DOCTORAL THESIS

Characterization of the "sporadically lurking HAP1-immunoreactive (SLH) cells" in the hippocampus, with special reference to the expression of steroid receptors, GABA, and progenitor cell markers

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Abstract

Huntingtin-associated protein 1 (HAP1) is a neural huntingtin interactor that is widely expressed as a core molecule of the stigmoid body (a neurocytoplasmic inclusion) in the limbic and hypothalamic regions and has putative protective functions against some neurodegenerative diseases (HAP1 protection hypothesis). Although HAP1 has been reported to be intimately associated with several steroid receptors, HAP1-immunoreactive (HAP1-ir) cells remain to be identified in the hippocampus, which is one of the major steroidal targets. In this study, we determined the distribution of hippocampal HAP1-ir cells in light and fluorescence microscopy and characterized their morphological relationships with steroid receptors, markers of adult neurogenesis and the γ-aminobutyric acid (GABA)-ergic system in adult male Wistar rats. HAP1-ir cells, which were sporadically distributed particularly in the subgranular zone (SGZ) of the dentate gyrus and in the interface between the stratum lacunosum-moleculare and stratum radiatum of Ammon's horn, were identified as the "sporadically lurking HAP1-ir (SLH)" cells. The SLH cells showed no clear association with neural progenitor/proliferating or migrating cell markers of adult neurogenesis, such as Ki-67, proliferating cell nuclear antigen, doublecortin, and glial fibrillary acidic protein in the SGZ, while all the SLH cells expressed a neuronal specific nuclear protein (NeuN). More than 90% of the SLH cells expressed nuclear estrogen receptor (ER) α, but neither ER β nor the androgen, while glucocorticoid receptor was differently stained in the SLH cells depending on the antibodies. More than 60% of them exhibited GABA immunoreactivity in the SGZ, suggestive of basket cells, but they were distinct from the ones expressing cholecystokinin or parvalbumin. We conclude that SLH cells, which should be stable against apoptosis due to putative HAP1 protectivity, might be involved

in estrogen-dependent maturation, remodeling and activation of hippocampal memory and learning functions via ERα and partly through GABAergic regulation.

Key words: Huntingtin-associated protein 1, dentate gyrus, Ammon's horn, neurogenesis, estrogen receptor α, immunohistochemistry

2. Introduction

2.1. HAP1 is an essential component of the stigmoid body

Huntingtin-associated protein 1 (HAP1) was first identified as a polyglutamine (polyQ) length-dependent interactor with huntingtin (Htt), which is the gene product responsible for Huntington's disease (Li et al., 1995). In normal rodent brains, HAP1 is uniquely localized to a distinct neurocytoplasmic inclusion, the stigmoid body (STB) (Li et al., 1998 a; Gutekunst et al., 1998; Fujinaga et al., 2007, 2009). The STBs were originally detected by the immunohistochemistry for human placental antigen X-P2 (hPAX-P2) and identified as which was first identified as a spherical-to-ovoid, non-membrane-bound inclusion (0.5–3μm in diameter) with a granular, fuzzy texture and moderate-to-low electron density in rat brains (Shinoda et al., 1992, 1993). HAP1 induces the formation of STB by its complementary DNA (cDNA) transfection into HEK293 cells (Li et al., 1998a). Subsequently, HAP1 was detected in STB of the rat brain in light and electron microscopy (Gutekunst et al., 1998). The STB has none of the aggresomal characteristics, such as being ubiquitinated and surrounded by vimentin, indicating that the STB is physiological entity distinct from pathological aggresome (Fujinaga et al., 2009). The distribution of HAP1 mRNA expression in the rat (Page et al., 1998) and mouse (Fujinaga et al., 2004) brains corresponds to STB's distribution. Recent studies have shown that STB formation is inhibited in the brain of *Hap1* (+/-) mice (Chan et al., 2002). All these data strongly suggest that HAP1 is an essential component of the STB in the neuronal cytoplasm *in vivo* and *in vitro*.

2.2. STB/HAP1 protection hypothesis

STB/ HAP1 has been widely detected in several brain regions, such as the medial amygdaloid, septal, and preoptic-hypothalamic areas as well as related brainstem regions (Shinoda et al., 1992, 1993; Fujinaga et al., 2004, 2009), which tend to be spared from neurodegeneration in Huntington's disease. HAP1 interacts with huntingtin in polyQ dpendent manner (Li et al., 1995) and it has a protective effect on neurons against apoptosis induced by polyQ-expanded huntingtin (Kamei et al., 2001; Li et al., 2003; Fujinaga et al., 2004; Metzger et al., 2008). STB/HAP1 also interacts with PolyQ-expanded androgen receptor (AR) in polyQ-depended manner through a ligand-binding domain, and it suppresses apoptosis induced by a polyQ-expanded AR derived from spinal and bulbar muscular atrophy (SBMA) (Takeshita et al., 2006). HAP1 has also been reported to interact with other polyQ disease-related gene products, including TATA-binding protein in spinocerebellar ataxia (SCA) type 17 (Prigge and Schmidt, 2007) and ataxin-3 in SCA3 (Machado–Joseph disease) (Takeshita et al., 2011). Furthermore, HAP1 can interact with Abelson helper integration site 1 (Sheng et al., 2008), mutations in which lead to Joubert syndrome (Dixon-Salazar et al., 2004; Ferland et al., 2004) and schizophrenia (Amann-Zalcenstein et al., 2006; Ingason et al., 2007). These findings emphasize the important role of HAP1 in the pathology underlying polyQ diseases and other neurodevelopmental and psychiatric disorders. The line of data suggests that STB/HAP1 could play a protective role in Huntington's disease, SBMA, SCA3, SCA17, and Joubert syndrome, leading to the "STB/HAP1 protection hypothesis" that STB/HAP1 expression raises the threshold of vulnerability for neurodegeneration and renders more beneficial stability to neurons with STB/HAP1 than those without it (Fujinaga et al., 2004).

2.3. Physiological functions of STB/HAP1

The precise functions of STBs under physiological conditions are not fully understood, despite the fact that they are widely present in normal rat and mouse brains (Shinoda et al., 1992; Nagano and Shinoda, 1994; Li et al., 2003; Sheng et al., 2006; Fujinaga et al., 2007, 2009). Several studies using Hap1-knockout mice have indicated that Hap1 plays important roles in hypothalamic functions, including the maintenance of neuronal survival (Li et al., 2003), the regulation of food intake and body weight (Chan et al., 2002; Dragatsis et al., 2004; Sheng et al., 2006; Lin et al., 2010), and the control of locomotor activity (Lin et al., 2010). A new evidence for HAP1 and its associated proteins is emerging that supports a role for HAP1 in intercellular trafficking. Intracellular transport of membrane organelles requires the molecular motors kinesin and dynein, which are involved in anterograde and retrograde transport, respectively. HAP1 interacts with p150 (Glued) subunit of dynactin (Engelender et al., 1997; Li et al., 1998a), an essential component of dynein motor complexes (Holleran et al., 1998; King and Schroer, 2000), suggesting HAP1 is involved in retrograde transport along microtubules.

2.4. STB/HAP1 and steroid receptors:

Since the amygdaloid and preoptic-hypothalamic areas are regarded as the most prominent targets for sex steroids, we have previously demonstrated the coexistence of STB with estrogen receptor (ER) α (Nagano and Shinoda, 1994) or AR (Fujinaga et al., 2005) in the same neurons in these areas. Recently, we reported that HAP1/STB directly interacts with steroid receptors and regulates their nuclear translocation *in vitro* (Takeshita et al., 2006; Fujinaga et al., 2011).

2.5. Purpose of the present study

The hippocampus is another important target for the actions of steroid hormones (Prange-Kiel and Rune, 2006; Ogiue-Ikeda et al., 2008; Hajszan et al., 2008; Nishi, 2011). Gonadal steroids have been reported to modulate hippocampal neurogenesis (Galea et al., 2006). It has also been suggested that ERα may mediate estrogen actions in γ-aminobutyric acid (GABA)-ergic neurons (Hart et al., 2001), which has been considered to play diverse roles in hippocampal functions (Jinno and Kosaka, 2003). Hippocampal HAP1 expression has been reported in mice by *in situ* hybridization (Fujinaga et al., 2004) and in rats by Western blotting (Li et al., 1998a), reverse transcriptase-polymerase chain reaction (Li et al., 2000), immunohistochemistry (Gutekunst et al., 1998), and *in situ* hybridization (Page et al 1998; Li et al., 1996). In the human and monkey hippocampus, HAP1 expression has been observed in Northern and Western blots (Li et al., 1998b). Thus, HAP1/STB might be involved in hippocampal neurogenesis, stress regulation, and/or memory functions through steroid-sensitive and/or GABAergic systems. With respect to their relationship with these systems, however, the detailed distribution and histochemical characterization of hippocampal HAP1-immunoreactive (HAP1-ir) cells remain to be determined.

The objectives of the present study are:

1. To clarify of the detail distribution of STB/HAP1-ir cells in the rat hippocampus.
2. To evaluate the morphological relationship of STB/HAP1with hippocampal steroid receptors, markers of neurogenesis and the GABAergic system.

3. Materials and Methods

3.1.Primary antibodies

A goat polyclonal anti-HAP1 antibody (R19) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody was raised against a peptide that mapped to the C-terminus of HAP1 of rat origin. A rabbit polyclonal anti-ERα antibody (MC20), a rabbit polyclonal anti-AR antibody (N20), a rabbit polyclonal anti-glucocorticoid receptor (GR) antibody (M20) and a mouse monoclonal anti-GR antibody (3D5) were also purchased from Santa Cruz Biotechnology. Each one was raised against a peptide that mapped to the C-terminal ERα sequence of mouse origin, the N-terminal AR sequence of mouse origin, the N-terminal GR sequence of mouse origin (M20) or a peptide fragment of a well conserved region of human GR sequence (3D5), respectively. A rabbit anti-ERβ polyclonal antibody (Z8P) against an 18-amino acid synthetic peptide that corresponded to the C-terminus of mouse origin was obtained from Zymed Laboratories, Inc. (South San Francisco, CA, USA). Mouse monoclonal antibodies to proliferating cell nuclear antigen (PCNA, PC10) and neuronal specific nuclear protein (NeuN, A60) (Millipore, Billerica, MA, USA) were raised against recombinant rat PCNA, and purified cell nuclei from mouse brain, respectively. Rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP, G9296) and GABA (A2052) (Sigma-Aldrich Co., Saint Louis, MO, USA) were developed using purified human brain GFAP and bovine serum albumin-conjugated GABA, respectively. In addition, we used a rabbit polyclonal anti-Ki67 antibody that was raised against a recombinant protein corresponding to a Ki67 motif (NCL-Ki67p; Novocastra, Laboratories Ltd., Newcastle, UK) and a rabbit polyclonal antibody to doublecortin (DCX) that was raised against a Keyhole Limpet Hemocyanin-coupled synthetic

peptide corresponding to human DCX (Cell Signaling Technology Inc., Danvers, MA USA). We also used a rabbit polyclonal anti-cholecystokinin antibody that was produced using synthetic sulfated cholecystokinin (CCK) (26-33) amide (sulfated CCK-8) as immunogen (C2581, Sigma-Aldrich Co) and a mouse monoclonal anti-parvalbumin antibody that was made using purified frog muscle parvalbumin as immunogen (P3088, Sigma-Aldrich Co). For detecting 5-bromo-2′-deoxyuridine (BrdU), we used a mouse monoclonal anti-BrdU antibody (NCL-BrdU; Novocastra Laboratories Ltd.).

3.2 Animals and tissue preparation

Wistar male (n = 20) and female (n = 8) rats (10 weeks old; Japan SLC Inc., Shizuoka, Japan) were used in this study. They were group housed (3 - 4 rats/cage) at a constant temperature (22°C) with a 12 h-12 h light-dark cycle (lights on 08:00-20:00) and provided food and water ad libitum. All experimental protocols were approved by the Committee on the Ethics of Animal Experimentation at Yamaguchi University School of Medicine, and conducted according to the guidelines for Animal Research of Yamaguchi University School of Medicine and the Law (No. 105) and Notification (No. 6) of the Japanese Government. The animals were transcardially perfused with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) under anesthesia with sodium pentobarbital (60-80 mg/kg i. p.). For GABA staining, the animals were perfused with 0.125% glutaraldehyde mixed with 4% paraformaldehyde in PB, as previously reported (Shinoda et al., 1987; Jinno and Kosaka, 2010). Brains were removed and post-fixed for 24 h in the same fixative used for perfusion. Brains were then soaked in cold 0.1 M PB containing 30% sucrose until they sank followed by freezing in powdered dry ice. The tissue was frontally sectioned at a thickness of 30 μm

on a cryostat. Frozen sections were collected in icè-cold 0.02 M sodium phosphate buffered saline (PBS; pH 7.4) containing 0.1% sodium azide, and stored at 4° C until use.

3.3 BrdU injections

Three adult male Wistar rats were injected intraperitoneally with the thymidine analog BrdU (B9285-1G, Sigma-Aldrich Co., 300 mg/kg at a concentration of 15 mg/mL in saline) once in a single dose. The rats were then perfused, as described above, 24 h after the injection (Cameron and McKay, 2001).

3.4 Immunohistochemistry

3.4.1 Pretreatment of sections

In order to visualize Ki-67, PCNA and BrdU by immunofluorescence staining, sections were heated in 0.1 M citric acid buffer (pH 6.0) at 90 °C for 10 min and denatured with 2N HCl for 30 min at room temperature (Cameron and McKay, 2001; Olariu et al., 2007; Snyder et al., 2009) before blocking with 10% normal serum containing 0.3% Triton X-100 in PBS. For immunoperoxidase staining of Ki-67 and PCNA, the pretreatment step was omitted because optimal signals were obtained without pretreatment.

3.4.2 Single immunoperoxidase staining

Immunohistochemistry was performed as previously described (Sheng et al., 2003, 2004; Fujinaga et al., 2007, 2009; Zhao et al., 2007). The sections were pre-incubated for 2 h with PBS containing 10% normal donkey serum (NDS) and 0.3% Triton X-100 , followed by bleaching for 1 h with 50% methanol and 1.5% hydrogen peroxide diluted with double-distilled water at 4° C. The sections were washed three times each for 10 min with PBS containing 0.3% Triton X-100 and 0.05% NDS (PBST-NDS). The

sections were then incubated with antibodies to HAP1 (1: 2,000), AR (1: 2,000), GR M20 (1: 500), ERα (1: 5,000), ERβ (1: 500), Ki-67 (1: 2,000), PCNA (1: 2,000) or DCX (1: 2,000), for 5 days at room temperature. For the HAP1 pre-adsorption test, the diluted antibody was incubated overnight at 4° C with a specific blocking peptide (Santa Cruz Biotechnology Inc.). After the primary antibody immunoreaction, the sections were washed with PBST-NDS three times each for 15 min and then incubated overnight at 4° C in PBS containing 1% NDS with biotinylated donkey anti-goat, anti-rabbit or anti-mouse secondary antibody, according to the animal species used for the primary antibodies (Millipore; 1:500 diluted). Subsequently, the sections were washed twice for 10 min with PBS containing 0.05% NDS and, once for 10 min with PBS, followed by incubation for 3 h at 20° C with peroxidase-conjugated streptavidin (1:500 diluted in PBS; DakoCytomation, Glostrup, Denmark). The sections were washed three times each for 10 min in 0.05 M Tris-HCl buffer (pH 7.6), followed by a violet-to-black color reaction with 0.02% 3, 3 diaminobenzidine (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.6% nickel ammonium sulfate (Sigma-Aldrich, Tokyo, Japan) in 0.05 M Tris-HCl buffer containing 0.0008% H_2O_2 (nickel-enhanced DAB reaction) for 10- 30 min at room temperature. After washing in 0.05 M Tris-HCl buffer and PBS, the sections were mounted on glass slides coated with 0.6% gelatin solution, air-dried, dehydrated using a graded series of ethanol, immersed in xylene, and then embedded with Entellan Neu (Merck KgaA, Darmstadt, Germany).

3.4.3 Double-label immunoperoxidase staining

After the sections were stained by the nickel-enhanced DAB reaction method (violet-to-black color) using a rabbit anti-Ki-67 antibody, they were thoroughly washed with PBST-NDS and subsequently incubated with a goat anti-HAP1 antibody (1:500

diluted in PBS containing 1% NDS and 0.3% Triton X-100) for 5 days at 4° C. The sections were then washed with PBST-NDS, followed by incubation with an un-labeled donkey anti-goat antibody (Millipore; 1:500 diluted in PBS containing 1% NDS) overnight at 4° C. After washing the sections in PBS containing 0.05% NDS, they were incubated for 3 h at 20° C with goat or rabbit peroxidase anti-peroxidase (DakoCytomation; 1:500 diluted in PBS). The sections were then washed in 0.05 M Tris-HCl buffer, and colored by a DAB reaction without nickel enhancement (0.08% DAB, 0.002%H_2O_2) to visualize HAP1 as a brown color.

3.4.4 Double-label immunofluorescence staining

Sections were blocked with PBS containing 10% NDS and 0.3% Triton X-100 for 3 h at 4° C. The sections were then washed twice for 10 min in PBST-NDS, followed by incubation with a goat anti-HAP1 antibody in combination with a rabbit anti-Ki-67, rabbit anti-DCX, rabbit anti-GFAP (1: 2,000), rabbit anti-GABA (1: 200), rabbit anti-ERα, rabbit anti-GR M20 (1:500), rabbit anti-GR 3D5 (1:500),rabbit anti-CCK (1: 1,000), mouse anti-parvalbumin (1: 1,000), mouse anti-PCNA, or mouse anti-NeuN (1: 500) antibody for 5 days at 20°C. After washing the sections three times each for 10 min with PBST-NDS, they were subjected to secondary immunoreactions with a mixture of Alexa Fluor 594-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-rabbit or anti-mouse IgG (Molecular Probes, Eugene, OR, USA; 1:500 diluted in PBS containing 1%NDS) for 5 h at 20°C. After washing with PBS, the sections were mounted on glass slides coated with a 0.6% gelatin solution, followed by air-dried and embedded with Fluoromount/Plus (Diagnostic BioSystems, Pleasanton, Inc., CA, USA).

3.4.5 Triple-label immunofluorescence staining

After the sections were blocked with PBS containing 10% NDS and 0.3% Triton X-100, they were incubated with a mixture of goat anti-HAP1, rabbit anti-Ki-67 and mouse anti-BrdU (1:500) antibodies or a combination of goat anti-HAP1, rabbit anti-DCX and mouse anti-BrdU antibodies for 5 days at 20°C. The sections were then incubated with a mixture of Alexa Fluor 594-conjugated donkey anti-goat IgG, Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Alexa Fluor 647-conjugated anti-mouse IgG.

3.5 Immuno-electron microscopy

Tissue preparation for immuno-electron microscopy was performed as previously described (Shinoda et al., 1994). Adult male Wistar rats were anesthetized, as described above and, intracardially perfused with ice-cold saline, followed by 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M PB. The trimmed slabs of brain blocks containing the hippocampus were cut into 30 μm thick sections on a vibrating blade microtome (LEICA VT 1000S, Leica, Microsystems Inc.) in 0.1M PB. The free floating sections were stained by the nickel-enhanced DAB reaction method, as described above, using the goat anti-HAP1 antibody. HAP1-stained sections were postfixed for 1 h with 1% osmium tetroxide in 0.1 M PB, block-stained for 1 h with 2% uranyl acetate in distilled water, dehydrated with a graded series of ethanol rinses, infiltrated in propylene oxide, placed in a mixture of propylene oxide and Epon 815 (1:1), and then flat-embedded on Aclar film (Nisshin EM, Tokyo, Japan) in Epon 815. Ultrathin sections were obtained from the trimmed slab containing the dentate gyrus with HAP1-ir structures using Reichert-Jung Ultracut OmU4 ultramicrotome and mounted on copper grids. The sections were observed under a Hitachi H-7500 or FEI

Tecnai G2 BioTwin electron microscope operated at 80 kV or 120kV without uranyl acetate or lead staining.

3.6 Tissue analysis and cell counting

For cytoarchitectonic analysis, neighboring sections were Nissl-stained with cresyl violet (Merck KgaA). The coexpression ratios for HAP1/ ERα or ERα/ HAP1 (Table 1) were calculated from the real numbers of HAP1-ir and ERα-ir cells, and those of cells double-immunolabeled for HAP1 and ERα. The total numbers of immunoreactive cells were counted in the SGZ of the dentate gyrus as well as in the interface between stratum lacunosum-moleculare and stratum radiatum (IFLMR) of Ammon's horn using forty eight sections from four adult male and female rats (six sections from each rats) at Bregma levels of approximately from −2.86 to−4.16 mm (Paxinos and Watson,1998). Values were shown as mean ± SEM (n = 4). The data were analyzed using the Mann-Whitney U test, with $P < 0.05$ indicating statistical significance between male and females. The coexpression ratios for HAP1/ GABA or GABA/ HAP1 (Table 2) were also estimated using twenty four sections from four male rats.

3.7 Photomicrographs

Photomicrographs were taken using a DXM1200 color digital camera (Nikon, Tokyo, Japan) equipped with an Eclipse E80i photomicroscope (Nikon). Fluorescence images were acquired using a laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany) in the Institute for Biomedical Research and Education, Yamaguchi University Science Research Center. Single optical sections were obtained (1024 × 1024 pixels). All images were transferred to Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA, USA), and the brightness and contrast were adjusted. No other adjustments were made.

4. Results

4.1 Distribution of HAP1-ir cells in the hippocampus

HAP1-ir cells were sporadically distributed in the hippocampus, including the dentate gyrus and Ammon's horn [Fig. 1A, B (Nissl staining)]. In the dentate gyrus, the majority of HAP1-ir cells with strong immunoreactivity were observed in the hilar interface between the granule cell layer, which corresponds to the SGZ (Fig. 1A, C). A few HAP1-ir cells were also found in the deeper parts of the granule-cell layer, molecular-cell layer and hilar region of the dentate gyrus. These HAP1-ir cells were bipolar, pyramidal or fusiform in shape unlike the typical granule cells that showed no clear HAP1 expression. In the Ammon's horn, most of the HAP1-ir cells with strong immunoreactivity were sporadically distributed along the interface between stratum lacunosum-moleculare and stratum radiatum (IFLMR) (Fig. 1A, D). Sparse cells with weaker immunoreactivity were also detected in the stratum oriens, stratum pyramidale and stratum radiatum of Ammon's horn. Typical pyramidal cells, however, showed no clear HAP1 immunoreactivity. The distribution pattern was basically the same throughout the rostrocaudal extent of the hippocampus. Majority of the positive cells showed clear HAP1-ir STB in the diffusely immunoreactive cytoplasm, while the others showed only diffuse HAP1 immunoreactivity in the cytoplasm with no clear STB (Fig. 1C, D). Both types of HAP1 immunoreactivity were clearly hampered in the pre-adsorption test with a blocking peptide against anti-HAP1 antibody (Fig. 1E). The HAP1-ir dot-like structures were proven to be STBs in immuno-electron microscopy (Fig. 1F, G). Similar distribution pattern of the HAP1-ir cells was observed in both male and female hippocampus.

4.2 Expression of steroid receptors in the HAP1-ir cells

In order to evaluate the morphological relationships between HAP1 and steroid receptors (AR, GR, ERα and ERβ), their distribution patterns in the dentate gyrus and Ammon's horn were immunohistochemically compared. Prominent AR expression was exclusively observed in pyramidal cells in the CA1 field (Fig. 2A) but was not clearly detected in other cells in the dentate gyrus or Ammon's horn. Strong GR expression (using anti-GR M20) was also found in the pyramidal cells in the CA1 field (Fig. 2B). GR expression was moderate in the granule cell layer of the dentate gyrus, weak in pyramidal cells in the CA2 and CA3 fields, and weak or very weak in sporadic non-pyramidal cells in the hilar region and in other parts of the dentate gyrus and Ammon's horn. ERα-ir cells with strong immunoreactivity were sporadically present in SGZ of the dentate gyrus and IFLMR of the Ammon's horn (Fig. 2C-E). A small number of ERα-ir cells with moderate immunoreactivity were solitarily observed in the deeper granule cell layer, the molecular cell layer and hilar region of the dentate gyrus and in the stratum oriens, stratum pyramidale and stratum radiatum of Ammon's horn. Neither typical granule cells nor pyramidal cells showed any clear ERα expression. No ERβ-ir cells were detected in any part of the dentate gyrus or Ammon's horn, although ERβ-ir cells were strongly stained in previously reported areas (the medial amygdaloid nucleus, the bed nucleus of the stria terminalis etc.) (data not shown). From the viewpoint of distribution, ERα expression resembled that of HAP1 in the hippocampus. In addition, since most of the hippocampal cells expressed GR, HAP1-ir cells as well as non-HAP1-ir cells seemed to express GR in the hippocampus. Because AR was exclusively expressed in CA1 pyramidal cells and ERβ was not clearly expressed in any

part of the hippocampus, HAP1-ir cells, which were not present in the pyramidal cells, were thought to express neither AR nor ERβ. Thus we focused on the relationship of HAP1 with GR and ERα.

Double-immunostaining of HAP1 and ERα clearly indicated that most of the HAP1-ir cells expressed ERα in the dentate gyrus (Fig. 3A-C) and Ammon's horn (Fig. 3D-F). Results of the cell counting indicated that in the SGZ of dentate gyrus, the coexpression ratio of ERα in HAP1-ir cells was 91.5 ± 1.5% and that of HAP1 in the ERα-ir cells was 94.6 ± 0.9 % in male, while in female, the coexpression ratio of ERα in HAP1-ir cells was 92.9 ± 1.2% and that of HAP1 in the ERα-ir cells was 95.4 ± 0.3% (Table. 1). In the IFLMR of Ammon's horn, coexpression ratio of ERα in the HAP1-ir cells was 93.8 ± 1.5% and that of HAP1 in the ERα-ir cells was 91.6 ± 1.0% in male, whereas in female, coexpression ratio of ERα in the HAP1-ir cells was 94.2 ± 0.9% and that of HAP1 in the ERα-ir cells was 91.2 ± 0.8% (Table. 1). There was no significant difference between male and female in coexpression ratios either in the SGZ or IFLMR. In the other hippocampal regions, although the counted cell numbers were limited, the coexpression ratios of HAP1 and ERα also showed similar results. With respect to GR expression, when the anti-GR M20 antibody was used, although most of the cells in the dentate gyrus seemed to express GR, HAP1-ir cells showed very weak GR expression in their nuclei in the SGZ of the dentate gyrus (Fig. 3G-I) and in the IFLMR of Ammon's horn (Fig. 3M-O). In contrast, when the anti-GR 3D5 antibody was used, HAP1-ir cells showed moderate-to strong GR expression in their nuclei of the neurons particularly in the SGZ of the dentate gyrus (Fig. 3J-L) and in the IFLMR of Ammon's horn (Fig. 3P-R).

4.3 Expression of molecular markers for different stages of neurogenesis in the HAP1-ir cells

In order to address the question whether HAP1-ir cells are involved in adult neurogenesis in the SGZ of dentate gyrus, the distribution pattern of HAP1-ir cells was compared with those of the cells expressing molecular markers of different stages of neurogenesis (Fig. 4, Ki-67 and PCNA as proliferating cell markers and DCX as a migrating cell marker). Immunohistochemically, Ki-67 and PCNA were expressed in the nuclei of some disperse cells in the SGZ. Each immunoreactive cell group for Ki-67 and PCNA often formed sporadic cell clusters composed of two or three Ki-67-ir or PCNA-ir cells. DCX was localized in the cytoplasm of numerous cells located in a line along the SGZ. DCX-ir cells were much larger in number than Ki-67-ir or PCNA-ir cells, but they rarely assembled like Ki-67-ir or PCNA-ir cells.

In double immunostaining of HAP1 and each proliferating cell marker, HAP1-ir cells were sometimes found to be located close to or next to the clusters of Ki-67-ir or PCNA-ir nuclei, but they themselves did not express any of the markers (Fig. 5A, B-D for Ki-67; 5E-G for PCNA). With respect to the migrating cell marker, HAP1-ir cells did not express DCX (Fig. 5H-K). In addition, intraperitoneal BrdU injections resulted in BrdU labeling of the proliferating (Ki-67-ir) cells and some of migrating (DCX-ir) cells (inset of Fig. 6H), but no such labeling of HAP1-ir cells or most of migrating (DCX-ir) cells in the SGZ (Fig. 6). The results clearly indicate that cell division evidenced by BrdU uptake occurred in both Ki-67-ir cells and a few putative immature migrating DCX-ir cells, but in neither HAP1-ir cells nor most of migrating DCX-ir cells in the SGZ at the time of injection (adulthood).

Finally, in double immunofluorescence staining for HAP1 and GFAP (a marker of astrocytes or early progenitor cells) (Fig. 7A-C) or NeuN (a postmitotic neuronal cell marker) (Fig. 7D-F), almost all the HAP1-ir cells clearly showed NeuN-immunoreactivity in their nuclei but not GFAP immunoreactivity. The data indicated that HAP1-ir cells in the SGZ exhibited attributes of mature neurons but did not share any proliferating or migrating features of glial or subgranular progenitor cells.

4.4 Coexpression of GABA and related basket-cell markers in HAP1-ir cells

Double immunofluorescence staining of HAP1 and GABA indicated that 63.2 ± 2.2% of HAP1-ir cells showed GABA immunoreactivity and 57.7 ± 1.2% of GABA-ir neurons showed HAP1-immunoreactivity in the SGZ of the dentate gyrus (Fig. 8, Table. 2). In the IFLMR of Ammon's horn, cell counting indicated that 64.3 ± 1.5% of HAP1-ir cells showed GABA immunoreactivity and 56.7 ± 2.5% of GABA-ir neurons showed HAP1immunoreactivity (Table. 2). In the other hippocampal regions, the number of GABA-ir cells was much larger than that of HAP1-ir cells. Therefore a minor proportion of GABA-ir cells expressed HAP1. These results indicated that hippocampal HAP1-ir cells were composed of two distinct subtypes, GABAergic and non-GABAergic HAP1-ir cells. In order to clarify the relationship with CCK-ir or parvalbumin-ir cells, which are parts of GABAergic cells in the hippocampus (Kosaka et al., 1985, 1987), double-immunostainings for HAP1 and CCK or parvalbumin were performed. As a result, each of the two GABAergic basket-cell markers were never expressed in the HAP1-ir cells (Fig. 9), indicating that the population of GABAergic HAP1-ir cells is different from that of GABAergic CCK- or parvalbumin-expressing cells.

Table 1 Coexpression ratios of HAP1/ERα and ERα/HAP1 in the two major HAP1 expression zones in the hippocampus of male and female rats.

		HAP1/ERα ratio (%)	ERα/HAP1 ratio (%)
SGZ	Male	94.6 ± 0.9	91.5 ± 1.5
	Female	95.4 ± 0.3	92.9 ± 1.2
IFLMR	Male	91.6 ± 1.0	93.8 ± 1.5
	Female	91.2 ± 0.8	94.2 ± 0.9

Values represent the mean ± SEM (n = 4). ERα, estrogen receptor α; HAP1, huntingtin-associated protein 1; IFLMR, interface between stratum lacunosum-moleculare and stratum radiatum; SGZ, subgranular zone

Table 2 Coexpression ratios of HAP1/GABA and GABA/HAP1 in the two major HAP1 expression zones in the hippocampus of male rats.

	HAP1/GABA ratio (%)	GABA/HAP1 ratio (%)
SGZ	57.7 ± 1.2	63.2 ± 2.2
IFLMR	56.7 ± 2.5	64.3 ± 1.5

Values represent the mean ± SEM (n = 4). GABA, γ-aminobutyric acid; HAP1, huntingtin-associated protein 1; IFLMR, interface between stratum lacunosum-moleculare and stratum radiatum; SGZ, subgranular zone

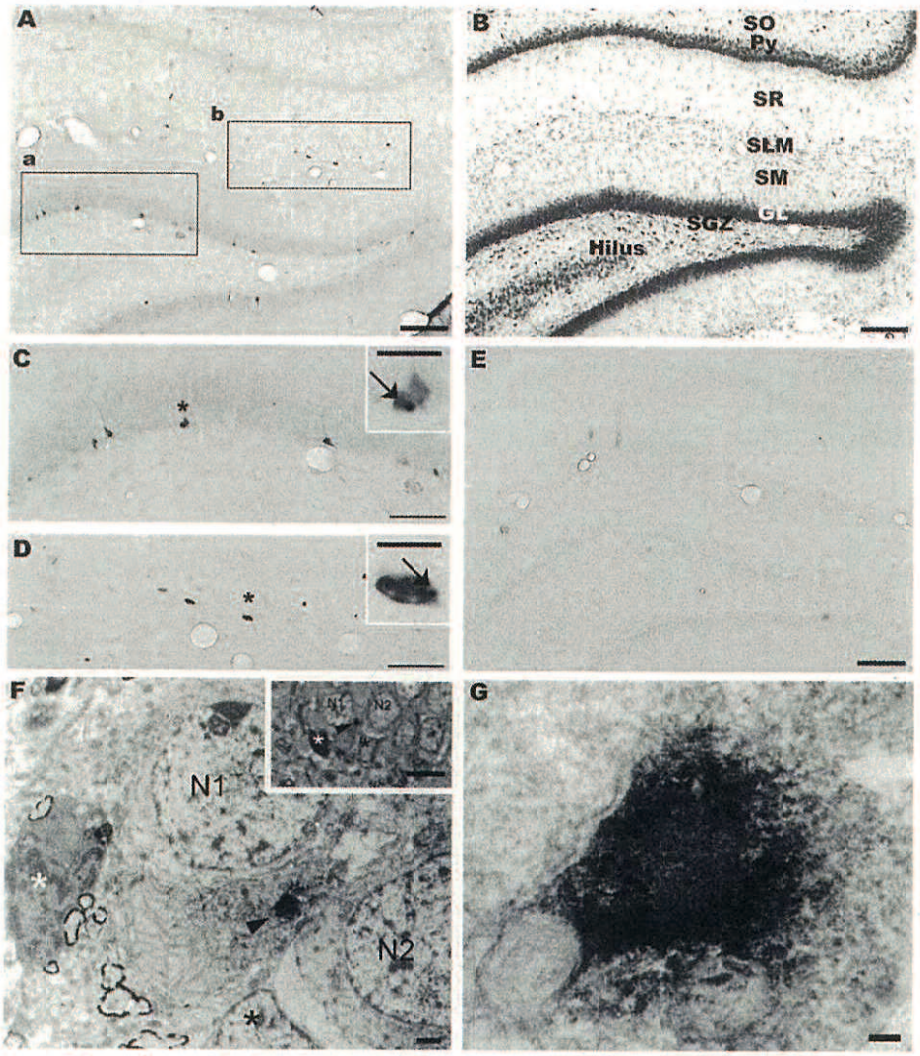

Figure 1. (A) Photomicrograph showing huntingtin-associated protein 1-immunoreactive (HAP1-ir) cells in the subgranular zone (SGZ) of the dentate gyrus and in the interface between the stratum lacunosum-moleculare and the stratum radiatum (IFLMR) of Ammon's horn of a male rat. **(B)** Nissl staining of a neighboring section of A. SO, stratum oriens; Py, pyramidal cell layer; SLM, stratum lacunosum-moleculare; SM, stratum molecular; SR, stratum radiatum; GL, granule cell layer. **(C)** Enlargement of box a in A for the SGZ. **(D)** Enlargement of box b in A for the IFLMR. Insets in C and D are the enlargement of HAP1-ir cell indicated by asterisks in C and D, respectively. Arrows indicate the HAP1-ir stigmoid body (STB). **(E)** Result of pre-adsorption test with blocking peptide against the anti-HAP1 antibody. **(F)** Correlative light and electron microscopy of HAP1-ir cells in the SGZ. Inset shows a toluidine blue-stained semithin section. White asterisk indicates an oligodendrocyte, and black asterisk indicates an astrocyte. N1 and N2 show neurons. Arrowheads indicate HAP1-ir STB. **(G)** Enlargement of HAP1-ir STB in F. Scale bars = 200 μm in A, B, C, D, and E; 10 μm in the insets in C, D and F; 1μm in F; 100 nm in G.

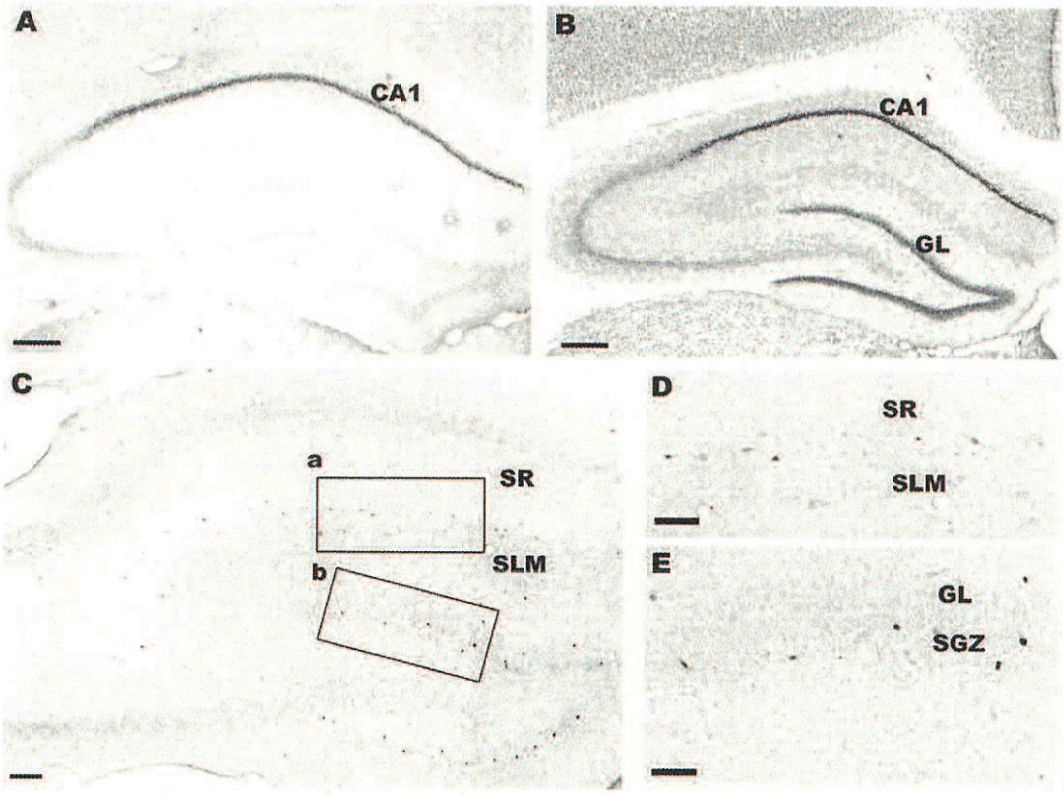

Figure 2 Photomicrographs showing the comparative distribution of steroid receptors in the hippocampus of male rats, including **(A)** the androgen receptor (AR), **(B)** the glucocorticoid receptor (GR), and **(C-E)** the estrogen receptor α (ERα). **(D, E)** Enlargements of box a in C for the IFLMR and box b in C for the SGZ, respectively. Scale bars = 200 μm in A-B; 100μm in C, and 50μm in D-E.

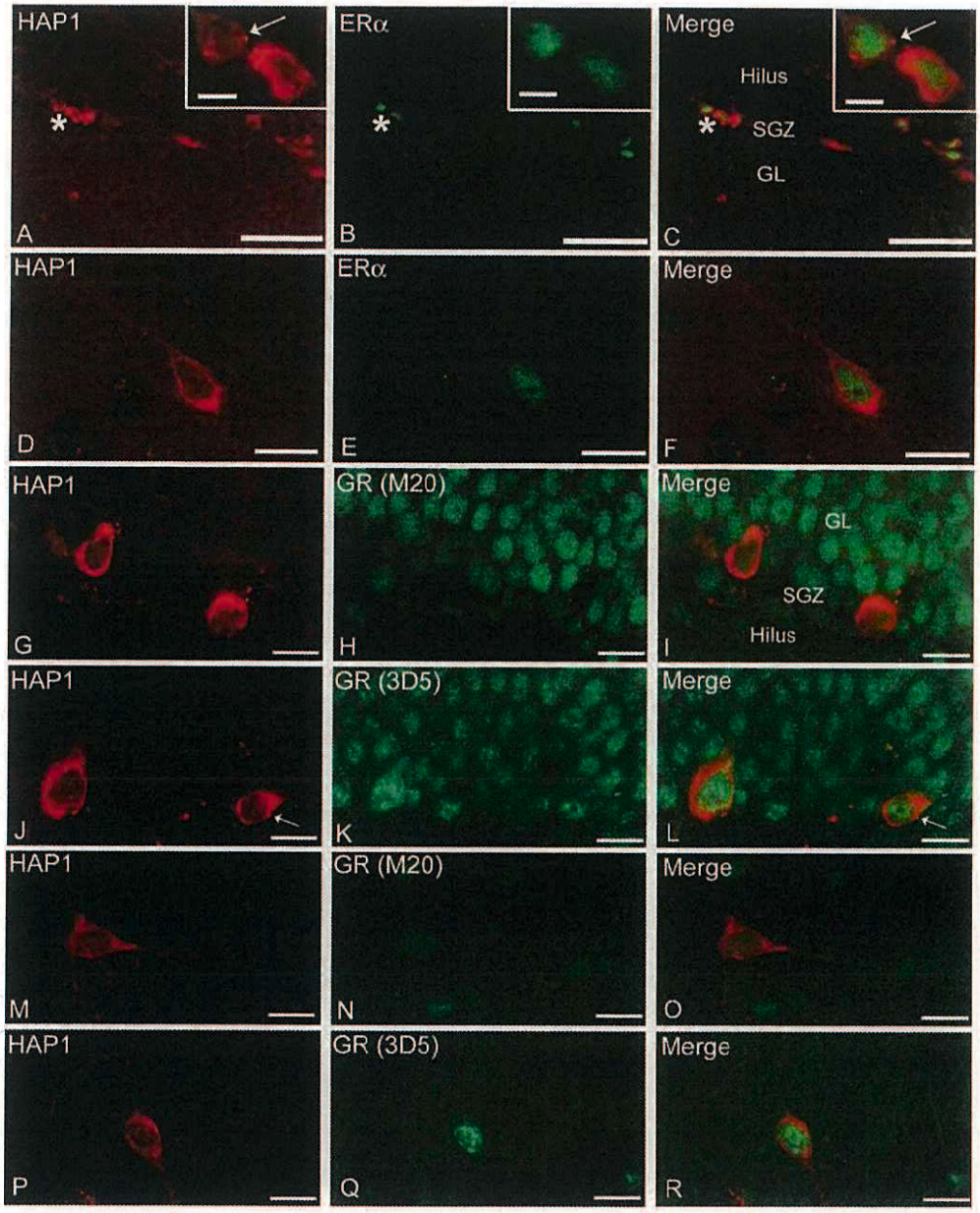

Figure 3 Double-label immunofluorescence staining of HAP1 and ERα or GR in the SGZ of dentate gyrus (**A-C** for ERα; **G-I** for GR M20; **J-L** for GR 3D5) or in the IFLMR of Ammon's horn (**D-F** for ERα; **M-O** for GR M20; **P-R** for GR 3D5) of male rats. Insets in A-C are enlargements of cells indicated by asterisks. GL, granule cell layer. Arrows indicate STB. Scale bars = 20 μm in A-L; 10 μm in the insets.

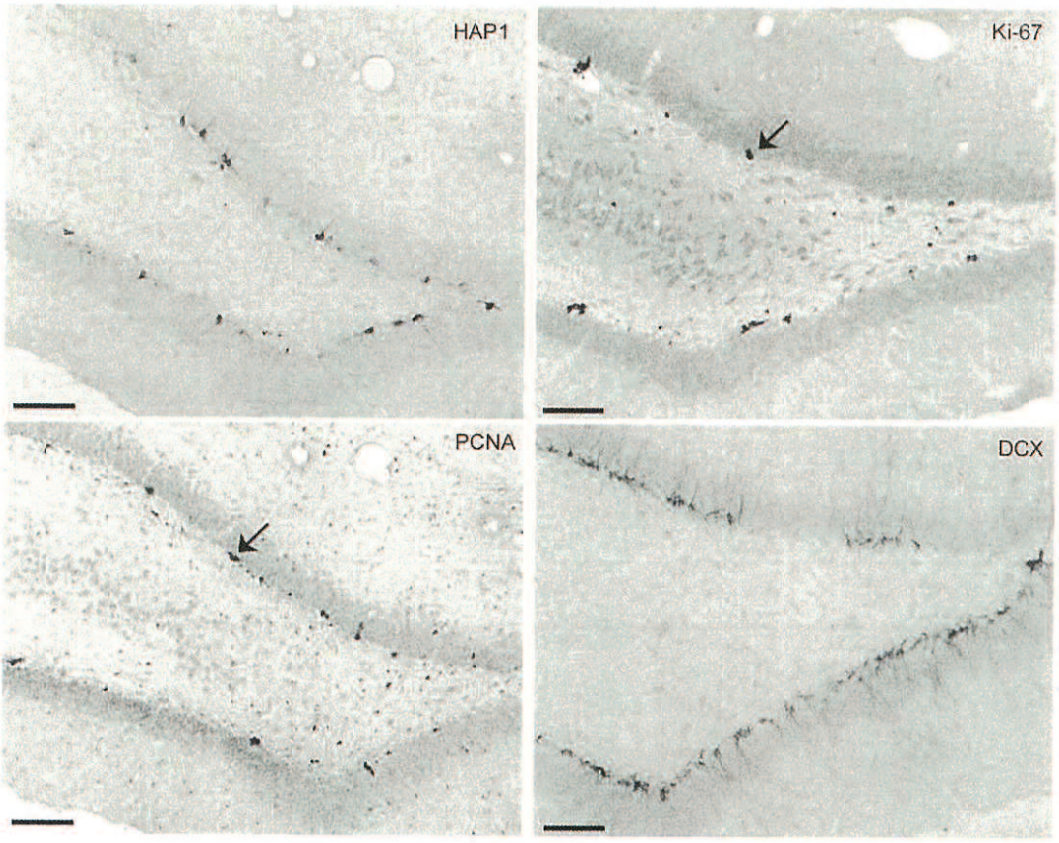

Figure 4. Photomicrographs showing the comparative distribution of cells immunoreactive for HAP1, Ki-67 (a proliferating cell marker), proliferating cell nuclear antigen (PCNA; proliferating cell marker), and doublecortin (DCX; migrating cell marker) in the SGZ of the dentate gyrus of a male rat. Scale bars = 100 μm. Arrowheads indicate cell clusters that are positive for Ki-67 or PCNA.

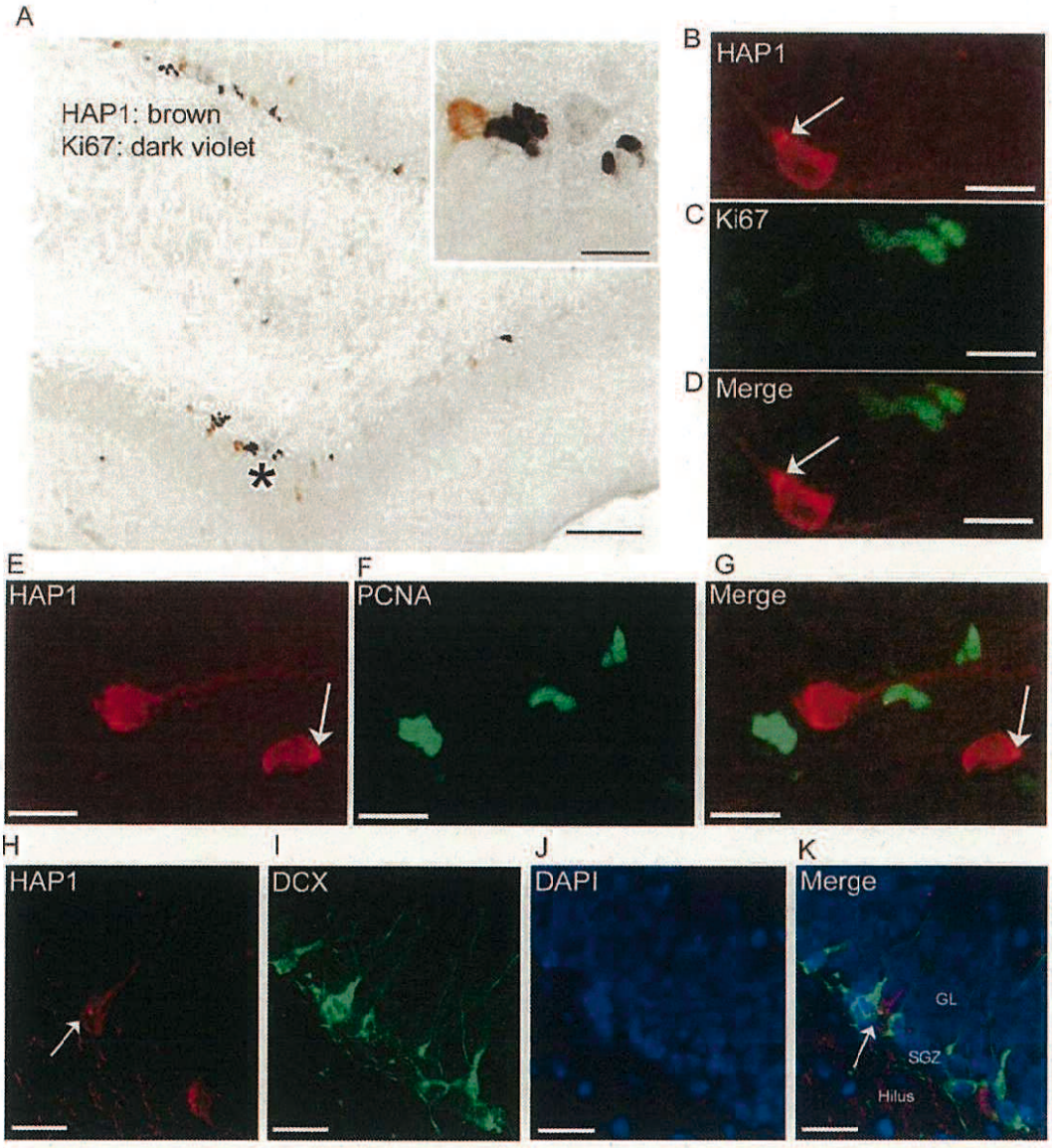

Figure 5 Double-label immunoperoxidase **(A)** and double-label immunofluorescence **(B-D)** staining of HAP1 and Ki-67 in the SGZ of a male rat. Inset in A is an enlargement of asterisk. **(E-G)** Double-label immunofluorescence staining of HAP1 and PCNA in the SGZ. **(H-K)** Double-label immunofluorescence staining of HAP1 and DCX with DAPI in the SGZ. Arrows indicate STBs. GL, granule cell layer. Scale bars = 100 μm in A; 20 μm in the inset in A and B–K.

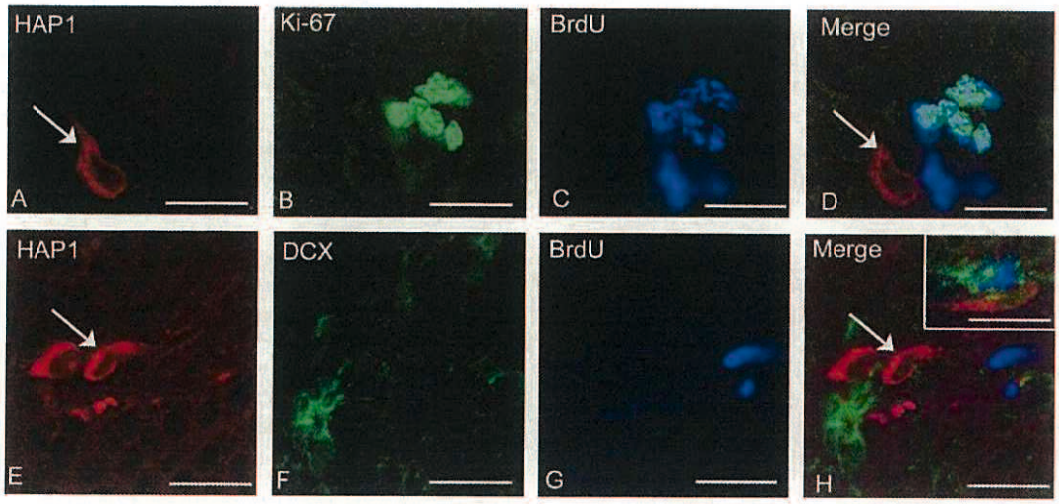

Figure 6 **(A-D)** Triple-label immunofluorescence staining of HAP1, Ki-67 and 5-bromo-2′-deoxyuridine (BrdU) in the SGZ of a male rat. **(E-H)** Triple label-immunofluorescence staining of HAP1, DCX and BrdU in the SGZ of a male rat. Arrows indicate STBs. Inset in H is the independent image showing triple label-immunofluorescence staining of HAP1, DCX and BrdU in the SGZ of a male rat. Scale bars = 20 μm.

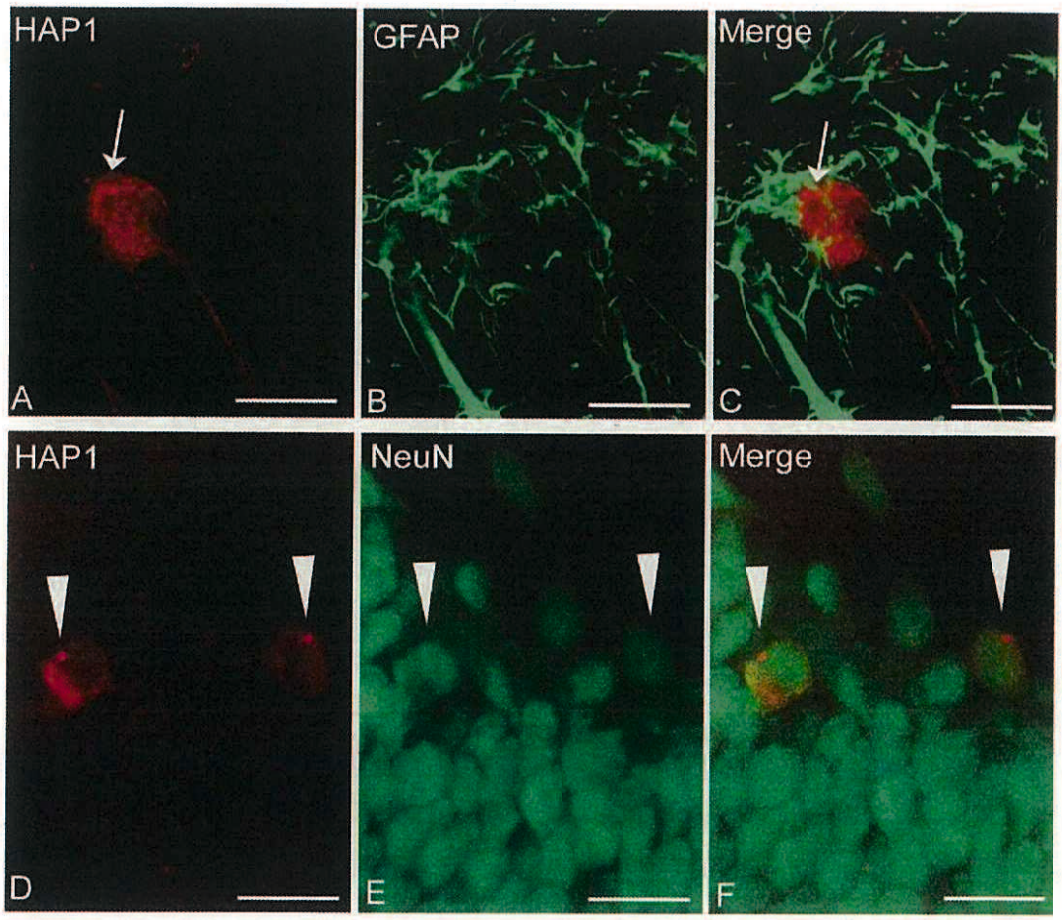

Figure 7 Double-label immunofluorescence staining of HAP1 and glial fibrillary acidic protein (GFAP) **(A-C)** or neuronal nuclear antigen (NeuN) **(D-F)** in the SGZ of a male rat. Arrows indicate the STB. Arrowheads show cells that are positive for both HAP1 and NeuN. Scale bars = 20 μm.

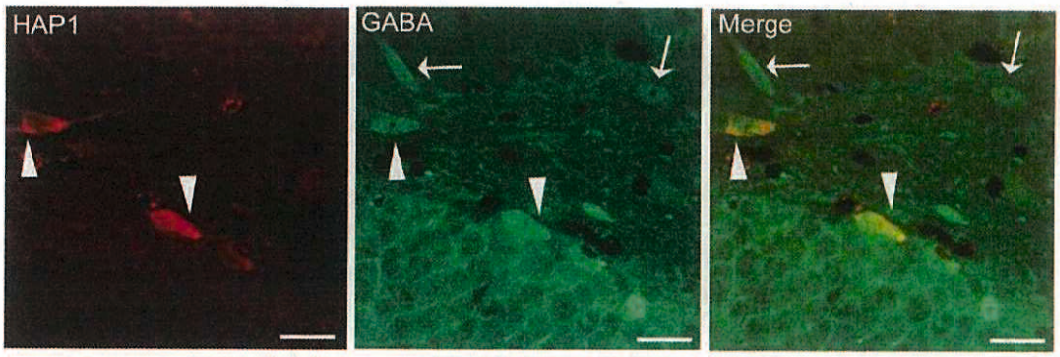

Figure 8 Double-label immunofluorescence staining for HAP1 and γ-aminobutyric acid (GABA) in the SGZ of a male rat. Arrowheads indicate cells that are double positive for both HAP1 and GABA. Arrows indicate cells that are positive only for GABA. Scale bars = 20μm.

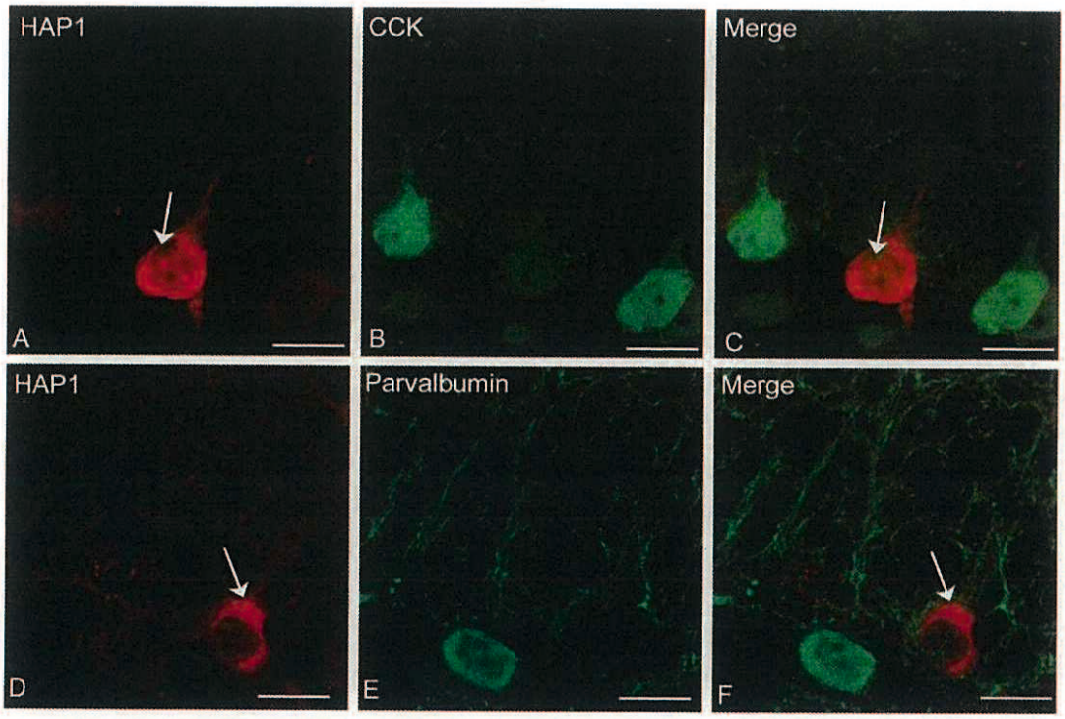

Figure 9 Double-label immunofluorescence staining for HAP1 and Cholecystokinin (CCK) **(A-C)** or Parvalbumin **(D-F)** in the SGZ of a male rat. Arrows indicate STBs. Scale bars = 20μm.

5. Discussion

5.1 HAP1-ir cells are sporadically lurking mature neurons

Previous *in situ* hybridization studies on rats (Page et al., 1998) and mice (Fujinaga et al., 2004) have shown that HAP1 mRNA is expressed in the hippocampus. However, the HAP1-expressing entity has never been morphologically determined. In this study, HAP1-ir cells were first demonstrated to be sporadically present in the hippocampus, particularly in the SGZ of the dentate gyrus and the IFLMR of the Ammon's horn. We called them "sporadically lurking HAP1-ir (SLH)" cells. In most of the SLH cells, HAP1-ir STBs were detected in the cytoplasm as previously observed in other brain regions (Fujinaga et al., 2007, 2009). The emergence of STBs depends on the intracellular expression ratio of two HAP1 isoforms, HAP1A/HAP1B (Li et al., 1998a; Fujinaga et al., 2007). However, since the current immunohistochemistry could not distinguish staining of HAP1A from that of HAP1B, we were unable to determine how far the SLH cells had STBs in the hippocampus.

The scattered distribution of the HAP1-ir cells in the SGZ of dentate gyrus was reminiscent of the patterns exhibited by neural progenitor cells that have the potential for adult neurogenesis, which generally consists of mitotic (proliferation) and post mitotic (differentiation, migration and maturation) stages (Von Bohlen and Halbach, 2007). We observed that HAP1-ir cells did not exhibit BrdU uptake in adulthood. In addition, they expressed NeuN which is a marker of postmitotic or mature neuronal cells (Mullen et al., 1992), and did not express PCNA (a proliferating cell markers; Jin et al., 2001), Ki67 (a proliferating cell marker; Scholzen and Gerdes, 2000), DCX (a marker of microtubule-associated protein in migrating and young neurons; Gleeson et al., 1999) or GFAP (a marker of early progenitor or astroglial cells; Seri et al., 2001). As

shown in our results, DCX-ir cells were basically devoid of BrdU labeling, although a few DCX-ir cells seemed to be marked with BrdU. However, HAP1-ir cells have never been labeled by BrdU so far. A few DCX-ir cells have also previously been reported to transiently express NeuN and they were regarded as older stages of DCX-ir cells (Brown et al., 2003). Though the possibility cannot completely be excluded that very limited numbers of the HAP1-ir cells also express DCX, coexpression of DCX and HAP1 has never been detected in our current results. Considering that early postmitotic cells continue to express DCX for possibly up to 4 weeks (Brown et al., 2003), the HAP1-ir cells are so far thought to be sporadically lurking mature neurons that can be distinguished from the adult progenitor cells occurring in the SGZ.

5.2 The SLH neurons exclusively express ERα

The most striking findings in this study were that more than 90% of sporadic HAP1-ir cells exclusively expressed ERα in their nuclei, more than 90% of sporadic ERα-ir cells expressed HAP1. Taking the methodological limitations of immunohistochemistry into account, the two groups could be regarded as the same entity. The HAP1-ir cells were devoid of ERβ and AR expression. Estrogen has been reported to modulate adult neurogenesis (Galea et al., 2006), particularly facilitating cellular proliferation. But the present results strongly suggested that these putative effects were not directly mediated by nuclear ERα in the progenitor cells. Such a modulation, if any, might be due to indirect trans-synaptic effects on the progenitor cells from ERα-ir SLH cells or directly mediated by membrane estrogen receptors possibly located on the progenitor cells themselves.

With respect to GR expression, when the anti-GR M20 antibody was used, the

SLH cells showed very weak GR expression, making a sharp contrast with the great majority of granule cells. Thus they are also distinct from the general granule cells in GR immunostaining by anit-GR M 20 antibody. However, when anti-GR 3D5 antibody was used, the SLH cells were found to show moderate-to-strong GR immunoreactivity like neighboring granule cells. The discrepancy cannot cogently be explained, but it has recently been warned that different types of anti-GR antibody showed different staining patterns *in vivo* (Sarabdjitsingh et al., 2010). Different staining intensity might be due to a different GR conformation reflecting a different phase of GR function. Alternatively, GR expressed in the SLH cells might possibly be one of some GR variants or fragments, which should be distinct from the general GR as expressed in the ordinary granule cells and differently stained depending on the antibodies. For example, C-terminus variant GR (GRβ), which can bind to GR responsive element but cannot bind ligands unlike classical GR (GRα), has been reported to be present in the human hippocampus and hypothalamus and shown to block GRα-mediated function as a "dominant negative inhibitor" (Hollenberg et al., 1985; Encio and Detera-Wadleigh, 1991; Bamberger et al., 1995, 1996; Oakley et al., 1996; De Castro et al., 1996). Concerning mineralocorticoid receptor (MR), we could not unfortunately obtain any reliable antibody good for immunohistochemistry from commercial source (though some antibodies were available for cultured cells). Indeed, there has been no critical study on definitive immunohistochemical distribution of MR-ir cells in the hippocampus probably because of no anti-MR antibody appropriate for immunohistochemistry; one group has exceptionally reported that all the neurons showed MR-immunoreactivity (Ito et al. 2000; Han et al. 2005), while other one demonstrated that MR is more intensively expressed in the CA1 (Zhe et al., 2008). MR might have some structural difficulty in

producing anti-MR antibody available for immunohistochemistry.

Recently, STB/HAP1 has been shown to be closely associated with nuclear steroid receptors (Shinoda et al., 1992; Nagano and Shinoda, 1994; Fujinaga et al., 2004, 2011; Takeshita et al., 2006). Although a high coexpression ratio (approximately 80%) of STB/ ERα has also been reported in neurons in the medial preoptic and medial amygdaloid areas (Nagano and Shinoda, 1994), the neural group with STB and the one with ERα are not the same entity in these areas. The association between ERα and HAP1 is in the hippocampus is considered to be exceptionally intimate. Interestingly, HAP1 was recently shown to bind to the ligand-binding domains (LBDs) of steroid receptors, including ERα, ERβ, AR, GR and the MR *in vitro* (Fujinaga et al., 2011). In particular, it interacts with the full length of AR and GR and suppresses or regulates their cytoplasm-to-nuclear translocation (Fujinaga et al., 2011). Furthermore, we have demonstrated that HAP1 interacts with polyQ-expanded AR in a polyQ -length dependent manner and that HAP1 overexpression suppresses the apoptosis induced by SBMA-derived extraordinarily expanded polyQ-AR (Takeshita et al., 2006). HAP1 has also been reported to interact with other polyQ -disease-related gene products, including Htt in Huntington disease (Li et al., 1995; Li et al., 2003; Metzger et al., 2008), TATA-binding protein in SCA17 (Prigge and Schmidt, 2007) and ataxin-3 in SCA3 (Takeshita et al., 2011). HAP1 is thought to regulate nuclear translocation of the causative products in several neurodegenerative diseases, raise the cellular threshold for apoptosis, and provide stability to HAP1-expressing cells which probably results in protecting against apoptosis or cell death (Fujinaga et al., 2004; Takeshita et al., 2006, 2011). Unfortunately, the full length of ERα has not been shown to bind to HAP1 under routine cell culture conditions, but it could interact with HAP1 through its LBD under

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