

# DNA Methyltransferases 1 (DNMT1) and 3a (DNMT3a) Colocalize with GAD67-Positive Neurons in the GAD67-GFP Mouse Brain

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## ABSTRACT

DNA methylation is an epigenetic regulatory mechanism commonly associated with transcriptional silencing. DNA methyltransferases (DNMTs) are a family of related proteins that both catalyze the de novo formation of 5-methylcytosine and maintain these methylation marks in cell-specific patterns in virtually all mitotic cells of the body. In the adult brain, methylation occurs in progenitor cells of the neurogenic zones and in postmitotic neurons. Of the DNMTs, DNMT1 and DNMT3a are most highly expressed in postmitotic neurons. While it has been commonly thought all postmitotic neurons and glia express DNMTs at comparable levels, the coexpression of selected DNMTs with markers of distinct neurotransmitter phenotypes has not been previously examined in detail in the mouse. To this end, we analyzed the expression of DNMT1

and DNMT3a along with GAD67 in the brains of the glutamic acid decarboxylase67-enhanced green fluorescent protein (GAD67-GFP) knockin mice. After first confirming that GFP-immunopositive neurons were also GAD67-positive, we showed that in the motor cortex, piriform cortex, striatum, CA1 region of the hippocampus, dentate gyrus, and basolateral amygdala (BLA), GFP immunofluorescence coincided with the signal corresponding to DNMT1 and DNMT3a. A detailed examination of cortical neurons, showed that  $\approx 30\%$  of NeuN-immunopositive neurons were also DNMT1-positive. These data do not exclude the expression of DNMT1 or DNMT3a in glutamatergic neurons and glia. However, they suggest that their expression is low compared with the levels present in GABAergic neurons. *J. Comp. Neurol.* 000:000–000, 2011.

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**INDEXING TERMS:** conditional knockout; DNA methylation; epigenetics; GABAergic neuron; gene regulation; schizophrenia

DNA methylation is a major factor involved in the epigenetic regulation of neuronal gene expression underlying changes in synaptic plasticity associated with learning and memory processes (Day and Sweatt, 2010, 2011). Aberrant methylation of candidate gene promoters affects the expression of downstream transcription units. Pathogenic mechanisms associated with psychiatric disorders including mood and cognition disorders are linked to neuronal mRNA changes (Veldic et al., 2004; Abdolmaleky et al., 2005; Grayson et al., 2005, 2006; Ruzicka et al., 2007; Mill et al., 2008; Poulter et al., 2008; Higuchi et al., 2011).

The enzymes that catalyze the transfer of a methyl group from S-adenosyl-methionine to the 5' position of the C moiety of CpG dinucleotides form a small family of structurally related proteins called the DNA methyltransferases (DNMTs). These include DNMT1, DNMT3a, DNMT3b, and the more recently described DNMT3L (see

Goll and Bestor, 2005; Cheng and Blumenthal, 2008; Jurkowska et al., 2011). DNMT1 is the most active of the DNMTs, and while it has a preference for hemi-methylated sites, it also catalyzes de novo methylation in CpG islands (Jair et al., 2006; Cheng and Blumenthal, 2008). DNMTs-3a and -3b are typically known as de novo DNMTs and each show distinct developmental patterns of expression in the brain. DNMT3b is expressed in neuroprogenitor cells and is likely important for establishing

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methylation patterns during neurogenesis, while DNMT3a is expressed in postmitotic, newly differentiated and mature neurons (Feng et al., 2005). DNMT3a may be required for the establishment of tissue-specific methylation patterns of the genome (Watanabe et al., 2006). DNMT3L is catalytically inactive and acts to enhance the activities of DNMT3a and DNMT3b (Van Emburgh and Robertson, 2011).

Interestingly, DNMT1 and DNMT3a are highly expressed in adult mammalian postmitotic neurons, whereas DNMT3b expression is low (Inano et al., 2000; Veldic et al., 2004; Feng et al., 2005; Zhubi et al., 2009). DNMT1 is more highly expressed in GABA neurons of the human cortex and less so in pyramidal neurons or glia (Veldic et al., 2004). DNMT1 is also increased in GABAergic neurons of cortical layers I and II in schizophrenia (SZ) and bipolar disorder patients (BPD) (Veldic et al., 2004, 2005; Ruzicka et al., 2007) and is upregulated in GABAergic medium spiny neurons of the striatum of SZ but not BPD patients (Veldic et al., 2007). More recently, an examination of DNMT3a mRNA in postmortem human cortex showed higher numbers of neurons reaching detection in layers I and II in SZ patients (Zhubi et al., 2009).

Conditional double knockout mice in which DNMT1 and DNMT3a are absent in forebrain excitatory neurons show abnormal long-term plasticity in the CA1 region of the hippocampus and deficits in learning and memory (Feng et al., 2010). In addition, a large number of genes that are deregulated in glutamatergic neurons in response to the loss of DNMT function have been identified. The observed decrease in DNA methylation in the double knockout neurons compared with the single conditional knockout mice suggests the possibility that DNMT1 and DNMT3a have overlapping roles in postmitotic neurons. The studies described in the preceding paragraph with respect to DNMT localization in GABA neurons of the human brain and the loss of function studies carried out in excitatory forebrain neurons sug-

gest that the localization of DNMT1 and 3a with respect to neurons of a defined neurotransmitter phenotype is still not entirely clear.

Only limited studies have been carried out in the mouse brain to show the localization of DNMT1 with markers of GABAergic or pyramidal neurons. For example, Satta et al. (2008) showed that DNMT1 and GAD67 colocalize in layers I and II of the mouse frontal cortex. However, the localization of DNMT3a with respect to neurons of a defined neurotransmitter phenotype is still unknown. The goal of the current study was to provide a comprehensive analysis of the distribution of DNMT1 and DNMT3a in the mouse brain with respect to neurotransmitter phenotype. To this end, we studied the glutamic acid decarboxylase67-enhanced green fluorescent protein (GAD67-GFP) knockin mouse (Tamamaki et al., 2003). This mouse was developed by targeting GFP to the ATG start codon of the GAD67 primary transcript by homologous recombination such that GFP expression is under control of the endogenous GAD67 promoter. These GAD67-GFP mice have previously been examined with respect to the colocalization of GFP and GAD67 and various additional markers of GABAergic neurons such as parvalbumin, calretinin, and somatostatin (Tamamaki et al., 2003). Establishing the distribution of DNMT1 and DNMT3a in these mice will allow us the possibility of using fluorescence-activated cell sorting of neurons to study changes in GABA neuron-specific methylation patterns following different developmental, environmental, and pharmacological manipulations. As a first step, we determined the neurotransmitter phenotype of neurons expressing DNMT1 and DNMT3a by colocalizing the respective immunohistochemistry with markers for GABAergic neurons (GAD67) in the motor and piriform cortices, striata, hippocampi, and BLA of GAD67-GFP knockin mice.

## MATERIALS AND METHODS

Animals were housed in a controlled environment and were genotyped prior to use. All experiments were conducted in accordance with the University of Illinois at Chicago Animal Care and Use Committee guidelines.

### Animals and tissue preparation

Adult mice, 25–30 g in body weight, were perfused intracardially with  $\approx 25$  ml saline (0.9% NaCl) followed by 40–60 ml of 4% paraformaldehyde (Liu et al., 2001). The brains were 1) postfixed for 72 hours in 4% paraformaldehyde and 2) embedded in 30% sucrose in 0.15 M phosphate-buffered saline (PBS), pH 7.4, at 4°C. All experiments were conducted in groups of three to five animals.

#### Abbreviations

|        |  |
|--------|--|
| BLA    | Basolateral amygdala                   |
| BPD    | Bipolar disorder                       |
| CA     | Cornu ammonis                          |
| CamK   | Calmodulin-kinase IIa                  |
| CNS    | Central nervous system                 |
| DAB 3, | 3'-Diaminobenzidine tetrahydrochloride |
| DNMT   | DNA methyltransferase                  |
| Emx1   | Empty spiracles homeobox               |
| GAD67  | Glutamic acid decarboxylase 67         |
| GFP    | Enhanced green fluorescent protein     |
| NeuN   | Neuronal nuclei                        |
| ori    | Stratum oriens                         |
| pyr    | Stratum pyramidale                     |
| rad    | Stratum radiatum                       |
| RELN   | Reelin                                 |
| PFC    | Prefrontal cortex                      |
| SZ     | Schizophrenia                          |
| VGLUT2 | Vesicular glutamate transporter        |

**TABLE 1.**  
**Primary Antibodies**

| Antibody | Immunogen  | Species | Source, cat. no., clone  | Dilution |
|----------|--|---------|--|----------|
| GFP      | Recombinant GFP protein  | Rabbit  | Abcam, Cambridge MA, ab290   | 1:250    |
| GAD67    | Recombinant GAD67 protein  | Mouse   | Millipore, Billerica MA, MAB5406, clone 1G10.2   | 1:500    |
| Reelin   | N-terminus of mouse reelin, amino acids 40–189   | Mouse   | Andre Goffinet, Fac Univ Notre-Dames de la Paix School of Medicine, Namur, Belgium (G10) | 1:500    |
| DNMT1    | Synthetic peptide corresponding to amino acids 637–650 (EKDDREDKENAFKR) of human Dnmt1 | Mouse   | Imgenex, San Diego CA IMG-261A, clone 60B1220.1  | 1:500    |
| DNMT3a   | Recombinant mouse Dnmt3a. The epitope is near the C-terminus (a.a. 705–908)            | Mouse   | Imgenex, San Diego CA IMG-268A, clone 64B1446  | 1:500    |
| NeuN     | Purified cell nuclei from mouse brain  | Mouse   | Imgenex, San Diego CA, MAB377, clone A60   | 1:500    |
| VGlut2   | Recombinant protein from rat VGLUT2.   | Mouse   | Millipore, Billerica MA, MAB5504, clone 8G9.2  | 1:500    |

For each antibody, from four to six sections were processed for each animal.

### GAD67-GFP knockin mice

For the experiments described we used heterozygous GAD67-GFP mice in which the cDNA encoding enhanced GFP is inserted into the GAD67 ATG start codon by homologous recombination (Tamamaki et al., 2003). Offspring of the original knockin mice were crossed with CAG-cre transgenic animals (Sakai and Miyazaki, 1997) to remove the loxP-flanked phosphoglycerokinase-neomycin resistance cassette used as a selectable marker (Tamamaki et al., 2003). GAD67-GFP ( $\Delta$ neo) mice were used throughout this study and were backcrossed and maintained in the C57BL/6 background. GAD67 is one of two genes encoding isoforms of the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD). Heterozygous GAD67-GFP breeder males were mated with wild-type C57BL/6 females and offspring were genotyped at birth. Two primer pairs were used for genotyping to identify both alleles (GAD67-GFP and GAD67/GAD67) of all offspring (for primer sequences, see Tamamaki et al., 2003). The GFP insertion disrupts the GAD67 reading frame so that mice homozygous for the GAD67-GFP knockin allele die just prior to birth (Asada et al., 1997; Condie et al., 1997). For the experiments described, 60–80-day-old animals of either sex were used. GAD67 wild-type littermates were used as controls. Coronal slices (nominally 20–25  $\mu$ m thick) of fixed mouse brains were cut using a cryostat (Richard Allen Scientific, Kalamazoo, MI). Sections were directly used to view native GFP fluorescence. GFP fluorescence was observed; however, to enhance the signal rabbit anti-GFP antibodies were used in the colocalization studies. All fixed slices were washed in PBS (three washes at 10-minute intervals), then proc-

essed for immunohistochemistry and immunofluorescence experimental analysis.

### Antibodies

See Table 1 for a complete list of all antibodies, sources, and dilutions used in the present study. Secondary antibodies included: Cy5-labeled goat antimouse, antirabbit IgG (diluted 1:1,000; Amersham Biosciences, Piscataway, NJ), or Cy2-labeled streptavidin (diluted 1:1,000; Amersham Biosciences) were used as described below.

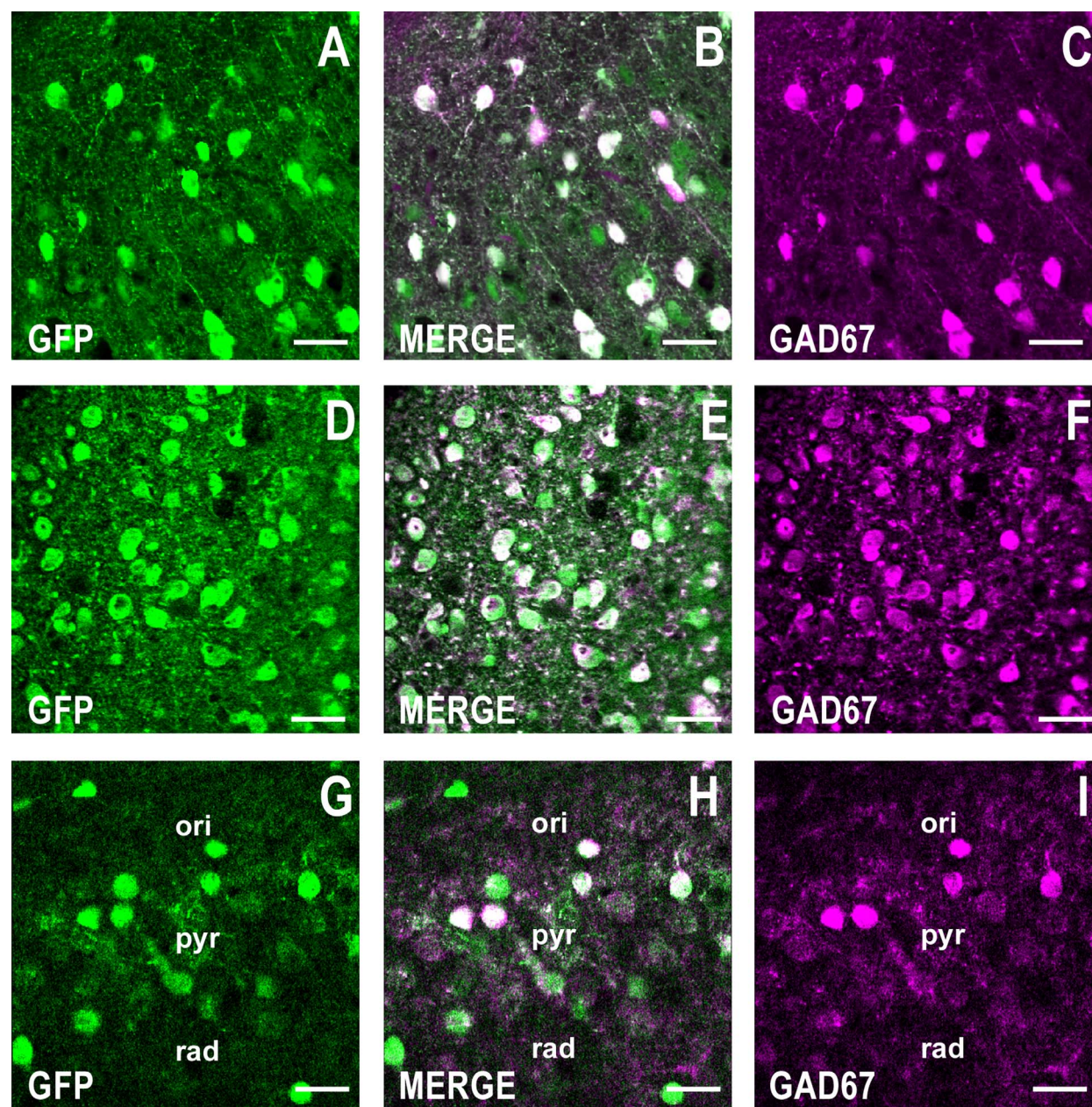
### Antibody characterization

Antibody specificity was evaluated by western blot analyses of cortical extracts. Following separation on 4–12% sodium dodecyl sulfate-polyacrylamide gels and blotting to nitrocellulose, major immunoreactive bands of the expected molecular size were detected with all antibodies used.

The GFP polyclonal antibody (directed against purified recombinant GFP protein) detects the enhanced green fluorescent protein in formalin-fixed sections according to the manufacturer's technical information. Comparison of the confocal images (Fig. 1) from the GAD67-GFP mouse with those of Tamamaki et al. (2003) indicates that these antibodies recognize virtually the same neurons in both studies. When slices from wildtype C57BL/6 mice are incubated with both primary antibody and secondary antibodies, no signal is observed.

The GAD67 (clone 1G10.2) monoclonal antibody, directed against recombinant GAD67 protein, reacts with GAD67 from rat, mouse, and human. There is no detectable crossreactivity with GAD65 based on western blot analysis of brain lysates when compared with blots probed with antibodies that react with both GAD65 and GAD67 (manufacturer's data sheets). Immunostaining of





**Figure 1.** GFP is coexpressed with GAD67 (the prototypic GABAergic marker) in telencephalic neurons from a GAD67-GFP knockin mouse. Confocal double fluorescence images corresponding to GFP (green), GAD67 (magenta), and merged images (white). **A–C:** Motor cortex, layers I/II. Coronal section corresponding roughly to bregma +1.4 mm is shown. **D–F:** Striatum. Coronal plane shown as described for **A–C**. **G–I:** CA1 region of the hippocampus showing the stratum oriens (ori), stratum pyramidale (pyr), and stratum radiatum (rad). Coronal section corresponds roughly to bregma –2 mm. Note that in the three brain areas studied, more than 90% of GFP-positive cells are also positive for GAD67. Scale bars = 40  $\mu$ m.

mouse cortical slices showed selective neuronal staining similar to the pattern previously published (Liu et al., 2001).

The reelin (clone G-10) monoclonal antibody was directed against the hinge region of the protein (amino acids 40–189) and recognizes multiple reelin related products based on western analysis (de Bergueyck et al., 1998). The antibody stained Cajal-Retzius cells in the

embryonic forebrain of mice (de Bergueyck et al., 1997). Immunostaining of adult wildtype cortical slices is consistent with previous findings (Liu et al., 2001) and no staining was visible in slices processed from the *reeler* mouse.

The DNMT1 (clone 60B1220.1) monoclonal antibody (directed against amino acids 637–650) has been purified by protein G chromatography and recognizes a single

band of  $\approx 190$  kd based on western blot analysis (Kundakovic et al., 2007; manufacturer's data sheet). The antibody was generated against amino acids 637–650 of the human protein but also crossreacts with mouse DNMT1. Preincubation of the antibody with the antigen eliminates the signal. This antibody has been used to detect DNMT immunohistochemically in cortical neurons (Satta et al., 2008) and in olfactory receptor neurons following formalin fixation (MacDonald et al., 2005).

The DNMT3a (clone 64B1446) monoclonal antibody was generated against recombinant mouse DNMT3a and has been purified by G protein chromatography. The epitope was subsequently mapped to the carboxyl terminus (Chen et al., 2002). Western blot analysis shows a single band that is eliminated upon preabsorption with recombinant DNMT3a (manufacturer's data sheets). Immunohistochemistry data have been reported using this antibody with paraformaldehyde fixed mouse cortical slices (Feng et al., 2005) and olfactory receptor neurons (MacDonald et al., 2005) during development and in the adult.

The NeuN (clone A60) monoclonal antibody was generated against purified brain cell nuclei and recognizes the neuron-specific nuclear protein NeuN (Mullen et al., 1992; Wolf et al., 1996). The antibody recognizes two to three similar-sized bands in the range of 46–48 kd on western blots (manufacturer's data sheets). The antibody has been used extensively to identify neurons in brain slices from a wide variety of species. Immunohistochemical staining is primarily localized in the nucleus of neurons with lighter staining in the cytoplasm. Of those neurons not recognized by the antibody (Purkinje, mitral, and photoreceptor neurons), none are known to be located in coronal slices of the motor cortex (as in Fig. 8).

The VGlut2 (clone 8G9.2) monoclonal antibody was generated using recombinant rat vesicular glutamate transporter 2. The protein A purified antibody recognizes a single band of  $\approx 65$  kd on SDS gels following western blotting and antibody preabsorbed with the VGlut2 immunizing antigen eliminated the signal (Agis-Balboa et al., 2006). Immunohistochemistry in the absence of the primary antibody showed no detectable product (Agis-Balboa et al., 2006; Griffin et al., 2010). We have previously used this antibody to stain glutamatergic neurons in multiple regions of the mouse brain (Agis-Balboa et al., 2007).

### Confocal double fluorescence microscopy

Immunofluorescence labeling was performed by following a modification of the procedure described by Pesold et al. (1998), Veldic et al. (2007), and Agis-Balboa et al. (2007). After labeling with the primary antibodies, slices were incubated with Cy5-labeled goat antimouse or anti-rabbit IgG (diluted 1:1,000; Amersham Biosciences) to

produce red fluorescent staining or Cy2-labeled streptavidin (diluted 1:1,000; Amersham Biosciences) to produce green fluorescent staining. The reactions were carried out in 3% normal goat serum, and 1% bovine serum albumin (BSA) in PBS for 1 hour.

The number of cells in which green and red fluorescence colocalize compared with the number of cells that express only green or only red fluorescence was quantified using confocal microscopy (Leica, Bannockburn, IL) at a magnification of  $40\times$  in a counting box of  $100 \times 100 \times 20 \mu\text{m}$ . The red images were subsequently converted to magenta as indicated below.

### Immunohistochemistry

To obtain neuron-specific nuclear protein (NeuN) and DNMT1 immunolabeling, 20- $\mu\text{m}$  floating sections were incubated in parallel with 1) monoclonal anti-DNMT1 antiserum (1:500; Imgenex, San Diego, CA) or 2) monoclonal anti-NeuN antiserum (diluted 1:500; Chemicon, Billerica, MA). The sections were incubated with the primary antibody for 48 hours at  $4^\circ\text{C}$  and 2 hours at room temperature. After addition of the secondary antibody, sections were stained following a previously described protocol and sections were reacted with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) with nickel-ammonium sulfate to obtain a dark-brown reaction product (Satta et al., 2008). To estimate the relative density of NeuN- and DNMT1-positive neurons in motor cortex, coronal cortical blocks (from 1.4 to +1 mm anterior to bregma) were studied. For each brain area, five to six sections were taken (one every fourth slice) and cells were counted using an "assumption-based" bidimensional cell counting method (Benes and Lange, 2001). The NeuN and DNMT1 counts were performed blindly in three randomly selected squares in each of five to six sections; thus, a total of 15–18 squares per sample were counted. All analyses were carried out in comparable areas ( $100 \times 100 \mu\text{m}$ ) under the same optical and illumination conditions (for example, light intensity was set to 8 on a scale of 1–10, and exposure time was set to 1.5 ms) in a Zeiss Microlmager. Black and white images were digitized and viewed on a computer using AxioVision software.

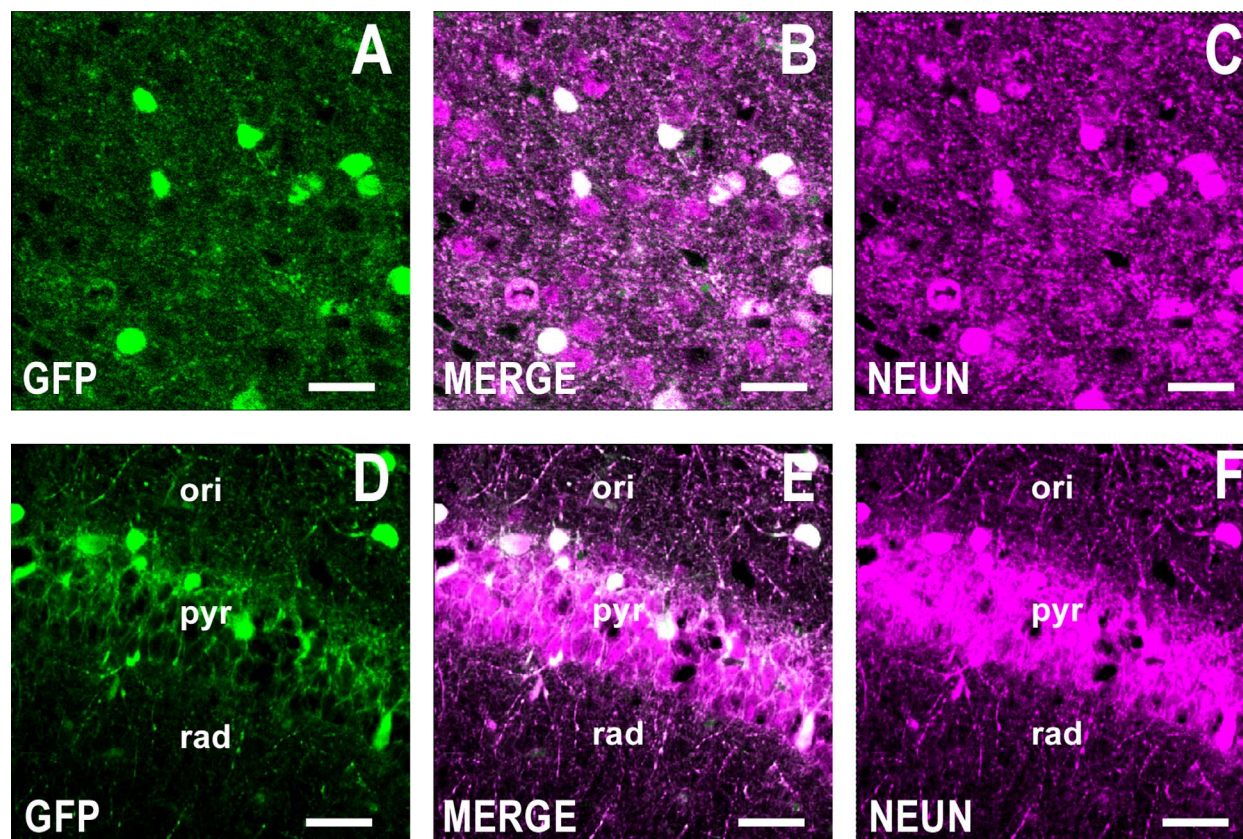
### Statistical analysis

Results are expressed as the mean  $\pm$  SE. Analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used. The criterion for significance was  $*P < 0.05$ .

### Digital photomicrography and production

DAB stained images were captured by AxioVision 3.1 (Zeiss), while confocal immunofluorescent images were captured using a Leica Confocal Microscope (Leica





**Figure 2.** GFP is expressed in NeuN-positive cells of the GAD67-GFP knockin mouse. Roughly 100% of GFP-positive cells are positive for NeuN. Confocal double immunofluorescence labeling of GFP coded in green (left panels), NeuN is color-coded in magenta (right panels), and merged images in white (center panels). A–C: Motor cortex, layers I/II. Coronal section corresponds roughly to bregma +1.4 mm. D–F: CA1 field of the hippocampus. Coronal section corresponds roughly to bregma –2 mm. Scale bars = 40  $\mu$ m.

Microsystems). Photoshop CS4 (Extended v. 11.0, Adobe Systems, San Jose, CAQ) was used to adjust contrast, brightness, and sharpness of the captured images. Red-green fluorescent images were converted to magenta-green images also using Adobe Photoshop CS4. Finally, images were imported into CanvasX System v. 10.5.8 for final figure production and subsequent conversion to tif-format.

## RESULTS

### Colocalization of GFP and GAD67

To confirm the original findings with respect to the colocalization of GFP and GAD67 in the knockin mice, we performed double immunofluorescence histochemistry using antibodies directed against GFP and GAD67. In this first group of experiments we examined the colocalization of these antigens in the frontal cortex, striatum, and hippocampus of heterozygous GAD67-GFP knockin mice with confocal microscopy. As shown in Figure 1, the extent of overlap between GAD67 and GFP fluorescence was greater than 90%. The colocalization of GFP and GAD67 was extended to include additional brain regions

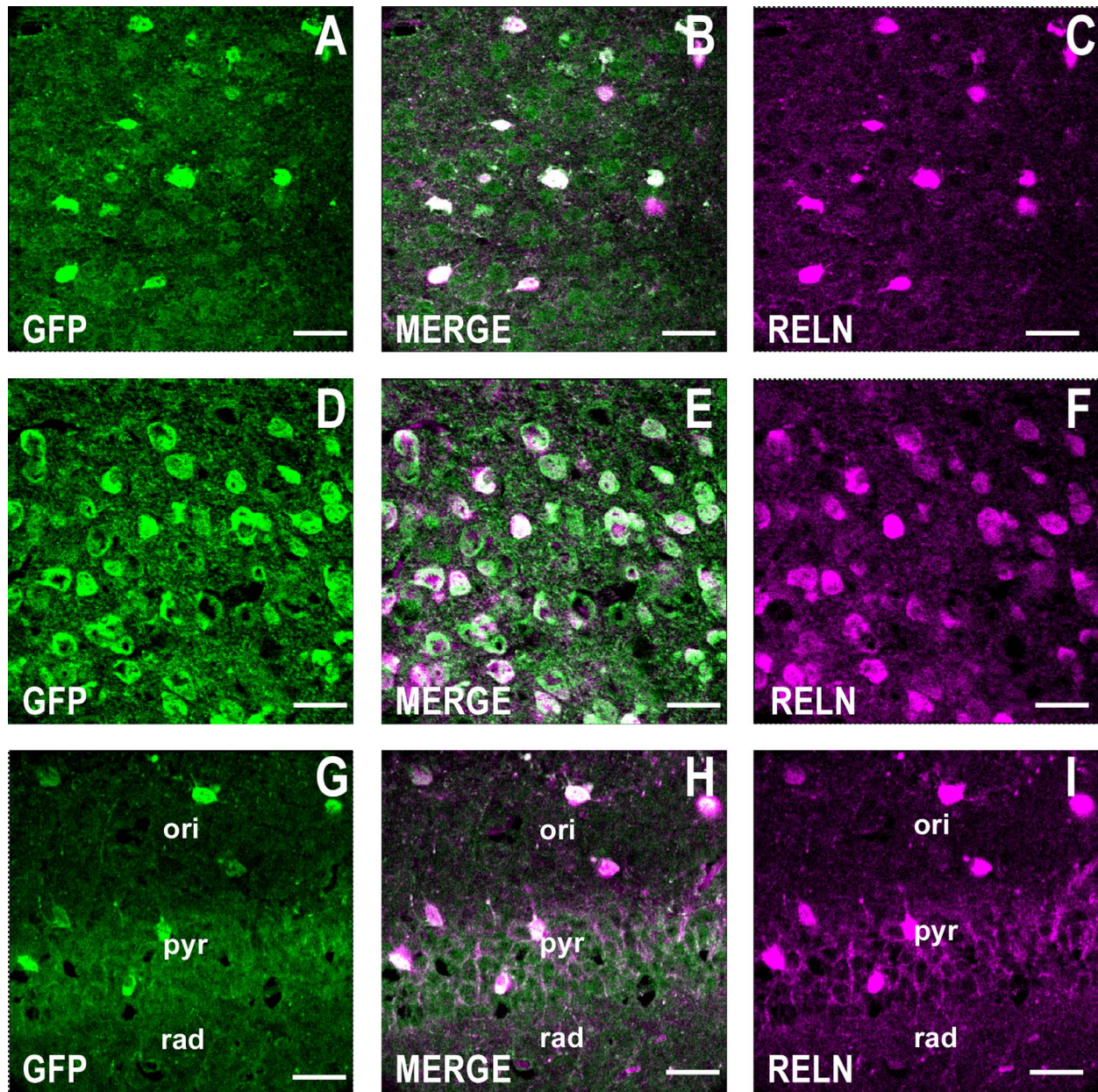
including the piriform cortex and BLA. Cerebellar Purkinje cells showed inconsistent staining, as previously reported (Tamamaki et al., 2003; data not shown).

To confirm previous findings regarding neuronal expression of GFP in the GAD67-GFP knockin mouse (Tamamaki et al., 2003), we examined the colocalization of GFP and NeuN by immunofluorescence histochemistry. In the cortex and hippocampus, virtually all of the GFP-positive cells coexpress NeuN, indicating that GFP is expressed in neurons and not in glial cells (Fig. 2). Collectively, these results demonstrate that GFP expression is regulated by the endogenous GAD67 promoter and that GFP-positive cells are indeed GABAergic neurons.

In the following experiments we used GAD67-GFP mice to investigate whether GFP colocalizes with RELN and the vesicular glutamate transporter 2 (VGLUT2). While RELN is primarily expressed in telencephalic GABAergic neurons in the adult brain (Pesold et al., 1999), VGLUT2 is mostly expressed in glutamatergic neurons in cortical layers IV and VI (Fremeau et al., 2001), in layer V pyramidal neurons (Agis-Balboa et al., 2007), and in principal excitatory neurons in other telencephalic areas. Similar

F2





**Figure 3.** RELN is coexpressed with GFP in telencephalic GABAergic neurons in the GAD67-GFP knockin mouse. A large proportion of the GFP-positive GABAergic neurons are also positive for RELN. Confocal double immunofluorescence labeling of GFP coded in green (left panels), RELN color coded in magenta (right panels), and merged images in white (center panels). **A–C:** Motor cortex, layers I/II. Coronal sections correspond to bregma +1.4 mm. **D–F:** Striatum. Coronal sections are as described in A–C. **G–I:** CA1 field of the hippocampus. Coronal section corresponds roughly to bregma –2 mm. Scale bars = 40  $\mu$ m.

to GAD67, RELN immunofluorescence is only expressed in GFP-positive neurons (Fig. 3). As anticipated, VGLUT2-positive neurons fail to express GFP immunofluorescence (Fig. 4) or RELN (data not shown).

### Colocalization of GFP and DNMTs

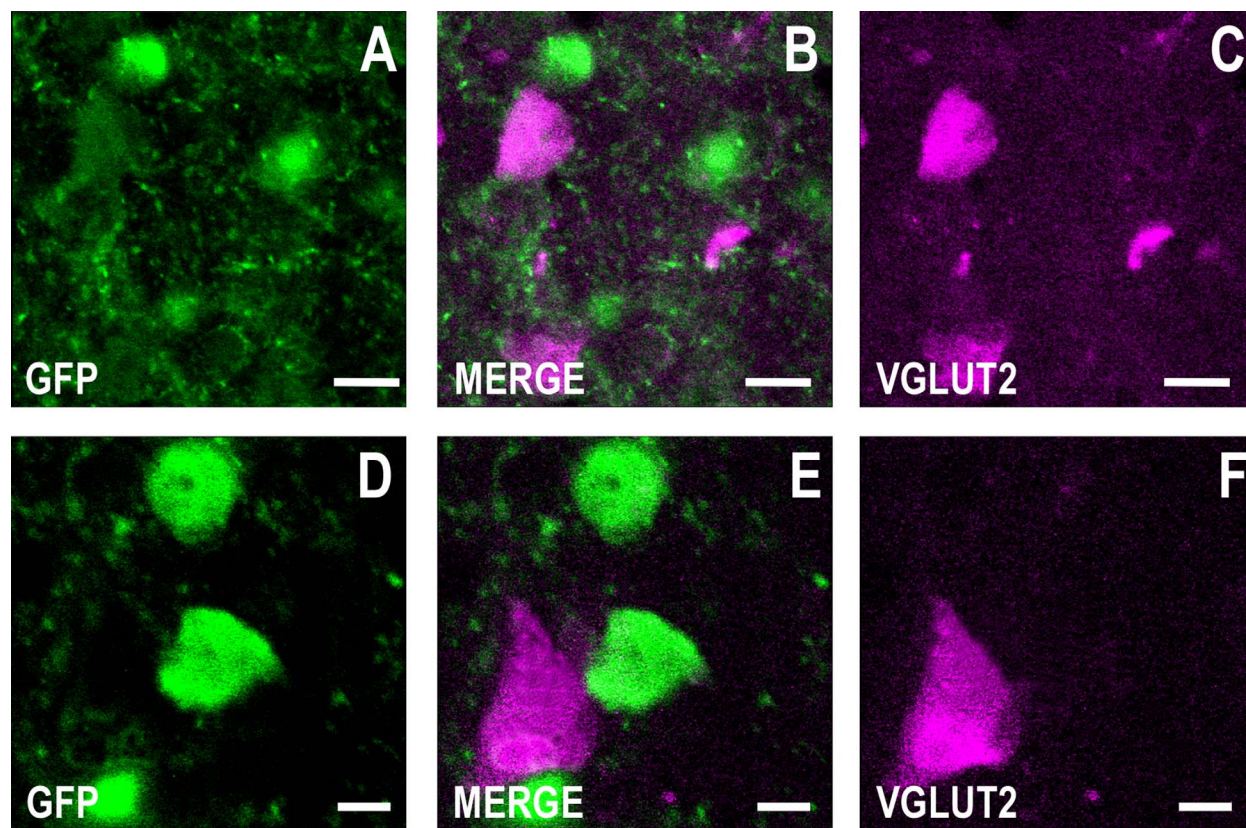
We next examined the colocalization of DNMT1 with GFP in the GAD67-GFP mouse (Fig. 5). In the motor cortex, piriform cortex, striatum, CA1 region of the hippo-

campus, dentate gyrus, and BLA, GFP immunofluorescence coincides with the signal corresponding to DNMT1. It is noteworthy that at larger magnifications GFP immunofluorescence is present throughout the neuronal somata and also includes the neuropil (axon terminals and dendrites), whereas DNMT1 fluorescence is mostly confined to the somata of the GFP-positive cells (Fig. 6).

We also report that similar to DNMT1, in the motor cortex, striatum, hippocampus, and amygdala, GFP

F6





**Figure 4.** GFP is not expressed in cortical glutamatergic neurons in the GAD67-GFP knockin mouse. Note the absence of colocalization between GFP and VGLUT2 immunofluorescence. Confocal double immunofluorescence labeling of GFP coded in green (left panels), RELN color-coded in magenta (right panels), and merged images in white (center panels). A–C: Motor cortex, layer II. D–F: Motor cortex, layer III. Coronal sections correspond roughly to bregma +1.4 mm. Scale bars = 20  $\mu$ m in A–C; 5  $\mu$ m in D–F.

immunofluorescence colocalizes with the fluorescent immunohistochemical signal detected with the DNMT3a F7 antibody (Fig. 7).

### NeuN- and DNMT1-positive neurons in the frontal cortex

F8 In Figure 8 we show that NeuN-immunopositive cells are significantly more abundant than DNMT1-positive cells, particularly in cortical layers III to V. The data reflect a ratio of  $\approx 30\%$  between DNMT1 (GABAergic interneurons) and the total number of neurons in this brain area.

### DISCUSSION

The expression and activity of DNMTs is abundant in dividing cells and is very high, particularly during development. Previous studies have reported that in adult mammalian brains, which primarily contain postmitotic terminally differentiated neurons and glial cells, the expression levels of DNMT1 and DNMT3a mRNA and proteins are evident but, interestingly, their neuronal/cellular distribution is uneven (Inano et al., 2000; Veldic et al., 2004, 2007; Feng et al., 2005). For example, immunohistochemical studies and biochemical measurements of DNMT1 in laser microdissected neurons from prefrontal cortex (PFC)

**Figure 5.** DNMT1 is coexpressed with GFP in corticolimbic GABAergic neurons in the GAD67-GFP knockin mouse. Confocal double immunofluorescence labeling of GFP coded in green (left panels), DNMT1 color coded in magenta (right panels), and merged images in white (center panels). A–C: Layers I and II of the motor cortex. Coronal sections correspond roughly to bregma +1.4 mm. D–F: Layers I and II of the piriform cortex. Coronal section as described in A–C. G–I: Striatum. Coronal section as described in A–C. J–L: CA1 field of the hippocampus. Coronal section corresponding roughly to bregma  $-2$  mm. M–O: Dentate gyrus. Coronal section as in J–L. P–R: Basolateral amygdala (BLA). Coronal section as described in M–O. Note that in all corticolimbic areas studied, greater than 90% of GFP-positive cells are also positive for DNMT1. Scale bars = 40  $\mu$ m.



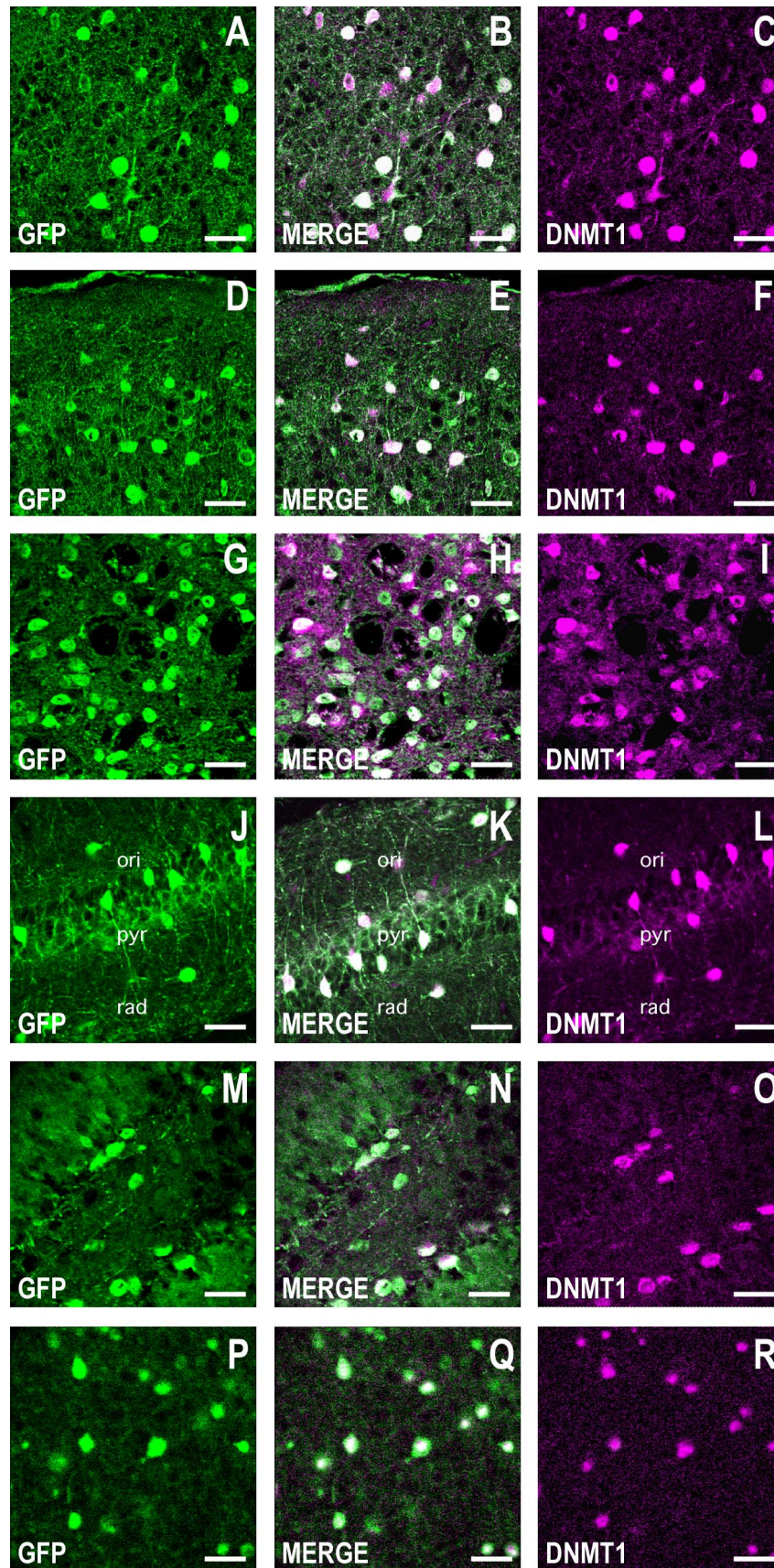
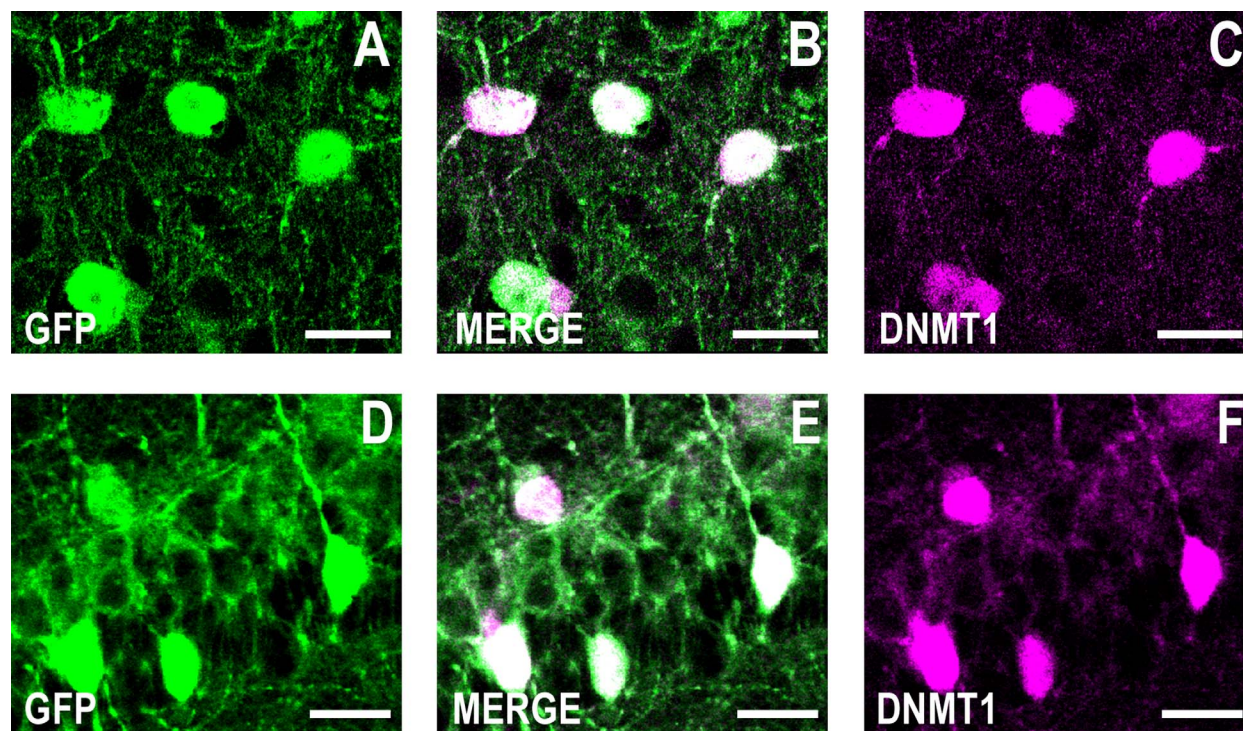


Figure 5.





**Figure 6.** Larger magnification of GFP and DNMT1 immunofluorescence showing that both colocalize to the neuronal somata. Confocal double immunofluorescence labeling of GFP in green (left panels), DNMT1 in magenta (right panels), and merged images in white (middle panels). **A–C:** Larger magnification of Figure 2D–F showing double labeling of GFP and DNMT1 immunofluorescence in the piriform cortex. Coronal section corresponding roughly to bregma +1.4 mm. **D–F:** Larger magnification of Figure 2J–L showing double labeling of GFP and DNMT1 immunofluorescence in the CA1 hippocampal formation. Coronal sections corresponding to bregma –2 mm. Note that while DNMT1 immunofluorescence is primarily limited to the somata, GFP immunofluorescence is also abundant in the neuropil. Scale bars = 20  $\mu$ m.

postmortem human brain indicate that DNMT1 is highly expressed in GABAergic interneurons but, in contrast, is expressed at very low levels in pyramidal neurons and glial cells (Veldic et al., 2005, 2007; Ruzicka et al., 2007; Zhubi et al., 2009).

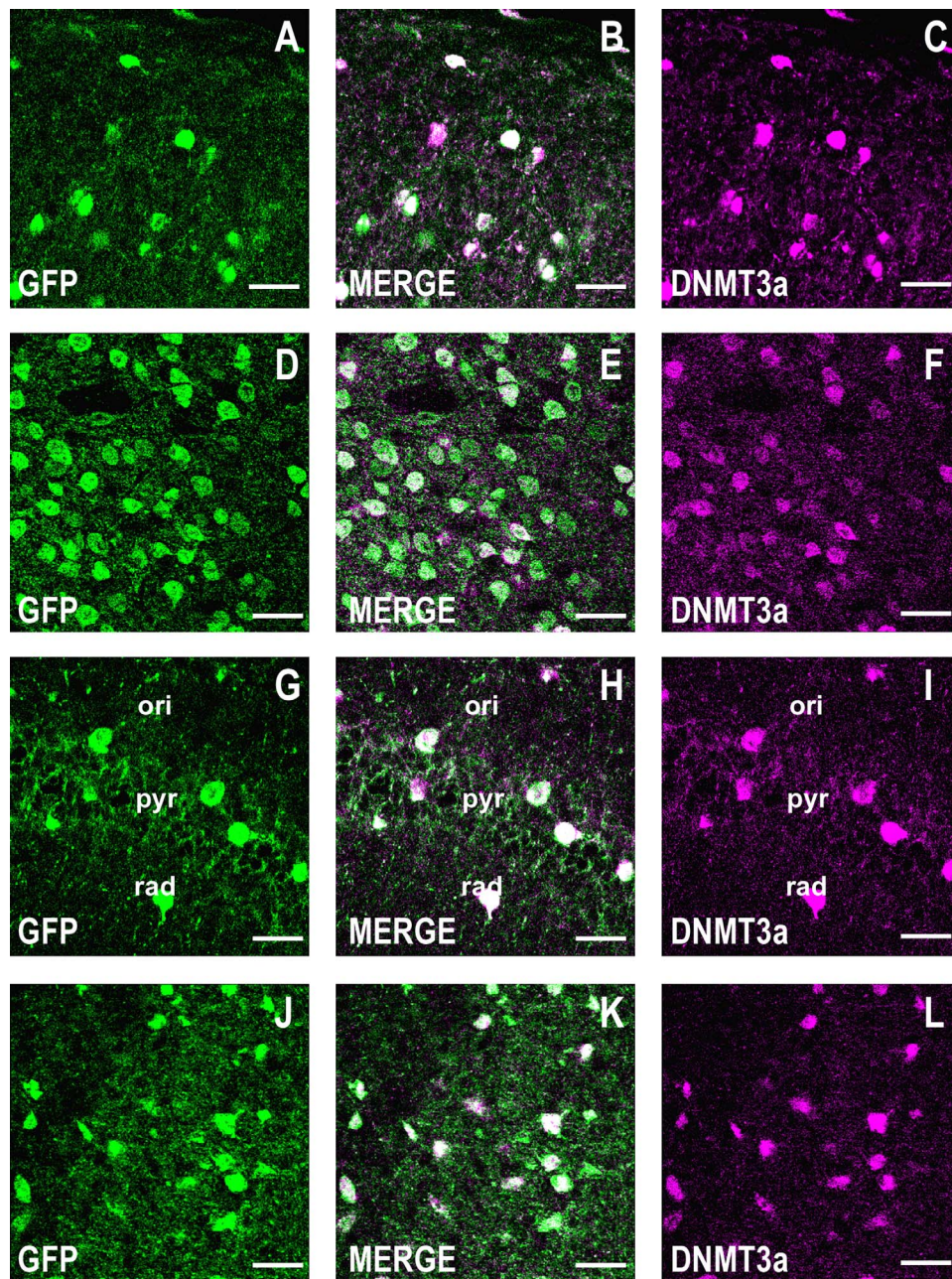
Our data show that the neuronal localization of DNMT1 in adult GAD67-GFP mice is consistent with previous studies of DNMT1 and DNMT3a in mouse neurons (Satta et al., 2008). DNMT1 and DNMT3a are highly expressed in the GAD67-GFP mouse brain and, more important, similar to reports of studies of the human brain, the expression of these proteins is almost exclusively localized to GABAergic interneurons (Veldic et al., 2004, 2005, 2007; Zhubi et al., 2009). For example, virtually all GABAergic GFP-positive neurons in cortex, striatum, hippocampus, and BLA also express high levels of DNMT1 and 3a immunoreactivity. DNMT immunostaining is virtually absent in pyramidal and other glutamatergic principal neurons in the telencephalon of these mice.

We have previously shown in the adult telencephalon of mice (Liu et al., 2001), Patas monkeys (Rodriguez et al., 2002), and humans (Veldic et al., 2005; Ruzicka

et al., 2007) that RELN and GAD67 colocalize. This was further explored in the current study in which we demonstrate a high percentage of GFP-positive neurons are also RELN-positive. In contrast, there is no overlap between GFP and VGLUT2, one of two excitatory amino acid transporters and a marker for glutamatergic neurons (Freneau et al., 2001; Agis-Balboa et al., 2007). Collectively, these data suggest that DNMT1 and DNMT3a may play important roles in the epigenetic regulation of GABAergic gene expression.

In future experiments, we will take advantage of GAD67/GFP mice to isolate populations of cells enriched in GABAergic neurons to study the role of DNMT in the epigenetic control of GABAergic function. GABAergic interneurons are involved in complex tasks such as shaping receptive fields of pyramidal neurons, synchronizing the activity of cortical neurons, controlling information processing within the PFC, and more generally, controlling principal neuron excitability in the cortex, hippocampus, and amygdala (Mountcastle, 1998). To accomplish these functions, GABAergic interneurons express unique patterns of electrical activity and are easily recognized compared with





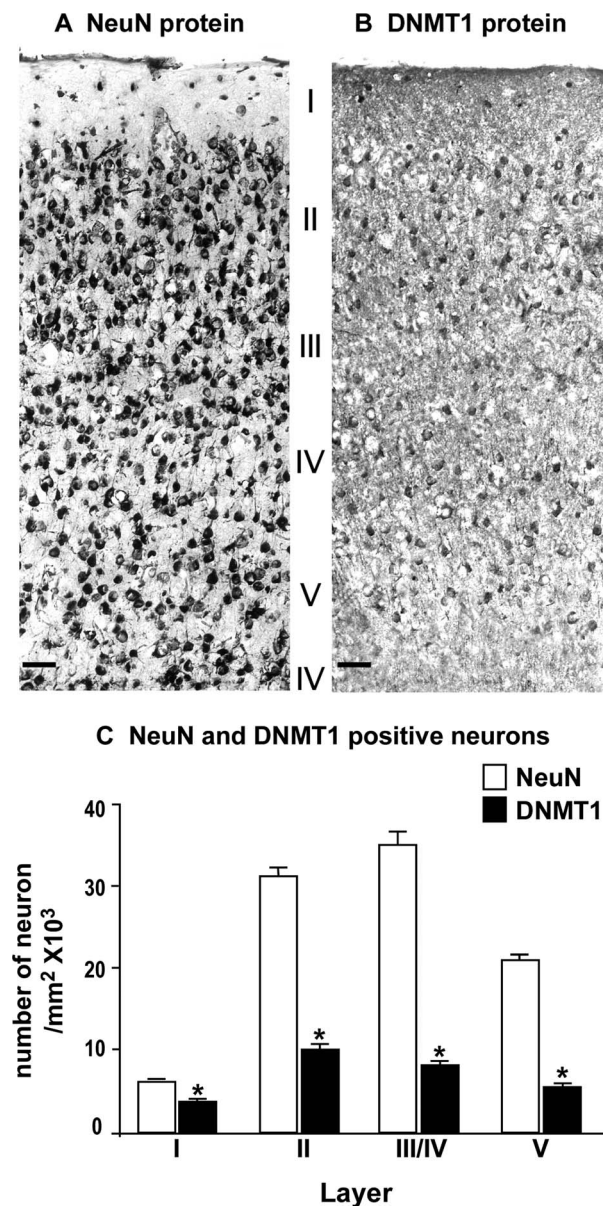
**Figure 7.** DNMT3a is coexpressed with GFP in corticolimbic GABAergic neurons of the GAD67-GFP knockin mouse. Confocal double immunofluorescence labeling of GFP (green), DNMT3a (magenta), and merged images in white (center panels). **A–C:** Layers I and II of the motor cortex. Coronal sections correspond roughly to bregma +1.4 mm. **D–F:** Striatum. Coronal sections as described in A–C. **G–I:** CA1 field of the hippocampus. Coronal sections correspond roughly to bregma –2 mm. **J–L:** Basolateral amygdala. Coronal section corresponding roughly to bregma –2 mm. Scale bars = 40  $\mu$ m.

other types of neurons because of their fast-spiking discharge rate in response to sudden changes in synaptic activity (Gonzalez-Burgos and Lewis, 2008). Hence, a possible role for the high levels of DNMTs in telencephalic GABAergic interneurons may be that of controlling specific GABAergic genes via epigenetic mechanisms regulating transcription. This would include promoters corresponding to genes such as GAD67, RELN, members of the NMDA re-

ceptor subunits, various  $\text{Ca}^{2+}$  binding proteins, and GABA-transporter-1, all of which contribute to the control of neuronal excitability and maintenance of synaptic plasticity (Guidotti et al., 2011).

The absence of detectable DNMT immunoreactivity in glutamatergic principal neurons or glia does not exclude the presence of lower levels of DNMT in these cells. Rather, it indicates that in pyramidal neurons and glia, the levels of





**Figure 8.** Photomicrographs showing examples of DNMT1 and NeuN immunolabeling in a coronal section of motor cortex (bregma +1.4 mm) from the GAD67-GFP knockin mouse. **A:** NeuN immunostaining. **B:** DNMT1 immunostaining. **C:** Average numbers of NeuN- and DNMT1-positive neurons in different cortical layers of a coronal block of motor cortex included between 1.4 mm and 1.0 mm anterior to bregma. Each bar is derived from the mean  $\pm$  SE of 5 mice per group. \* $P < 0.01$  for NeuN vs. DNMT1. ANOVA followed by Bonferroni multiple comparison. Scale bars = 40  $\mu$ m.

DNMT are below the threshold for immunohistochemical detection and are low compared to the level of DNMTs in GABAergic interneurons. In fact, recent studies suggest that DNMTs are present in cortical pyramidal neurons and their presence may play an important role in dendritic arborization and synaptic plasticity (Feng et al., 2010).

To study DNMT1 and DNMT3a loss of function in neurons, conditional mutant mice have been generated and analyzed (Fan et al., 2001; Nguyen et al., 2007). Various cre-recombinase-expressing mouse lines have been used to target DNMT deletions to distinct neurons at specific times during development and in the adult. The promoters that have been used to drive cre expression include nestin (Fan et al., 2001; Nguyen et al., 2007), empty spiracles homeobox-1 (Emx1, Golshani et al., 2005; Hutnick et al., 2009), and CamK (Fan et al., 2001; Feng et al., 2010). Nestin is expressed in many types of cells during early development and in neuronal precursor cells (Dubois et al., 2006). Emx1 is expressed in differentiating and mature glutamatergic cortical neurons (Bishop et al., 2003) and CamK is selectively expressed in a subpopulation of excitatory neurons that show no overlap with GAD67-expressing neurons (Jones et al., 1994). The conditional mutant mice generated with each of these cre-driver lines show clear phenotypes that have been associated with alterations in DNA methylation and the downregulation of affected mRNAs. In Emx1-cre/DNMT1 mutant mice, loss of DNMT1 in Emx1-positive neurons disrupts development of the somatosensory barrel cortex (Golshani et al., 2005) and impairs postnatal neuronal maturation (Hutnick et al., 2009). The double DNMT1/3a conditional knockout in CamK-expressing neurons shows deficits in synaptic plasticity, learning, and memory (Feng et al., 2010). While our results show that GABAergic neurons express much higher amounts of both DNMT1 and 3a, the above studies indicate a function for DNMT1 and DNMT3a in glutamatergic neurons during development and following maturation.

As both DNMT1 and DNMT3a mRNAs are more abundantly expressed in GABAergic neurons in all brain regions examined, the availability of GAD67 cre (Higo et al., 2009)- and parvalbumin cre (Tanahira et al., 2009)-mice provides a unique opportunity to examine the function of DNMTs in GABAergic neurons. Our previous studies demonstrating the increased expression of DNMT1 and DNMT3a in GABAergic neurons of schizophrenia patients (see Guidotti et al., 2005, 2011, for recent reviews) provide a compelling rationale for examining DNMT function in these neurons to gain novel insight into the pathogenesis of this devastating psychiatric disorder.

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