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Neurogenic role of Gcm transcription factors is conserved in chicken spinal cord

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Although *glial cells missing* (*gcm*) genes are known as glial determinants in the fly embryo, the role of vertebrate orthologs in the central nervous system is still under debate. Here we show for the first time that the chicken ortholog of fly *gcm* (herein referred to as *c-Gcm1*), is expressed in early neuronal lineages of the developing spinal cord and is required for neural progenitors to differentiate as neurons. Moreover, *c-Gcm1* overexpression is sufficient to trigger cell cycle exit and neuronal differentiation in neural progenitors. Thus, *c-Gcm1* expression constitutes a crucial step in the developmental cascade that prompts progenitors to generate neurons: *c-Gcm1* acts downstream of proneural (neurogenin) and progenitor (Sox1-3) factors and upstream of NeuroM neuronal differentiation factor. Strikingly, this neurogenic role is not specific to the vertebrate gene, as fly *gcm* and *gcm2* are also sufficient to induce the expression of neuronal markers. Interestingly, the neurogenic role is restricted to post-embryonic stages and we identify two novel brain neuronal lineages expressing and requiring *gcm* genes. Finally, we show that fly *gcm* and the chick and mouse orthologs induce expression of neural markers in HeLa cells. These data, which demonstrate a conserved neurogenic role for Gcm transcription factors, call for a re-evaluation of the mode of action of these genes during evolution.

KEY WORDS: *Drosophila melanogaster*, Chicken, *glial cells missing*, Neurogenesis

INTRODUCTION

Glial cells missing transcription factors (Gcm and Gcm2) are considered as the glial determinants in the fly embryonic central nervous system (CNS) (for a review, see Van De Bor and Giangrande, 2002). Their absence leads to glia-to-neuron transformation, and ectopic expression of *gcm* or *gcm2* leads to differentiation of supernumerary glia at the expense of neurons, indicating a role as a molecular switch between neuronal and glial fates (Alfonso and Jones, 2002; Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001; Vincent et al., 1996).

Although structurally conserved throughout evolution, the function of these two genes in vertebrate gliogenesis has remained elusive (Altshuller et al., 1996; Anson-Cartwright et al., 2000; Basyuk et al., 1999; Kanemura et al., 1999; Kim et al., 1998; Schreiber et al., 2000). Previous studies have shown that *Gcm1*-deficient mice die during embryogenesis owing to developmental defects of the placenta (Anson-Cartwright et al., 2000; Schreiber et al., 2000), whereas *Gcm2*-deficient mice are viable but lack a parathyroid gland (Gunther et al., 2000). No defects were found in the CNS; however, *Gcm1*-knockout mice die by E10, thereby preventing functional analyses in the nervous system. Moreover, RT-PCR experiments revealed the presence of *Gcm1* transcripts in mouse brain, starting from E12 (Iwasaki et al., 2003).

We and others have recently shown that fly *gcm* genes are required in a specific neuronal population of the larval visual system (Chotard et al., 2005; Yoshida et al., 2005). During optic lobe development, neuroblasts produce lamina precursor cells (LPCs), which divide once to produce lamina neurons (Selleck and Steller, 1991). *gcm* and *gcm2* are coexpressed in LPCs and, in their absence, lamina neurons are not produced, indicating that these two genes are required in neuronal precursors to induce neuronal fate (Chotard et al., 2005; Yoshida et al., 2005). This puzzling observation raises the possibility that vertebrate *gcm* genes might also be required for neuronal differentiation, and calls for the role of *gcm* genes in evolution to be re-evaluated.

We here show that the chicken ortholog of fly *gcm* (herein referred to as *c-Gcm1*), but not the chicken ortholog of fly *gcm2* (herein referred to as *c-Gcm2*), is expressed in early neuronal lineages of the developing chick spinal cord. Moreover, full neuronal differentiation is prematurely induced upon *c-Gcm1* overexpression and inhibited upon blocking its pathway, thus revealing a neurogenic, rather than a gliogenic, role. We show that *c-Gcm1* is required for precursors to enter the post-mitotic state, acting downstream of the neurogenin and *Sox1-3* genes and upstream of *NeuroM*. Thus, we demonstrate for the first time that a vertebrate *gcm* gene is expressed and required in the CNS. We also show that, in flies, the neurogenic role of the *gcm* genes extends to the newly identified interneurons of the central brain. Moreover, *gcm* or *gcm2* overexpression can induce ectopic neuronal differentiation after embryonic development. Finally, we show that fly, chick and mouse *gcm* genes induce expression of neuronal and glial markers in transfected HeLa cells. Thus, *gcm* genes have a double potential that is conserved during evolution.

MATERIALS AND METHODS

Fly strains

The wild-type strain used was *Sevelen*. Flies were raised at 25°C, except for experiments with *tub-gal80^{ts}* lines. *gcm-gal4* carries a *gal4 P*-element inserted into the *gcm* promoter. *UAS-gcm^{DN}* was used to block *gcm-gcm2* function with *UAS-gcm^{N7-4DN}* as a control (Soustelle et al., 2004). *UAS-ncGFP* targets GFP to the nucleus and cytoplasm, whereas *UAS-mCD8GFP*

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targets GFP to the membrane. *actin-gal4* was used for overexpression experiments with *UAS-gcm* (Bernardoni et al., 1998) or *UAS-gcm2* (Kammerer and Giangrande, 2001).

In situ hybridization

The *c-Gcm1* RNA probe was synthesized from chicken EST pgr1n.pk002.g21 (Chicken EST project, Delaware Biotechnology Institute, Newark, USA). *NeuroM* and *Sox2* probes were generated from specific PCR fragments to avoid cross-reactivity with transgenes. The *c-Gcm2* probe was synthesized from the partial cDNA template described by Okabe and Graham (Okabe and Graham, 2004). Chicken embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). In situ hybridization was performed according to Braquart-Varnier (Braquart-Varnier et al., 2004). Sections were digitalized and analyzed using Zeiss software, and images were manipulated using Adobe Photoshop. In situ hybridizations were performed according to Kammerer and Giangrande (Kammerer and Giangrande, 2001), except that *Drosophila* brains were fixed overnight at 4°C in 4% paraformaldehyde. Double in situ hybridization was performed using probes labeled with either digoxigenin or fluorescein. Mouse anti-digoxigenin (1:100, Boehringer Mannheim) or rabbit anti-FITC (1:10,000, Molecular probes) and fluorescent secondary antibodies were used for immunolabeling.

Immunohistochemistry

The *Drosophila* CNS was immunolabeled as described previously (Ceron et al., 2001). Primary antibodies used were: mouse anti-Repo (1:100, DSHB), rat anti-Elav (1:200, supernatant from DSHB), rabbit anti-phospho-Histone H3 (1:500, Upstate Biotechnology), rabbit anti-GFP (1:500, Molecular probes), mouse anti-Acj6 (1:50, DSHB). Secondary antibodies conjugated with FITC, Cy3 or Cy5 (Jackson) were used at 1:500. DAPI was used at 100 ng/ml for nuclei counterstaining. Brains were mounted in Vectashield (Vector) mounting medium.

Chicken tissue vibratome sections fixed in 4% formaldehyde were processed using standard protocols. Primary antibodies used were: rabbit anti-Pax6 (1:150, Covance), mouse (1:2000) and rabbit (1:1000) anti-βIII-tubulin (Covance), mouse anti-Mnr2 (1:4, DSHB), mouse anti-Pax7 (1:2, DSHB), mouse anti-Lim1/2 (1:2, DSHB), mouse anti-BrdU (1:2000, DSHB), mouse anti-HuC/D (1:500, Molecular Probes), rabbit anti-GFP (1:500, Torrey). Rabbit anti-Sox1 (1:800), anti-Sox2 (1:4000) and anti-Sox3 (1:500) were used, but as similar results were obtained from each only data relative to Sox3 are shown. Secondary antibodies used were: anti-mouse Ig Alexa-546, anti-rabbit Ig Alexa 647 (1:1000, Molecular Probes). Sections or brains were analyzed using confocal microscopes (Leica).

Ectopic expression experiments

actin-gal4,tub-gal80^{ts},UAS-gcm or *UAS-gcm2* LII larvae were incubated at 30°C and brains dissected at LIII. The *c-Gcm1* coding region (Hashemolhosseini et al., 2004) was cloned into the pCIG vector. The c-Gcm1BD-ER vector was constructed by cloning a PCR-amplified fragment (equivalent to amino acids 1-193 of c-Gcm1) into the CMV-based vector pCS2, which contains the repressor domain (amino acids 1-298) of the *D. melanogaster* Engrailed protein (Smith and Jaynes, 1996). The coding region of *Ngn2* was cloned into expression plasmid pAdRSV-Sp. In ovo chicken electroporation was performed as previously described (Danesin et al., 2006; Itasaki et al., 1999). Expression vectors (1 μl) were used at 2 μg/μl, except for the c-Gcm1BD-ER construct which was used at 3 μg/μl, and were coinjected with control vectors pEGFP-N1 (Clontech, 0.6 μg/μl) or pCIG to visualize the transfected area. Flat-mounted spinal cord explants were cultivated in an organotypic culture system as previously described (Agius et al., 2004). Embryos or spinal cord explants were fixed in 4% formaldehyde in PBS for 2 hours for immunohistochemistry, or overnight for in situ hybridization, and dehydrated in an ethanol series. Cell proliferation was evaluated by bromodeoxyuridine (BrdU, Roche) incorporation. BrdU (10 mM) was injected into the lumen of the chicken neural tube and embryos were harvested 1 hour later. BrdU immunolabeling was performed on sections treated with 2N HCl in 0.2% Triton X-100/PBS for 30 minutes, after blocking and before incubation with primary antibodies.

Cell quantification

Quantifications were performed by calculating the percentage of electroporated (GFP-positive) cells labeled with a particular marker, divided by the total number of GFP-positive cells. Counts were performed on at least ten sections from three embryos in each experiment. Data were obtained from at least three independent experiments, in each of which at least 150 cells were counted. Results are expressed as the mean percentage±s.e.m. of labeled electroporated cells. Significance was analyzed using the Student's *t* test and *P* values are indicated in the figure legends.

HeLa cell transfection

HeLa cells were grown in DMEM supplemented with fetal bovine serum and antibiotics and transfected with Effecten Transfection Reagent (Qiagen). Each cDNA was cloned into the pCIG vector. After 48 hours, transfected cells were analyzed by immunolabeling, using rabbit anti-Tuj1 (1:1000, Covance), mouse anti-GFAP (1:100, ICN Biomedicals), fluorescent secondary antibodies (1:600) and DAPI as above. Preparations were mounted in Polymount medium (Polysciences) and analyzed by confocal microscopy. Labeled cells were counted from three independent transfection assays. Results are expressed as the mean of counted cells.

RESULTS

c-Gcm1 is expressed in the developing chicken CNS

To investigate the role of vertebrate *gcm* genes during neural development, we determined the expression profile of the two chicken orthologs *c-Gcm1* (Hashemolhosseini et al., 2004) and *c-Gcm2* (Okabe and Graham, 2004). *c-Gcm1* RNA was observed as early as the primitive streak stage, in the presumptive neural plate (Fig. 1A). *c-Gcm1*-positive cells were found in the ectoderm (Fig. 1A,A′) and were included in the domain expressing *Sox2* (Fig. 1B), a marker of early neural progenitors (Bylund et al., 2003; Graham et al., 2003; Uwanogho et al., 1995). At the onset of rostral neural tube folding, high levels of transcripts were detected at all rostrocaudal levels of the developing CNS along the entire dorsoventral axis (Fig. 1C,D), except for the dorsal-most region (Fig. 1E). At E2.5, when neuronal differentiation has started (McConnell and Sechrist, 1980; Sechrist and Bronner-Fraser, 1991), *c-Gcm1* expression was seen to be markedly reinforced in cells located in the lateral wall of the forming mantle layer, which are likely to correspond to newly generated neurons (Fig. 1F). From E3, *c-Gcm1* RNA was not detected in the ventricular zone nor in the forming mantle layer (Fig. 1G and data not shown), indicating that *c-Gcm1* expression is extinguished in late neuronal progenitors, before the appearance of glial progenitors (Rowitch, 2004). By contrast, *c-Gcm2* RNA was never detected in the developing spinal cord at any of the stages analyzed, from E1 to E8 (see Fig. S1 in the supplementary material). However, in E1.5 embryos, *c-Gcm2* was expressed in a restricted rostrocaudal domain of the hindbrain (see Fig. S1B,B′ in the supplementary material).

These results reveal for the first time the expression profile of *gcm* genes in the embryonic vertebrate CNS.

c-Gcm1 overexpression promotes neuronal differentiation

To directly assess the function of *c-Gcm1*, we in ovo electroporated a *c-Gcm1*-expressing vector at E1.5 and analyzed neural tubes using markers specific to progenitor or post-mitotic cells. Electroporation of the *c-Gcm1* vector cell-autonomously suppresses the expression of *Sox1-3*, which code for HMG-box transcription factors specific to CNS proliferating progenitors (Fig. 2A,A′,G) (Bylund et al., 2003; Graham et al., 2003; Uwanogho et al., 1995). Only 15% of *c-Gcm1*-overexpressing cells coexpressed *Sox1-3*, as compared with

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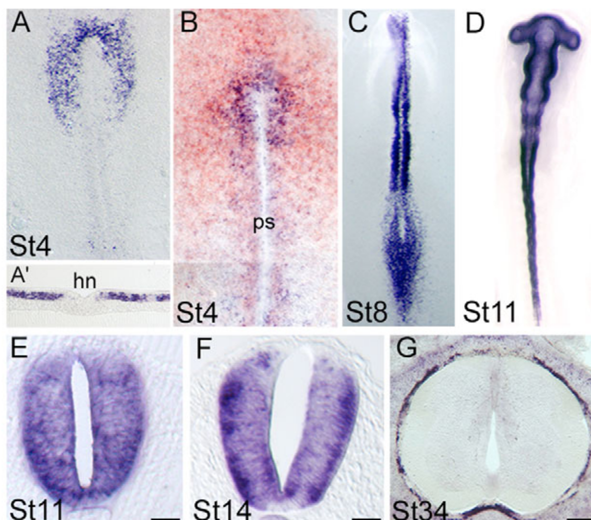


Fig. 1. Expression of *c-Gcm1* in developing chicken embryonic CNS. *c-Gcm1* in situ hybridization on whole chick embryos (**A-D**) and on transverse sections of embryonic spinal cord (**E-G**). (A,B) Dorsal views showing that *c-Gcm1* expressing cells (purple) are detected in the anterior neural plate at stage 4, in an area corresponding to the epiblast as shown on transverse section in A′. (B) Colabeling shows that *c-Gcm1*-expressing cells are included in the Sox2 expression domain (red). (C) During neurulation, *c-Gcm1* is strongly expressed throughout neural tube and neural plate. (D,E) Starting from E1.5 (stage 11), *c-Gcm1* is expressed in the neural tube (D), along the entire dorsoventral axis except in the dorsal-most region (E). (F) At E2.5 (stage 14), *c-Gcm1* expression is downregulated in neural progenitors lining the lumen whereas it is maintained in cells located in the newly formed intermediate mantle layer. (G) At E8 (stage 34), *c-Gcm1* RNA is no longer detected in the spinal cord. hn, Hensen's node; ps, primitive streak. Scale bars: 60 μm in E; 70 μm in F; 150 μm in G.

51% when control vector was electroporated (Fig. 2G). The effects of *c-Gcm1* overexpression on *Sox1-3*, which were observed as early as 6 hours after electroporation, suggest that they may be direct (Fig. 2A,A′) and concomitant with upregulation of *NeuroM* (Fig. 2B,B′), a marker for early post-mitotic neurons (Roztocil et al., 1997).

To further demonstrate that *c-Gcm1* is involved in directing neural progenitors towards a neuronal fate, we analyzed the proliferation profile of *c-Gcm1*-overexpressing neural progenitors that were subjected to a 1-hour BrdU incorporation pulse 30 hours after electroporation. Only 9% of *c-Gcm1* electroporated cells were BrdU-positive (Fig. 2F,H), as compared with 24% for the control (Fig. 2E,H). Thus, *c-Gcm1* overexpression causes cell cycle exit. To determine whether this phenotype is associated with loss of proliferative neural progenitors, we analyzed the expression of Pax6 and Pax7, homeodomain transcription factors expressed in intermediate and dorsal progenitors of the ventricular zone, respectively (Briscoe and Ericson, 2001; Ericson et al., 1997). Electroporation with *c-Gcm1* cell-autonomously suppressed Pax6 (Fig. 2I,K,K′,M) and Pax7 (Fig. 2N,N′,P) expression. These data show that *NeuroM* induction upon *c-Gcm1* overexpression is linked to a reduced rate of proliferation in the neural tube and to cell-autonomous loss of progenitor status.

Vertebrate neurogenesis is driven by proneural bHLH transcription factors such as the neurogenins (Ngns), which direct cell cycle exit of neural progenitors by repressing the expression of Sox1-3, and which promote neuronal differentiation by inducing the expression of NeuroM and NeuroD (Bertrand et al., 2002). Interestingly, *Ngn2* overexpression upregulated *c-Gcm1* (Fig. 2D,D′), whereas the reverse was not true (Fig. 2C,C′), placing *c-Gcm1* downstream of proneural and upstream of neuronal differentiation factors.

To determine whether premature engagement of neural progenitors towards a neuronal fate results in a full neuronal phenotype, we analyzed the expression of the pan-neuronal markers class III β-tubulin (Tuj1, Fig. 3A-F) and HuC/D (Fig. 3G-H′), as well as that of interneuron marker Lim1/2 (Fig. 3F,I-J′). Twenty-four hours after electroporation, 55% of *c-Gcm1*-overexpressing cells expressed βIII-tubulin and 40% were Lim1/2-positive, whereas only 9% of cells electroporated with a control vector differentiated into neurons in the same time window (Fig. 3F). Overexpression of *c-Gcm1* also triggered neuronal differentiation in the embryonic brain, as assessed by premature expression of βIII-tubulin in the forebrain (Fig. 3D). Neuronal progenitors normally exit the ventricular zone when they leave the cell cycle and start expressing neuronal differentiation markers. This behavior was retained in neural cells electroporated with a control vector (Fig. 3A,A′,G,G′,I,I′), whereas *c-Gcm1*-induced neurons were also found in the ventricular zone (Fig. 3B,B′,H,H′,J,J′) and no longer expressed Pax7 (Fig. 3E), a feature indicative of premature differentiation.

These data demonstrate that *c-Gcm1* overexpression prompts neural progenitors to differentiate prematurely by downregulating neural progenitor genes and upregulating a repertoire of neuronal characteristics.

c-Gcm1 overexpression does not promote glial differentiation

We then asked whether *c-Gcm1* has the ability to induce glial cells in the embryonic spinal cord. We used Glast and O4 to identify astrocyte and oligodendrocyte precursors, respectively. Both markers start to be expressed in precursor cells of the neuroepithelium from E5/E6, corresponding to initiation of gliogenesis in chicken (Agius et al., 2004; Soula et al., 2001).

First, *c-Gcm1* was electroporated at E1.5 and glial marker expression was analyzed 1.5 to 4 days later. Irrespective of the stage of phenotypic analysis (E3 or E5.5), *c-Gcm1* overexpression did not trigger premature and/or ectopic expression of glial markers (Fig. 4A,B and data not shown). Strikingly, O4-positive cells were not detected in the ventral domain of the neuroepithelium (Fig. 4B).

Second, we overexpressed *c-Gcm1* at late stages in a gliogenic context. E4.5/E5 spinal cords were electroporated ex ovo, plated in an organotypic culture system and expression of glial markers assessed three days later on transverse sections of spinal cord explants (Danesin et al., 2006). As with early electroporation, late *c-Gcm1* overexpression did not induce ectopic expression of glial markers (Fig. 4C-G), and a reduction of the O4-expressing domain was observed (Fig. 4E). Accordingly, we found that *c-Gcm1*-overexpressing cells invariably expressed Lim1/2 (Fig. 4H,I) and most had left the neuroepithelium, indicating that they had adopted a neuronal fate. Altogether, our data show that *c-Gcm1* overexpression induces neurogenesis at both early and late stages of spinal cord development.

A repressive form of the *c-Gcm1* DNA-binding domain prevents neuronal differentiation

To further investigate the function of *c-Gcm1* during neurogenesis, we generated a construct containing the repressor domain (ER) of the *D. melanogaster* Engrailed protein fused to the DNA-binding domain of c-Gcm1 (c-Gcm1BD-ER). ER fusion constructs have

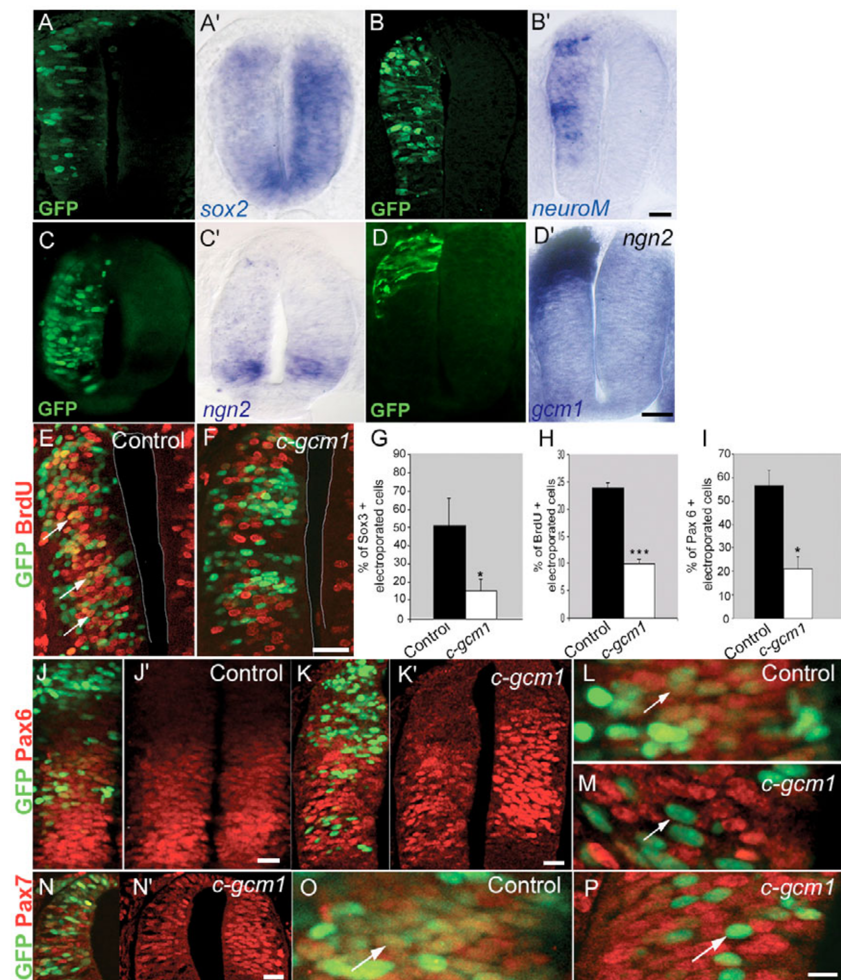


Fig. 2. Overexpression of *c-Gcm1* leads to depletion of proliferative neural progenitors. (**A-F**,**J-P**) Transverse sections after electroporation with control or *c-Gcm1* expression vectors at the level of the truncal neural tube of E1.5 chicken embryos. For all images, the electroporated side is on the left; electroporated cells were detected by GFP immunolabeling or by GFP fluorescence (green in both cases). (A-B′) GFP immunolabeling (A,B) and in situ hybridization using *Sox2* (A′) and *NeuroM* (B′) probes. Note the decrease in *Sox2* (A′) and the increase in *NeuroM* (B′) expression 6 hours after *c-Gcm1* overexpression. (C-D′) *Ngn2* expression domain is not modified upon *c-Gcm1* overexpression (C,C′), whereas *Ngn2* overexpression induces *c-Gcm1* expression (D,D′) 24 hours after electroporation. Note that signal development was stopped before detection of endogenous *c-Gcm1* mRNA in D′. (E,F) BrdU (red) and GFP colabeling in embryos subjected to a 1 hour BrdU pulse performed 30 hours after electroporation of control (E) or *c-Gcm1* (F) expression vectors. Many of the cells electroporated with the control expression vector are in a proliferative state (arrows in E), whereas overexpression of *c-Gcm1* markedly reduces the number of transfected BrdU-positive cells (F). Pax6 (J-M) and Pax7 (N-P) immunolabeling performed 24 hours after electroporation of control (J,J′,L,O) or *c-Gcm1* (K,K′,M,N,N′,P) expression vectors. *c-Gcm1* overexpression strongly reduces the number of Pax6 (K,K′,M) and Pax7 (N,N′,P) expressing progenitors in the intermediate and dorsal neural tube, respectively. High magnifications of neural tube sections show GFP (L,M,O,P) and Pax6 (red in L,M) or Pax7 (red in O,P) colabeling. Neural progenitors electroporated with control vector maintain the expression of Pax6 and Pax7 (arrows in L,O), whereas neural progenitors electroporated with *c-Gcm1* do not (arrows in M,P). (**G-I**) Percentage of transfected cells expressing Sox3 (G), incorporating BrdU (H) or expressing Pax6 (I), after electroporation of control or *c-Gcm1* expression vectors. Asterisks indicate significant differences ($P<0.05$ in G, $P<0.0001$ in H; $P<0.01$ in I). Scale bars: 40 μm in A-F,J-K′,N-O; 10 μm in L,M,O,P.

been successfully used to produce dominant-negative mutations that reproduce loss-of-function phenotypes in vertebrates and in flies (Feig, 1999).

Electroporation of c-Gcm1BD-ER invariably lead to a marked reduction in the number of cells expressing βIII-tubulin (Fig. 5A,A′), Lim1/2 (Fig. 5B,B′,E,F,I) and the motoneuron marker Mnr2 (Fig. 5J) (Tanabe et al., 1998). Similarly, *NeuroM* expression was reduced as compared with electroporation with a control vector (Fig. 5C-D′). Thus, c-Gcm1 activity is required to induce expression of neuronal differentiation genes both in ventral and dorsal regions of the neural tube. A similar proportion of GFP-positive cells incorporated BrdU after electroporation of c-Gcm1BD-ER or control vectors (Fig. 5G,H,M), indicating that antagonizing c-Gcm1 activity does not affect the proportion of cells in S phase. Similarly, the expression patterns of Sox1-3, Pax6 and Pax7 were not modified in neural tubes electroporated with c-Gcm1BD-ER as compared with the control (Fig. 5K,L,N-O′ and data not shown). In addition, no apoptosis was observed by TUNEL assay in cells electroporated with control or c-Gcm1BD-ER expression vectors (data not shown). Thus, repressing c-Gcm1 target genes prevents expression of neuronal differentiation genes but does not impede cell cycle exit nor does it affect progenitor identity. Moreover, c-Gcm1 does not control spatial patterning in the chick spinal cord. The finding that c-Gcm1BD-ER does not affect general developmental pathways excludes the possibility that this construct induces non-specific effects, as also confirmed by the fact that coexpressing wild-type c-Gcm1 and c-Gcm1BD-ER is sufficient to rescue the loss of neurons induced by c-Gcm1BD-ER alone (see Table S1 in the supplementary material).

gcm and *gcm2* display a neurogenic potential during post-embryonic development

The puzzling finding that c-Gcm1 induces neuronal differentiation prompted us to revisit fly gain-of-function phenotypes, as *gcm* and *gcm2* are known to induce gliogenesis when overexpressed in the embryo (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Ubiquitous *gcm* or *gcm2* expression [shift at restrictive temperature during second larval instar (LII)] induced a discrete increase in glial cell number in the CNS but did not induce ectopic expression of neuronal markers (data not shown). By contrast, numerous cells positive for the Elav neuronal marker were induced outside the CNS (for example, see the wing disc in Fig. 6), indicating that *gcm* as well as *gcm2* (data not shown) can trigger the neuronal fate during post-embryonic development. Moreover, a subset of Elav-positive cells

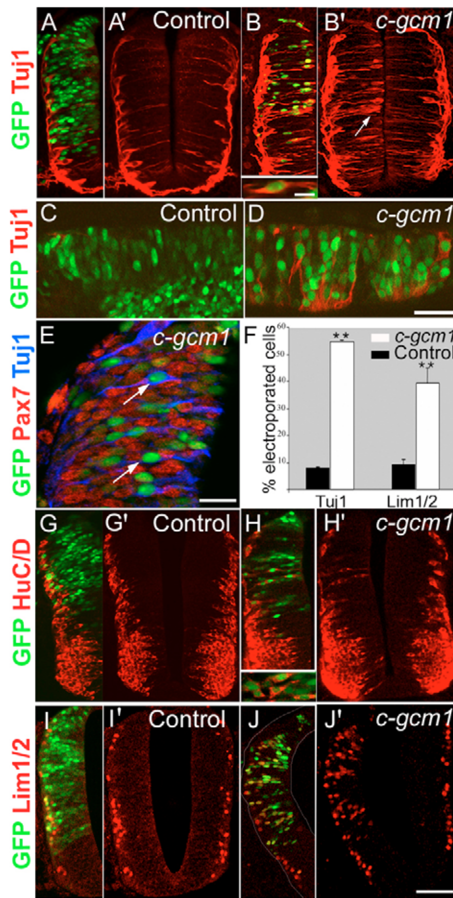


Fig. 3. Overexpression of *c-Gcm1* promotes premature neuronal differentiation. Electroporation with control or *c-Gcm1* expression vectors in truncal neural tube (**A-B′**,**E-J′**) or telencephalon (**C**,**D**) of E1.5 embryos. Immunolabeling with βIII-tubulin (Tuj1 in A-E), HuC/D (G-H′) and Lim1/2 (I-J′) neuronal markers were performed 24 hours after electroporation. (A-H′) Electroporation of control vector (green in A,C,G,I) does not modify the expression pattern of βIII-tubulin (red in A,A′,C) or HuC/D (red in G,G′), whereas *c-Gcm1* overexpression (green in B,D,E,H,J) leads to ectopic differentiation of Tuj1-positive (red in B,B′,D; blue in E, arrows) and HuC/D-positive (red in H,H′) neurons in the neuroepithelium. Inserts in B and H are high magnifications of *c-Gcm1*-overexpressing cells that coexpress βIII-tubulin and HuC/D, respectively. (E) βIII-tubulin (blue) and Pax7 (red) coimmunolabeling shows that *c-Gcm1*-overexpressing cells coexpress βIII-tubulin but not Pax7 (arrows). (I-J′) Compared with control (I,I′), *c-Gcm1* overexpression induces the generation of Lim1/2-positive neurons within the ventricular zone (J,J′). (F) Percentage of transfected cells expressing βIII-tubulin (Tuj1) and Lim1/2 after electroporation of control or *c-Gcm1* expression vectors. Differences between the percentage of *c-Gcm1* transfected cells expressing neuronal markers versus controls are statistically significant (asterisks, P<0.001). Scale bars: 40 μm in A-D,G-J′; 10 μm in insert in B,H; 20 μm in E.

expressed Acj6, an interneuron marker (Certel et al., 2000) that is not expressed in wild-type imaginal discs (Fig. 6D-F″). This indicates that at least two different neuronal subpopulations are induced following *gcm* or *gcm2* ectopic expression. Discs also displayed ectopic glial marker labeling, which did not colocalize with neuronal labeling (Fig. 6A-C″). Elav-positive cells were observed adjacent to Repo-positive cells or in clusters that only expressed the neuronal marker (Fig. 6B″,C″), indicating that the two

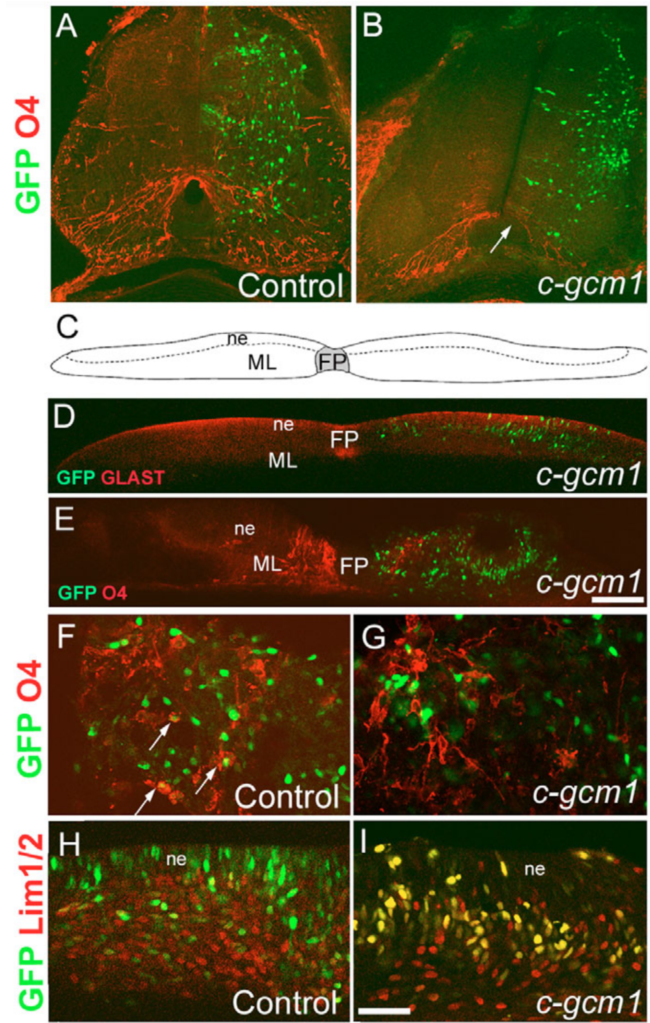


Fig. 4. Overexpression of *c-Gcm1* promotes neuronal but not glial differentiation in spinal cord. (**A**,**B**) E1.5 neural tube electroporated with control or *c-Gcm1* expression vectors (green) and analysis of O4 expression (red) three days later (E5/E5.5). (A) Electroporation of control vector does not modify the pattern of O4 decorating oligodendrocyte progenitors in the ventral neuroepithelium and no ectopic expression of O4 is observed. (B) Note that the endogenous O4-positive domain is strongly reduced (arrow) and no ectopic expression of O4 is detected when the *c-Gcm1*-expressing vector is used. (**C-I**) Electroporation with control (F,H) or *c-Gcm1* (D,E,G,I) expression vector was performed in E4.5/E5 embryonic spinal cord that was further dissected, opened dorsally and plated in culture with neuroepithelial precursors up, as depicted in C. Glial and neuronal differentiation was assessed three days later by immunolabeling using Glast (D, red), O4 (E,F,G, red) and Lim1/2 (H,I, red). (D,E) Transverse sections of open-book spinal cords showing that *c-Gcm1* overexpression does not induce ectopic Glast-positive (D) or O4-positive (E) cells. Note in E that the O4-positive domain is strongly reduced in the ventral spinal cord. (F,G) High magnifications show that some cells electroporated with control vector have adopted an O4-positive fate (F, arrows), whereas no *c-Gcm1*-overexpressing cells adopt such a fate (G). (H,I) High magnifications of explants showing that most cells electroporated with control vector are located in the neuroepithelium, and only a few of them reach the mantle layer and express Lim1/2 (H). By contrast, most *c-Gcm1*-overexpressing cells have left the neuroepithelium and all of them express Lim1/2 (I). ne, neuroepithelium; ML, mantle layer; FP, floor plate. Scale bars: 120 μm in A-E; 40 μm in F-I.

DEVELOPMENT

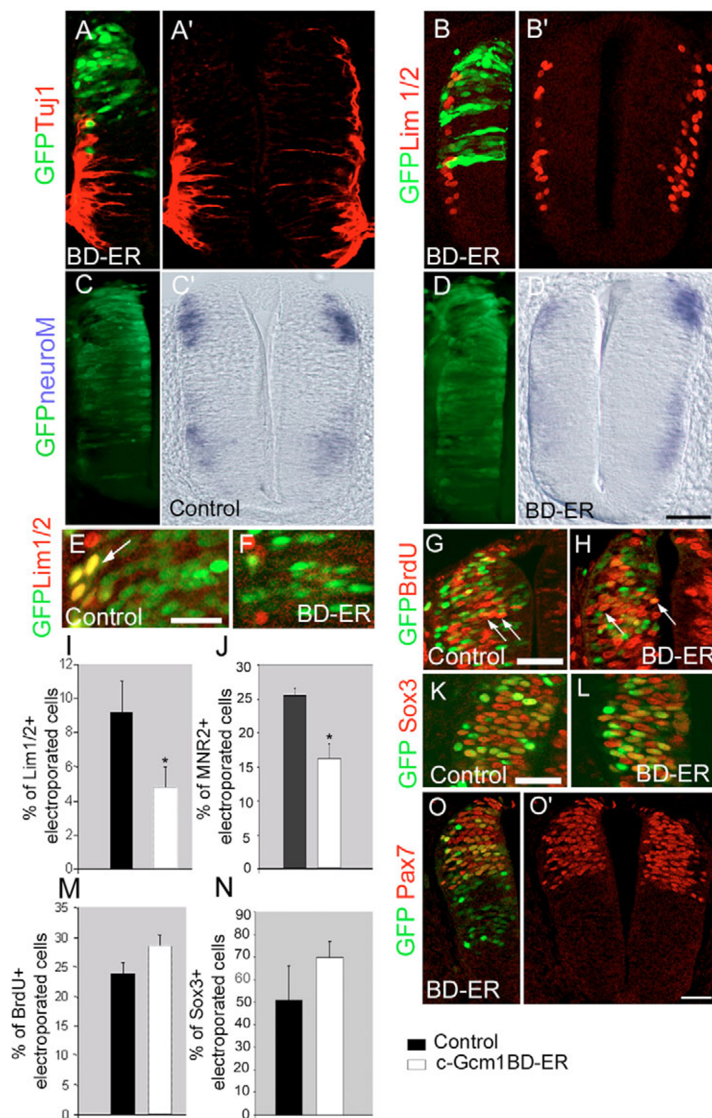


Fig. 5. Repression of c-Gcm1 targets inhibits neuronal differentiation without affecting cell cycle exit. (**A-O′**) Electroporation of E1.5 embryos with control (C,C′,E,G,I-K,M,N) or c-Gcm1BD-ER (BD-ER; A-B′,D,D′,F,H-J,L-O′) expression vectors. Immunolabeling and in situ hybridization on transverse sections were performed 24 hours after electroporation using anti-βIII-tubulin (Tuj1 in A,A′) or Lim1/2 (B,B′,E,F) antibodies or *NeuroM* probe (C-D′). (A-B′) Overexpression of c-Gcm1BD-ER inhibits the generation of terminally differentiated neurons. (E,F) High magnification of electroporated neural tube showing that cells overexpressing c-Gcm1BD-ER do not express Lim1/2, whereas a fraction of cells electroporated with a control vector express Lim1/2 (arrow in E) as they reach the mantle layer. (I,J) Percentage of transfected cells expressing Lim1/2 (I) or MNR2 (J) after electroporation of control or c-Gcm1BD-ER expression vectors; asterisks indicate significant difference ($P<0.01$). (G,H) BrdU (red) and GFP immunolabeling on transverse sections obtained from embryos subjected to a 1 hour BrdU pulse performed 24 hours after electroporation of control (G) or c-Gcm1BD-ER (H) expression vectors. Note the presence of double-labeled cells in both cases (arrows). (M,N) Percentage of transfected cells incorporating BrdU (M) or expressing Sox3 (N) after electroporation of control or c-Gcm1BD-ER expression vectors. Note that values are not significantly different. (K,L,O,O′) Similar profile of Sox3 (red in K,L) and Pax7 (red in O,O′) expression upon electroporation of control (K) or c-Gcm1BD-ER (L,O,O′) expression vectors. Scale bars: 50 μm in A-D′,O,O′; 20 μm in G,H; 60 μm in I,J; 40 μm in K,L.

phenotypes can be induced independently. Similar data were obtained in all the other imaginal discs (data not shown). Altogether, these results show that *gcm* and *gcm2* are able to trigger glial as well as neuronal differentiation during post-embryonic development.

gcm and *gcm2* are expressed and required in neuronal lineages of the larval central brain

In situ hybridization and immunolabeling using independent *gcm*-specific enhancer trap lines (*rA87*, *gcm-gal4*) showed that *gcm* and *gcm2* are coexpressed in two clusters (dorsolateral and medial) per central brain hemisphere (Fig. 7A-C′, Fig. 8A, and see Figs S2-S4 in the supplementary material). Each of these clusters, which we will refer to as central brain clusters (or cbcs), contains some forty neurons that persist in the adult, where they represent the only labeled cells of *gcm-gal4,UAS-GFP* animals (Fig. 7C,C′ and see Figs S3, S4 in the supplementary material). Cbcs appeared and coexpressed *gcm-gcm2* at early LII, reaching their final size by the end of the third instar larval stage (LIII) (data not shown). At no stage did cbc cells express the Repo glial marker (see Figs S2, S4 in the supplementary material). Interestingly, phospho-Histone H3 labeling revealed the presence of one mitotically active cell (Fig. 7D-D‴). Altogether, these data imply that cbcs originate from pure neuronal progenitors and that progenitors as well as neurons express *gcm-gcm2*, suggesting that the neurogenic role of *gcm-gcm2* is not restricted to the visual system.

Expression of a dominant-negative construct (gcm^{DN}) containing the Gcm DNA-binding domain and the repressor domain of Engrailed blocked both Gcm and Gcm2 activities, producing the same phenotypes as those observed in the double *gcm-gcm2* deficiency (Chotard et al., 2005; Soustelle et al., 2004; Yoshida et al., 2005). By contrast, gcm^{DN} expression in neurons that do not express *gcm-gcm2* did not induce any defect (Fig. 8C,D), further confirming that the gcm^{DN} construct acts by blocking the Gcm pathway rather than by non-specifically repressing gene expression.

This construct provided us with a unique opportunity to explore the role of *gcm-gcm2* in cells that can be specifically visualized by the *gcm-gal4* driver. As *gcm-gal4;UAS-*gcm^{DN} animals die as embryos, we crossed *gcm-gal4;UAS-*gcm^{DN} flies with *tub-*$Gal80^{ts}$ transgenic flies, which ubiquitously express a temperature-sensitive Gal80 protein that represses Gal4 at 18°C (McGuire et al., 2003). *gcm-gal4,tub-*$gal80^{ts}$*;UAS-*gcm^{DN} animals are viable and fertile when grown at 18°C but do not hatch when grown at 30°C. Flies expressing gcm^{DN} (shift at early LII) showed the same visual system

DEVELOPMENT

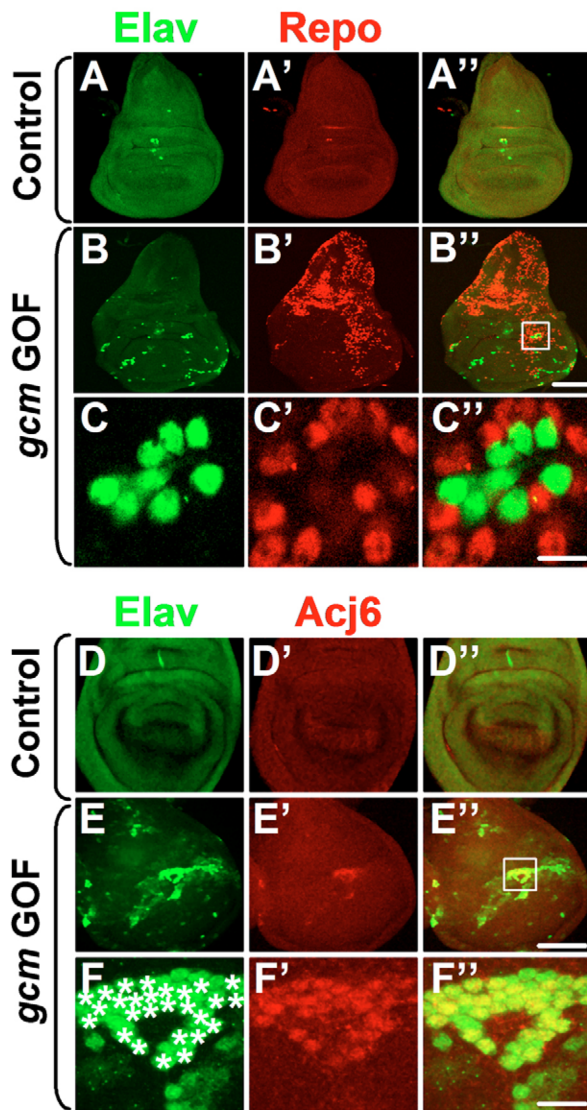


Fig. 6. Context-dependent role of *gcm* during post-embryonic development. (**A-C″**) Elav (green) and Repo (red) coimmunolabeling on wing imaginal discs. Right panels (A″,B″,C″) show merge of Elav (A,B,C) and Repo (A′,B′,C′) immunolabeling. *gcm* overexpression leads to neuronal and glial differentiation (compare B-C″ with A-A″). Note that some ectopic neurons and glial cells are closely associated but neuronal and glial markers never colocalize (see C″). (**D-F″**) Elav (green) and Acj6 (red) coimmunolabeling on wing imaginal discs. Right panels (D″,E″,F″) show merge of Elav (D,E,F) and Acj6 (D′,E′,F′) immunolabeling. Note that some ectopic Elav-positive neurons coexpress Acj6 (white asterisks in F). C-C″ and F-F″ are magnifications of squares shown in B″ and E″, respectively. Scale bars: 50 μm in A-B″,D-E″; 200 μm in C-C″,F-F″.

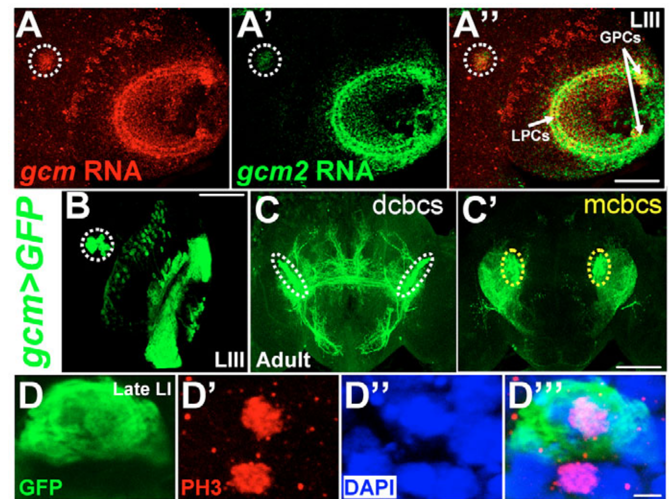


Fig. 7. *gcm* and *gcm2* are expressed in central brain neuronal lineages. (**A-A″**) *gcm* (A) and *gcm2* (A′) double in situ hybridization on wild-type *Drosophila* LIII brain hemispheres. (A″) Merge of *gcm* (red) and *gcm2* (green) labeling. *gcm* and *gcm2* RNAs are coexpressed in lamina neuronal [lamina precursor cells (LPCs)] and glial [glial precursor cells (GPCs)] progenitors as well as in a central brain cluster (area encircled by dotted line in A-A″). (**B-C′**) GFP immunolabeling on *gcm-gal4,UAS-mCD8GFP* (*gcm>GFP*) LIII (B) or whole-mount adult brains (C,C′). Note that the GFP expression profile mimics *gcm* expression pattern in larvae. Anterior (C) and posterior (C′) views of the same adult brain are shown. Neuronal somata of dorsolateral (dcbcs) and medial (mcbcs) clusters are encircled by white (C) and yellow (C′) dotted lines, respectively. (**D-D‴**) GFP (green), phospho-Histone H3 (red) and DAPI (blue) colabeling at late LI *gcm-gal4,UAS-ncGFP* larva. (D‴) Merge of D-D″; note that a single GFP-positive cell undergoes mitosis as shown by phospho-Histone H3 expression. Scale bars: 40 μm in A-A″; 40 μm in B; 50 μm in C-C′; 400 μm in D-D‴.

neuronal and glial loss (see Fig. S5 in the supplementary material) as observed in the *gcm-gcm2* double deficiency (Chotard et al., 2005), providing a final validation of the approach. Importantly, these phenotypes were not observed in animals containing a *gcm*$^{N7\text{-}4DN}$ transgene, which carries a point mutation abolishing DNA binding (Soustelle et al., 2004; Vincent et al., 1996). All cbc neurons were missing in *gcm-gal4,tub-gal80*^{ts}*;UAS-gcm*^{DN}*,UAS-ncGFP* animals (Fig. 8B), whereas they were still present in *gcm-gal4,tub-gal80*^{ts}*;UAS-gcm*$^{N7\text{-}4DN}$*,UAS-ncGFP* control animals (Fig. 8A). Interestingly, the cbc phenotype was observed when using one *gcm*^{DN} transgene, whereas defects in the visual system were only induced when using two transgenes, suggesting that different cells display distinct requirements for the Gcm pathway and further confirming the specificity of *gcm*^{DN} phenotypes.

Altogether, these data indicate that the Gcm pathway is necessary for the differentiation of specific neuronal populations of the larval brain.

Fly, chick and mouse *gcm* genes induce the expression of neuronal and glial markers in HeLa cells

The finding that fly *gcm* genes induce different cell fates, whereas *c-Gcm1* only induces neuronal differentiation, prompted us to determine the potential of fly and vertebrate *gcm* genes in a cellular context. We transfected HeLa cells, a human cell line of non-neural origin, with an expression vector carrying fly *gcm* or *c-Gcm1*. Because neurogenic potential had never been reported in mammals, we also performed the same type of experiments using a mouse *Gcm1* expression vector.

Strikingly, the three *gcm* genes induced the expression of βIII-tubulin in almost half of the transfected HeLa cells, indicating that all three genes share neurogenic potential (data not shown). Because previous studies in mouse fibroblasts showed that mouse *Gcm1*

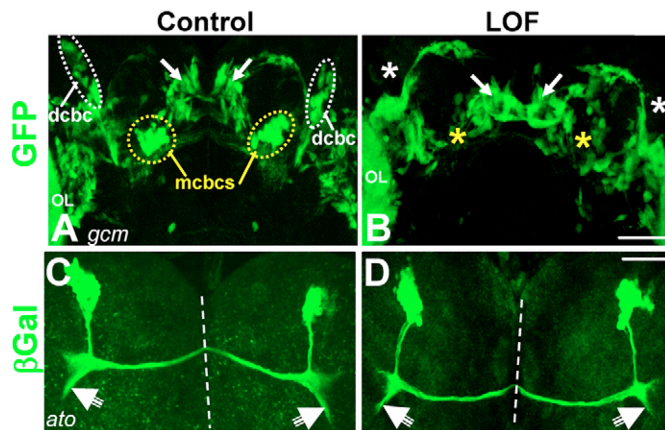
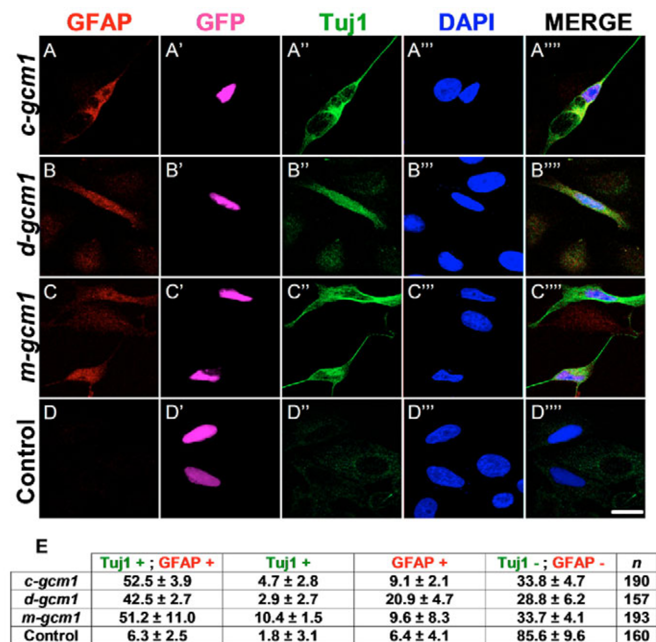


Fig. 8. Neuronal differentiation requires Gcm activity in central brain. GFP immunolabeling in (**A**) control (*gcm-gal4,tub-gal80*^{ts}*;UAS-ncGFP;UAS-gcm*$^{N7\text{-}4DN}$) and (**B**) *gcm-gcm2* loss-of-function (LOF) (*gcm-gal4,tub-gal80*^{ts}*;UAS-ncGFP;UAS-gcm*^{DN}) LIII CNS. Note that dcbcs and mcbcs (encircled by white and yellow dotted lines, respectively) are missing in *gcm-gcm2* LOF but not in control animals as marked by the white and yellow asterisks in B. In these conditions of a single dose of the *UAS-gcm*^{DN} transgene, lamina development (neurons and glia) is less affected than with two doses of *UAS-gcm*^{DN} transgene (see Fig. S5 in the supplementary material). Arrows in A,B indicate processes of glia at the interhemispheric junction. (**C**,**D**) Development of central brain atonal-positive lineage is not affected by the *gcm*^{DN} construct. GFP immunolabeling in control (*atonal-gal4,UAS-mCD8GFP* in C) and *gcm-gcm2* LOF (*atonal-gal4,UAS-mCD8GFP,UAS-gcm*^{DN} in D) LIII CNS shown in dorsal view. In control (C) as well as in *gcm-gcm2* LOF (D) animals, atonal lineage includes two clusters of 20-30 neurons that are connected by a commissure crossing the midline (dashed line) and which extend a bundle of ipsilateral axons (arrows) into the optic lobes. Thus, *gcm*^{DN} expression does not affect the development of atonal-positive neurons. Scale bars: 100 μm in A,B; 180 μm in C,D.



	Tuj1 + ; GFAP +	Tuj1 +	GFAP +	Tuj1 - ; GFAP -	n
c-gcm1	52.5 ± 3.9	4.7 ± 2.8	9.1 ± 2.1	33.8 ± 4.7	190
d-gcm1	42.5 ± 2.7	2.9 ± 2.7	20.9 ± 4.7	28.8 ± 6.2	157
m-gcm1	51.2 ± 11.0	10.4 ± 1.5	9.6 ± 8.3	33.7 ± 4.1	193
Control	6.3 ± 2.5	1.8 ± 3.1	6.4 ± 4.1	85.6 ± 9.6	160

Fig. 9. Fly, chick and mouse *gcm* genes induce expression of neuronal and glial markers in HeLa cells. GFAP (**A**,**B**,**C**,**D**), GFP (**A′**,**B′**,**C′**,**D′**), Tuj1 (**A″**,**B″**,**C″**,**D″**) and DAPI (**A‴**,**B‴**,**C‴**,**D‴**) colabeling and merges (**A‴′**,**B‴′**,**C‴′**,**D‴′**) of HeLa cells transfected with expression vectors carrying *c-Gcm1* (A-A‴′), *gcm* (*d-gcm1* in B-B‴′), mouse *Gcm1* (*m-gcm1* in C-C‴′) or vector as control (D-D‴′). Scale bar: 20 μm. (**E**) Percentage of transfected cells expressing neuronal/glial markers. *n* indicates the number of GFP-positive cells counted. Note that all *gcm1* genes induce the expression of GFAP and Tuj1 markers and that these two markers colocalize in most transfected cells.

induces expression of an astrocytic marker (Iwasaki et al., 2003), we also assayed the gliogenic role of the three *gcm* genes in a similar transfection assay and indeed observed GFAP expression in almost two-thirds of the transfected HeLa cells (data not shown). We then asked whether *gcm* genes induce the expression of neuronal and glial markers in the same cells, and indeed found colabeling upon transfection of fly, chicken and mouse *gcm*/*Gcm1* genes (Fig. 9A-D‴). Interestingly, upon quantification of labeling, we found that most cells expressed both GFAP and βIII-tubulin (60% to 80%, depending on the species), the remainder expressing either marker (Fig. 9E). Thus, expression in a naive context reveals the double neurogenic and gliogenic potentials of *gcm* genes throughout evolution. These findings allow us to reconcile the apparent discrepancy between the gliogenic potential previously described for fly (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996) and mouse (Iwasaki et al., 2003) genes and their neurogenic potential in fly and chicken (Chotard et al., 2005; Yoshida et al., 2005) (see also present study). Interestingly, transfection of *gcm* genes does not induce expression of the O4 antigen, which is specific to oligodendrocyte precursors (data not shown), indicating that the observed increase in GFAP and βIII-tubulin labeling is not due to a general, non-specific enhancement of gene expression. Altogether, in vitro and in vivo data support the hypothesis that the Gcm class of transcription factors plays versatile roles in the fly and vertebrate CNS.

DISCUSSION

The present study provides the first evidence for a vertebrate *gcm* gene, *c-Gcm1*, being expressed and required for neuronal differentiation in the CNS. Fly *gcm* and *gcm2* are also coexpressed and required in central brain neurons and are able to induce neuronal differentiation. In addition, *gcm* genes from fly, chicken and mouse are all able to induce the expression of neuronal markers in vitro. These data point to an unpredicted and conserved neurogenic role of *gcm* genes and further our understanding of the multistep process that builds the nervous system.

Data concerning the neurogenic role of fly *gcm* genes and expression of *c-Gcm1* at the onset of neurogenesis formed the basis for a functional analysis in the chicken spinal cord. We here show that *c-Gcm1* overexpression leads to the downregulation of genes specifically expressed in proliferative neural progenitors of the ventricular zone and concomitantly induces the full neuronal differentiation program. Conversely, blocking the c-Gcm1 pathway does not modify the proliferation rate and expression of progenitor genes, but prevents neuronal differentiation gene expression. *c-Gcm1* acts as a neuronal differentiation gene, downstream of proneural genes and upstream of *NeuroM*. Neuronal differentiation relies on the repression of the *Sox* genes, which has been proposed to be mediated by neurogenin (Bertrand et al., 2002; Bylund et al., 2003; Graham et al., 2003; Uwanogho et al., 1995). Blocking *Sox* gene expression, however, leads cells to acquire an early post-mitotic

phenotype, but does not allow these cells to fully differentiate into neurons (Bylund et al., 2003), indicating that late events require other Ngn-dependent pathways. *c-Gcm1* is likely to constitute a relay of the neurogenin pathway that is indispensable to trigger complete neuronal differentiation.

The widespread expression of *c-Gcm1* and its overexpression phenotype strongly argue in favor of a general neurogenic role. Indeed, altering the c-Gcm1 protein does not modify expression of ventral, intermediate or dorsal markers (Fig. 5 and data not shown), although further experiments are required to definitively exclude a role for c-Gcm1 in specifying neuronal subpopulations. Absence of *c-Gcm2* expression in the spinal cord excludes the possibility that the two *c-Gcm* genes play a redundant role in this tissue, although we cannot exclude a *c-Gcm2* neurogenic/gliogenic role in other regions/stages.

Our study provides the first evidence for *c-Gcm1* playing an essential role in the neurogenic process and points to an unexpected conservation of *gcm* gene function in neuronal differentiation in chicken and *Drosophila*. The question now is whether *gcm* neurogenic expression and function are also conserved in mammals. Our HeLa cell transfection data showing that neuronal characteristics are induced by fly *gcm*, *c-Gcm1* and mouse *Gcm1* support the hypothesis of conservation. These results may explain the stimulation of secondary neurulation observed in transgenic mice overexpressing mouse *Gcm1* in the developing tail bud (Nait-Oumesmar et al., 2002). In the light of present data, and based on the fact that the expression of mouse and human orthologs is detected by RT-PCR in developing brains (Altshuller et al., 1996; Iwasaki et al., 2003; Kim et al., 1998), it will be crucial to determine the expression profile of mammalian *gcm* genes within the CNS and score for neuronal defects in *Gcm*-knockout mice.

Fly *gcm* genes are expressed and required in the neurons of the central brain. Owing to the fact that the only available tool to identify and target cbcs is the *gcm-gal4* line, it is not possible to assess the specific roles of *gcm* and *gcm2*. Their similar levels of expression, however, suggest that both genes are required, as demonstrated in the visual system (Chotard et al., 2005). *gcm* genes are necessary for neuron generation rather than maintenance, in line with the early expression of *gcm-gcm2* and with the lack of any phenotype in experiments removing *gcm-gcm2* function in post-mitotic neurons (data not shown). Generating independent markers for the two cbcs will be crucial for characterizing the *gcm* pathway and the role of these neurons, which integrate information from different centers of the fly brain (see Fig. S3 in the supplementary material).

Our data show that fly Gcm/Gcm2 transcription factors induce glia or neuron differentiation depending on the cellular context. First, Gcm or Gcm2-induced neuron to glia transformation is more effective in the embryonic than in the larval CNS (data not shown). Second, Gcm or Gcm2 overexpression induces both Repo and Elav expression outside the CNS, but only Repo expression within the CNS. This implies that most neural cells express factors that do not allow Gcm to induce a neuronal pathway, or contain a positive factor that induces Gcm to trigger the glial pathway.

Although fly *gcm* genes are also necessary and sufficient for glial differentiation, *c-Gcm1* overexpression does not induce gliogenesis in vivo and indeed *c-Gcm1* activity inversely correlates with the production of glia in the spinal cord. Similarly, no glial defect has been reported for the *Gcm1 and Gcm2* knockout mice (Anson-Cartwright et al., 2000; Gunther et al., 2000; Kim et al., 1998; Schreiber et al., 2000) and the only reported in situ expression (mouse *Gcm1*) concerns cells that do not correspond to known glial lineages (Iwasaki et al., 2003). On the other hand, the gliogenic potential is conserved in vitro because *gcm* genes induce glial markers in HeLa cells. We therefore speculate that *gcm* genes play a gliogenic role in discrete cell populations, in line with mouse *Gcm1* overexpression inducing the expression of glial markers in brain cultures (Iwasaki et al., 2003), but not in retina (Hojo et al., 2000). The observation that most cells coexpress neuronal and glial markers in vitro, a situation that is not observed in vivo, strongly suggests that HeLa cells provide a naive context in which both potentials of *gcm* genes are revealed.

Altogether, the present data merit revisiting the role and potential of the classically defined fly glial master genes. We also identify a conserved neurogenic potential in this class of transcription factors and define a novel step in the pathways leading to neuronal differentiation. Finally, based on in vivo and in vitro data, we propose that fly as well as vertebrate *gcm* genes induce neurons and/or glial cells depending on the cellular context. The reiterated use of Gcm developmental pathways within and outside the CNS further emphasizes the need for cell-specific factors. In the future, one of the most challenging issues will be to perform screens to identify cues that are necessary for the neuronal program and to determine common versus specific features of the different Gcm pathways. This will enable us to understand how spatio-temporal patterning regulates and integrates these pathways to control cell specificity.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/3/625/DC1

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DEVELOPMENT

Table S1. Rescue of c-Gcm1BD-ER-induced neuronal loss by co-electroporation with *c-Gcm1* wild-type-expressing vector

Electroporation	Percentage of GFP^+ and Lim^+ cells
c-Gcm1WT	57.7
c-Gcm1BD-ER + *c-Gcm1WT* (ratio 2:3)	53
c-Gcm1BD-ER + *c-Gcm1WT* (ratio 3:2)	43
c-Gcm1BD-ER	7.3

Note that the degree of rescue is directly proportional to the amount of *c-Gcm1* wild-type-expressing vector used in the co-electroporation (comparing data obtained from the 2:3 versus 3:2 ratios).