Development 134, 1679-1689 (2007) doi:10.1242/dev.02835

Clathrin-mediated endocytic signals are required for the regeneration of, as well as homeostasis in, the planarian CNS

Takeshi Inoue[1,2], Tetsutaro Hayashi[1,*], Katsuaki Takechi[1,†] and Kiyokazu Agata[1,3,‡]

Planarians have a well-organized central nervous system (CNS), including a brain, and can regenerate the CNS from almost any portion of the body using pluripotent stem cells. In this study, to identify genes required for CNS regeneration, genes expressed in the regenerating CNS were systematically cloned and subjected to functional analysis. RNA interference (RNAi) of the planarian *clathrin heavy chain* (*DjCHC*) gene prevented CNS regeneration in the intermediate stage of regeneration prior to neural circuit formation. To analyze *DjCHC* gene function at the cellular level, we developed a functional analysis method using primary cultures of planarian neurons purified by fluorescence-activated cell sorting (FACS) after RNAi treatment. Using this method, we showed that the *DjCHC* gene was not essential for neural differentiation, but was required for neurite extension and maintenance, and that *DjCHC*-RNAi-treated neurons entered a TUNEL-positive apoptotic state. *DjCHC*-RNAi-treated uncut planarians showed brain atrophy, and the *DjCHC*-RNAi planarian phenotype was mimicked by RNAi-treated planarians of the *mu-2* (*μ2*) gene, which is involved in endocytosis, but not the *mu-1* (*μ1*) gene, which is involved in exocytosis. Thus, clathrin-mediated endocytic signals may be required for not only maintenance of neurons after synaptic formation, but also axonal extension at the early stage of neural differentiation.

KEY WORDS: Planarian, Regeneration, Central nervous system (CNS), Clathrin heavy chain, Endocytosis, AP-2, Neuronal homeostasis, *Dugesia japonica*

INTRODUCTION

Planarians are free-living *Platyhelminths*, and are among the most primitive animals possessing a CNS, which is composed of an inverted U-shaped brain in the anterior region of the animal and two longitudinal ventral nerve cords along the body (Agata et al., 1998). The planarian CNS is relatively simple but well organized, consisting of several functional and structural domains, as defined by the discrete expression of three *otd*/*Otx*-related homeobox genes and a complex set of other regulatory genes (Umesono et al., 1997; Umesono et al., 1999; Cebrià et al., 2002b; Cebrià et al., 2002c; Mineta et al., 2003; Nakazawa et al., 2003; Koinuma et al., 2003; Inoue et al., 2004). Furthermore, planarians possess a remarkable regenerative ability that derives from pluripotent somatic stem cells (Agata and Watanabe, 1999; Saló and Baguñà, 2002; Agata et al., 2003; Reddien and Sánchez Alvarado, 2004), whereby even a tiny fragment cut from most parts of the body can regenerate the entire body with a functional nervous system within 5 days (Inoue et al., 2004). To identify genes involved in functional brain regeneration, we systematically cloned genes expressed in the regenerating brain (Nakazawa et al., 2003; Mineta et al., 2003) and analyzed their expression timing during brain regeneration (Cebrià et al., 2002c). Those studies revealed that planarian brain regeneration consists of at least five steps: blastema formation, brain induction, patterning, neural circuit formation and functional recovery. Several candidate genes involved in each step have been identified. *Djnlg*, a planarian noggin homologue, and *DjvlgA*, a *vas*-related gene, are the earliest genes found to be involved in blastema formation (Ogawa et al., 2002; Shibata et al., 1999). The second-earliest gene is *nou-darake*, a dominant-negative-type fibroblast growth factor receptor (FGFR) that is activated in only the anterior blastema within 24 hours after amputation to form a brain rudiment (Cebrià et al., 2002a). *DjotxA*, *DjotxB* and *Djotp*, brain-specific homeobox genes, are activated in the brain rudiment 36 hours after amputation and act to create patterns in the brain (Umesono et al., 1997; Umesono et al., 1999). After patterning, *netrin* and several immunoglobulin superfamily neural cell adhesion molecule genes (IgCAMs) are activated in the brain approximately 72 hours after amputation to form neural circuits (Cebrià et al., 2002b; Cebrià and Newmark, 2005; Fusaoka et al., 2006). Finally, 5 days after amputation, a functional brain is recovered (Inoue et al., 2004). The structure and function of the CNS depend on precisely controlled interactions among the neural cells, but the mechanisms of intercellular interaction required to construct and maintain the functional brain during brain regeneration are unclear. Here, we demonstrate that the *clathrin heavy chain* gene (*DjCHC*) of a planarian, *Dugesia japonica*, was required for proper brain regeneration and investigate at what point it acts during brain regeneration.

Clathrin-mediated membrane trafficking plays an important role in cell signaling and is involved in numerous cell functions, including nutrient uptake, regulation of the number of signaling receptors on the cell surface and the recycling of synaptic vesicles at nerve terminals (Takei and Haucke, 2002; Murthy and DeCamilli, 2003; Le Roy and Wrana, 2005). Functional analyses of clathrin genes has been performed in cultured cells, fungi, trypanosomes and yeast, and have provided insights into the functions of clathrins and their interactions with other proteins in cell signaling (Seeger et al., 1992; Ruscetti et al., 1994; Niswonger and O'Halloran, 1997b; Wettey et al., 2002; Motley et al., 2003; Allen et al., 2003; Hinrichsen et al., 2003). However, much remains unknown about

[1]Group for Evolutionary Regeneration Biology, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan. [2]KAN Research Institute, Inc., Kobe MI R&D Center, 6-7-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan. [3]Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan.

*Present address: Genome Resource and Analysis Subunit, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan
†Present address: Faculty of Science, Kumamoto University, 2-39-1, Kurokami, Kumamoto, 860-8555, Japan
‡Author for correspondence (e-mail: agata@mdb.biophys.kyoto-u.ac.jp)

Accepted 13 February 2007

the developmental, molecular and cellular processes that determine the onset and maintenance of intercellular communication and the role of such communication in physiological responses of the nervous system. Here, by in vitro and in vivo analyses, we show that *DjCHC*-RNA interference (RNAi) inhibits neuronal survival, and neurite outgrowth and maintenance, and that *DjCHC* is required for proper CNS regeneration and homeostasis.

MATERIALS AND METHODS

Animals

A clonal strain (GI) of the planarian *Dugesia japonica* maintained in autoclaved tap water at 22-24°C was used in this study. In all experiments, 8-10 mm-long planarians that had been starved for 2 weeks were used. For regeneration studies, animals were cut posterior to the auricle and pharynx on ice. To prepare X-ray-irradiated planarians, worms were irradiated with 12R X-rays using an X-ray generator B-4 (Softex).

cDNA cloning of *DjCHC*, *Djmu-1* and *Djmu-2*

cDNA clones (Dj_aH_000_01290HH, Dj_aH_507_P09, Dj_aH_402_I20, Dj_aH_302_F09 and Dj_aH_316_I10) encoding the respective proteins (DjCHC, Djmu-1, Djmu-2, Djunc13A and DjsbpA) were first identified in a previously constructed database of expressed sequence tags (ESTs) (Mineta et al., 2003). The longest cDNA of *DjCHC* was obtained by the stepwise dilution method (Watanabe et al., 1997) from a cDNA library constructed from poly A(+) RNA of whole planarians in the ZAPII vector (Stratagene). The sense primer 5′-TGATCATCGAAACTTACAAAACC-3′ and antisense primer 5′-ATACCCATATGTGCTCGTTCTAA-3′ corresponded to the sequence of Dj_aH_000_01290HH. The positive cDNA clone with the longest insert was re-cloned into pBluescript SK(–) (Stratagene).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out using 20 ng/ml of digoxygenin (DIG)-labeled riboprobes (Roche), as previously described (Umesono et al., 1997; Agata et al., 1998).

Preparation of antibody against DjCHC

A fragment of Dj_aH_000_01290HH (amino acids 1190-1462) was inserted into pQE30 vector (Qiagen) and the construct was introduced into *E. coli* strain JM109. The fusion protein with a histidine tag was induced with isopropyl-β-D-thiogalactopyranoside and purified according to the supplier's protocol (Qiagen). For further purification, the gel slice containing the target protein was cut out after SDS-PAGE, and the purified protein was collected using an electroeluter (Amicon). The purified recombinant DjCHC protein was injected with Titer Max Gold adjuvant (CytRx) into mice (Balb/c) three times at intervals of 1 month. The antiserum against the fusion protein was prepared according to a standard procedure (Orii et al., 2002).

Whole-mount immunostaining

Whole-mount immunostaining was performed as described previously (Cebrià et al., 2002c). Planarians were stained using mouse antibodies: 1/2000 anti-planarian synaptotagmin (anti-DjSYT) (Tazaki et al., 1999); 1/2000 anti-DjCHC; 1/2000 anti-planarian G-protein β subunit (anti-DjGβ) (AB245430); 1/5000 VC-1 monoclonal antibody (Sakai et al., 2000); and 1/200 anti-phospho histone H3 antibody (anti-H3P) (Hendzel et al., 1997) (Upstate Biotechnology), all of which were diluted in 10% goat serum in 0.1% Triton X-100-containing PBS (TPBS).

RNA interference

Double-stranded RNA (dsRNA) was synthesized essentially as previously described (Sánchez Alvarado and Newmark, 1999). Control animals were injected with dsRNA for green fluorescence protein (GFP), a protein not found in planarians. At 4 hours after the third injection, planarians were amputated immediately posterior to the auricles and pharynxes, and the resulting pieces were used for various assays.

BrdU labeling and detection in RNAi-treated planarians

BrdU labeling was performed by microinjection basically as described previously (Newmark and Sánchez Alvarado, 2000). RNAi-treated planarians (RNAi planarians) were prepared as described above and then cut into three pieces. At 3 hours after amputation, 10 mg/ml of BrdU was injected once into the intestinal tract of the trunk-piece. At either 4 or 48 hours after the injection, planarians were fixed and BrdU was detected immunohistochemically (Agata et al., 1998).

FACS and the culturing of sorted primary neurons

Fluorescence-activated cell sorting (FACS) and the culturing of planarian neural cells were performed basically as previously described (Asami et al., 2002). In total, 50 RNAi planarians were cut into head, trunk and tail pieces. After 4 days, the regenerated heads of the trunk and tail pieces (total of 100 pieces) were collected and used for FACS. The collected cells were maintained statically in a humidified incubator at 22°C for 3 days in vitro (DIV).

Immunocytochemistry

Cultured cells were fixed in 4% PFA, 5/8 Holtfreter's solution for 60 minutes at 4°C, washed three times with TPBS and blocked in 10% goat serum in TPBS for 30 minutes at 4°C. The cells were then incubated with 1/2000 diluted mouse anti-DjCHC, mouse anti-DjSYT, mouse anti-DjGβ or hamster anti-planarian 14-3-3ϵ (whose signal was distributed throughout the whole cell; AB245429; used as counterstaining) for 60 minutes at 4°C. The samples were washed with 10% goat serum in TPBS for 5 minutes three times and signals were detected with 1/400 Alexa Fluor 488-conjugated goat anti-mouse IgG(H+L) (Invitrogen) or 1/400 Alexa Fluor 546-conjugated goat anti-hamster IgG(H+L) (Invitrogen) diluted 1/400 in 10% goat serum in TPBS for 60 minutes in the dark. After the samples were washed three times with TPBS, cell nuclei were labeled with Hoechst 33342 (Invitrogen). Fluorescence was detected with an LSM 510 confocal microscope (Carl Zeiss).

TUNEL assay of regenerating planarian neural cells

The head-abundant cell fraction on day 4 of regeneration (R4HAC) was cultured and collected at 0, 1, 2 or 3 DIV, and TUNEL reactions were performed (Hwang et al., 2004). The signal was enhanced using 1/400 Alexa Fluor 488-conjugated anti-fluorescein (Invitrogen). Immunostaining was performed using 1/400 hamster anti-planarian 14-3-3ϵ for counterstaining and 1/400 Alexa Fluor 546-conjugated anti-hamster IgG(H+L) (Invitrogen) as a counterstain.

Statistical evaluation

Quantitative data were analyzed by one-way analysis of variance (ANOVA) and the statistical significance of differences between test results was determined by Student's *t*-test; *P* values greater than 0.05 were taken as not significant (NS). For in vitro analysis, data from at least eight images were averaged.

RESULTS

Identification and characterization of planarian *clathrin heavy chain* gene

The full-length cDNA for *DjCHC* containing a 5381-bp insert with an open reading frame (ORF) encoding 1683 amino acids was screened by stepwise dilution screening (Watanabe et al., 1997) and named *DjCHC*. Sequencing analysis revealed that *DjCHC* has five highly conserved functionally distinct domains – the globular N-terminal domain, knee region, distal domain, proximal domain and C-terminal end (Ybe et al., 1999; Fotin et al., 2004; Wilbur et al., 2005) – and seven clathrin heavy-chain repeat (designated CHCR1-CHCR7) motifs (residues 546-1583) (see Fig. S1A in the supplementary material) (Ybe et al., 1999; Wilbur et al., 2005). Phylogenetic analysis using the full-length DjCHC showed high homology of this protein to the clathrin heavy chains (CHCs) of other eukaryotes (see Fig. S1B in the supplementary material).

Expression of *DjCHC* mRNA and proteins

Intact planarians were examined for the expression pattern of *DjCHC*. In agreement with previous reports, whole-mount in situ hybridization showed strong and ubiquitous expression of *DjCHC*

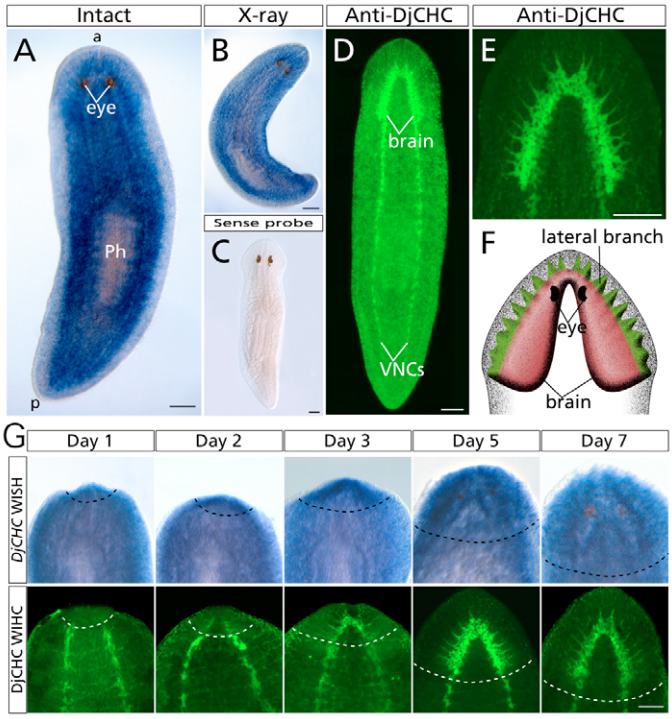

Fig. 1. Expression patterns of *Dj*CHC mRNA and protein. (**A-C**) *DjCHC* mRNA, analyzed by whole-mount in situ hybridization. (A) Dorsal view. a, anterior; p, posterior; Ph, pharynx. (B) *DjCHC* expression at 7 days after X-ray irradiation. (C) *DjCHC* sense probe. (**D**) DjCHC protein, analyzed by whole-mount immunostaining. VNCs, ventral nerve cords. (**E**) Higher magnification of a single section of the confocal image of the head region shown in D. (**F**) Schematic illustration of the head region of planarian. The planarian CNS, consisting of an inverted U-shaped spongy region (red) and nine lateral branches (green). (**G**) Upper panels show whole-mount in situ hybridization and lower panels show whole-mount immunostaining of planarians at 1, 2, 3, 5 or 7 days after amputation. Scale bars: 100 μm.

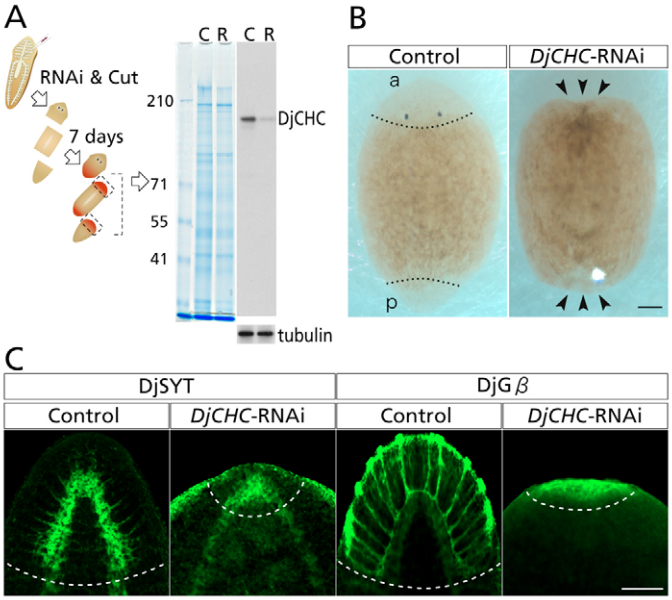

Fig. 2. *DjCHC*-RNAi planarian could not regenerate the CNS. (**A**) Equal protein loadings of homogenates of 7-day-regenerated head region of either control or *DjCHC*-RNAi-treated planarians were subjected to SDS-PAGE using 7% gels, and western blots were probed with antibodies against the indicated protein. C, control; R, *DjCHC*-RNAi treated. (**B**) Dorsal view of the injected organisms (bright-field images). The left (control) and right (*DjCHC*-RNAi) panels show the 7-day regenerates of trunk pieces. The control planarian regenerated the tail and the head, as indicated by the dashed lines. The *DjCHC*-RNAi-treated planarian did not regenerate (arrowheads). a, anterior; p, posterior. (**C**) Defects of *DjCHC*-RNAi-treated planarians 7 days after amputation were detected in the CNS architecture, as revealed by immunostaining with anti-DjSYT and anti-DjGβ. The dashed line indicates the border between the newly formed region and old stump region. Scale bars: 100 μm.

transcripts (Fig. 1A). In X-ray-irradiated planarians, weaker *DjCHC* signals were detected than in intact planarians, suggesting that *DjCHC* was expressed in various tissues, including in stem cells (Fig. 1B). To analyze the expression pattern of DjCHC protein, anti-DjCHC antibody was produced. DjCHC protein was also detected ubiquitously, but was detected especially strongly in the spongy region composed of the neurites of the neural cells (Fig. 1D). Confocal microscopy of the brain revealed much higher expression of DjCHC protein in the CNS compared with in other organs (Fig. 1E). Thus, the *DjCHC* gene might play a major role in the planarian CNS. Expression of the *DjCHC* gene within the newly formed blastema was first detected at day 2 of regeneration as a weak signal in two clusters of cells, which probably corresponded to the primordium of the inverted U-shaped brain region (Fig. 1G).

Silencing of *DjCHC* perturbs CNS regeneration

To identify genes involved in regeneration of the planarian brain, we performed expression analysis of the genes expressed in the regenerating CNS and a functional screen of these genes by RNAi, and thereby found that the *DjCHC* gene was essential for regeneration. Fig. 2A shows western blot analysis of equal protein loadings of the newly regenerated head region at 7 days after amputation of control and *DjCHC*-RNAi-treated planarians. In the controls, the *DjCHC* gene was expressed at a high level, whereas only a weak signal was detected in *DjCHC*-RNAi-planarians, indicating that RNAi-treatment downregulated DjCHC protein expression. The *DjCHC*-RNAi-treated planarians (139/139) seemed to be unable to regenerate their head or tail (Fig. 2B). When we cut planarians at levels posterior to the auricle and the pharynx into three pieces (head, trunk and tail pieces), extensive degeneration of the head pieces was observed (see Fig. S2A in the supplementary material). By contrast, almost all of the trunk pieces and tail pieces survived beyond 4 weeks, although they could not regenerate their heads. This suggests that *DjCHC* gene function is specifically required for regeneration and maintenance of the head region. Immunostaining of *DjCHC*-RNAi-treated planarians with anti-DjSYT as a pan-neural marker (Tazaki et al., 1999) and anti-DjGβ, which stains the lateral branches of the brain, revealed that *DjCHC*-RNAi-treated planarians could not regenerate their brain normally (Fig. 2C), which was confirmed by the fact that they could not recognize the direction of light and they hardly moved (see Fig. S2C in the supplementary material). To determine whether *DjCHC* gene function is specific to the brain, we investigated the effects of *DjCHC*-RNAi in other tissues and organs by using molecular markers, including a tail-specific homeobox gene, *DjAbd-Ba* (Nogi and Watanabe, 2001). Interestingly, the homeobox gene was

expressed with the normal spatial pattern, but the regenerating ventral nerve cords (VNCs) in the posterior blastema were strongly disorganized (Fig. 3A). No disorganization was detected in the newly formed pharynx (Fig. 3B) or in the regenerating intestinal duct or body-wall musculature (Nogi and Levin, 2005; Orii et al., 2002) (Fig. 3C,D).

CNS abnormality caused by silencing of *DjCHC* is independent of stem cell function

Head atrophy was also seen in X-ray-irradiated or RNAi-treated planarians with targeting of stem cell-related genes (Reddien et al., 2005a; Reddien et al., 2005b; Salvetti et al., 2005). Therefore, we speculated at first that the phenotype of the *DjCHC*-RNAi-treated planarians was caused by a stem cell defect, because *DjCHC* is expressed ubiquitously (including in stem cells). To test this possibility, we compared the phenotypes of planarians in which the stem cells were ablated by X-ray irradiation (Wolff and Dubois, 1948; Baguñà et al., 1989), *DjCHC*-RNAi-treated planarians and planarians that had undergone both treatments. It is known that the cell number in planarians is determined by the balance between cell proliferation and cell death, and even starved planarians can maintain cell proliferation while their body is shrinking in size via 'tissue homeostasis' (Baguñà and Romero, 1981; Sánchez Alvarado, 2006). We investigated the phenotype of *DjCHC*-RNAi-treated planarians whose stem cells were destroyed by X-ray irradiation. Although, in control and X-ray-irradiated planarians, we could not find any defects through 14 days after irradiation or RNAi-treatment, CNS atrophy could be detected in *DjCHC*-RNAi-treated planarians from 5 day after RNAi-treatment, and the number of CNS-regressed planarians progressively increased thereafter until almost all planarians showed head atrophy by 14 days after RNAi-treatment (Fig. 4A,B). Furthermore, ablation of the stem cells by X-irradiation enhanced the CNS atrophy of the *DjCHC*-RNAi-treated planarians, perhaps because it caused a decrease in the cell number in the CNS (Baguñà and Romero, 1981; Hwang et al., 2004). These results support the notion that the CNS atrophy caused by *DjCHC*-RNAi treatment was due to effects on differentiated neuronal cells rather than on stem cells.

To investigate the stem cells of *DjCHC*-RNAi-treated planarians in greater detail, the cell division and migration of stem cells were assessed using BrdU (Newmark and Sánchez Alvarado, 2000). The number and distribution of BrdU-positive stem cells (which might migrate to the blastema) did not differ between control and *DjCHC*-RNAi-treated planarians at 4 or 48 hours after BrdU injection (Fig. 4C). Furthermore, analysis of the number of mitotic stem cells using anti-H3P (Newmark and Sánchez Alvarado, 2000) in uncut *DjCHC*-RNAi-treated planarians 14 days after RNAi treatment showed that H3P-positive cells were present in *DjCHC*-RNAi-treated planarians and survived for over 3 weeks even though the CNS was atrophied (Fig. 4D, and see Fig. S2B in the supplementary material). There

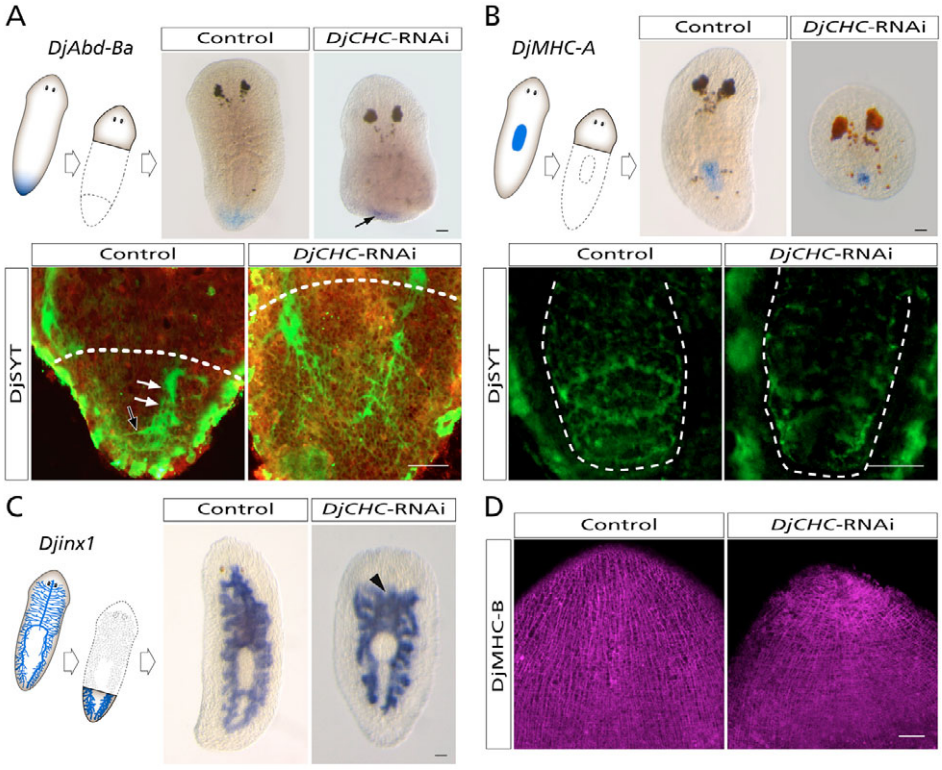

Fig. 3. No effects on the tail, pharynx, intestinal tract or muscles of *DjCHC*-RNAi-treated planarian. (**A**) Control and *DjCHC*-RNAi-treated planarian head piece at 7 days after amputation showing tail regeneration. Upper panels show *DjAbd-Ba* expression (blue) visualized using whole-mount in situ hybridization. Lower panels show VNCs of regenerated head pieces visualized with anti-DjSYT. White arrows, VNCs; black arrows, commissure neurons. VNCs of *DjCHC*-RNAi-treated planarians were perturbed. The dashed line indicates the border of the newly formed region and old stump region. (**B**) Pharynx regeneration of the head piece of the control and *DjCHC*-RNAi-treated planarian 7 days after amputation. The upper panels show pharynx-specific-gene *DjMHC-A* expression (blue) by whole-mount in situ hybridization. The lower panels show nerves of the pharynx visualized using anti-DjSYT. The dashed line indicates the pharynx. (**C**) Regeneration of the intestinal tract of a tail piece of a *DjCHC*-RNAi-treated planarian 7 days after amputation. Schematic drawings in A-C show the original expression patterns. (**D**) Body-wall musculature of a control and *DjCHC*-RNAi-treated planarian 7 days after amputation visualized with anti-DjMHC-B. Scale bars: 50 μm.

were around 130 H3P-positive cells in an 8-mm-long planarian in both control (130.3±6.5 cells) and *DjCHC*-RNAi-treated (128.2±7.0 cells) planarians (not significantly different) (Fig. 4D,E). On the other hand, in planarians at 14 days after X-ray irradiation, when half of the treated animals had died (see Fig. S2B in the supplementary material), there were no H3P-positive cells and the CNS was normal (Fig. 4D,E). However, Reddien et al. (Reddien et al., 2005b) reported that a planarian homolog of the *piwi* gene was involved in supporting the generation of cells that promote regeneration and homeostasis, but not the division or migration of stem cells; we therefore performed further histological analyses using several molecular markers under *DjCHC*-knockdown conditions to identify the stage at which *DjCHC* is required for proper brain formation during head regeneration.

Silencing of *DjCHC* does not perturb the early regeneration process

To test the ability of *DjCHC*-RNAi-treated planarians to form the blastema and brain rudiment, expression analysis was performed for *Djnlg* [a planarian *noggin* homologue rapidly induced by dorsoventral interaction after amputation to provide a positional cue to stem cells (Ogawa et al., 2002)], for *DjvlgA* expressed in the early blastema and stem cells (Shibata et al., 1999), and for *nou-darake* (*ndk*) expressed in the predicted brain region in the anterior blastema (Cebrià et al., 2002a). *Djnlg*, *DjvlgA* and *ndk* were detected in *DjCHC*-RNAi-treated planarians as in control planarians (Fig. 5A-C), indicating that *DjCHC* was not required for the formation of the blastema or brain rudiment early in regeneration.

Moreover, expression analysis was performed using the *DjotxA*, *DjotxB* and *Djotp* genes, which are thought to be involved in patterning of the brain 48 hours after decapitation (Umesono et al., 1997; Umesono et al., 1999). The expression patterns of these genes were not grossly different between control and *DjCHC*-RNAi-treated planarians, suggesting that brain patterning was not affected by *DjCHC*-RNAi (Fig. 5D,E).

Silencing of *DjCHC* perturbs the proper formation of the CNS after patterning and cell differentiation

Immunostaining using anti-DjSYT and anti-DjGβ 72 hours after amputation revealed conspicuous abnormalities of the regenerating brain. Positive signals were detected in the newly formed blastema region (Fig. 5F), but the brain architecture was disorganized. Although the regenerated brain was not properly organized, some normal features, such as an inverted U-shaped structure and lateral branches, were observed. Staining of visual cells with specific antibody against visual neurons (VC-1) (Sakai et al., 2000) revealed that eye regeneration was also perturbed. Planarian eye regeneration can be divided into three steps: formation of two visual-cell clusters in the dorsal side of the anterior blastema; projection of the left and right visual neurons to the opposite ones; and connection of the optic nerves onto the

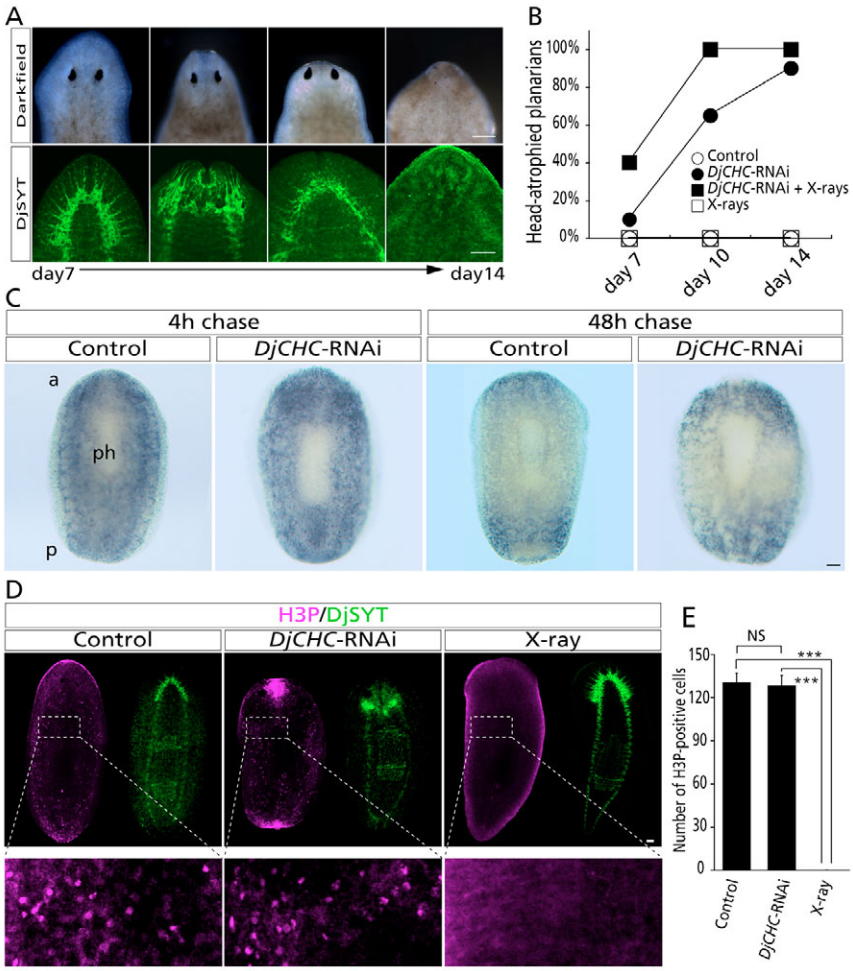

Fig. 4. *DjCHC*-RNAi treatment caused CNS atrophy. (**A**) *DjCHC*-RNAi treatment caused atrophy of the head and brain from 7 to 14 days after the injection of intact animals. (**B**) Numbers of head-atrophied planarians in control, X-ray-irradiated, *DjCHC*-RNAi-treated and *DjCHC*-RNAi plus X-ray-irradiated planarians. *n*=30. (**C**) BrdU-labeling of the trunk piece of control and *DjCHC*-RNAi-treated planarians. Samples were fixed 4 or 48 hours after BrdU treatment. a, anterior; p, posterior; ph, pharynx. (**D**) Frequency of mitosis in uncut planarians detected using anti-H3P. Confocal single projection of the fluorescent image of H3P-positive cells (magenta) and the CNS of the identical animal visualized using anti-DjSYT (green) are shown. Control, *DjCHC*-RNAi-treated and X-ray-irradiated planarians were fixed 14 days after RNAi treatment or irradiation. Animals are oriented with anterior towards the top. (**E**) The number of labeled mitoses determined using anti-H3P in planarians. Each column represents the average number (±s.e.m.) of anti-H3P-positive cells from 12 planarians. Similar data were obtained in experiments using BrdU. ****P*<0.01. NS, not significant. Scale bars: 100 μm.

brain (Inoue et al., 2004). Although the differentiation of visual neurons was not inhibited by *DjCHC* silencing, projection of left and right visual neurons to the opposite ones and connection of the optic nerves onto the brain were inhibited (Fig. 5G), implying that *DjCHC* might be required for the projection of axons during CNS regeneration.

DjCHC silencing inhibits the outgrowth and maintenance of neurites in planarian neuronal cells

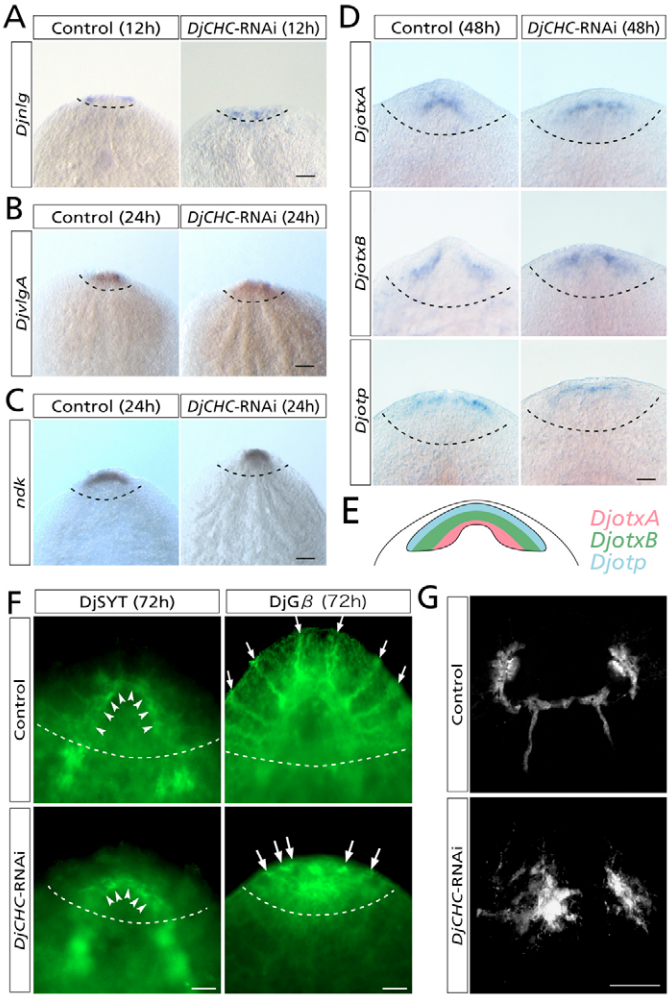

Fig. 5. Molecular analysis of *DjCHC*-RNAi-treated planarians during regeneration. (**A-D**) Expression patterns of *Djnlg* (A), *DjvlgA* (B), *ndk* (C) and brain-specific homeobox genes (D) were analyzed by whole-mount in situ hybridization 12, 24 or 48 hours after amputation, as indicated. *DjCHC*-RNAi-treated planarians showed no differences in the expression patterns of *Djnlg*, *DjvlgA*, *ndk*, *DjotxA*, *DjotxB* or *Djotp* compared to those of control animals. (**E**) Schematic illustration of the discrete expression pattern of the brain-specific homeobox genes. (**F**) Defects of *DjCHC*-RNAi-treated planarians 72 hours after amputation were detected in the brain architecture using anti-DjSYT and anti-DjGβ. Arrowheads, inverted U-shaped structure; arrows, lateral branches in regenerated blastema. The dashed line indicates the border of the newly formed region and old stump region. (**G**) Whole-mount immunostaining with VC-1 anti-planarian visual-neuron monoclonal antibody 7 days after regeneration. Scale bars: 50 μm.

To address the gene function of *DjCHC* in CNS regeneration and homeostasis at the cellular level, we used a primary culture system of planarian neural cells (HAC; head abundant cells) purified using fluorescence-activated cell sorting (FACS) and compared the head (with abundant neural cells) with the body (with few neural cells) (Asami et al., 2002). The *DjCHC*-RNAi-treated HAC contained DjCHC protein (data not shown) due to the fact that RNAi does not affect already expressed proteins. The RNAi technique, combined with the strong regenerative ability of planarians, makes it possible to disrupt the gene expression specifically in newly formed differentiated structures (T. Takano, J. Pulvers, T. Inoue, H. Tarui, H. Sakamoto, K.A. and Y. Umesono, unpublished). We used the newly formed head of *DjCHC*-RNAi-treated planarians to collect the head-abundant cell fraction on day 4 of regeneration (R4HAC) by FACS. This method is optimal for adjusting the start point of the RNAi effect, and therefore to perform time-course analysis.

Fig. 6A shows a comparison of the FACS profiles of the body and head fractions. The specific cell fraction designated R4HAC (Fig. 6A, black ellipse in middle panel) was detected in cells from the head but not from the body (Fig. 6A, left panel). R4HAC accounted for approximately 10% of the total cells in the head fraction in both control (10.9±2.9%) and *DjCHC*-RNAi-treated (9.3±3.5%) planarians (not significantly different) (Fig. 6B). Over 95% of the collected control and *DjCHC*-RNAi-treated R4HAC expressed the neural marker DjSYT (data not shown). Immunostaining of R4HAC at 0 days in vitro (DIV) using anti-DjCHC showed that 90.6±1.4% of control R4HAC were positive for DjCHC protein. *DjCHC*-RNAi treatment drastically reduced the percentage of DjCHC-protein-positive cells to 23.5±4.6%, and even positive cells expressed lower levels of DjCHC protein (Fig. 6C).

R4HAC began to extend neurites soon after culturing (Fig. 6D). Although the neurites of some cells regressed after 1 DIV, those of other cells showed additional elongation and branching by 3 DIV. In the cultures of *DjCHC*-RNAi-treated R4HAC, projection of neurites was observed, but almost all of the extended neurites regressed during culturing. The neurites of the *DjCHC*-RNAi-treated R4HAC (11.0±1.6 μm) were shorter than those of the control R4HAC at 1 DIV, and the length was further shortened to 2.1±0.3 μm at 3 DIV (Fig. 6F). The percentage of neurite-extending cells in *DjCHC*-RNAi-treated R4HAC was similarly decreased (Fig. 6E). Thus, neuronal cells of *DjCHC*-RNAi-treated planarians could not progressively extend the growth of or maintain their neurites.

To further evaluate the molecular features of *DjCHC*-RNAi-treated R4HAC, immunocytochemical analysis was performed using antibodies against DjCHC and DjSYT. DjCHC protein was strongly detected in neurites in R4HAC, but was not detected in *DjCHC*-RNAi-treated R4HAC, at 3 DIV. More than 95% of the control R4HAC at 3 DIV still expressed DjSYT protein, which was accumulated in neurites, consistent with the expression pattern in vivo (Figs 2-4) (Tazaki et al., 1999). DjSYT expression was detected at least until 3 DIV (Fig. 6G), suggesting that the *DjCHC*-RNAi-treated R4HAC might maintain their neural identity.

DjCHC-silencing induces neuronal apoptosis

To investigate how *DjCHC* mediates neuronal cell survival, the TdT-mediated dUTP nick-end labeling (TUNEL) assay (Hwang et al., 2004) was performed on R4HAC to detect apoptosis. *DjCHC*-RNAi treatment induced an increase in apoptosis (Fig. 7A-D). The degree of neuronal apoptosis was also quantified by measuring the percentage of TUNEL-positive cells among the total cells, and the data showed that the increase peaked at 3 DIV, when over 80% of the neurons were either dying or dead (Fig. 7A,B,E) and exhibited typical features of

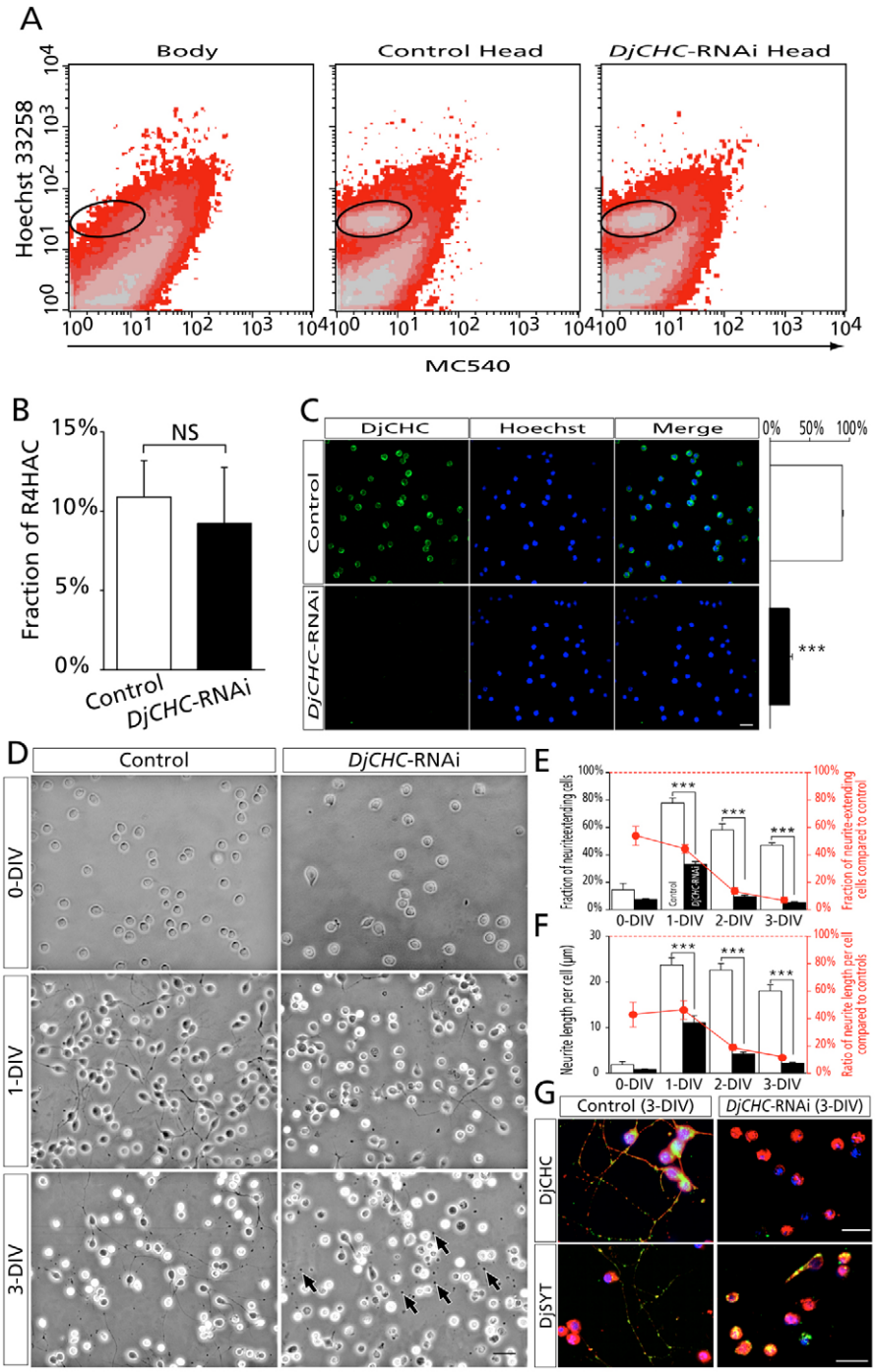

Fig. 6. Primary cultured neurons (R4HAC) obtained from control and *DjCHC*-RNAi-treated planarians by FACS. (**A**) FACS profiles of R4HAC from control body (left), control regenerated head (middle) and regenerated head of the *DjCHC*-RNAi-treated (right) planarians at 4 days of regeneration. Black ellipses indicate the R4HAC-specific peak of the head region. (**B**) Percentage (±s.e.m.) of R4HAC among the total cells. (**C**) Immunostaining with anti-DjCHC (green) and nuclear staining with Hoechst 33342 (blue) of R4HAC of control (upper panels) and *DjCHC*-RNAi (bottom panels) planarians. The graphs show the percentage (±s.e.m.) of DjCHC protein-expressing cells. (**D**) Phase-contrast views of R4HAC from control and *DjCHC*-RNAi-treated planarians. Arrowheads, debris remaining after neurite regression. (**E**,**F**) The percentage (±s.e.m.) of neurite-extending cells and the average neurite length per cell of control (white) and *DjCHC*-RNAi-treated (black) R4HAC. The percentage (±s.e.m.) of neurite-extending cells and average neurite length per cell of *DjCHC*-RNAi R4HAC compared to those of the control are indicated by the red lines. Data from 200-300 cells from the in vitro analysis were averaged. (**G**) Immunostaining of R4HAC using anti-DjCHC and DjSYT (green). For counterstaining, planarian 14-3-3ε was stained with a specific antibody (red). Nuclei were stained with Hoechst 33342 (blue). ***P<0.01. NS, not significant; DIV, days in vitro. Scale bars: 10 μm.

apoptosis (Fig. 6G, Fig. 7A-D). To examine whether *DjCHC*-silencing specifically caused apoptosis in DjCHC-protein-negative cells, we performed double TUNEL staining and immunostaining using anti-DjCHC. In the DjCHC-positive cell fraction, there was no significant difference in the fraction of TUNEL-positive cells between the control and *DjCHC*-RNAi-treated R4HAC, whereas, in the DjCHC-negative cell fraction, *DjCHC*-RNAi treatment markedly elevated the fraction of TUNEL-positive cells (Fig. 7C,D,F,G). Furthermore, all TUNEL-positive cells in the *DjCHC*-RNAi-treated R4HAC lacked neurites (Fig. 7B). Thus, dysfunction of the *DjCHC* gene might induce cell-autonomous apoptosis in neuronal cells.

Dysfunction of the clathrin-associated endocytic gene can lead to a phenotype mimicking that induced by *DjCHC*-RNAi treatment

Neurons have extensive and specialized requirements for protein trafficking, in order to transduce signals as well as to form the extremely polar shape of neurons. One of the unique functions of endocytosis in neurons is synaptic-vesicle recycling (Murthy and DeCamilli, 2003; Wu, 2004). When we knocked-down the planarian unc13 homolog (*Djunc13A*; AB281585) planarian syntaxin-binding protein homolog (*DjsbpA*; AB281586) and the Djsyt genes involved in synaptic vesicle transmission, we could not find any abnormalities in the regeneration or maintenance of CNS architecture, although behavioral abnormalities were detected in these gene-knockdown

planarians (Fig. 8A). This suggested that clathrin-mediated synaptic-vesicle recycling might not be important for the regeneration and maintenance of the CNS.

Clathrin functions in exocytosis at the endosome and trans-Golgi network (TGN) as well as in endocytosis at the plasma membrane (Hirst and Robinson, 1998). Thus, it was difficult to distinguish whether CNS abnormalities were caused by exocytic or endocytic dysfunction. To address the function of *DjCHC* necessary for neuronal survival, we compared the effects of *DjCHC* with those of the *adaptor protein complex-1* (*AP-1*) gene, whose protein product is found on the TGN and endosomes, and the *AP-2* gene, which is specifically involved in endocytosis (Boehm and Bonifacino, 2001; Robinson, 2004). First, we isolated the planarian *mu-1* gene, encoding a subunit of *AP-1*, and the *mu-2* gene, encoding a subunit of *AP-2*, and named them *Djmu-1* and *Djmu-2*, respectively (Fig. 8B). They are expressed ubiquitously (Fig. 8C,D). Then, we performed loss-of-function analysis by RNAi. Like *DjCHC*-RNAi-treated planarians, *Djmu-2*-RNAi-treated planarians could not regenerate their brain properly, although they showed a relatively weak phenotype (Fig. 8E). By contrast, no gross effects were detected in the brain regeneration of *Djmu-1*-RNAi-treated planarians. Moreover, gene knockdown of the γ and $\sigma 1$ genes, which encode the other subunits of *AP-1* (Hirst and Robinson, 1998) had no effect on CNS regeneration (data not shown). No additive phenotype was seen when double knockdown of *Djmu-1* and *Djmu-2* or of *DjCHC* and *Djmu-2* was performed (Fig. 8E,F). These data suggest that AP-2- and clathrin-mediated endocytosis, but not protein trafficking from the TNG via AP-1, are essential for proper CNS regeneration and maintenance in planarians.

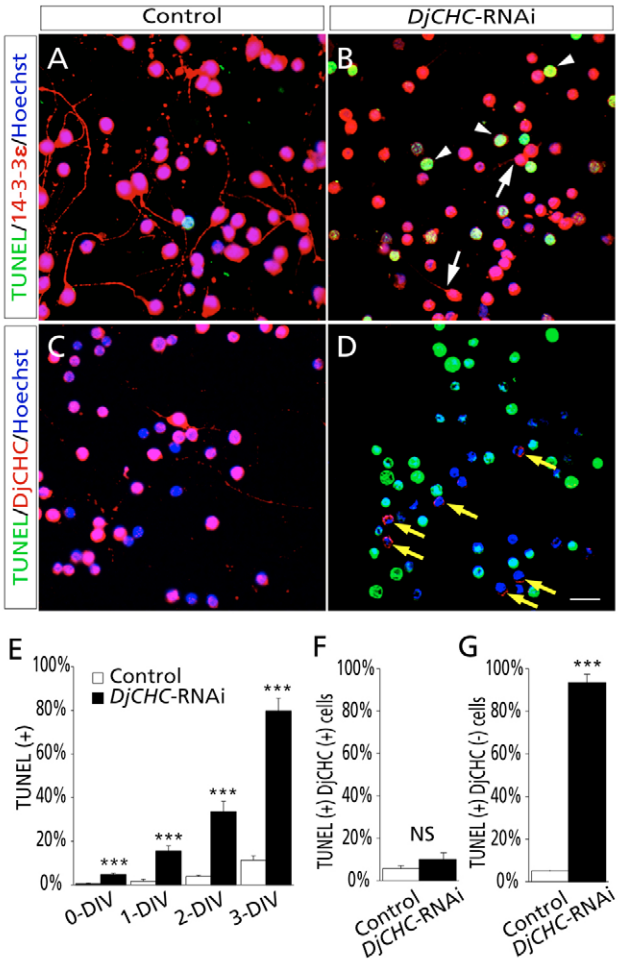

Fig. 7. TUNEL assay of control and *DjCHC*-RNAi-treated R4HAC. (**A-D**) TUNEL-positive signals (green) at 2 DIV were visualized in *DjCHC*-RNAi-treated R4HAC. Antibody staining showed the expression of planarian 14-3-3ε or DjCHC as counterstaining (red), and Hoechst 33342 staining showed nuclei (blue). White arrows indicate neurite-extending TUNEL-negative cells. White arrowheads indicate neurite-less TUNEL-positive cells. Yellow arrows indicate the DjCHC-positive cells in *DjCHC*-RNAi-treated R4HAC. Scale bar: 10 μm. (**E**) Percentage (±s.e.m.) of TUNEL-positive cells out of total cells during culturing. (**F**,**G**) The percentage (±s.e.m.) of TUNEL-positive cells in the DjCHC-protein-expressing and DjCHC-protein-negative cell fractions at 3 DIV. ***P<0.01; NS: not significant.

DISCUSSION

Clathrin is essential for regeneration as well as maintenance of the CNS

To investigate the function of clathrin-mediated signaling in neuronal cells leading to proper regeneration of the brain in planarians, we isolated a full-length *DjCHC* cDNA. The *Djmu-1* and *Djmu-2* genes, as well as the *DjCHC* gene, showed high structural similarity to the corresponding genes of other eukaryotes (Fig. 8B, and see Fig. S1 in the supplementary material) (Boehm and Bonifacino, 2001; Wilbur et al., 2005).

Clathrin silencing in the planarian prevented proper regeneration and maintenance of the CNS (Fig. 2). The headless or head-atrophied animals resulting from ablation of endocytic components did not die, because planarians have extraordinary regenerative potential. This feature of planarians was advantageous for analyzing the phenotype during the process of CNS formation and maintenance in vivo. Although the *DjCHC* transcripts were expressed ubiquitously, the DjCHC protein was strongly accumulated in neurites of CNS neurons (Fig. 1). Furthermore, gene knockdown of *DjCHC* without the regeneration process revealed that the CNS atrophied independently of stem cells (Fig. 4). These data imply that *DjCHC* might have an important role in neuronal cells in the formation and maintenance of the proper brain architecture. Notably, a tail, pharynx and intestinal tract were regenerated in the head- and tail-pieces of *DjCHC*-RNAi-treated planarians (Fig. 3A-C), indicating that intercalary regeneration, the principal manner of regeneration, generally occurred normally in *DjCHC*-depleted planarians (Kato et al., 1999; Agata et al., 2003). We could find no defects caused by silencing of *DjCHC* except for those in the CNS, despite the ubiquitous expression of this gene.

Clathrin dysfunction perturbs CNS formation after patterning and cell differentiation

We could find no deficiency in stem cell division, differentiation or migration, as indicated by BrdU, H3P and FACS analysis. Histological analysis using several genes involved in different steps of CNS formation revealed that blastema formation, brain induction, patterning and cell differentiation occurred normally until 48 hours after decapitation in *DjCHC*-RNAi-treated planarians (Fig. 5). At 72 hours after decapitation, *DjCHC*-RNAi-treated planarians could express a postmitotic neural-marker protein and could form a disorganized brain (Fig. 5F). Furthermore, although clathrin dysfunction did not disturb the differentiation or the distribution of visual neurons, projection of left and right visual neurons to the opposite ones and the connection of the optic nerves onto the brain were defective (Fig. 5G), implying that *DjCHC* is not needed for cell differentiation of the brain and eye, but rather for the projection of

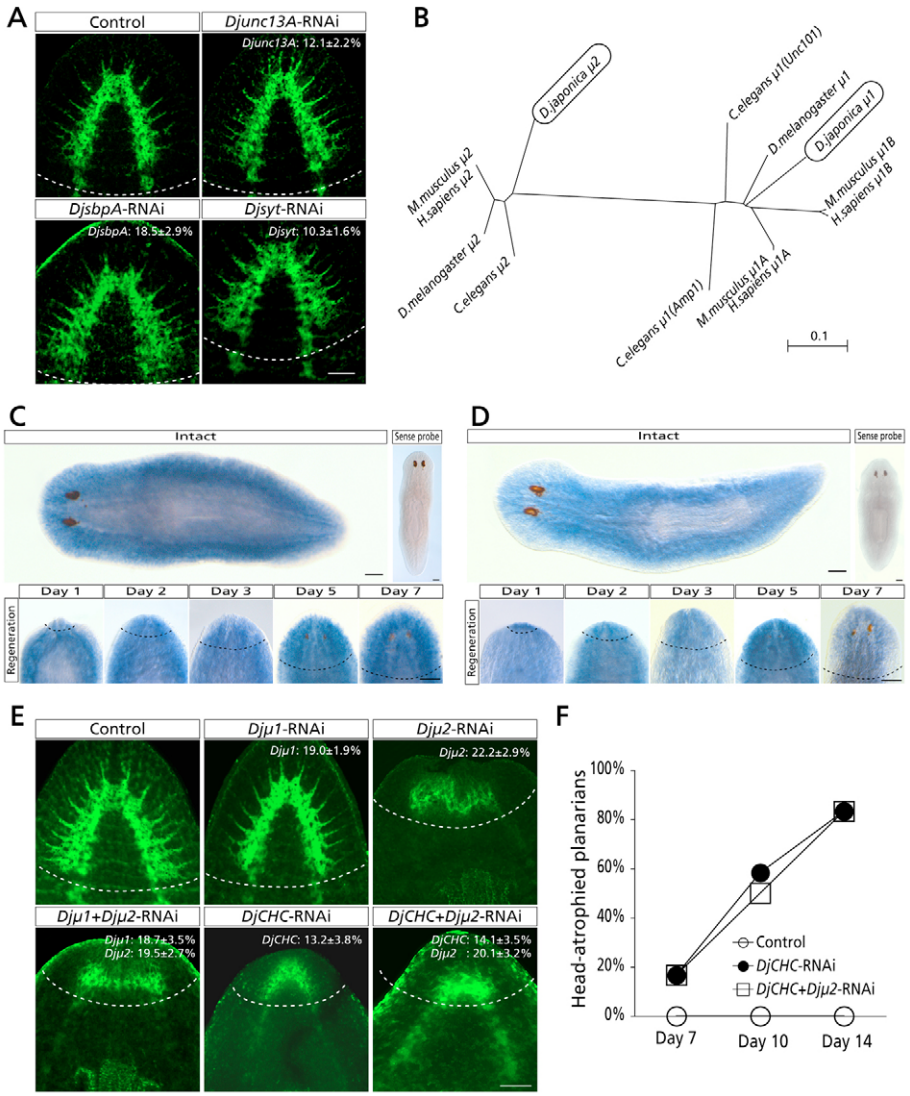

Fig. 8. Clathrin-associated endocytic genes are required for proper brain regeneration. (**A**) Whole-mount immunostaining with anti-PC2 (Agata et al., 1998) of planarians at 7 days of regeneration treated with dsRNA of *Djunc13A* (AB281585), *DjsbpA* (AB281586) and *Djsyt*. (**B**) The phylogenetic tree was constructed by the neighbor-joining method using the full sequences. The tree includes the amino acid sequences of the *mu-1* (*μ1*) and *mu-2* (*μ2*) genes derived from *Dugesia japonica* (AB243058, AB243059), Homo sapiens (Q9BXS5, Q9Y6Q5, Q96CW1), Mus musculus (P35585, Q9WVP1, P84091), *Drosophila melanogaster* (EAL28715, AAF56001) and *Caenorhabditis elegans* (NP491572, AAA72418, P35603). (**C**,**D**) The expression pattern of *Djmu-1* (*Djμ1*; C) and *Djmu-2* (*Djμ2*; D). (**E**) Whole-mount immunostaining with anti-DjSYT of planarians at 7 days of regeneration that had been treated with RNAi for *DjCHC*, *Djmu-1*, *Djmu-2*, *Djmu-1* plus *Djmu-2*, or *DjCHC* plus *Djmu-2*. The numbers in each panel represent the downregulated expression level of each gene analyzed by real-time PCR. The dashed line indicates the border between the newly formed region and the old stump region. Scale bars: 100 μm. (**F**) The numbers on the *y* axis indicate the percentage of head-atrophied planarians on each day after dsRNA treatment (*x* axis). *n*=30.

the axons and formation of the proper CNS. Thus, clathrin is essential for the construction of the proper architecture of the CNS after patterning and neuronal cell differentiation, and before neural circuit formation during CNS regeneration, as well as for the maintenance of the proper architecture of the CNS after synaptic connection in planarians (Fig. 9).

Clathrin is essential for neurite outgrowth and maintenance, and for neuronal cell survival

In vitro analysis using primary cultured planarian neurons revealed that the numbers of control and *DjCHC*-RNAi-treated R4HAC collected were not significantly different, suggesting that differentiation and mitosis occurred normally in *DjCHC*-RNAi-treated planarians (Fig. 6B), in contrast to *Dictyostelium* cells and HeLa cells, in which clathrin inhibition blocks cytokinesis (Niswonger and O'Halloran, 1997a; Motley et al., 2003). The combination of RNAi and the in vitro analysis described here should prove useful for the functional characterization of neural genes at the cellular level during the regeneration and maintenance of the planarian CNS. At 3 DIV, almost none of the *DjCHC*-RNAi-treated R4HAC could lengthen or maintain their neurites, which seemed to be regressed but not retracted (Fig. 6D-F). These observations are consistent with the features of RNAi-treated planarians in vivo and suggest that *DjCHC*-silenced neurons could extend some neurites, but could not further extend or maintain these neurites, and seemed to undergo cell death after the completion of differentiation.

Interestingly, although the percentage of neurite-extending cells and the neurite length per cell of the control R4HAC peaked by 1 DIV and were subsequently reduced, the length of the neurite-extending cells tended to be slightly increased (from 30.5±1.9 μm at 1 DIV to 41.5±6.5 μm at 3 DIV) (data not shown). It is tempting to speculate that some mechanisms related to appropriate synaptic connections or trophic factors might be important for neurite outgrowth and maintenance in this organism (Vaudry et al., 2002; Howe and Mobley, 2005). In contrast to the findings in control R4HAC, both the percentage of neurite-extending cells and neurite length of *DjCHC*-RNAi-treated R4HAC progressively decreased during culturing, indicating that dysfunction of clathrin-mediated endocytosis might accelerate neurite regression (Fig. 6E,F). Previous reports suggested that neurotrophic factors such as nerve growth factor are produced and released in target tissues to activate receptors on the presynaptic elements of innervating neurons, thereby signaling to regulate the neuronal survival of these neurons

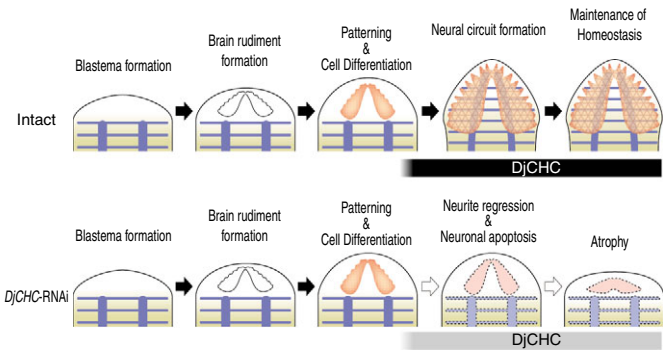

Fig. 9. Schematic illustration of CNS maintenance and regeneration. In normal planarian regeneration (upper panel), blastema formation is followed by patterning and cell differentiation of neural cells, and then the neural cells are organized into neural circuits, which are maintained by homeostasis. In *DjCHC*-silenced planarians (lower panel), the patterning and cell differentiation of neural cells occur normally, but neurites regress and neural cells die via apoptosis, resulting in atrophy of the CNS.

at nerve terminals involved in mediating endocytosis (Casaccia-Bonnefil et al., 1999; Ye et al., 2003; Yano and Chao, 2004; Howe and Mobley, 2005).

We tested whether clathrin dysfunction interferes with cellular functions such as cell survival by examining apoptosis in R4HAC, and found that *DjCHC*-RNAi treatment increased apoptosis (Fig. 7). This is consistent with the fact that chicken B cell line DT40 cells deficient in the clathrin gene undergo apoptosis (Wettey et al., 2002). However, clathrin-depleted HeLa cells do not show any apparent increase in apoptosis (Motley et al., 2003; Hinrichsen et al., 2003). These discrepancies will be resolved by further analyses revealing the details of the mechanism of endocytosis in each cell type.

Clathrin-mediated endocytic signals are essential for proper CNS regeneration

Besides clathrin, one of the most important components of clathrin-mediated endocytosis is AP-2, which binds both clathrin and cytoplasmic tyrosine-based signals on transmembrane proteins (Boehm and Bonifacino, 2001; Robinson, 2004). AP complexes involved in cargo selection for inclusion into coated vesicles in the late secretory and endocytic pathways are widely distributed among eukaryotes (Boehm and Bonifacino, 2001; Boehm and Bonifacino, 2002). Our finding that the disruption of endocytic genes caused defective CNS regeneration, but disruption of an exocytic gene, *Djmu-1*, did not, suggested that clathrin-mediated endocytosis at the plasma membrane, not protein trafficking at the TNG, is necessary for CNS formation (Fig. 8E). This is consistent with our finding that the DjSYT and DjGβ proteins were detected in neurites in *DjCHC*-RNAi-treated planarians, indicating that protein trafficking occurred without the *DjCHC* gene (Fig. 2C, Fig. 5F). However, the possibility that AP-1-independent alternative factors in the clathrin-coated pits at the TGN are involved in proper CNS formation and maintenance could not be completely ruled out.

Interestingly, the gene-knockdown animals for *DjCHC* and *Djmu-2* did not show exactly the same phenotypes: *DjCHC*-RNAi treatment caused a more-severe phenotype than *Djmu-2*-RNAi treatment. The distribution of α-adaptin and epsin and the cellular morphologies are different in CHC-siRNA- and *mu-2*-siRNA-treated HeLa cells (Motley et al., 2003; Hinrichsen et al., 2003). Furthermore, clathrin-mediated endocytosis can still occur in the absence of AP-2, and AP-2 may not be essential for the recruitment and assembly of clathrin at the plasma membrane (Motley et al., 2003; Hinrichsen et al., 2003; Conner and Schmid, 2003). Our data, together with these previous findings, lead us to speculate that clathrin, AP-2 and alternative adaptors may co-assemble at the plasma membrane, bringing cargo with different types of internalization signals into the coated pit. In the absence of AP-2, alternative adaptors are still recruited onto the plasma membrane and co-assemble with clathrin (Mishra et al., 2001; Mishra et al., 2002; Motley et al., 2003; Hinrichsen et al., 2003; Sever, 2003). Furthermore, simultaneous silencing of *Djmu-1* and *Djmu-2* or *DjCHC* and *Djmu*-2 did not cause additive effects on CNS formation (Fig. 8E,F), indicating that the more-severe phenotype of *DjCHC*-RNAi-treated planarians was probably not caused by inhibition of both endocytosis and trafficking at the TNG. In planarians, alternative adaptors in addition to AP-2 in the clathrin-coated pits mediating endocytosis may be necessary for proper CNS regeneration and maintenance.

In summary, our data demonstrate that clathrin-mediated endocytosis is essential for neuronal cell survival, and for neurite outgrowth and maintenance, after the completion of neuronal differentiation to enable the formation as well as the maintenance of the proper functional CNS in planarians in vivo (Fig. 9). Studies of this enormously interesting organism will advance our understanding of the physiological roles, and the cellular and molecular mechanisms of intercellular communication and cellular signaling in maintaining the proper function of the nervous system.

We thank Elizabeth Nakajima and Kosei Takeuchi for careful reading of the manuscript. We also thank Hidefumi Orii (Department of Life Science, University of Hyogo) for his generous gift of anti-DjMHC-B antibody. This work was supported by a research grant from the Special Postdoctoral Researcher Program of RIKEN to T.I., a Grant-in-Aid for Creative Scientific Research to K.A. (17GS0318), a Grant-in-Aid for Scientific Research on Priority Areas to K.A., and the Grant for Biodiversity Research of the 21st Century COE (A14) to K.A.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/9/1679/DC1

References

Agata, K. and Watanabe, K. (1999). Molecular and cellular aspects of planarian regeneration. *Semin. Cell Dev. Biol.* **10**, 377-383.
Agata, K., Soejima, Y., Kato, K., Kobayashi, C., Umesono, Y. and Watanabe, K. (1998). Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers. *Zool. Sci.* **15**, 433-440.
Agata, K., Tanaka, T., Kobayashi, C., Kato, K. and Saitoh, Y. (2003). Intercalary regeneration in planarians. *Dev. Dyn.* **226**, 308-316.
Allen, C., Goulding, D. and Field, M. (2003). Clathrin-mediated endocytosis is essential in Trypanosoma brucei. *EMBO J.* **22**, 4991-5002.
Asami, M., Nakatsuka, T., Hayashi, T., Kou, K., Kagawa, H. and Agata, K. (2002). Cultivation and characterization of planarian neuronal cells isolated by fluorescence activated cell sorting (FACS). *Zool. Sci.* **19**, 1257-1265.
Baguñà, J. and Romero, R. (1981). Quantitative analysis of cell types during growth, degrowth and regeneration in the Planarians Dugesia mediterranea and Dugesia tigrina. *Hydrobiologia* **84**, 181-194.
Baguñà, J., Saló, E. and Romero, R. (1989). Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians. *Int. J. Dev. Biol.* **33**, 261-266.
Boehm, M. and Bonifacino, J. (2001). Adaptins: the final recount. *Mol. Biol. Cell* **12**, 2907-2920.
Boehm, M. and Bonifacino, J. (2002). Genetic analyses of adaptin function from yeast to mammals. *Gene* **286**, 175-186.
Casaccia-Bonnefil, P., Gu, C. and Chao, M. (1999). Neurotrophins in cell survival/death decisions. *Adv. Exp. Med. Biol.* **468**, 275-282.
Cebrià, F. and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* **132**, 3691-3703.
Cebrià, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Sánchez Alvarado, A. et al. (2002a). FGFR-related gene nou-darake restricts brain tissues to the head region of planarians. *Nature* **419**, 620-624.

Cebrià, F., Kudome, T., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T. and Agata, K. (2002b). The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. *Mech. Dev.* **116**, 199-204.
Cebrià, F., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T. and Agata, K. (2002c). Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. *Dev. Growth Differ.* **44**, 135-146.
Conner, S. and Schmid, S. (2003). Differential requirements for AP-2 in clathrin-mediated endocytosis. *J. Cell Biol.* **162**, 773-779.
Fotin, A., Cheng, Y., Sliz, P., Grigorieff, N., Harrison, S., Kirchhausen, T. and Walz, T. (2004). Molecular model for a complete clathrin lattice from electron cryomicroscopy. *Nature* **432**, 573-579.
Fusaoka, E., Inoue, T., Mineta, K., Agata, K. and Takeuchi, K. (2006). Structure and function of primitive immunoglobulin superfamily neural cell adhesion molecules: a lesson from studies on planarian. *Genes Cells* **11**, 541-555.
Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.
Hinrichsen, L., Harborth, J., Andrees, L., Weber, K. and Ungewickell, E. (2003). Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J. Biol. Chem.* **278**, 45160-45170.
Hirst, J. and Robinson, M. S. (1998). Clathrin and adaptors. *Biochem. Biophys. Acta* **1404**, 173-193.
Howe, C. and Mobley, W. (2005). Long-distance retrograde neurotrophic signaling. *Curr. Opin. Neurobiol.* **15**, 40-48.
Hwang, J., Kobayashi, C., Agata, K., Ikeo, K. and Gojobori, T. (2004). Detection of apoptosis during planarian regeneration by the expression of apoptosis-related genes and TUNEL assay. *Gene* **333**, 15-25.
Inoue, T., Kumamoto, H., Okamoto, K., Umesono, Y., Sakai, M., Sánchez Alvarado, A. and Agata, K. (2004). Morphological and functional recovery of the planarian photosensing system during head regeneration. *Zool. Sci.* **21**, 275-283.
Kato, K., Orii, H., Watanabe, K. and Agata, K. (1999). The role of dorsoventral interaction in the onset of planarian regeneration. *Development* **126**, 1031-1040.
Koinuma, S., Umesono, Y., Watanabe, K. and Agata, K. (2003). The expression of planarian brain factor homologs, DjFoxG and DjFoxD. *Gene Expr. Patterns* **3**, 21-27.
Le Roy, C. and Wrana, J. (2005). Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat. Rev. Mol. Cell Biol.* **6**, 112-126.
Mineta, K., Nakazawa, M., Cebrià, F., Ikeo, K., Agata, K. and Gojobori, T. (2003). Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. *Proc. Natl. Acad. Sci. USA* **100**, 7666-7671.
Mishra, S., Agostinelli, N., Brett, T., Mizukami, I., Ross, T. and Traub, L. (2001). Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins. *J. Biol. Chem.* **276**, 46230-46236.
Mishra, S., Keyel, P., Hawryluk, M., Agostinelli, N., Watkins, S. and Traub, L. (2002). Disabled-2 exhibits the properties of a cargo-selective endocytic clathrin adaptor. *EMBO J.* **21**, 4915-4926.
Motley, A., Bright, N., Seaman, M. and Robinson, M. (2003). Clathrin-mediated endocytosis in AP-2-depleted cells. *J. Cell Biol.* **162**, 909-918.
Murthy, V. and DeCamilli, P. (2003). Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.* **26**, 701-728.
Nakazawa, M., Cebrià, F., Mineta, K., Ikeo, K., Agata, K. and Gojobori, T. (2003). Search for the evolutionary origin of a brain: planarian brain characterized by microarray. *Mol. Biol. Evol.* **20**, 784-791.
Newmark, P. and Sánchez Alvarado, A. (2000). Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. *Dev. Biol.* **220**, 142-153.
Niswonger, M. and O'Halloran, T. (1997a). Clathrin heavy chain is required for spore cell but not stalk cell differentiation in Dictyostelium discoideum. *Development* **124**, 443-451.
Niswonger, M. and O'Halloran, T. (1997b). A novel role for clathrin in cytokinesis. *Proc. Natl. Acad. Sci. USA* **94**, 8575-8578.
Nogi, T. and Watanabe, K. (2001). Position-specific and non-colinear expression of the planarian posterior (Abdominal-B-like) gene. *Dev. Growth Differ.* **43**, 177-184.
Nogi, T. and Levin, M. (2005). Characterization of innexin gene expression and functional roles of gap-junctional communication in planarian regeneration. *Dev. Biol.* **287**, 314-335.
Ogawa, K., Ishihara, S., Saito, Y., Mineta, K., Nakazawa, M., Ikeo, K., Gojobori, T., Watanabe, K. and Agata, K. (2002). Induction of a noggin-like gene by ectopic DV interaction during planarian regeneration. *Dev. Biol.* **250**, 59-70.
Orii, H., Ito, H. and Watanabe, K. (2002). Anatomy of the planarian Dugesia japonica I. The muscular system revealed by antisera against myosin heavy chains. *Zool. Sci.* **19**, 1123-1131.
Reddien, P. and Sánchez Alvarado, A. (2004). Fundamentals of planarian regeneration. *Annu. Rev. Cell Dev. Biol.* **20**, 725-757.
Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R. and Sánchez Alvarado, A. (2005a). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev. Cell* **8**, 635-649.
Reddien, P. W., Oviedo, N. J., Jennings, J. R., Jenkin, J. C. and Sánchez Alvarado, A. (2005b). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* **310**, 1327-1330.
Robinson, M. (2004). Adaptable adaptors for coated vesicles. *Trends Cell Biol.* **14**, 167-174.
Ruscetti, T., Cardelli, J., Niswonger, M. and O'Halloran, T. (1994). Clathrin heavy chain functions in sorting and secretion of lysosomal enzymes in Dictyostelium discoideum. *J. Cell Biol.* **126**, 343-352.
Sakai, F., Agata, K., Orii, H. and Watanabe, K. (2000). Organization and regeneration ability of spontaneous supernumerary eyes in planarians - eye regeneration field and pathway selection by optic nerves. *Zool. Sci.* **17**, 375-381.
Saló, E. and Baguñà, J. (2002). Regeneration in planarians and other worms: New findings, new tools, and new perspectives. *J. Exp. Zool.* **292**, 528-539.
Salvetti, A., Rossi, L., Lena, A., Batistoni, R., Deri, P., Rainaldi, G., Locci, M. T., Evangelista, M. and Gremigni, V. (2005). DjPum, a homologue of Drosophila Pumilio, is essential to planarian stem cell maintenance. *Development* **132**, 1863-1874.
Sánchez Alvarado, A. (2006). Planarian regeneration: its end is its beginning. *Cell* **124**, 241-245.
Sánchez Alvarado, A. and Newmark, P. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* **96**, 5049-5054.
Seeger, M. and Payne, G. (1992). A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO J.* **11**, 2811-2818.
Sever, S. (2003). AP-2 makes room for rivals. *Dev. Cell* **5**, 530-532.
Shibata, N., Umesono, Y., Orii, H., Sakurai, T., Watanabe, K. and Agata, K. (1999). Expression of vasa(vas)-related genes in germline cells and totipotent somatic stem cells of planarians. *Dev. Biol.* **206**, 73-87.
Takei, K. and Haucke, V. (2001). Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends Cell Biol.* **11**, 385-391.
Tazaki, A., Gaudieri, S., Ikeo, K., Gojobori, T., Watanabe, K. and Agata, K. (1999). Neural network in planarian revealed by an antibody against planarian synaptotagmin homologue. *Biochem. Biophys. Res. Commun.* **260**, 426-432.
Umesono, Y., Watanabe, K. and Agata, K. (1997). A planarian orthopedia homolog is specifically expressed in the branch region of both the mature and regenerating brain. *Dev. Growth Differ.* **39**, 723-727.
Umesono, Y., Watanabe, K. and Agata, K. (1999). Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. *Dev. Genes Evol.* **209**, 31-39.
Vaudry, D., Stork, P., Lazarovici, P. and Eiden, L. (2002). Signaling pathways for PC12 cell differentiation: making the right connections. *Science* **296**, 1648-1649.
Watanabe, K., Sakai, F. and Orii, H. (1997). Stepwise dilution screening of a cDNA library by polymerase chain reaction. *Anal. Biochem.* **252**, 213-214.
Wettey, F., Hawkins, S., Stewart, A., Luzio, J., Howard, J. and Jackson, A. (2002). Controlled elimination of clathrin heavy-chain expression in DT40 lymphocytes. *Science* **297**, 1521-1525.
Wilbur, J., Hwang, P. and Brodsky, F. (2005). New faces of the familiar clathrin lattice. *Traffic* **6**, 346-350.
Wolff, E. and Dubois, F. (1948). Sur la migration des cellules de regeneration chez les planaires. *Rev. Suisse Zool.* **55**, 218-227.
Wu, L. (2004). Kinetic regulation of vesicle endocytosis at synapses. *Trends Neurosci.* **27**, 548-554.
Yano, H. and Chao, M. (2004). Mechanisms of neurotrophin receptor vesicular transport. *J. Neurobiol.* **58**, 244-257.
Ybe, J., Brodsky, F., Hofmann, K., Lin, K., Liu, S., Chen, L., Earnest, T., Fletterick, R. and Hwang, P. (1999). Clathrin self-assembly is mediated by a tandemly repeated superhelix. *Nature* **399**, 371-375.
Ye, H., Kuruvilla, R., Zweifel, L. and Ginty, D. (2003). Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. *Neuron* **39**, 57-68.