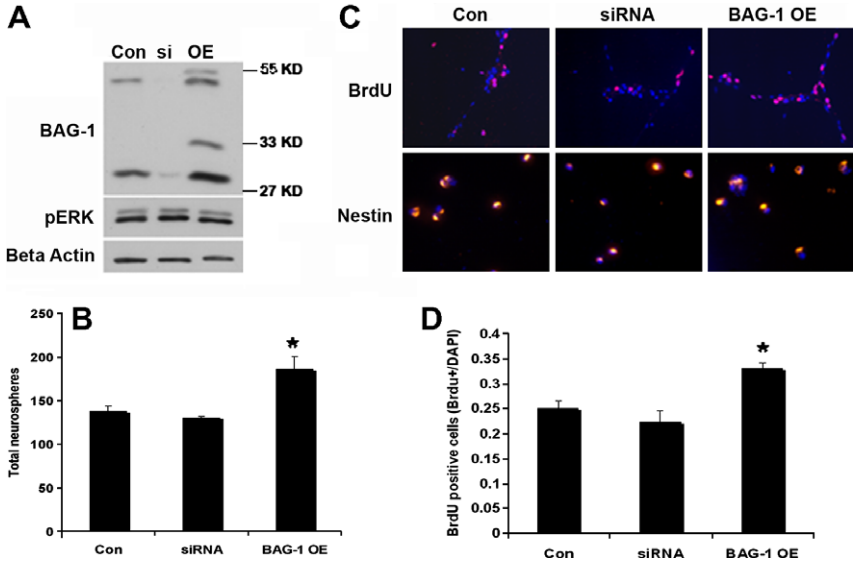

Fig. 4. BAG-1 overexpression increases proliferation rates of NPCs. (A) Neuronal precursor cell cultures were infected with lentiviruses containing empty vector, siRNA targeting BAG-1, or cDNA overexpressing BAG-1 (BAG-1 OE). Western blot analysis shows the levels of BAG-1 after infection. (B) Infected NPCs were grown in culture for four days, and then total neurospheres formed were counted from each culture ($n = 5$, $^{*}P < 0.01$). (C) Neuronal precursor cells infected with viruses were incubated with BrdU for 12 h, and then immunostained for BrdU and the neuronal precursor marker Nestin. (D) Statistical analysis of BrdU+ cells from Fig. 4C ($n = 5$, $^{*}P < 0.01$).

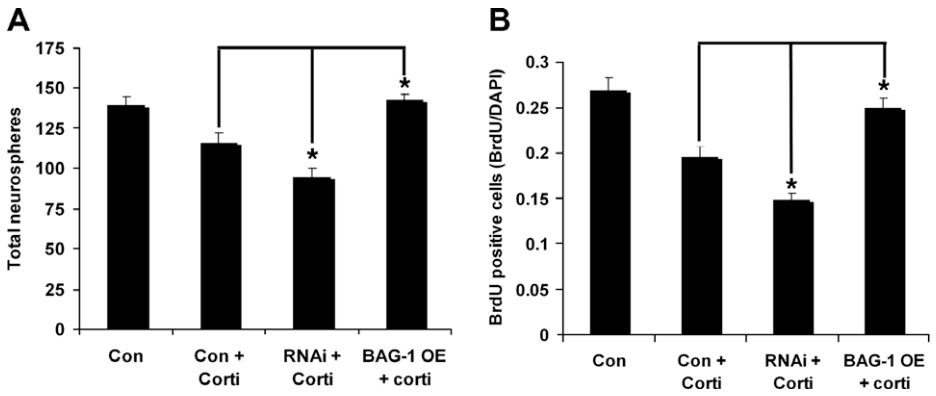

Fig. 5. BAG-1 regulates response of NPCs to corticosterone. (A) Infected NPCs were grown in culture ± corticosterone (1 μM) for 4 days, and then total neurospheres in culture were counted ($n = 5$, $^{*}P < 0.01$). (B) Infected NPCs were grown in culture ± corticosterone, incubated with BrdU for 12 h, and then subjected to immunocytochemistry to determine BrdU incorporation ($n = 5$, $^{*}P < 0.01$).

ened the downregulation of proliferation by corticosterone, while BAG-1 overexpression abolished the effect (Fig. 5B). Therefore, endogenous BAG-1 is necessary to buffer the effect of corticosterone on proliferating cells, and BAG-1 overexpression can completely attenuate the effect.

4. Discussion

BAG-1 has been implicated in processes of neuronal development. Until now, there has been little study on the role of endogenous BAG-1 in the brain after development. The data from this work suggest that BAG-1 maintains its role as a factor in neuronal development and neurogenesis in the adult mouse brain. BAG-1 is found in relatively high levels in NPCs in both niches of adult neurogenesis, the subventricular zone and dentate gyrus. In addition, the overall decrease in BAG-1 levels after mouse development further suggests that the main role of BAG-1 in the brain is developmental, and may play only more minor roles in the mature neurons. This is further supported by our findings that BAG-1 is downregulated in the olfactory neurons during the same developmental timepoint that NeuN is expressed.

Using lentiviral mediated depletion and overexpression of BAG-1 in NPCs, we determined that an overexpression of BAG-1 increases the proliferation rate of NPCs in vitro. This correlates well with an established pro-proliferation function of BAG-1 in other cell types. However, siRNA depletion had no effect on proliferation rates. We hypothesized that endogenous BAG-1 may be necessary to protect NPCs from stress signals, such as glucocorticoids, that are not found normally in the cell culture medium. BAG-1 had previously been shown to inhibit the glucocorticoid receptor in neuronal cells. Therefore, we determined the effects of depletion and overexpression of BAG-1 in the presence of corticosterone, a member of the glucocorticoid family. RNAi depletion of BAG-1 induced a decrease of proliferation in the presence of corticosterone, compared to corticosterone treatment alone. Therefore, endogenous BAG-1 is necessary to partially attenuate the effects of glucocorticoids on NPC proliferation.

Earlier research has suggested that BAG-1 may induce neuronal differentiation through interactions with Raf-1. In our studies, BAG-1 overexpression did not induce a neuronal phenotype of NPCs, but rather affected cell proliferation. We also note that Nestin staining, a marker for undifferentiated NPCs, did not change after BAG-1 overexpression. However, the continued expression of BAG-1 in the migrating cells of the rostral migratory stream (Fig. 2) suggests that BAG-1 may also play important roles in differentiating cells.

Transgenic mice overexpressing BAG-1 in neurons are less susceptible to glutamate toxicity [7] and are able to recover quickly from manic-like and depressive-like behaviors, compared to control mice [14]. Therefore, replenishing the BAG-1 levels in mature neurons may have some positive behavioral effects. However, BAG-1 has also been shown to increase intracellular levels of tau protein [10], which is an important protein leading to Alzheimer's Disease pathology. Therefore, the decrease in BAG-1 levels upon maturation may be necessary to prevent tau accumulation.

In summary, we report a decrease in brain BAG-1 expression during mouse development, and a preferential expression of BAG-1 in NPCs in the adult brain. BAG-1 overexpression by lentiviral infection induced an increase in NPC proliferation in vitro. Depletion of BAG-1 by siRNA induced a decrease of NPC proliferation in the presence of corticosterone. Therefore, a major role for BAG-1 in the adult mouse brain is the continued maintenance of NPCs.

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