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Megane/Heslike is required for normal GABAergic differentiation in the mouse superior colliculus

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The mouse Mgn protein (Helt) is structurally related to the neurogenic *Drosophila hairy* and *Enhancer of split* [*h/E(spl)*] proteins, but its unique structural properties distinguish it from other members of the family. *Mgn* expression shows a spatiotemporal correlation with GABAergic markers in several brain regions. We report here that homozygous *Mgn*-null mice die between the second and the fifth postnatal week of age, and show a complete depletion of *Gad65* and *Gad67* expression in the superior colliculus and a reduction in the inferior colliculus. Other brain regions, as well as other neural systems, are not affected. The progenitor GABAergic cells appear to be generated in right numbers but fail to become GABAergic neurons. The phenotype of the mice is consistent with reduced GABAergic activity. Thus, our in vivo study provides evidence that Mgn is the key regulator of GABAergic neurons, controlling their specification in the dorsal midbrain. Another conclusion from our results is that the function of Mgn shows a previously unrecognized role for *h/E(spl)*-related transcription factors in the dorsal midbrain GABAergic cell differentiation. Vertebrate *h/E(spl)*-related genes can no longer be regarded solely as a factors that confer generic neurogenic properties, but as key components for the subtype-neuronal identity in the mammalian CNS.

KEY WORDS: bHLH transcription factor, GABAergic neurons, Gad65 (Gad2), Gad67 (Gad1), *Megane* (*Heslike*, *Helt*), Superior colliculus, Midbrain, Specification, Mouse

INTRODUCTION

The GABAergic neurons are the principal inhibitory neurotransmitter system in the mammalian central nervous system (CNS) and their impairment is involved in the aetiology of several neurological disorders. At the molecular level, much of our knowledge about their specification comes from cerebral cortical developmental studies. In the forebrain, these neurotransmitter-identified neurons arise from proneural bHLH *Mash1* (*Ascl1* – Mouse Genome Informatics)-expressing progenitors in the subventricular zone (SVZ) of the ganglionic eminence and migrate tangentially into the cerebral cortex (Corbin et al., 2001; Parnavelas, 2000). Loss-of-function studies suggest a key role for the *Nkx2.1* and *Dlx1/2* homeobox gene families: providing the positional information needed to commit the undifferentiated subpallial progenitor cells into a GABAergic interneuron precursor (Ross et al., 2003). The transcription factor Ptf1a is also associated with specification of GABAergic neurons in the cerebellum (Hoshino et al., 2005). By contrast, the molecular mechanisms underlying the development of midbrain GABAergic neurons remains elusive because, among other reasons, most of the players mentioned above are not expressed in the midbrain, which suggests different pathways towards this cell fate.

bHLH transcription factors comprise an evolutionarily ancient group of proteins conserved across many species that mainly function as key regulators of cell-fate decisions and neuronal differentiation in the developing CNS of invertebrates and vertebrates (Jan and Jan, 1994; Kageyama and Nakanishi, 1997; Lee, 1997; Guillemot, 1999; Bertrand et al., 2002). In *Drosophila*, a most prominent group of neurogenic bHLH factors are the members of the *Drosophila hairy* and *Enhancer of split* [*E(spl)*] family. These proteins function as transcriptional repressors to maintain neural progenitors in a proliferative state and generally antagonize the activity of proneural bHLH proteins (e.g. Mash1, neurogenin 1, neurogenin 2 and neurogenin 3), promoting or suppressing neuronal determination-differentiation programs (Heitzler and Simpson, 1991; Kageyama and Nakanishi, 1997; Fisher and Caudy, 1998). In mammals, accumulating evidence suggests that, as in *Drosophila*, neurogenesis also relies upon *h/E(spl)*-related factors and proneural factors, and is associated with comparable functions (Davis and Turner, 2001). Recent evidence suggests that vertebrate proneural bHLH genes not only confer generic neuronal properties, but also play a crucial role as determinants of neuronal identity, controlling the appearance of cell type-specific traits (Brunet and Ghysen, 1999; Parras et al., 2002; Nakada et al., 2004). However, it has not been demonstrated so far whether *h/E(spl)* genes can also promote neuronal subtype identities.

We isolated a new member of the *h/E(spl)*-related bHLH protein referred to as megane (*Mgn*; *Helt* – Mouse Genome Informatics) (Guimera et al., 2006; Miyoshi et al., 2004; Nakatani et al., 2004). *Mgn* shows an expression pattern restricted to the midbrain at mouse developmental day 9.5 (E9.5). *Mgn* mRNA displays spatiotemporal transcriptional correlation with that of 65 kDa and 67 kDa isoforms of glutamic acid decarboxylase (*Gad65* and *Gad67*, respectively; *Gad2* and *Gad1* – Mouse Genome Informatics), which are two independent biosynthetic enzymes for the neurotransmitter GABA (Erlander et al., 1991; Erlander and Tobin, 1991). A neuron is GABAergic if it contains either isoform of GAD (the rate-limiting enzyme in GABA synthesis).

Gain-of-function experiments suggest a possible involvement of *Mgn* in GABAergic neurogenesis (Miyoshi et al., 2004); however, the processes requiring *Mgn* in vivo as well as the involvement of *Mgn* in any specific GABAergic lineage remained unknown. To address this issue, we have performed loss-of-function studies using a gene targeting approach. Homozygous

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Mgn mutant mice show normal brain histology and morphology. However, expression of *Gad65* and *Gad67* is completely abolished in the superior colliculus (SC), and homozygous mutant animals display a cramping phenotype and symptoms resembling tonic-clonic seizures preceding postnatal death. Thus, our results demonstrate a key role of Mgn in the proper development of GABAergic neurons of the SC and, in addition, suggest a vital role of Mgn in early postnatal survival. Hence, we designated this bHLH gene as *Mgn* (mesencephalic GABAergic neurons *h/E(spl)*-related gene).

MATERIALS AND METHODS

Construction of an Mgn targeting vector and production of *Mgn* mutant mice

An Mgn gene targeting vector (Fig. 1B) was designed to delete the bHLH-O domain (part of exon 2 and exon 3). *Mgn* genomic fragments were cloned from a 129SV-derived BAC clone. The targeting vector contains a 1.8 kb fragment of 5′ homologous genomic DNA and a 5.2 kb fragment of 3′ homologous genomic DNA flanking the deletion. A tau-*lacZ* (tZ) fusion protein was inserted in frame with the 12th codon in exon 2, followed by a neomycin cassette flanked by two *loxP* sites for subsequent removal by Cre recombinase-mediated excision. The plasmid was linearized with *Spe*I, electroporated into embryonic stem cells (129SVderived) and selected with G418. Genomic DNA was isolated from surviving ES cells, digested with *Bam*HI and screened by Southern blot with 5′ and 3′ external probes, in parallel experiments. Two correctly targeted ES cell clones were identified out of 1360 ES clones analysed, then injected individually into blastocysts derived from C57BL/6 mice to produce chimaeric mice. Chimaeric pups were identified by their agouti coat colour. Chimaera males were bred to C57BL/6 and 129SV/J females and germline transmission was determined by Southern blot analysis of tail genomic DNA (Fig. 1E,F) as described above. Later, a PCR-based strategy was designed to facilitate the genotyping of the progeny. Primers for the wild-type band were MM108 and MM109, whereas primers for the mutant allele were MM107 and tlacZ. The PCR assay generated a 334 bp fragment for the wild-type allele and a 395 bp fragment for the mutant allele (Fig. 1G). Null allele animals were confirmed by the absence of the PCR fragment when specific primers for the deletion were used (MM111D and MM112R) (Fig. 1H). The mutation is maintained on both a 129SV/J and C57BL/6 genetic background. The neo-selectable marker was removed by crossing mice heterozygous for the *Mgn* floxed neo allele with C57BL/6 inbred CMV-Cre transgenic mouse line (Schwenk et al., 1995). The resulting progeny were analysed for the excision of the neo cassette by Southern blot (Fig. 1F). The animal experiments were conducted under federal guidelines for the use and care of laboratory animals and were approved by the GSF Institutional Animal Care and Use Committee.

Whole mount in situ hybridization and *lacZ* staining

Embryos were obtained from natural matings of wild-type C3H mice. Noon on the day when the vaginal plug was detected was considered to be embryonic day 0.5 of gestation (E0.5). Embryos were collected and staged precisely according to Theiler (Theiler, 1989). Embryos and dissected brains from different stages (E9-E14) were fixed overnight at 4°C in 4% paraformaldehyde. Whole-mount in situ hybridization was performed as described by Sporle and Schughart (Sporle and Schughart, 1998). Antisense and sense digoxigenin (DIG)-labelled riboprobes transcribed from linearized plasmids containing a partial cDNA for *Brn3a* (*Pou4f1* – Mouse Genome Informatics) (bp 614-938; XM_135366); *Chat1* (*Slc5a7* – Mouse Genome Informatics) (bp 27-1769; NM_022025); *Dat* (*Slc6a3* – Mouse Genome Informatics) (bp 2332-2725; AF109072); *Ebf2* (bp 653-1195; BC050922); *Gad65* (*Gad2* – Mouse Genome Informatics) (bp 753-1600; BC018380); *Gad67* (*Gad1* – Mouse Genome Informatics) (bp 934-1786; NM_008077); *Gat1* (*Slc6a1* – Mouse Genome Informatics) (bp 1381-2053; M92378); islet 1 (*Isl1*) (bp 1013-1322; NM_021459); *Mgn* (*Helt* – Mouse Genome Informatics) (bp 166-1000, DQ294234); *Pitx2* (bp 1172-1863; NM_011098); *Plp1* (bp 106-857; AK077564); *Th* (bp 23-788; NM_009377); and *Vglut1* (*Slc17a7* – Mouse Genome Informatics) (bp 1720-2332; NM_182993) were produced using a DIG-RNA labelling kit (Roche). *lacZ* expression was monitored by a colour reaction catalysed by the *lacZ* gene product β-galactosidase as described (Gossler and Zachgo, 1993).

Immunohistochemistry

For immunohistochemistry, tissue sections were stained overnight with the following primary antibodies: rabbit anti-phospho-histone H3 (1:500; Upstate Biotechnology), which recognizes only M-phase nuclei as a mitosis marker (Mahadevan et al., 1991; Ajiro et al., 1996); anti-NeuN (specific marker for neuronal nuclei) (1:1000, Chemicon); anti-calbindin (1:2000; Swant); and anti-cleaved caspase 3 (1:200; Cell Signaling), which recognizes cleaved caspase 3 cell death protease activated during apoptosis (Krajewski et al., 1997). Subsequently sections were incubated with a biotinylated goat-anti-rabbit antibody (1:300, Dianova) followed by incubation with an HRP-coupled ABC complex according to the manufacturer's protocol (Vector Laboratories). Staining was visualized using diaminobenzidine as a chromogen.

Histological analysis

Brains and whole embryos were obtained from animals that were transcardially perfused with 4% paraformaldehyde, then fixed by immersion in 4% paraformaldehyde overnight at 4°C. Some of the postnatal brains were shock frozen on dry ice. Perfused brains were either cut on a cryostat at 30 μm or paraffin embedded and cut on a microtome at 4-8 μm. Frozen brains were cut on a cryostat at 18 μm and processed for in situ hybridization. In situ hybridization of frozen and paraffin sections was performed according to a modified version of the procedure described by Dagerlind et al. (Dagerlind et al., 1992). Following in situ hybridization, sections were counterstained with Cresyl Violet.

Oligonucleotides

MM107 (5′-cagagactggaaggagagtccttg-3′); MM108 (5′-gcggaatgcagagctct-gag-3′); MM109 (5′-gaacgagctgggcaagacag-3′); MM111D (5′-cccggtt-tctcataaagtgatag-3′); MM112R (5′-gcactcgtggtaaccgtagtg-3′); and tlacZ (5′-agcatgatcttccatcacgtcg-3′) oligonucleotides were used. *Mgn* cDNA was taken from GenBank (Accession Number DQ294234).

BrdU cell proliferation assay and TUNEL assay

Pregnant females were intraperitoneally injected with BrdU 15 minutes and 2 hours before they were sacrificed. Incorporation and detection of BrdU into cellular DNA were carried out with BrdU labelling and detection kit II (Roche), according to the manufacturer's instructions. For the TUNEL assay, paraffin sections of midgestational embryos were dewaxed and apoptosis was detected using In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions.

Statistical analysis

To compare the body weight between wild-type and heterozygous mice, the autocorrelation of the data from several mice for each mouse line was taken into account. A weighted linear model was fitted (weighted ANOVA with AR_1-autocorrelated errors) using the gls (generalized least squares) function in the nlme-package. The R statistical software was used to perform the statistical analysis (Pinheiro and Bates, 2000).

RESULTS

Production of mutant mice

An *Mgn* gene targeting vector was designed to replace the entire bHLH and the nuclear localization signal domains with a tau-lacZ fusion protein by homologous recombination (Fig. 1A-D). Two targeted ES cell clones gave rise to virtually 100% chimaeric mice that transmitted the mutant allele into the germline (50% of descendents) by mating chimaeric mice with both C57BL/6 and 129SV/J mice. The resulting heterozygous mice ($Mgn^{+/tZ}$) generated from those two independently targeted ES clones were viable, fertile and not distinguishable from wild-type littermates. The *Mgn* mutant mouse was consecutively back crossed 11

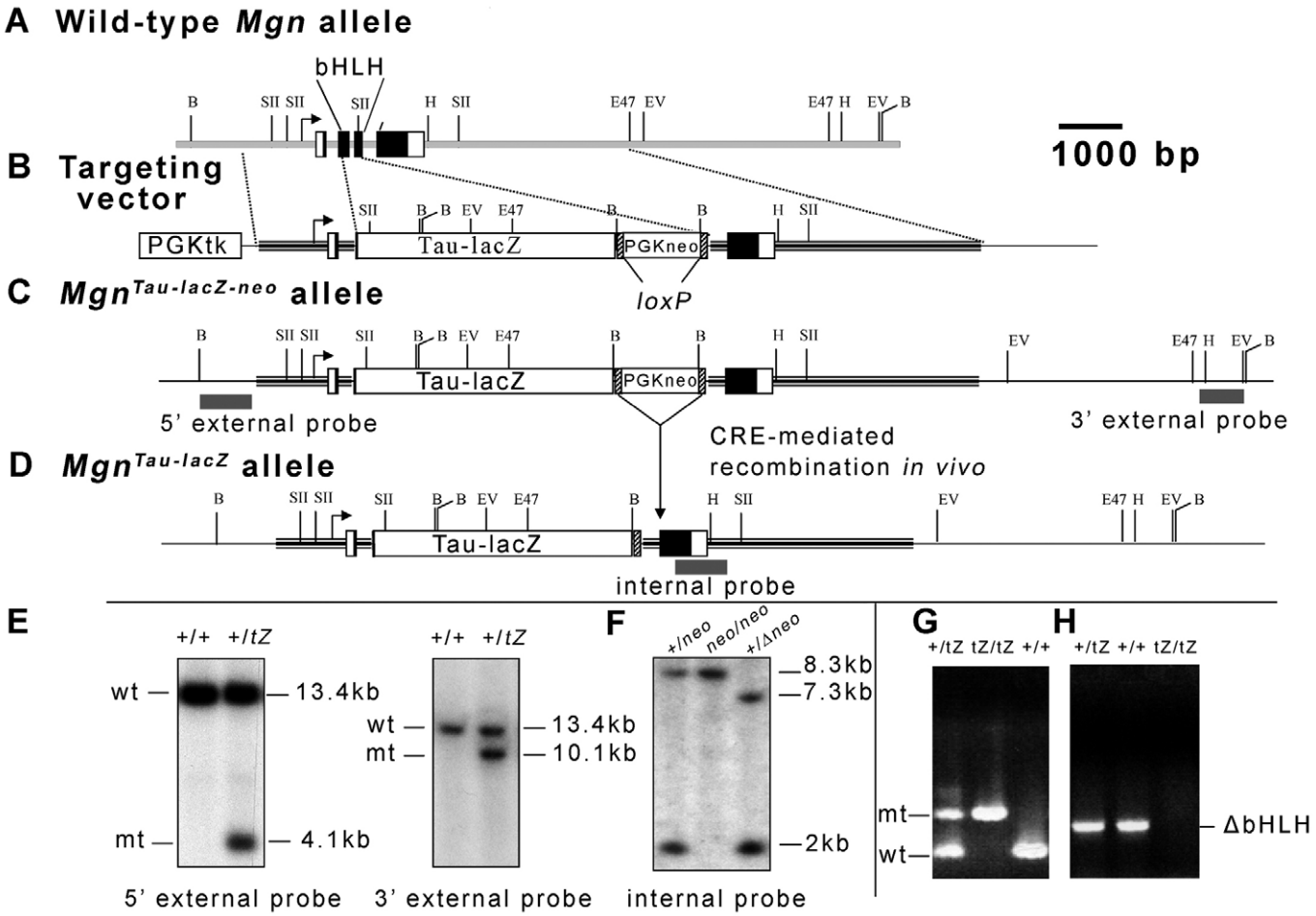

Fig. 1. Schematic representation of the *Mgn^{tZ}* targeting vector and recombination at the *Mgn* locus. (**A**) Restriction map of the wild-type *Mgn* locus. Broken lines indicate regions of homology in the targeting vector. (**B**) Restriction map of the *Mgn^{tZ}* targeting vector. (**C**) The predicted structure of a mutated *Mgn* allele following homologous recombination. The horizontal bars (5′ and 3′ external probe) indicate the DNA fragment used for Southern blot analysis. (**D**) Targeted locus after removing neomycin cassette (neo) by Cre-mediated excision at the *loxP* sites. (**E**) Southern blot analysis of restriction enzyme-digested DNA from targeted *Mgn^{tZ}* (+/*tZ*) and wild-type (+/+) animals with 5′ and 3′ external probes (the same strategy was used to detect littermates from heterozygous intercrosses). wt and mt indicate the position of the wild-type and the mutated allele, respectively. (**F**) Southern blot containing genomic DNA upon *Sac*II digestion and probed with an internal probe, indicating proper excision of the neomycin gene (Δneo). (**G**) Heteroduplex PCR (primers for the mutant allele, MM107 and tlacZ; primers for the wild-type allele, MM108 and MM109) used to identify mutated embryos/mice. (**H**) RT-PCR with specific primers for the bHLH domain of *Mgn* (MM111D and MM112R). The 250 bp band is missing in the *Mgn$^{tZ/tZ}$* mice.

generations to C57BL/6. Corresponding homozygous mutant mice were produced and analysed. RT-PCR analysis revealed that *Mgn* full-length mRNA is not present in the brains of *Mgn$^{tZ/tZ}$* mice (Fig. 1H). Next, *Mgn* expression was determined by in situ hybridization on mouse embryo sections. *Mgn* mRNA was detected in wild-type mice, but not in *Mgn$^{tZ/tZ}$* mice (Fig. 7C,D). The gene targeting strategy used in creating the *Mgn^{tZ}* allele deletes intron II. Thus, it is possible that the lack of this intronic sequence could disrupt a transcriptional regulatory element. To examine transcription from the *Mgn* locus, *lacZ* staining was performed at different midgestational times on *Mgn* mutant embryos and compared with the *Mgn* expression in wild-type embryos, as determined by in situ hybridization. The findings suggest that the *tlacZ* transcripts are essentially expressed in an *Mgn*-specific pattern in *Mgn* heterozygous and homozygous mutant embryos. Thus, this mouse provides a marker that reflects the endogenous expression of *Mgn*.

Mgn expression and GABAergic markers

To determine the potential role of *Mgn* in neurogenesis, we first compared the *Mgn* expression with that of markers for the major midbrain neurotransmitter pathways. This analysis showed that in the mesencephalon *Mgn* is expressed in close vicinity to dopaminergic neurons, as indicated by the presence of tyrosine hydroxylase (*Th*). However, the two expression domains do not overlap and the dopaminergic cell population extends more ventrally than the *Mgn*-expressing cells (data not shown). Strikingly, however, *Mgn* expression starts in the ventral aspect of the midbrain and later also appears in the dorsal aspect. This change correlates with the appearance of GABAergic neurons in the midbrain. The spatiotemporal expression of *Mgn* with GABAergic neurons is further evidenced by expression analysis of *Gad65* and *Gad67* (Fig. 2C-H; data not shown). *Mgn* is expressed in the ventricular/subventricular zone of the mesencephalon underlying the mantle layer in which GABAergic neurons are

detectable. *Mgn*, *Gad65* and *Gad67* are not co-expressed, but their expression domains are adjacent in the ventral and dorsal midbrain, suggesting that *Mgn* may be expressed in a population of progenitor cells destined to become GABAergic neurons. In addition, when GABAergic neurons arise in the mantle zone of the dorsal mesencephalon, *Mgn* expression can be detected in the ventricular zone of this region. This spatial relationship of expression of GABAergic markers and *Mgn* holds true from the first appearance of GABAergic neurons (E10.5). In other regions of the brain, specifically in the zona limitans intrathalamica (ZLI), a close correlation between the expression of *Mgn*, *Gad65* and *Gad67* can also be detected. *Mgn* expression is found in the VZ/SVZ, but never detected in the mantle zone of the developing nervous system, indicating that *Mgn* is expressed in neural progenitor cells.

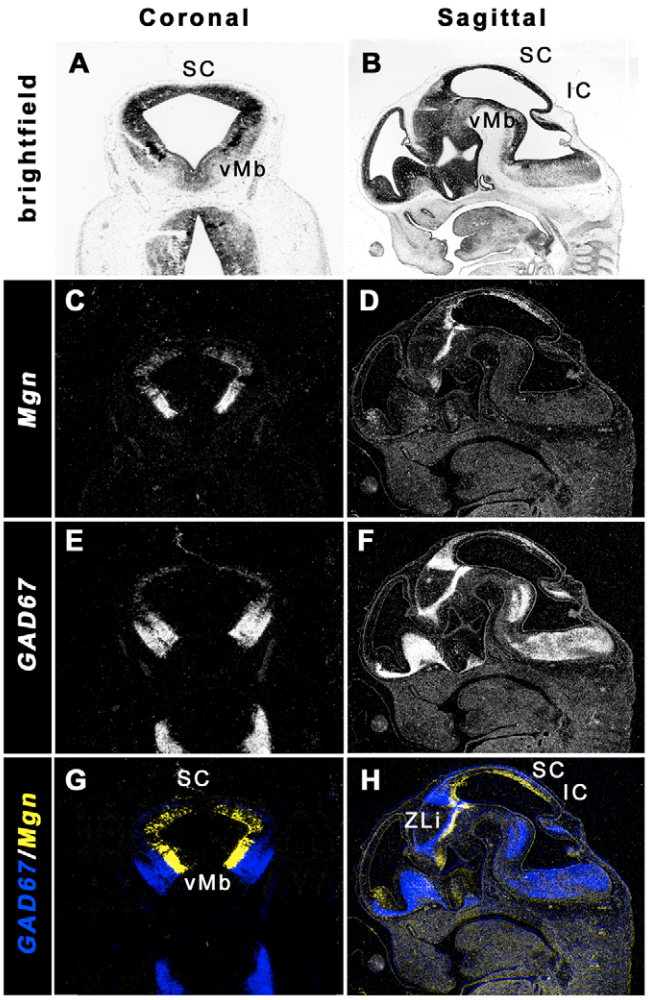

Fig. 2. Colocalization analysis of *Mgn* expression with GABAergic markers in the developing mesencephalon. (**A-H**) Expression of *Mgn* (C,D) and *Gad67* (E,F) in adjacent coronal (A,C,E,G) and sagittal (B,D,F,H) sections of E12.5 mouse embryos. (A,B) Bright-field images. (G,H) Superimposed images of the two adjacent sections hybridized in parallel with *Mgn* (in yellow) and *Gad67* (in blue) indicate that *Mgn* and *Gad67* are expressed in close vicinity but do not colocalize. At E12.5, *Mgn* is expressed in the ventricular zone underlying the mantle layer in which *Gad65/67* expression arises. However, no overlapping of the two expression domains can be observed. This spatial relationship between the two expression domains is especially intriguing in the developing mesencephalon (G), but also detectable in other expression domains such as the ZLi (H). SC, superior colliculus; IC, inferior colliculus; vMb, ventral midbrain.

Postnatal lethality in mice lacking *Mgn*

No dominant phenotypes were observed in mice heterozygous for $Mgn^{+/tZ}$. They appeared normal and were fertile. Heterozygote mice were intercrossed to generate homozygous $Mgn^{tZ/tZ}$ mutants. The genotype of offspring at P0 derived from $Mgn^{+/tZ}$ heterozygous intercrosses reveals that the mutant allele is inherited at the expected Mendelian frequencies (n=72: 18 +/+, 39 +/tZ, 15 tZ/tZ).

After birth, during the first postnatal week, newborn $Mgn^{tZ/tZ}$ mutant mice appeared healthy: the stomach and the intestine of homozygous mutants were filled with milk, pups were of similar size and weight to wild-type and heterozygous $Mgn^{+/tZ}$ littermate controls, and they displayed coordinated movements of the limbs and trunk without apparent neurological abnormalities. During the second week, the homozygous mutant mice displayed a gradual growth reduction, and the first deaths of mice were observed. Three to 4 weeks after birth, the body weight of those homozygous mutants that survived was approximately one-third that of wild-type littermate controls (Fig. 3D,F) because they did not nurse properly. Deaths continued to be observed during this week and no homozygous mutant mouse survived beyond 5 weeks (Fig. 3E). An initial examination at around P12 of the behaviour of $Mgn^{tZ/tZ}$ mice revealed the first signs of neurological impairment. Homozygous mutant mice retracted their fore/hindlimbs and digits when suspended by their tail – in contrast to wild-type mice, which extend them (Fig. 3A-C). Fore/-hindlimb clenching was consistently observed in all homozygous $Mgn^{tZ/tZ}$ knockout mice, but not in the wild-type or heterozygous $Mgn^{+/tZ}$ mice. Occasional convulsions in mice over 14 days of age preceded by a wild running phase, forelimb tonic-clonic spasms with hyperextended hindlimbs episodes, were observed in the homozygous mutant mice prior death, resembling a seizure-like phenotype. No spontaneous convulsions were observed in $Mgn^{+/tZ}$ mice.

Mgn is essential for the development of GABAergic neurons of the SC

To ascertain the cause of death of $Mgn^{tZ/tZ}$ mice, mutants and controls were prepared for morphological examination. The analysis of both embryonic and postnatal brain revealed no gross morphological alterations. Nissl and Luxol Fast Blue stains showed similar staining patterns in wild-type and homozygous mutant mice (data not shown). Given the expression of *Mgn* in the VZ/SVZ of the embryonic CNS and its striking spatiotemporal correlation with *Gad65* and *Gad67* markers (Fig. 2), we next addressed whether the expression of *Mgn* in the SVZ underlying GABAergic neurons has a role in the neuronal specification of this neurotransmitter population.

During postnatal stages, we found a complete loss of *Gad65* and *Gad67* expression in the SC of the mutant $Mgn^{tZ/tZ}$ mice, indicating a loss of GABAergic neurons (Fig. 4A,B; see Fig. S1B,E in the supplementary material). In the inferior colliculus (IC), a partial but not a complete absence of *Gad65* and *Gad67* expression was observed (Fig. 4C,D; data not shown). This lack of GABAergic neurons takes places in the most rostral and most caudal part of the IC (see Fig. S1B,E in the supplementary material), where *Mgn* is normally expressed (Guimera et al., 2006). Strikingly, in the ventral part of the postnatal mesencephalon and in other brain areas outside the colliculi, no obvious changes of the GABAergic neurons could be detected as determined by the presence of *Gad65* and *Gad67*

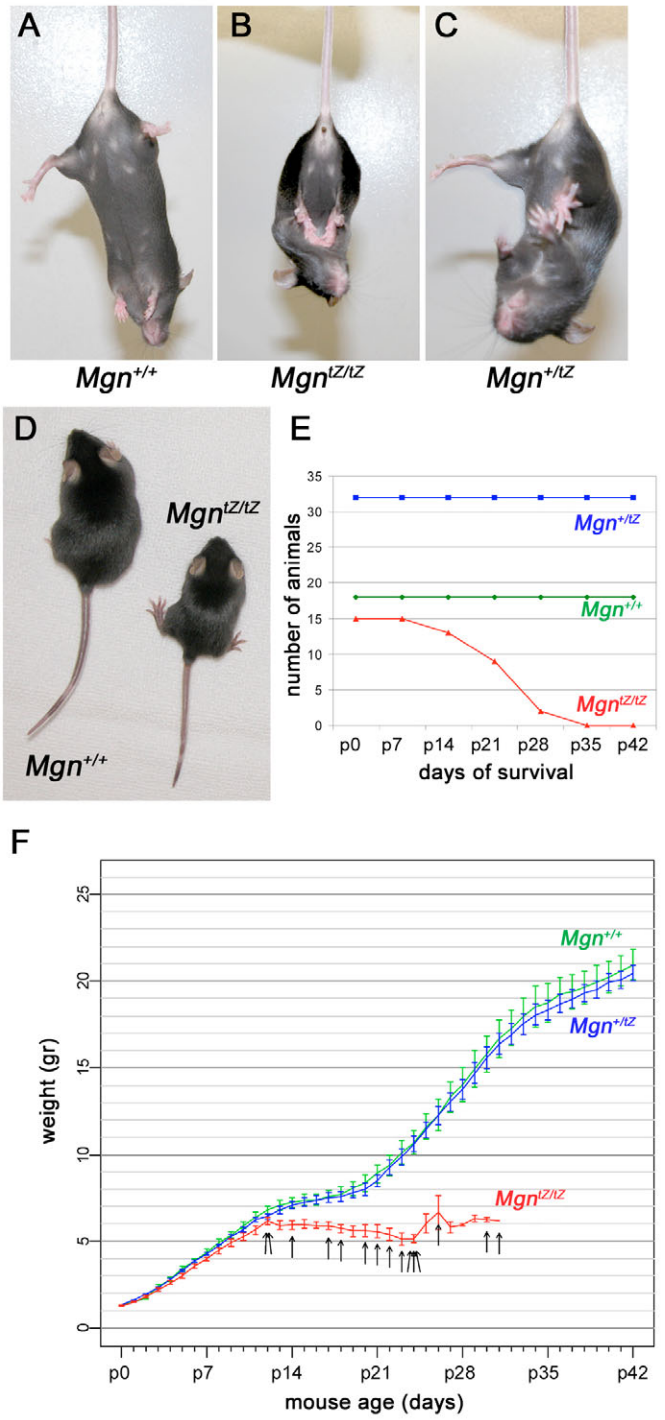

Fig. 3. Cramping, growth characteristics and lethal phenotype. (**A-C**) Fore/hindlimb cramping phenotype in postnatal homozygous $Mgn^{tZ/tZ}$ mutant at postnatal day 20 (P20). Wild-type (A), homozygous *Mgn* knockout (B) and heterozygous *Mgn* knockout (C) mice. When lifted by their tail, $Mgn^{tZ/tZ}$ mice displayed an obvious fore-/hindlimb clenching phenotype. Neither wild-type nor heterozygous $Mgn^{+/tZ}$ mice show this phenotype. (**D**) Phenotypic comparison of $Mgn^{+/+}$ and $Mgn^{tZ/tZ}$ mice at P32. Both animals were on C57BL/6 genetic background. (**E**) Survival curve of $Mgn^{tZ/tZ}$ mice. Days of survival of $Mgn^{tZ/tZ}$ (red line) compared with $Mgn^{+/+}$ (green line) and $Mgn^{+/tZ}$ (blue line) mice. (**F**) Representative growth curve comparison between littermates. Mean body weight at age P0-P45 is represented by a green line ($Mgn^{+/+}$), a blue line ($Mgn^{+/tZ}$) and a red line ($Mgn^{tZ/tZ}$) with error bars displaying the standard errors of the day-specific means. Arrows indicate the day of death for each of the $Mgn^{tZ/tZ}$ mice. The mean difference in weight between heterozygous and wild-type mice was 0.227 g (P=0.9475).

expression (see Fig. S1A-F in the supplementary material; data not shown). The cytoarchitecture of the SC appeared to be normal, as evidenced by immunohistochemical staining against the three Ca^{2+}-binding proteins (parvalbumin, calretinin and calbindin), which still revealed the typical layered and patched structure of the SC (Fig. 4E-H; data not shown). $Mgn^{+/tZ}$ animals were undistinguishable from wild type, indicating that one wild-type allele of *Mgn* is sufficient to promote *Gad65* and *Gad67* expression and a GABAergic phenotype.

These findings prompted us to address whether the induction of GABAergic neurons was affected. Analysis during early mouse embryonic development of $Mgn^{tZ/tZ}$ mutant brains (Fig. 5A-L) and later embryonic stages (E18.5; Fig. 5M-O) also showed a complete lack of *Gad65* and *Gad67* expression in the SC when compared with wild-type or heterozygous brains. By contrast, *Gad65* and *Gad67*-expressing cells are less numerous in the ventral aspect of the midbrain at E10.5-E12.5. Therefore, Mgn is the key regulator necessary for the proper specification of GABAergic neurons in the dorsal midbrain, as *Gad65* and *Gad67* are never expressed in the SC at any stage during development or postnatal brains.

We next focused on the fate of the *Mgn-lacZ* expressing cells in $Mgn^{tZ/tZ}$ mice around the time when the neurotransmitter phenotype appears. One hypothesis was that these cells undergo premature differentiation and/or cell death because of a misfunction during acquisition of generic neuronal properties, as expected for an *h/E(spl)*-related factor. By contrast, if *Mgn* encodes neuronal identity, a proper number of precursor cells should be achieved but without expressing *Gad65* and *Gad67,* bearing in mind that *Mgn*, *Gad65* and *Gad67* are never co-expressed in the same region. As a third possibility, transpecification can also be considered. To test the hypothesis that *Mgn*-expressing cells in the VZ/SVZ survive, despite their failure to be specified as GABAergic neurons, we performed apoptosis and proliferation assays during midgestational stages, the time period during which GABAergic neurons arise. No significant cell death was detected in the midbrain of mutant embryos between E10 and E13, as determined by immunohistochemical studies for cleaved caspase 3 (Fig. 6D,E), which is a specific marker for apoptosis. No reduced proliferation could be detected either, as determined using immunohistochemical staining for phosphohistone 3 (Fig. 6G-J), which is a marker for proliferating mitotic cells. We next confirmed the proliferation and apoptosis studies using BrdU incorporation and TUNEL assays, respectively (see Fig. S2 in the supplementary material). Using a BrdU immunohistochemistry assay, we compared cell proliferation in the dorsal midbrain of wild-type and $Mgn^{tZ/tZ}$ embryos at E10.5-E13.5. There was no obvious difference in cell proliferation among embryos of these genotypes on coronal sections. We next examined apoptotic cells death using a TUNEL assay on sections of embryos at E10.5-E13.5. TUNEL-positive cells were rarely detected in the SC in wild-type and $Mgn^{tZ/tZ}$ embryos.

Furthermore, no changes in the density of differentiated neurons within the mantle zone of the developing SC of mutant animals could be detected using the marker NeuN, which is a specific marker

for post-mitotic neurons in vertebrates (Fig. 6C,F). We could also detect no changes in the expression of *Ebf2* (a general marker for neuronal maturation). In addition, the expression of *Pitx2* [the expression of which essentially overlaps with that of *Gad65* and *Gad67* in the developing basal mesencephalon and SC (Katarova et al., 2000)], of Ca^{2+}-binding proteins (which mark subpopulations of *GAD* expressing cells in differentiated GABAergic neurons of the SC) and of $GABA_A$ transporter 1 (*Gat1*) was not altered (Fig. 7; see Fig. S1G-I in the supplementary material). These results indicate that the *Mgn*-expressing cells derived from the ventricular zone of the dorsal midbrain, which in the wild-type situation give rise to GABAergic neurons, are still present in the mutant but no longer express *Gad65* and *Gad67*, and therefore are not capable of producing GABA. This observation is supported by the fact that the *Gad65*/*Gad67* double-knockout mice completely lack GABA (Ji et al., 1999).

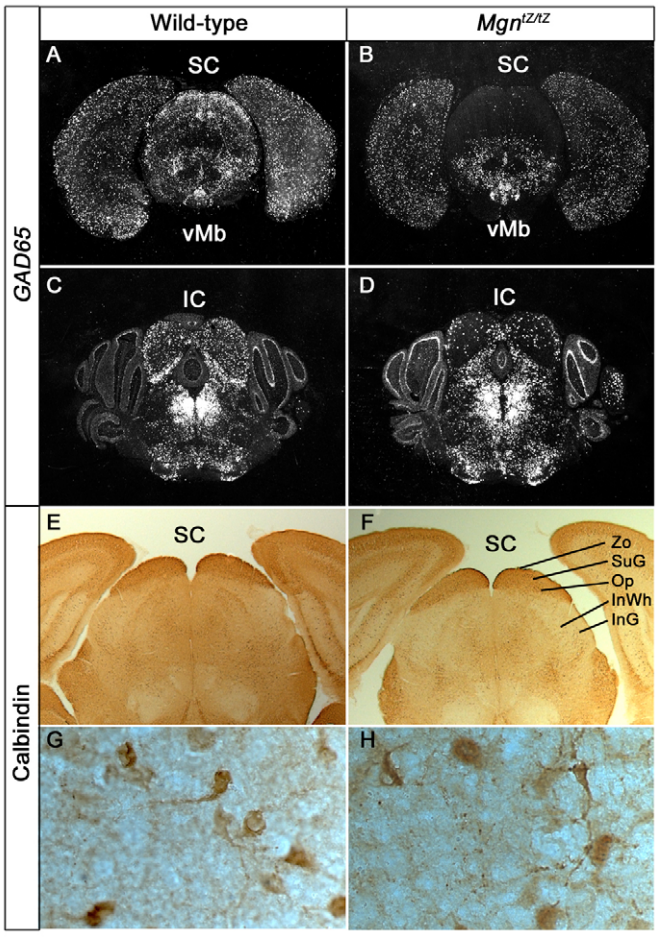

Fig. 4. Complete depletion of GABAergic neurons in the SC of postnatal mice. (**A-D**) *Gad65* expression in the postnatal superior colliculus (SC) of wild-type (A,C) and homozygous mutant mice (B,D). There is complete depletion of *Gad65*-expressing cells in the SC, whereas the ventral midbrain (vMb) and cortex are unaffected. *Gad65*-expressing cells in the inferior colliculus of homozygous mouse (D) are reduced compared with in the wild-type mouse (C). (**E-H**) Calbindin immunohistochemical staining of the SC of wild-type and homozygous mutant mice. The layers and patches characteristic of the cytoarchitecture of the superior colliculus are unaffected (E,F). (G,H) Higher magnification of the SC cytoarchitecture showing no obvious difference in the wild-type (G) and the $Mgn^{tZ/tZ}$ (H) mice. Abbreviations: Zo, zonal layer of the SC; SuG, superficial grey layer of the SC; Op, optic layer of the SC; InG, intermediate grey layer of the SC; InWh, intermediate white layer of the SC.

To further substantiate the hypothesis that progenitor cells in the ventricular zone do not die during later developmental stages because of an initial lack of *Mgn* expression, we took advantage of the presence of the *tZ* allele. The same pattern of *lacZ* staining was observed between homo- and heterozygote mutant mice. Moreover, the stability of the tZ protein allowed us to trace *Mgn*-expressing cells. We detected *lacZ*-positive cells in the outer zones of the SVZ of the SC (Fig. 6K-N), suggesting *Mgn*-deficient cells have embarked on their radial migration towards their final destination as neurogenesis ensues. The presence of the tau-*lacZ* knocked-in the *Mgn* locus allowed us to see the migratory streams of *Mgn*-expressing cells. It is clear that ventral midbrain GABAergic cells do not migrate dorsally, and the dorsal ones migrate radially from the SVZ into the superficial layers of the SC. These results are in good agreement with previous observations from Tsunekawa et al. (Tsunekawa et al., 2005). This migratory behaviour is different from the tangential migration of the neocortical GABAergic neurons described in the forebrain (Anderson et al., 1997; Tamamaki et al., 1997), suggesting that forebrain and midbrain have adopted not only different molecular pathways but also different mechanistic actions regulating the apparition of GABAergic neurons. Taken together, our results clearly imply that *Mgn-lacZ* expressing cells do not undergo apoptosis in the $Mgn^{tZ/tZ}$ mice at the time that the GABAergic phenotype appears and that the failure of GABAergic neuronal specification and differentiation does not result from a loss of progenitor cells. These results strongly support Mgn as a crucial determination factor for the cell fate of the GABAergic interneuron.

Non-GABAergic systems are not disturbed in $Mgn^{tZ/tZ}$ mice

As the lack of *Mgn* could have an effect in other neural lineages, we next investigated other neural subpopulations that, during development, arise close to the *Mgn* expression domain. To analyse an effect in the dopaminergic system, we studied the expression of *Th*, *Dat*, *Pitx3* and *Nurr1* (Nr4a2 – Mouse Genome Informatics) in embryos and postnatal tissue. The results revealed that all these four dopaminergic markers have no apparent change in expression in the $Mgn^{tZ/tZ}$ mutants (see Fig. S3E-J in the supplementary material; data not shown). Other non-GABAergic cell types, such as glutamatergic or cholinergic neurons, did not appear to be disturbed in the homozygous mutant mice, as determined by expression of the markers vesicular glutamatergic transporter 1/2 (*Vglut1/2*) and choline acetyltransferase transporter (*Chat1*), respectively (see Fig. S1J-L,M-O in the supplementary material; data not shown). Furthermore, we examined the oligodendrocytes, as it is known that the oligodendrocyte lineage is dependent on *Nkx2.2*, the expression of which overlaps with *Mgn* expression in the midbrain as well as in the ZLI (Guimera et al., 2006). We found that the oligodendrocyte system is not altered in the homozygous mutant brains at P15 using *Plp1*, *Olig1* and *Olig2* as markers (see Fig. S3K-M in the supplementary material; data not shown).

In addition, we did not observe any changes in the morphology of other midbrain regions, such as the red nucleus (marked by *Brn3a*) and the nuclei occulomotorius (marked by *Isl1*) (Fig. 7M,N; see Fig. S3A-D in the supplementary material). Altogether, we conclude that *Mgn* does not have any general patterning capacities in the midbrain; rather its function seems to be crucial for the acquisition of the neuronal GABAergic neurotransmitter subtype.

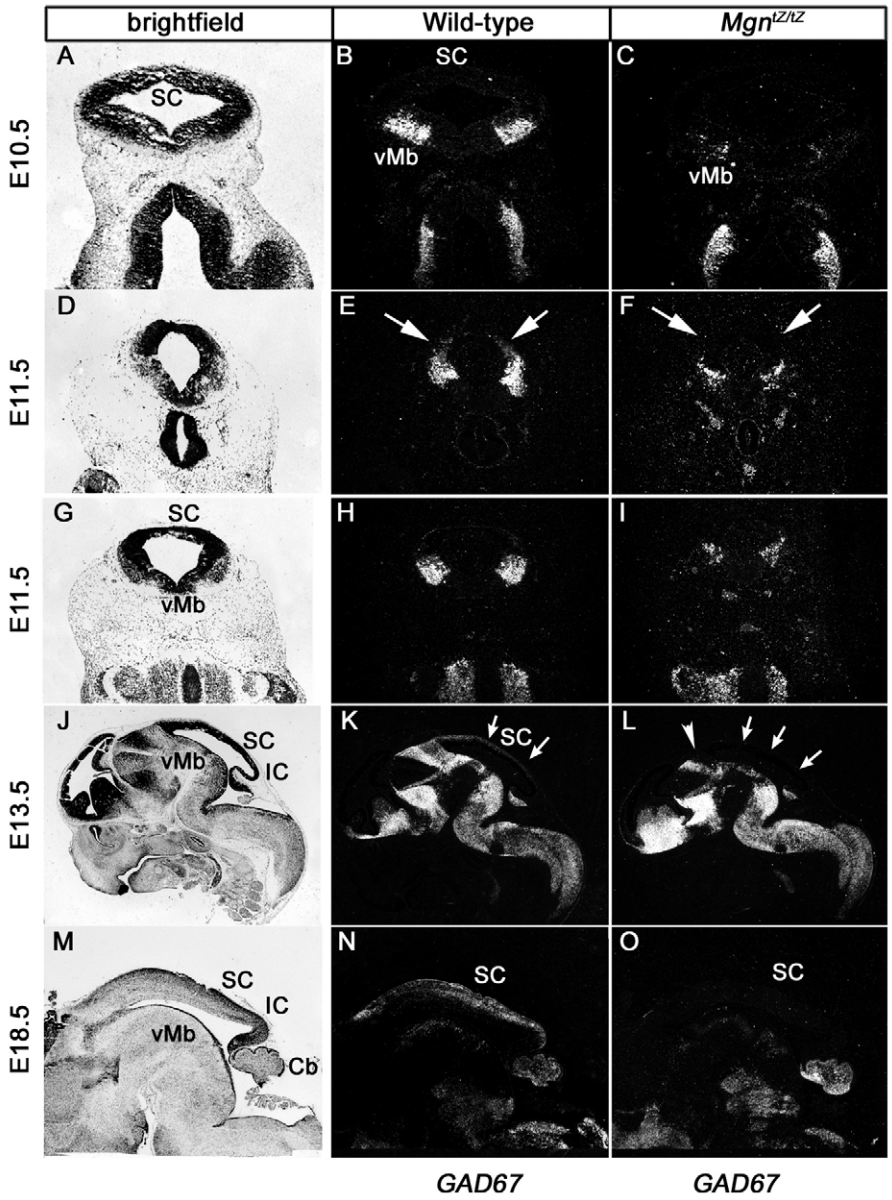

Fig. 5. GABAergic neurons in the dorsal mesencephalon are not induced. In situ hybridization with the *Gad67* riboprobe of coronal (A-I) and sagittal (J-O) sections, showing the mesencephalon of E10.5 (**A-C**), the anterior of E11.5 (**D-F**) and the caudal regions of E11.5 (**G-I**), 13.5 (**J-L**) and E18.5 (**M-O**) of wild-type (B,E,H,K,N) and $Mgn^{tZ/tZ}$ (C,F,I,L,O) embryos. (A,D,G,J,M) Bright-field images of wild type. (B,E) *Gad67* expression is prominent in the mantle zone of the developing ventral midbrain of wild-type embryo. (C,F) *Gad67* expression is reduced in the developing ventral midbrain of homozygous mutant mice, and dorsal expression is not detectable. Arrows in E,F indicate the presence (E) and absence (F) of dorsal *Gad67*-expressing cells. (H,I) *Gad67* expression is also reduced in the caudal region of the mutant SC at E11.5. (K) Expression of *Gad67* is present in the dorsal mesencephalic vesicle of wild-type (arrows), while the *Gad67* expression in the mutant (L) is completely absent in the dorsal mesencephalic vesicle (arrows); it is also reduced in the pretectal area (arrowheads) and slightly reduced in the ventral midbrain. *Gad67* expression in other areas of the brain (spinal cord, ventral hindbrain, cerebellum, thalamus, hypothalamus and the ventral telencephalon) is not affected. Arrows in K,L indicate the presence (K) and absence (L) of *Gad67*-expressing cells in the dorsal midbrain at E13.5 of the indicated genotype. (N-O) The mid/hindbrain region. Strong *Gad67* expression can be observed in the SC of the wild-type mouse (N). (O) In the mutant mouse, *Gad67* expression is completely absent in the dorsal midbrain, weaker in the ventral midbrain and not affected in the cerebellum and ventral hindbrain. Abbreviations: Cb, cerebellum; IC, inferior colliculus; SC, superior colliculus; vMB, ventral midbrain.

DISCUSSION

We have identified and characterized a bHLH factor referred to as megane (Mgn) related to the *Drosophila h/E(spl)* proteins (Guimera et al., 2006). In this study, we describe the function of *Mgn* in vivo using a knockout approach in mouse embryonic stem cells (ES). Homozygous $Mgn^{tZ/tZ}$ mutants are postnatal lethal between the second and fifth week of age. Histological analysis of the brain did not reveal any alteration. However, using GABAergic neuron specific markers, we could show that *Mgn* plays a key role controlling the acquisition of GABAergic neuronal identity in the SC.

Mgn protein and GABAergic neurogenesis

Expression of *Mgn* mRNA shows a specific and dynamic expression pattern in the embryonic CNS in a region-specific manner. In the midbrain, *Mgn* is expressed at a high level in the ventricular zone, next to the lumen, where neuroepithelial germinal cells proliferate to generate neural precursors, but it decreases rapidly as neural differentiation proceeds. These observations suggest *Mgn* might control early steps in the specification and/or differentiation of particular neural lineages in the CNS, rather than their maturation or functional maintenance. The developmentally controlled specific expression pattern of *Mgn* prompted us to investigate its correlation with GABA neurotransmitter markers and its function in cell differentiation. As determined by comparative expression analysis of in situ hybridization experiments on consecutive sections, *Mgn* mRNA expression was detected in a spatiotemporal correlation with *Gad65*- and *Gad67*-expressing cells. *Gad65* and *Gad67* expression appears when differentiating cells have migrated from the ventricular to the mantle layer. *Mgn* expression ceases once *Gad65* and *Gad67* expression becomes prominent in the mantle layer. Thus, *Mgn* and Gad expression are adjacent and not co-expressed. Expression of *Mgn* may demarcate neurogenic regions that give rise to GABAergic neurons. In postnatal brain, after neuronal differentiation has been completed, *Mgn* expression is strongly reduced in the CNS, and only maintained in scattered cells surrounding the lateral ventricles and olfactory ventricles.

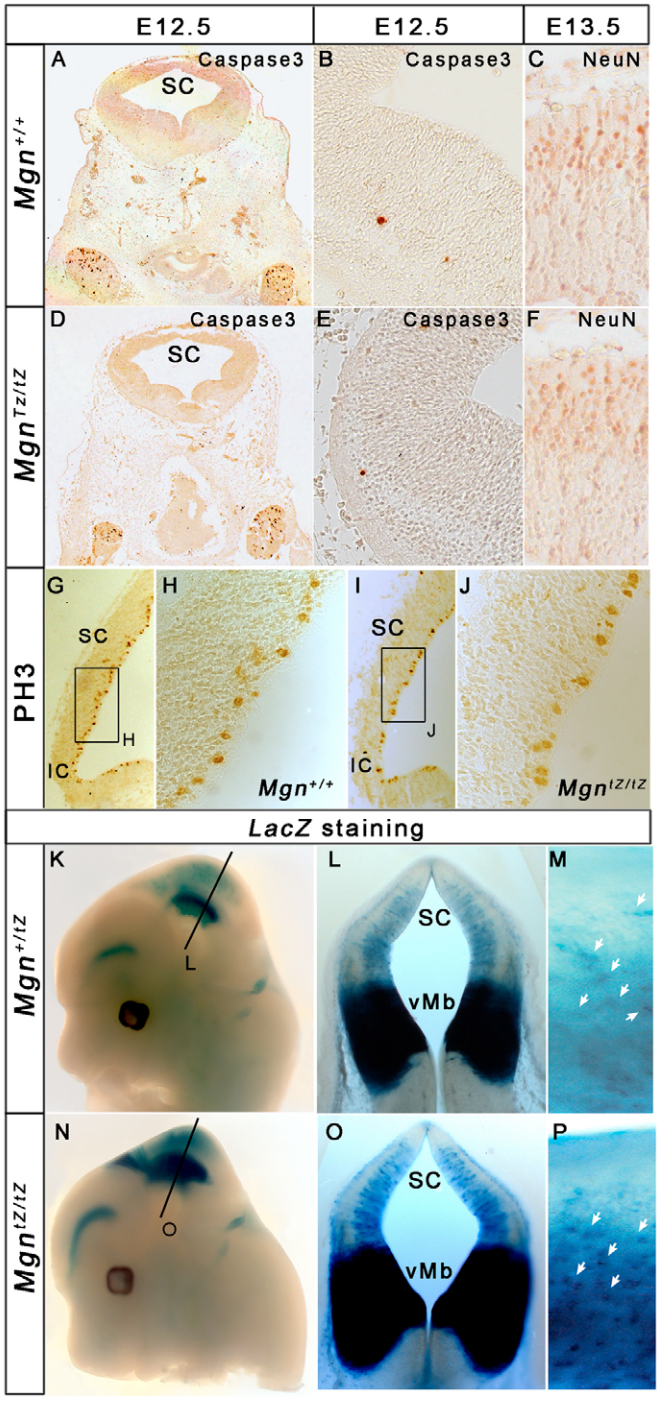

Fig. 6. Cells that failed to be GABAergic neurons are present in the midbrain of *Mgn*$^{tZ/tZ}$ embryos. (A,B,D,E) Immunohistochemical staining of activated caspase 3 in the mesencephalic vesicle at E12.5. In the mesencephalic vesicle, no increase in apoptosis can be observed in the mutant. Single cells could be found labelled in the wild-type (*Mgn*$^{+/+}$) (**A**,**B**) as well as in the mutant (*Mgn*$^{tZ/tZ}$) in this region (**D**,**E**). (**C**,**F**) Immunohistochemical staining of NeuN in the developing SC at E13.5. There are no alterations in the thickness of either the ventricular zone or the mantle zone in the mutant mice (F). (**G-J**) No differences are observed in the PH3-positive cells in the wild-type (G, magnified in H) when compared with the homozygous mutant (I, magnified in J). (**K-P**) *lacZ* expression of E13.5 mouse *Mgn*$^{+/tZ}$ (K-M) and *Mgn*$^{tZ/tZ}$ (N-P) embryos. Whole-mount *lacZ* expression is more intense in the *Mgn*$^{tZ/tZ}$ mice (N), whereas localization is identical to that in *Mgn*$^{+/tZ}$ mice (K). *lacZ* expression in a coronal section of the SC from *Mgn*$^{+/tZ}$ (L) and *Mgn*$^{tZ/tZ}$ (O) embryos at E13.5. (M,P) Horizontal sections of the SC are magnified, showing single cells migrating out from the SVZ into the differentiation zone (arrows).

Mgn determines the GABAergic phenotype in the mouse SC

Loss-of-function experiments by gene targeting in murine ES cells allowed us to determine the physiological relevance of *Mgn* during neuronal development in the mammalian brain. We could show that characteristic GABAergic neurons are missing in the superior colliculus of postnatal brain in homozygous *Mgn*$^{tZ/tZ}$ mutants. We have investigated whether these missing GABAergic neurons were initially: (1) not induced because of the absence of GABAergic precursor cells or as a consequence of improper cell-fate specification early in embryonic development; (2) induced but not maintained later in development due to cell death; or (3) induced but not developed properly because of defects in migration or maintenance. A priori, the fact that Mgn is a transcription factor expressed in the VZ/SVZ, when and where cell-fate decisions are taken, but is not expressed in the differentiation zone or in mature neurons, argues in favour of the last hypothesis. We could never detect *Gad67* or *Gad65* mRNA-positive cells in the SC of homozygous *Mgn*$^{tZ/tZ}$ mutants, ranging from the earliest appearance of these two markers until death, whereas the expression of *Gad65* and *Gad67* in the heterozygous mutant was indistinguishable from wild-type mice. This evidence suggests that the early determination of GABAergic neurons is disrupted in the SC and induction of the GABAergic phenotype does not take place. Therefore, the confined expression of *Mgn* in the VZ/SVZ adjacent to that of specific markers for the GABAergic neurons and the GABAergic neuronal identity phenotype of the homozygous mutant mice shows that *Mgn* determines early entry/specification of the precursor cells into the neuronal GABAergic phenotype.

Mgn plays a vital role for the development of the GABAergic system of the SC

Knockout mice were born at expected Mendelian frequencies. Therefore, no embryonic lethal phenotype can be attributed to the loss of the *Mgn* gene, which suggests GABAergic neurons of the SC do not play a vital role in mouse embryonic development. The physiological consequences of their differentiation failure are not obvious until the first postnatal week onwards, when the homozygous mutants do not nurse properly, leading to fatal dehydration/inanition. No obvious changes in the GABAergic system have been observed outside the SC. Beyond the GABAergic phenotype, specific markers for other neurotransmitter phenotypes and different CNS cell types (*Th*, *Dat*, *Pitx3* and *Nurr1* for the dopaminergic system; *Chat1* for the cholinergic system; *Oligo1*, *Oligo2* and *Plp1* for the oligodendrocytes; *Brn3a* for sensory neurons; *Isl1* for motoneurons; *Vglut1/2* for the glutamatergic neurons) were tested in the mutant mice without any apparent changes. We did not observe transpecification of the *Mgn*-deficient cells into glutamatergic cell fate, but even if *vGlut1/2* expression and that of other tested neurotransmitter markers is not changed, we cannot rule out that, in the absence of *Mgn*, these neurons may adopt some other non-GABAergic aspects of identity (e.g. glycinergic or serotonergic) and further cell-fate mapping experiments should be

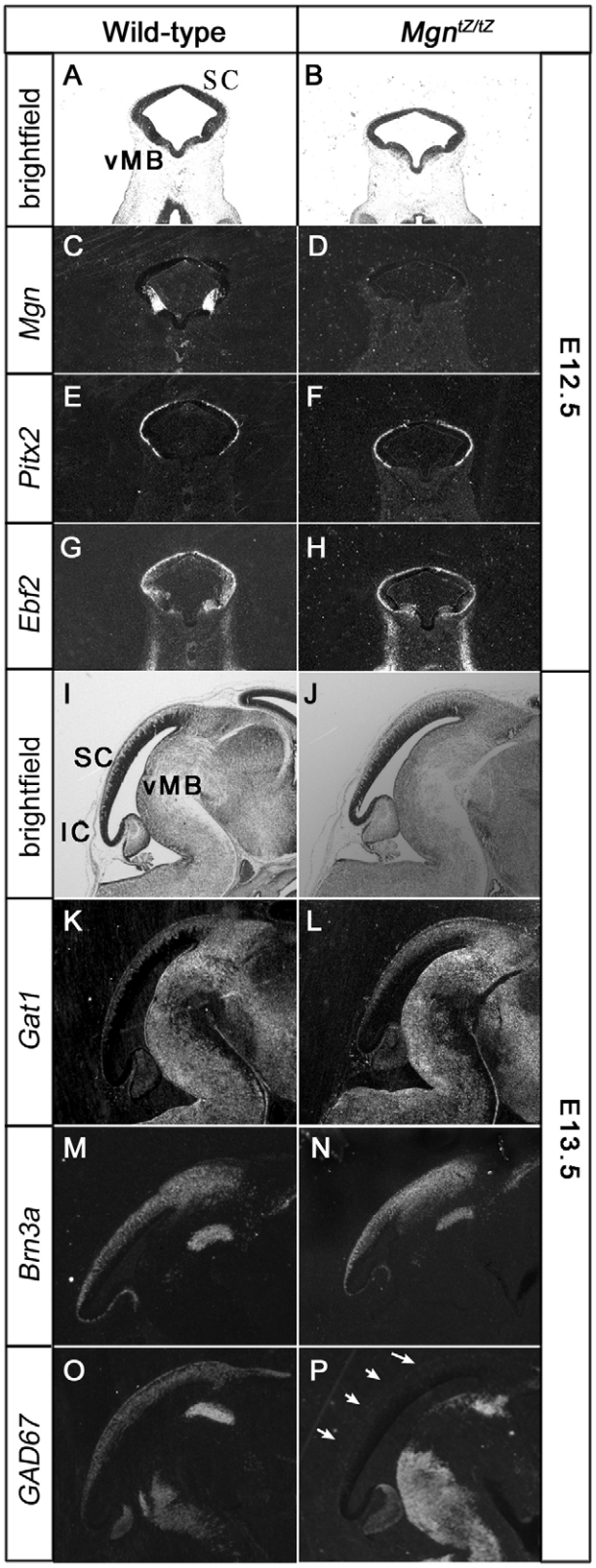

Fig. 7. Other GABAergic and non-GABAergic markers are present in the SC of *Mgn*$^{tZ/tZ}$ embryos. In situ hybridization with *Mgn*, *Pitx2* and *Ebf2* probes in adjacent coronal (**A-H**) at E12.5, or with *Gat1*, *Brn3a* and *Gad67* in sagittal (**I-P**) sections at E13.5 in wild-type and in *Mgn*$^{tZ/tZ}$ embryos. (A,B,I,J) Bright-field images. No changes for *Pitx2* (E,F) and *Ebf2* (G,H) could be observed when comparing wild-type and mutant mice. *Mgn* was absent in the mutant (compare C with D). At E13.5, no changes in the expression of *Gat1* (K,L) or *Brn3a* (M,N) were detectable in the SC of the mutant when compared with the wild type, whereas the expression of *Gad67* is totally abolished in the SC of the homozygous mutant mice (P, compare with O; see arrows).

conducted. The hypothesis that the lethal phenotype observed in the homozygous mutant mice might be intrinsic to the functions of the GABAergic neurons of the SC is supported by the observation that a similar phenotype, including cramping behaviour, seizure episodes and postnatal lethality, is described in other mice displaying a general specific disturbance of the GABAergic system (Asada et al., 1996; Asada et al., 1997; Condie et al., 1997; Homanics et al., 1997). Nevertheless, we cannot exclude the hypothesis that a subtle phenotype beyond the SC GABAergic phenotype may also contribute to the lethal or seizure-like phenotypes. Although we are a long way from understanding the physiological role of the GABAergic system, unravelling individual functions of the GABA signalling pathway should allow us to understand better the function of the SC in mammalian development. Therefore, *Mgn* homozygous mutants will offer a unique model to study the physiological roles of GABA in the SC during embryonic development and postnatally.

Homozygous *Mgn*$^{tZ/tZ}$ mutants display functional rather than morphologic defects

The morphological and anatomical structure of the homozygous mutant brains appeared normal in the embryos and newborns, as determined by histology. The SC also showed characteristic cytoarchitecture and normal laminated structures, and the usual pattern of cell distribution.

The presence of: (1) early neurogenesis markers (*Mash1*, *Ngn1/2/3*, *Hes5* and *Hey2*); (2) neuronal markers (*Ebf2* and *NeuN*); (3) GABAergic markers (*Pitx2* and *Gat1*); and (4) *lacZ*-positive cells in the in the SC of homozygous mutant brains compared with their control littermates, as well as apoptosis and proliferation assays, indicates that *Mgn-lacZ* expressing cells are present in the *Mgn*$^{tZ/tZ}$ mutants and do not undergo apoptosis, despite the fact that they do not express *Gad65* and *Gad67*. The partial loss of dorsal midbrain GABAergic identity in the *Mgn*$^{tZ/tZ}$ mice occurs from the onset of neuronal differentiation and is not due to alterations in cell death or cell proliferation during development. These results demonstrate that fully neural GABAergic identity specification failed in the SC of the *Mgn*$^{tZ/tZ}$ mutants. Presumptive GABAergic neurons lack their fully GABA identity, without the gain of an alternative neurotransmitter phenotype during embryonic and postnatal stages, retaining some GABAergic markers such as *Gat1* and *Pitx2*. Despite the hypothetical fact that a few postsynaptic membranes (p.e. astrocytes and glutamatergic neurons) may express *Gat1* in the midbrain is still not reported, the observation that *Gat1* expression is not reduced in the dorsal midbrain of the mutant mice suggests that the presumptive GABAergic neurons (the vast majority of *Gat1*-expressing cells in the wild-type midbrain – if not all) still express *Gat1* in the mutant. This suggests that different pathways are involved in specific aspects of GABAergic fate and *Mgn* impairment is not sufficient to produce a neurotransmitter identity switch.

Interestingly, *Mgn* and Gad mRNAs are not co-expressed, suggesting that *Mgn* does not regulate the expression of Gad genes directly. Alternatively, it is likely that Mgn induces expression of

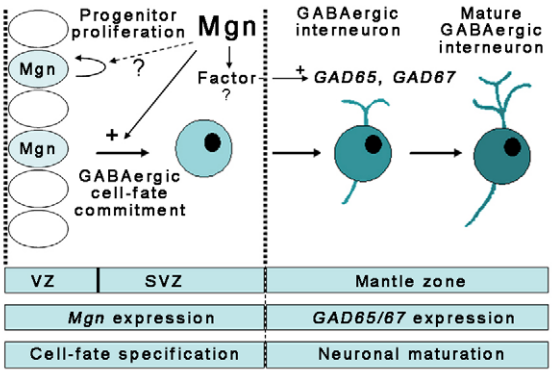

Fig. 8. Summary model of the *Mgn* expression and function regarding the specification of GABAergic neurons in the mouse SC. *Mgn* expression in the VZ/SVZ has an instructive role in the acquisition of GABAergic identity, as determined later on in the mantle zone by the appearance of two specific independent markers for GABAergic cells (*Gad65* and *Gad67*) in the postmitotic neurons. Given the adjacent and non-overlapping expression domains between *Mgn* and Gad genes, it is likely that the Mgn transcription factor activates downstream gene(s) that, in turn, transactivate the *Gad65* and *Gad67* promoters.

unknown transcription factors in the ventricular zone that might, in turn, regulate, among others, the expression of *Gad65* and *Gad67* in the mantle zone (Fig. 8).

Mgn displays a dorsoventral context-dependent activity

Another interesting result of our studies is the differences concerning the GABAergic neuronal development between the dorsal and the ventral aspect of the midbrain observed in the $Mgn^{tZ/tZ}$ mutants. The ventral GABAergic neurons appeared to be normal compared to a total absence in the SC. Distinct dorsoventral origins of mouse and human midbrain GABAergic neurons have been already postulated by Katarova et al. (Katarova et al., 2000) and by Verney et al., (Verney et al., 2001), respectively.

The most likely explanation for this difference is that other transcription factors in the ventral midbrain, e.g. *MashI* (which is not misregulated in the $Mgn^{tZ/tZ}$ mice; data not shown), are sufficient to compensate for the loss of *Mgn* in the ventral, but not in the dorsal, midbrain. Alternatively, Mgn and Mash1 proteins may collaborate with other transcription factors that modify their activity in controlling distinct genetic pathways for dorsoventral midbrain GABAergic neuron development. These observations support the hypothesis that the neuronal differentiation activity of the bHLH factors (e.g. Mash1, Ptf1a and Mgn) is strongly dependent on the regional and cellular context, and proneural information is combined with positional information to regulate different downstream genes to control the specification and differentiation of GABAergic neurons in distinct brain regions (Brunet and Ghysen, 1999; Bertrand et al., 2002; Miyoshi et al., 2004; Hoshino et al., 2005).

Mgn and mammalian neurogenesis

Mammalian neurogenesis depends essentially upon the balance between repressors of neurogenesis [represented mainly by the *h/E(spl)* factors] and activators of neurogenesis (represented mainly by the proneural genes). The level of expression of any of these two types of bHLH factors over the other one will determine whether the cells stay in an undifferentiated state or proceed with their cell-fate program (reviewed by Ross et al., 2003).

Despite the large number of transcription factors identified in the last years, vertebrate *h/E(spl)*-related genes with a specific role in the acquisition of neuronal identity have been shadowed by the role of the proneural genes. A major conclusion from our results is that *h/E(spl)* mammalian members are not only involved in controlling the proper number of precursor cells, but also in the acquisition of neuronal identity. Vertebrate *h/E(spl)*-related genes can no longer be regarded solely as a factors that confer generic neurogenic properties, but are also key components for the subtype-neuronal identity in the mammalian CNS.

The general function of *h/E(spl)* genes has been conserved from *Drosophila* to vertebrates. However, the unique expression pattern and the function of *Mgn* in vivo shows a previously unrecognized role for *h/E(spl)*-related genes in midbrain GABAergic cell differentiation. We failed to find any orthologue of *Mgn* in silico or by screening *Drosophila* cDNA libraries (Guimera et al., 2006). The molecular mechanisms in mammalian neurogenesis are largely controlled by genes related to those involved in *Drosophila* neurogenesis. However, neuronal development in mammals is still poorly understood compared with that of *Drosophila*, because, among other reasons, of the appearance of novel genes during vertebrate evolution that permit the addition of new functions during brain development. Therefore, bHLH mammalian factors with neuronal identity specification/differentiation activity without homologous counterparts in *Drosophila* should provide novel clues for understanding the genetic pathways of vertebrate neuronal development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/19/3847/DC1

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