The Spatiotemporal Segregation of GAD Forms Defines Distinct GABA Signaling Functions in the Developing Mouse Olfactory System and Provides Novel Insights into the Origin and Migration of GnRH Neurons

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ABSTRACT: **Gamma-aminobutyric acid (GABA) has a dual role as an inhibitory neurotransmitter in the adult central nervous system (CNS) and as a signaling molecule exerting largely excitatory actions during development. The rate-limiting step of GABA synthesis is catalyzed by two glutamic acid decarboxylase isoforms GAD65 and GAD67 coexpressed in the GABAergic neurons of the CNS. Here we report that the two GADs show virtually nonoverlapping expression patterns consistent with distinct roles in the developing peripheral olfactory system. GAD65 is expressed exclusively in undifferentiated neuronal progenitors confined to the proliferative zones of the sensory vomeronasal and olfactory epithelia In contrast GAD67 is expressed in a subregion of the nonsensory epithelium/vomeronasal organ epithelium containing the putative Gonadotropin-releasing hormone (GnRH) progenitors and GnRH neurons migrating from this region through the frontonasal mesenchyme into the basal forebrain. Only GAD67+, but**

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not GAD65+ cells accumulate detectable GABA. We further demonstrate that GAD67 and its embryonic splice variant embryonic GAD (EGAD) concomitant with GnRH are dynamically regulated during GnRH neuronal migration *in vivo* and in two immortalized cell lines representing migratory (GN11) and postmigratory (GT1–7) stage GnRH neurons, respectively. Analysis of GAD65/67 single and double knock-out embryos revealed that the two GADs play complementary (inhibitory) roles in GnRH migration ultimately modulating the speed and/or direction of GnRH migration. Our results also suggest that GAD65 and GAD67/EGAD characterized by distinct subcellular localization and kinetics have disparate functions during olfactory system development mediating proliferative and migratory responses putatively through specific subcellular GABA pools. Develop Neurobiol 00: 000–000, 2014

Keywords: **GAD forms; gamma-aminobutyric acid; GnRH; olfactory development; cell migration**

INTRODUCTION

In addition to being an inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) exerts excitation and acts as a trophic factor during development modulating many processes at the cellular and tissue level like proliferation, neuronal migration, differentiation and maturation (LoTurco et al., 1995; Barker et al., 1998; Owens and Kriegstein, 2002; Ben-Ari and Holmes, 2005).

The rate-limiting step in the synthesis of GABA is catalyzed by two highly homologous enzyme isoforms of GAD—GAD65 and GAD67 that are coexpressed in the GABAergic inhibitory neurons of the central nervous system (CNS) and differ by kinetic properties and subcellular distribution (Martin et al., 2000). Two alternatively spliced, developmentally regulated embryonic GAD (EGAD) messages are synthesized in the rat (Bond et al., 1990) and mouse (Szabo et al., 1994) and code for two truncated GAD proteins representing the N-terminal (GAD25) and C-terminal (GAD44) portions of GAD67 (Szabo et al., 1994). Both *Gad* genes are expressed from embryonic day 9.5 (E9.5) in the mouse, prior to the onset of GABAergic neuronal differentiation and show a specific spatiotemporal expression profile in the embryonic CNS and non-neuronal structures including the peripheral olfactory system (Katarova et al., 2000).

The development of the olfactory system in the mouse begins at E9.5 with the induction of the olfactory placodes—paired structures comprising a thickening of the rostral head epithelium (Balmer and LaMantia, 2005; Beites et al., 2005; Whitlock, 2005; Treloar et al., 2010; Wray, 2010) and proceeds through a highly coordinated sequence of events leading to formation of the olfactory pit, nasal epithelia, and olfactory bulb. During this period, the olfactory–vomeronasal epithelia go through different stages of elaborate folding within the frontonasal mesenchyme (FNM) concomitant with the generation of several neuronal lineages: the sensory neurons residing in the olfactory epithelium (OE) and vomeronasal epithelium (VNE), as well as different types of migratory neurons including the GnRH neurons (Treloar et al., 2010). The generation of different epithelia and neuronal cell types is thought to occur in a series of epithelial–mesenchymal (E/M) induction events regulated initially by retinoic acid (RA), which is expressed locally in the neural crest-derived mesenchyme underlying the olfactory placodes (Balmer and LaMantia, 2005) and the olfactory pit epithelium (Kawauchi et al., 2004). Three different types of neurons—olfactory and vomeronasal receptor neurons (ORN and VRN, respectively) and GnRH neurons—are born around the placodal stage (E9.5) and later (E10.5) become confined to different, partially overlapping domains of the olfactory pit epithelium that are defined by the expression of transcription factors known to regulate neural differentiation like *Pax6*, *Sox2*, *Mash1*, and neurogenin 1 (*Ngn1*) as well as fibroblast growth factor 8 (*Fgf8*; Beites et al., 2009; Rawson et al., 2010).

It has been shown that the GnRH neurons migrate out of the olfactory placode-derived vomeronasal organ (VNO) along the vomeronasal nerve (VNN) through the septum toward their final destination located in the septal-preoptic area of the ventral hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Mulrenin et al., 1999; Whitlock, 2005; Tobet and Schwarting, 2006; Wray, 2010; Wierman et al., 2011). The adult GnRH neurons project to the median eminence and release GnRH peptide into the hypophyseal portal system and—by stimulating the production and secretion of follicle stimulating hormone and luteinizing hormone—regulate the functions of the gonads, sexual development and reproduction (Wierman et al., 2011).

The GnRH neuronal migration is modulated by numerous factors like adhesion molecules, axonal guidance cues, neurotransmitters, neuropeptides, and growth factors that influence cell-surface attachment

and migration characteristics, such as speed and direction (Tobet and Schwarting, 2006; Cariboni et al., 2007; Cariboni et al., 2011; Cariboni et al., 2012; Messina and Giacobini, 2013). GABA has been shown to be present, presumably transiently in migratory GnRH neurons (Tobet et al., 1996; Fueshko et al., 1998a; Lee et al., 2008) and to inhibit the rate of GnRH neuronal migration in a paracrine fashion via GABA(A) receptor (GABA(A)R) activation *in vitro* (Fueshko et al., 1998b). Similarly, *in vivo* treatment of pregnant dams with the GABA(A)R agonist muscimol inhibits GnRH neuronal migration out of the nasal cavity and their fiber extension to the median eminence (Bless et al., 2000). In contrast, bicuculline (GABA(A)R antagonist) treatment results in dispersion of GnRH neurons within the brain due to dissociation of GnRH neurons from their guiding fibers leading to the suggestion that GABA acting on GABA(A)R modulates cell adhesion molecules and thereby the association of GnRH neurons with VNN axons (Bless et al., 2000). Indeed, a variety of adhesion molecules are involved in the regulation of the GnRH neuronal migration and their absence results in disorganized or impaired migration and consequently disruption of proper sexual development and reproductive cycle (Kottler et al., 2004; Tobet and Schwarting, 2006; Schwarting et al., 2007; Wray, 2010; Zhang et al., 2010; Wierman et al., 2011; Casoni et al., 2012). More recently, *in vitro* time-lapse studies have demonstrated that GABA promotes linear migration of the GnRH neurons out of embryonic nasal explants from E11.5 embryos since their movements are randomized in GAD67 knockout (KO) mice, in which GABA concentration is greatly reduced (Casoni et al., 2012).

GABA also increases the synthesis of the GnRH peptide (Fujioka et al., 2007) that is known in itself to modulate the rate of GnRH neuronal migration through the nasal compartment (Romanelli et al., 2004).

Previous studies using *in situ* hybridization (ISH) have shown that GAD67 is expressed in the olfactory pit/VNO and adjacent respiratory epithelium (RE) at stages E11.5–E16.5 mouse embryos with a low level of staining in axons crossing the nasal septum at E12.5, but not later stages (Wray et al., 1996; Katarova et al., 2000). Forced expression of GAD67 by the GnRH promoter in transgenic mice changes GnRH neuron trajectory but not their speed of migration in the brain resulting in the appearance of GnRH neurons at ectopic positions and causing altered GnRH, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) secretion, altered female estrous cycle and reproductive function (Heger et al., 2003). In GAD67 KO mice, GnRH neurons show altered migration speed compared to wild type (WT), but unchanged cell number (Lee et al., 2008).

Clearly, although the data so far show that GAD67 is expressed during GnRH neuronal migration, its precise spatiotemporal regulation and its exact role in this process is still obscure. Furthermore, the expression regulation and possible role(s) of the other GAD forms: the embryonic GAD derived from the *Gad1* gene (Szabo et al., 1994) and that of GAD65 in the developing peripheral olfactory system, especially in the development and migration of the GnRH neurons have not been elucidated so far.

Here we show that (1) GAD65 and GAD67 display segregated expression in the embryonic olfactory system associated with distinct properties: GAD65 is expressed in ORN/VRN progenitors anchored in the proliferative layers of the VNO/OE, while GAD67 is found in the nonsensory epithelium (NSE) of the VNO/RE border region and in migratory GnRH neurons throughout their journey; (2) GAD67+ VNO and GnRH cells but not the GAD65+ VRN/ORN progenitors accumulate GABA; (3) GAD67 and its splice variant EGAD are dynamically regulated *in vivo* and *in vitro* in the migrating GnRH; (4) despite their segregated expression, the GAD forms affect similarly, and therefore, have complementary roles in the precision and timing of GnRH migration by modulating intercellular interactions between GnRH and VRN/ORN axons.

METHODS

Animal use

Mice of strain FVB/Ant (Errijgers et al., 2007) housed in the Division of Medical Gene Technology of the Institute of Experimental Medicine were used. All experiments with animals were conducted in compliance with NIH (NIH Publication #85-23, 1985) and EC (86/609/EEC/2) guidelines and approved by in-house and national committees.

Transgenic and KO mice

Transgenic mice were derived by pronuclear microinjection into FVB/Ant fertilized eggs following standard protocols (Brinster et al., 1981; Costantini and Lacy, 1981). Two different GFP-green fluorescent protein (GAD65-GFP) transgenic mouse lines were used in this study. GAD65_2e/gfp (line #30) carries a 6336 bp segment of the mouse *Gad2* gene (encoding GAD65) spanning 5.1 kb of the 5′-upstream region, the first exon, first intron and a segment of the second exon with enhanced GFP (eGFP) fused inframe to the second exon and expressed as a fusion GAD65-eGFP protein (Supporting Information Fig. S1B). In this line,

GFP is expressed faithfully in the inhibitory GABAergic neurons of the CNS (Lopez-Bendito et al., 2004) and in non-neuronal tissues (Kwakowsky et al., 2007). The GAD65_1e-6e/gfp transgenic mouse line #5 was derived with a DNA construct containing additional 6.5 kb downstream in comparison to GAD65_2e/gfp and includes exons 1–6 with the respective introns. EGFP was fused into the first ATG codon of GAD65 found in exon 1 (Supporting Information Fig. S1B). Transgenic founders were identified by real-time PCR using GFP TaqMan® assay on genomic DNA obtained from tail biopsies. All transgenic progenies were routinely identified by GFP fluorescence at P0–P2 (postnatal day 0–2) using GFP-MDS-96/BN light system (BLS, Budapest, Hungary).

GAD65 KO (Yamamoto et al., 2004; Kubo et al., 2009) and GAD67-GFP KI (Tamamaki et al., 2003) (Supporting Information Fig. S1A) mice have been described previously and were genotyped by genomic PCR using specific primers (Supporting Information Table S1). The phenotype of the double GAD65/GAD67-GFP KI mice was identical to the previously described GAD65/GAD67 double KO mice (Ji et al., 1999) and these mice are herein referred to as GAD65/67 double KO.

GAD65, GAD67 single, and GAD65/67 double KO embryos were derived from pregnant dams upon mating between single or double heterozygous mice and genotyped using tail DNA; the day of the copulation plug was considered day 0.5 p.c. (post-coitum).

In Situ Hybridization

GAD65 and GAD67 cDNAs were subcloned into pGEM-T Easy vector (Promega, Madison, WI), plasmids were linearized and run on preparative 0.8% agarose gels in Tris–Acetate–EDTA (TAE) buffer. Linear DNA templates were isolated using GeneJET™ Gel Extraction Kit (Fermentas UAB, Vilnius, Lithuania). Digoxin-labeled GAD65 and GAD67 riboprobes were synthesized using a DIG RNA Labeling Kit (Roche Applied Science, IN) and 1 μg template DNA/reaction.

Embryos were derived by caesarean section from timed pregnant females, then rinsed immediately in ice-cold diethylpyrocarbonate (DEPC)-treated phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS, pH 7.4 overnight at 4°C. Following cryoprotection in graded sucrose solutions (10% then 20% sucrose in DEPC-PBS), the embryos were embedded in OCT (Tissue-Tek; Sakura Finetek) and sectioned on a cryostat (MICROM International GmbH, Walldorf, Germany). Sections of 20 μm thick were collected on silane (3-aminopropyltriethoxysilane; Sigma-Aldrich, St. Louis, MO)-subbed slides and air-dried. Slides were washed several times in DEPC-PBS, treated with Proteinase K (Sigma-Aldrich, 2 μg/mL in DEPC-PBS) for 10 min at room temperature (RT) and washed in DEPC-PBS. Prehybridization was at RT in hybridization buffer (50% formamide, 5× Saline-Sodium Citrate (SSC), 0.1% Tween-20, 10 μg/mL tRNA). Hybridization with GAD65 or GAD67 riboprobes (100 ng/mL in hybridization buffer) was performed in a humidified chamber at 56°C overnight. The next day sections were washed in 2× SSC-T (SSC+0.1% Tween-20), 0.2× SSC-T at 56°C for 20 min and then in PBS with 0.1% Tween-20 (PBST) at RT. Following blocking in 2% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 2 mg/mL bovine serum albumin (BSA; Sigma-Aldrich) in PBST, alkaline phosphatase (AP)-conjugated anti-DIG IgG (Roche, 1:4000 in 2% FBS) was applied overnight at 4°C. Sections were washed 3 × 10 min in PBST containing 2 m*M* levamisole (Sigma-Aldrich) at RT and stained with nitro-blue tetrasolium chloride (NBT)/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP) according to the protocol supplied by Roche. Slides were examined under Zeiss Axioskop-2 equipped with AxioCam HRc digital camera controlled by AxioVision 4.6 software (Carl-Zeiss, Jena, Germany).

Immunohistochemistry

GnRH. Whenever GnRH immunohistochemistry (IHC) was performed following ISH, the sections were washed with PBS for 3 × 5 min at RT and blocked with 10% horse serum—1% BSA in PBST for 1 h at RT. Subsequently sections were incubated with rabbit anti-GnRH antibody (Progen GmbH, Heidelberg, Germany; 1:1000) in 1% BSA-PBST overnight at 4°C. The specificity of the antibody has been verified by preadsorption with GnRH peptide (Ohlsson et al., 2010). Biotinylated goat IgG (Vector Labs, Burlingame, CA; 1:500) diluted in PBST was applied for two hours at RT followed either by ExtrAvidin–Horseradish peroxidase (HRP) (Sigma-Aldrich; 1:500) or Streptavidin-Cy3 (Life Technologies; Carlsbad, CA; 1:5000) in PBS for 90 min at RT. For HRP reaction, sections were washed in PBS and TBS (pH 7.4) and developed in 0.05 *M* 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) with 0.001% H_2O_2 in TBS. Sections were finally washed several times in PBS and coverslipped in DPX (Sigma-Aldrich).

GABA. For GABA IHC, fixation was performed in 4% PFA–0.1% glutaraldehyde–PBS overnight at 4°C. Rabbit polyclonal anti-GABA (Sigma-Aldrich; 1:5000), followed by anti-rabbit biotinylated (Vector; 1:500) antibodies and finally Streptavidin-Cy3 (Life Technologies; 1:5000) were applied essentially as described for GnRH.

Ki-67, OMP, Peripherin, GAD65. Mouse embryos were collected in ice-cold PBS, fixed in 4% PFA–0.1 *M* phosphate buffer (PB, pH 7.4) overnight at 4°C and cryoprotected in 20% sucrose–PBS. Cryostat sections (20 μm) were collected on gelatin-subbed slides and air-dried. Blocking was in 0.5% blocking reagent (Roche) in PBST for 1 h at RT. The rabbit polyclonal antibody Ki-67 (Abcam, Cambridge, UK; 1:1000), the monoclonal GAD65-specific antibody GAD-6 (see below), goat anti-olfactory marker protein (OMP) serum (Monti-Graziadei et al., 1977; Farbman and Margolis, 1980; Margolis, 1982), or rabbit anti-peripherin (Merck Millipore Billerica, MA; 1:500) were applied in 1% BSA-PBST overnight at 4°C. Following several washes in PBST, anti-rabbit Alexa 594-

conjugated or anti-goat Alexa 488-conjugated secondary antibodies (Life Technologies) were applied for 1 h at RT. Slides were washed in PBST and mounted in Mowiol (Calbiochem, San Diego, CA) containing DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma-Aldrich; 0.1 mg/mL).

Whole-Mount IHC of Mouse Embryos

The GAD65-specific mouse monoclonal antibody GAD-6 developed by Gottlieb et al. (1986) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Rabbit serum #6799 was previously characterized (Katarova et al., 1990). Mouse embryos stages E9.5 and E10.5 were derived as described above and fixed overnight at 4°C in 4% PFA–0.05% picric acid–0.1 *M* PB, pH 7.4 with gentle rocking, followed by 3 × 30 min washes in PBS and TBS. Blocking was for 2 h at RT with 0.5% blocking reagent (Roche) in TBS containing either 0.02% saponin (for GAD-6) or 0.1% Triton X-100 (6799; both from Sigma-Aldrich) followed by incubation with primary antibodies diluted in 1% BSA-tris-buffered saline +0.05% tween-20 (TBST) for 48 h at 4°C. The embryos were washed 4 × 30 min in TBST, then incubated with anti-rabbit (mouse)-Alexa 594 in 1% BSA-TBST overnight at 4°C, washed extensively in TBST and photographed under Zeiss LUMAR fluorescent stereomicroscope equipped with AxioCam MR camera (Carl Zeiss).

Cell Culture and Immunocytochemistry

The immortalized, GnRH-expressing cell lines GN-11 and GT1–7 were generously provided by Roberto Maggi (Institute of Endocrinology, University of Milan, Milano, Italy). Cells were routinely grown as monolayers in Dulbeco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% FBS, 4500 mg/L glucose, 1 mmol/L sodium pyruvate, and 2 mmol/L L-glutamine as previously described (Maggi et al., 2000; Lentini et al., 2008).

For immunocytochemistry, cells were seeded onto glass coverslips in 24-well plates and grown for 24–48 h, then rinsed with PBS and immediately fixed with either 4% PFA or 4% PFA–0.1% glutaraldehyde for GABA staining for 20 min at RT, followed by 3 × 5 min washes in PBS. For GAD and GnRH immunocytochemistry, cells were permeabilized with 0.2% Triton-X100 for 5 min at RT and blocked for 1 h at RT in 5% normal goat serum-PBST. Primary antibodies were applied as follows: mouse anti-GAD65 clone GAD-6 at 1:250; rabbit polyclonal sera #6799 (anti-GAD67; 1:500) and #8878 (anti-GAD44; 1:500); (Szabo et al., 1994); or rabbit anti-GnRH (1:1000, Progen) overnight at 4°C. Coverslips were washed 3 × 10 min at RT in PBST then anti-rabbit or anti-mouse biotinylated IgG (1:500; both from Vector) was applied for 90 min at RT. After 3 × 10 min washes in PBST coverslips were incubated with ExtrAvidin-HRP (1:500, Sigma-Aldrich) for 1 h at RT, washed 3 × 10 min in PBS, 1 × 5 min in TBS, and reacted with 0.3 mg/mL DAB (Sigma-Aldrich) in TBS, in the presence of 0.001% H_2O_2. Staining for GABA was performed as follows: blocking in 10% normal horse serum, 1% BSA in PBST for 30 min at RT followed by incubation with rabbit anti-GABA antibody (Sigma, 1:5000) overnight at 4°C, 3 × 10 min washes in PBST and incubation with secondary and tertiary antibodies as described for GAD antibodies.

RNA Preparation and RT-PCR

Transgenic Embryos. Cell biopsies were dissected along the migratory route of GnRH neurons from sections obtained from GAD65_1e-6e/gfp E13.5 embryos then fixed in 0.4% PFA–DEPC-treated PBS overnight at 4°C with gentle rocking. After cryoprotection in 20% sucrose, in DEPC-treated PBS, series of 25-μm-sagittal sections were cut on a cryostat, mounted on silane-subbed slides and briefly dried at RT. GFP+ cell groups from the VNO or GnRH neuronal migratory route were identified under Zeiss LUMAR fluorescent stereomicroscope (Carl Zeiss) and isolated manually as demonstrated schematically in Figure 6(A). Total RNA was prepared using the NucleoSpin® RNA XS (Macherey-Nagel, Düren, Germany) kit and reverse transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies). Amplification parameters and primers were as outlined in Supporting Information Table S2: denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s; annealing at 59.8°C (GAD65); 62°C (GAD67); 65°C (GnRH) for 45 s, extension at 72°C for 10 min. In some experiments, nested PCR was performed using primers listed in Supporting Information Table S2.

Cultured Cell Lines and Unfixed Tissue. Total RNA from brain or GN-11 or GT1–7 cell lines was isolated using TRI-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. Three micrograms of RNA were reverse-transcribed using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers according to the manufacturer's instructions. PCR amplification was performed with specific primers and cycle conditions outlined in Supporting Information Table S2, with final extension at 72°C for 10 min. β-actin was coamplified as control for 16–20 cycles either in the same tube or separately at identical conditions.

GnRH+ Cell Number Estimation in WT and KO Mouse Embryos

The number and spatial distribution of GnRH neurons was investigated in GAD65 and GAD67 KO and WT embryos at ages of E12.5 and E14.5. Three embryos were analyzed in each experimental group. GnRH IHC was performed as described above. Every GnRH+ neuron was counted in each consecutive sagittal section within two compartments: (1) the nasal septum and (2) the nasal–forebrain junction (NFJ). The number of cells per region and total cell number were estimated for each animal as the average of counted cells.

The distribution of GnRH+ neuronal clusters along the VNN and NFJ was obtained by counting all GnRH+ cell

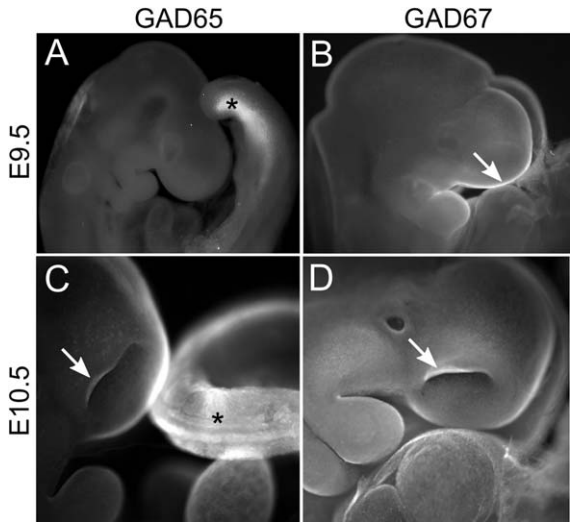

Figure 1 Expression of GAD65 and GAD67 in the frontonasal region of E9.5–E10.5 embryos: whole mount indirect immunofluorescence. (A, C) Staining with the anti-GAD65 antibody GAD-6. (B, D) staining was performed with anti-GAD67 serum #6799. Arrow in (B): strong staining of the olfactory placode. Arrows in (C, D) point to the partially overlapping expression in the olfactory pit labeled by GAD65 (C) or GAD67 (D) with the latter producing a much stronger signal. (*) marks the strong GAD65 signal in the tailbud.

profiles within a migratory group (cohort) along the VNN in E13.5 double GAD KO and WT embryos ($n = 3$). The cumulative size probability of GnRH+ cohorts was calculated and plotted for their visual representation. We defined a cohort as a group of migrating GnRH neurons with seemingly adjacent cell bodies.

The spatial distribution of the migratory GnRH+ neurons was obtained by 3D reconstruction using a computer-assisted morphometry system consisting of a Zeiss Axioskop 2 mot microscope equipped with an MS-88 two axis computer-controlled stepping motor system and a DV-47d color digital camera assisted by Neurolucida and NeuroExplorer software (MBF Bioscience, Williston, VT). All GnRH+ neurons were traced for reconstruction**.** Datasets of pair-matched adjacent sections were then exported to NeuroExplorer to generate 3D reconstructions of the GnRH neuronal migratory pathway.

RESULTS

Expression of GAD65 and GAD67 mRNAs in the Frontonasal Region of Mouse Embryos

GAD65 and GAD67 at E9.5–E10.5. Using whole-mount IHC we were unable to detect GAD65 in the olfactory placodes at stage E9.5, the earliest stage of peripheral olfactory system development [Fig. 1(A)]. In contrast, strong expression signal over the olfactory placode area with a faint staining in the underlying mesenchyme of GAD67 was observed [Fig. 1(B)]. At E10.5, weak GAD65 staining was detected over the rim of the lateral aspect of the olfactory pit invagination [Fig. 1(C)]. Sections for the same-age embryo hybridized with GAD65 antisense RNA revealed staining of the central olfactory pit epithelium as well. At this stage, GAD67 showed a much stronger expression in the rim of the olfactory pit and a diffuse staining in the underlying mesenchyme; the anterior (facial) surface ectoderm was also strongly labeled [Fig. 1(D)].

GAD65 (E13.5–E15.5). Our previous studies showed that GAD65 mRNA is detectable at E11.5 in the forming VNO/olfactory pit, but is absent from the nasal region of E12.5 embryo (Katarova et al., 2000). Here we have performed GAD65 DIG-ISH on series of sections from E13.5 and E15.5 mouse embryos, which demonstrated that at E13.5, GAD65 is expressed exclusively in the sensory epithelium of the VNO and OE [Fig. 2(B-D)]. No signal was observed over the nonsensory RE [Fig. 2(B)]. The hybridization signal at stage E13.5 was overall weak and visibly enriched over the basal and apical layers in both OE and VNO, although labeled cells were also detected scattered in the thickness of the epithelium. At E15.5 and later stages the hybridization signal became much stronger and shifted to basal layer expression in both OE and VNO [Fig. 2(A, E–I)]. A characteristic feature of the GAD65 expression pattern in the OE is the conspicuous patches comprised cell clusters interrupted by regions of low or no expression [Fig. 2(G, H)].

At these stages of development, the OE is a pseudostratified neuroepithelium that comprises cells positioned in pseudolayers as basal and apical pseudolayers containing proliferating globose basal (GBC) and supporting (sustentacular) cells, respectively, and the middle pseudolayer containing differentiating ORNs (Carson et al., 2006; Treloar et al., 2010) [Fig. 3(D)]. At E11–E14, the majority of dividing cells are found apically in the OE, while later in development proliferation occurs predominantly near the base of the OE (Rosenbaum et al., 2011). The ORNs differentiate from the self-renewing GBCs, which give rise to transit-amplifying achaete-scute homolog 1 (*Mash 1*) and neurogenin 1 (*Ngn1*)-expressing INPs that differentiate into postmitotic ORNs identified by staining for OMP (Margolis, 1982).

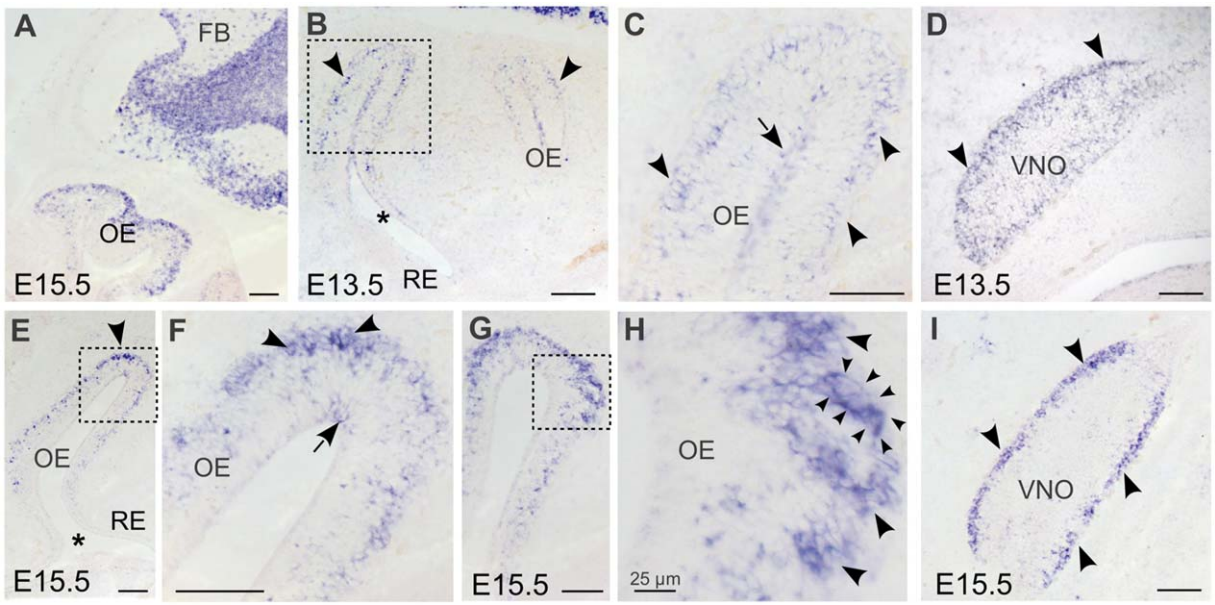

Figure 2 GAD65 expression in the developing nasal region of mouse embryos: ISH on sagittal sections. GAD65 gene is expressed in the olfactory epithelium (OE) and vomeronasal organ (VNO) of E13.5 (B–D) and E15.5 (A; E–I) mouse embryos. GAD65 expression is confined to the basal (arrowheads in B, C) and apical (arrows in B, C) layers of E13.5 OE and mostly to the basal layer (arrowheads in E, F, H) of E15.5 OE. In the VNO of E13.5, GAD65 is partially enriched (D, arrowheads), while at E15.5, it is confined exclusively to the basal layer (I, arrowheads). Within the OE, GAD65 signal was often found in vertical cell columns (F–H, arrowheads). No signal is detected over the respiratory epithelium (RE) (B, E). The lumen of the olfactory canal is marked with an asterisk. Boxed areas in B, E, and G are shown enlarged in C, F, and H, respectively. Scale bars: 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The GAD65 mRNA-expressing cells of the OE and VNO confine to the basal/apical layers at E13.5–E14.5 [Figs. 2 and 3(A)] or the basal layer alone at E15.5 and later stages (Fig. 2 and data not shown), which contain cells undergoing active proliferation (Carson et al., 2006; Beites et al., 2009; Tucker et al., 2010; Rosenbaum et al., 2011). Hence, we applied an antibody raised against the proliferation marker Ki-67, which delineates the borders of the proliferative zones (Rosenbaum et al., 2011). We found that at E14.5 GAD65+ cells codistribute with the Ki-67+ mitotically active progenitors to the basal/apical epithelial layers [Fig 3(A, B and scheme in D)], while at stages E15.5 or later, GAD65 was found exclusively in the basal layer containing undifferentiated ORN precursors only, the apical layer containing Ki-67+ sustentacular cell progenitors (Rosenbaum et al., 2011) was devoid of GAD65 signal (Fig. 2). However, only a subpopulation of GAD65+ cells of the basal layer appeared to be mitotically active and expressed Ki-67 (Supporting information Fig. S2). Postmitotic ORNs of the intermediate layer identified with an anti-OMP antibody (Keller and Margolis, 1975) [Fig. 3(C,D)] do not stain for GAD65 at any stage [compare to (A)].

Using of GAD65 ISH on sections from GAD65-GFP transgenic mice expressing GFP in immature and differentiated ORN and some post-mitotic progenitors, we detected GAD65 and GFP colocalization exclusively in the previously described GAD65+ cell clusters of the basal layers of VNO and OE containing rounded undifferentiated cells, but not in elongating cells with morphological features of differentiating neurons (Supporting Information Fig. S3).

GAD67 (E11.5–E15.5). Strong expression of GAD67 during E11.5–12.5 was detected in the ventral aspect of the olfactory pit, the forming VNO and an area of RE continuous with the VNO (Katarova et al., 2000). [Fig. 4(A–B)]. This pattern was preserved also at later stages [E13.5–E15.5; Fig. 4(D, K)]. In addition, GAD67 was also prominently expressed in a population of migratory cells in the nasal septum migrating by adhering to the olfactory (vomeronasal) nerve that

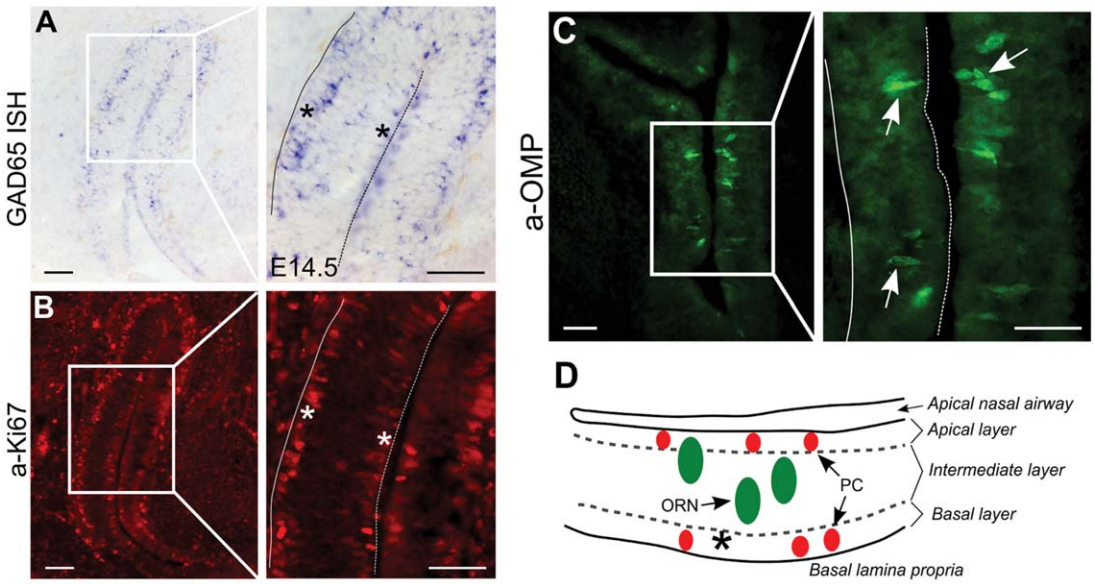

Figure 3 GAD65 is confined to the proliferative basal/apical layers of the OE. Right panels represent enlargements of the boxed areas in the left panels. Coronal sections from E14.5 embryos were processed for: (A) GAD65 ISH; (B) Immunofluorescence (IF) with antibody specific for the proliferation marker Ki-67 (a-Ki-67); (C) IF with the anti-olfactory marker protein (OMP) goat serum (a-OMP #255), a marker for postmitotic ORN; white arrows, OMP+ postmitotic ORNs. Apical and basal layers with borders defined by Ki67-labeled cells are marked with asterisks (A, B). (D) Schematic presentation of the cell type distribution in the E14.5 olfactory epithelium: proliferating cells (PC; in red) are confined to the apical and basal layer, respectively; postmitotic olfactory receptor neurons (ORN; in green) occupy the intermediate layer. Scale bars: 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

by location and appearance resemble the migratory GnRH neurons (Tobet et al., 1996) [Fig. 4(K–M)]. This observation was verified by a combined GAD67 ISH—GnRH IHC, which demonstrated an extensive overlapping between GAD67 and GnRH expression in the FNM [Fig. 4(I–J)]. Migratory neurons coexpressing GAD67 mRNA and GnRH peptide were found in both the nasal septum [Fig. 4(I, J, N)] and NFJ [Fig. 4(N), inset]. Along this route, some neurons expressed either GnRH peptide or GAD67 alone [Fig. 4(N-inset, O)]. Staining with anti-GABA antibody revealed accumulation of the neurotransmitter in a region of the ventral VNO distinguished by GAD67 ISH [Fig. 4(D)] and chain-migrating neurons staining for GnRH [Fig. 4(E, F)].

We also investigated the expression of GFP in GAD67-GFP KI (knock-in) embryos [Fig. 4(C, G, H)]. In this mutant mouse line, GFP is under regulation from the endogenous GAD67 promoter; (Tamamaki et al., 2003); targeting construct shown in Supporting Information Figure S1A. In the brain, it recapitulates the expression pattern of the cognate gene (Tamamaki et al., 2003). Similarly, the GFP expression in the VNO and adjacent NSE of E12.5–13.5 GAD67-GFP KI embryos [Fig. 4(C, G, H)] and in migratory cells (putative GnRH neurons) emerging from the VNO [Fig. 4(G, H)] was highly reminiscent to the pattern obtained by GAD67 ISH [Fig. 4(B, D, K-inset)]. The migratory cells seen in the vicinity of VNO were only weakly labeled by GFP at E13.5 [Fig. 4(G)] and barely, if at all detectable beyond this stage [Fig. 4(H)]. This early silencing of the GAD67 promoter is likely to result from disrupted epigenetic regulation caused by the GFP insertion.

GFP Provides an Useful Marker for Migratory GnRH+ Neurons in GAD65-GFP Transgenic Mice

Transgenic GAD65-GFP mice carrying fusion genes between GFP and the regulatory region of GAD65 were derived with two different constructs GAD65_1e-6e/gfp and GAD65_2e/gfp carrying different portions of the *Gad2* gene (Supporting Information Fig. S1B; Methods). The two lines exhibited almost identical patterns of expression in the nasal region, but differed substantially in the forebrain

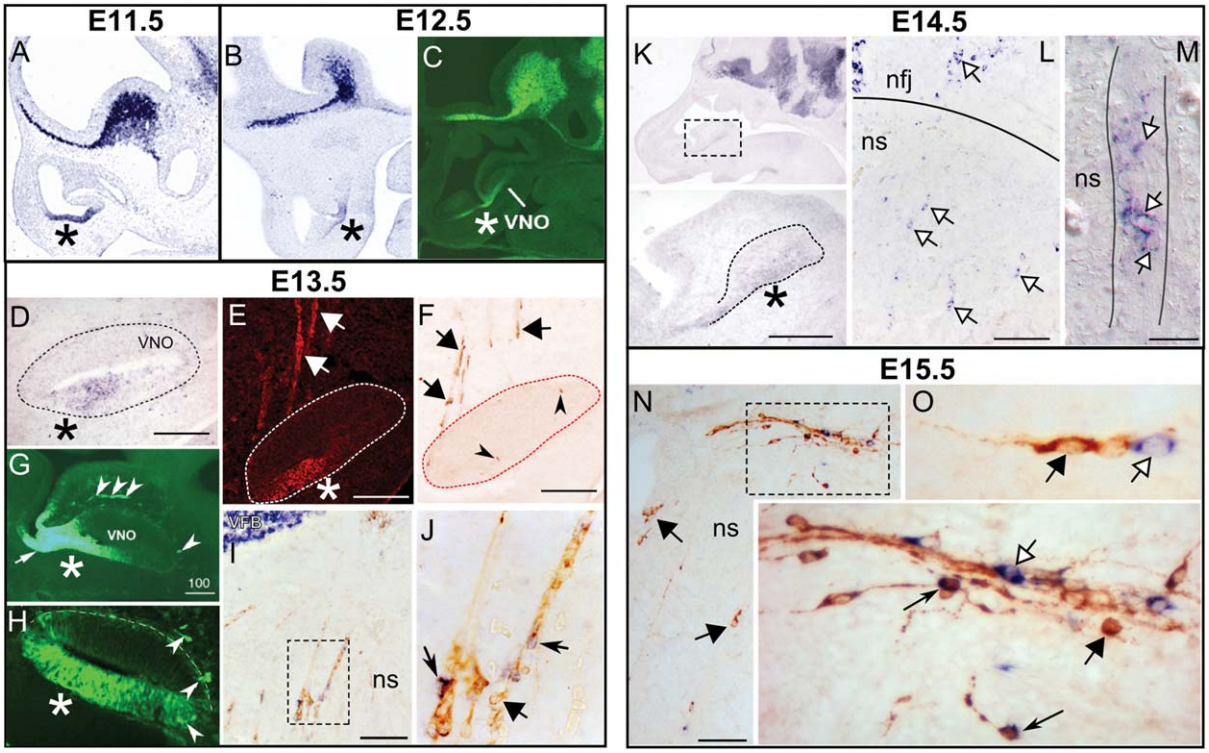

Figure 4 GAD67 expression in the nasal region of mouse embryos: relation to GABA and GnRH. (A, B, D, and K–M) ISH with GAD67 antisense probe; (F) IHC with a rabbit anti-GnRH antibody; (E) IF staining with anti-GABA antibody; (I, J, N, and O) GAD67 ISH-GnRH IHC; (C, G, and H) GFP fluorescence. At stages E11.5–E12.5, GAD67 and GFP in GAD67–GFP KI is expressed in the ventral region spanning the respiratory epithelium (RE)–vomeronasal organ (VNO) border (region above asterisk in A, B, and C, respectively). At E13.5–E14.5, GAD67 mRNA and GABA are detected in the ventral aspect of the VNO (labeled region above asterisk in D, E, G, H, and K) and in cell clusters migrating from this region in the FNM (G, H, white arrowheads; L, M, empty arrows), which also express GABA (E, short white arrows) and GnRH (F, short black arrows). Note, that on the territory of the VNO in only single migratory cells are stained for GnRH (F, black arrowheads), the GAD67/GABA-stained region marked by asterisk in (D, E) remains unstained. Lines in (M) delineate the bundle of VNN visualized by DIC (differential interference contrast) microscopy. GAD67+ cells migrating along the VNN at E13.5–E15.5 stain in the cytoplasm for GnRH (thin arrows in J, N). Few migratory cells stain exclusively for GnRH (thick arrows in J, N) or GAD67 (short empty arrows in N). (J) is an enlargement of the boxed area in (I). (K) the boxed area is shown enlarged in the lower panel. Inset in (N) is an enlargement of the boxed area and shows a cell cluster of migratory mostly GnRH+/GAD67+ cells at the nasal–FB junction (NFJ) of a E15.5 embryo. ns, nasal septum; Scale bars: 100 μm (A–D, F, and H) and 20 μm (E). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(FB): in GAD65_2e/gfp GFP strongly labeled the GABAergic neurons of the FB, whereas GAD65_1e-6e/gfp lacked expression in this region during late embryonic and early postnatal stages. Unlike the CNS, where the two lines showed correct expression pattern (Lopez-Bendito et al., 2004; data not shown) the GFP expression in the nasal compartment of GAD65-GFP transgenic mouse lines is ectopic and herein was used mainly for providing a strong fluorescent label for GnRH neurons.

Examination of histological sections from E12.5–P21 GAD65_1e-6e/gfp mice revealed that GFP-labeled cells migrating out of the VNO in a pattern highly reminiscent of the migratory GnRH neurons (Fig. 5; Supporting Information Fig. S4). Staining with an anti-GnRH antibody showed an extensive (more than 90%) overlap between GFP and GnRH (Fig. 5). From E12.5 on, migratory GFP+/GnRH+ cells were observed in the lamina propria of the nasal septum [Fig. 5(A, B, E)], crossing the cribriform

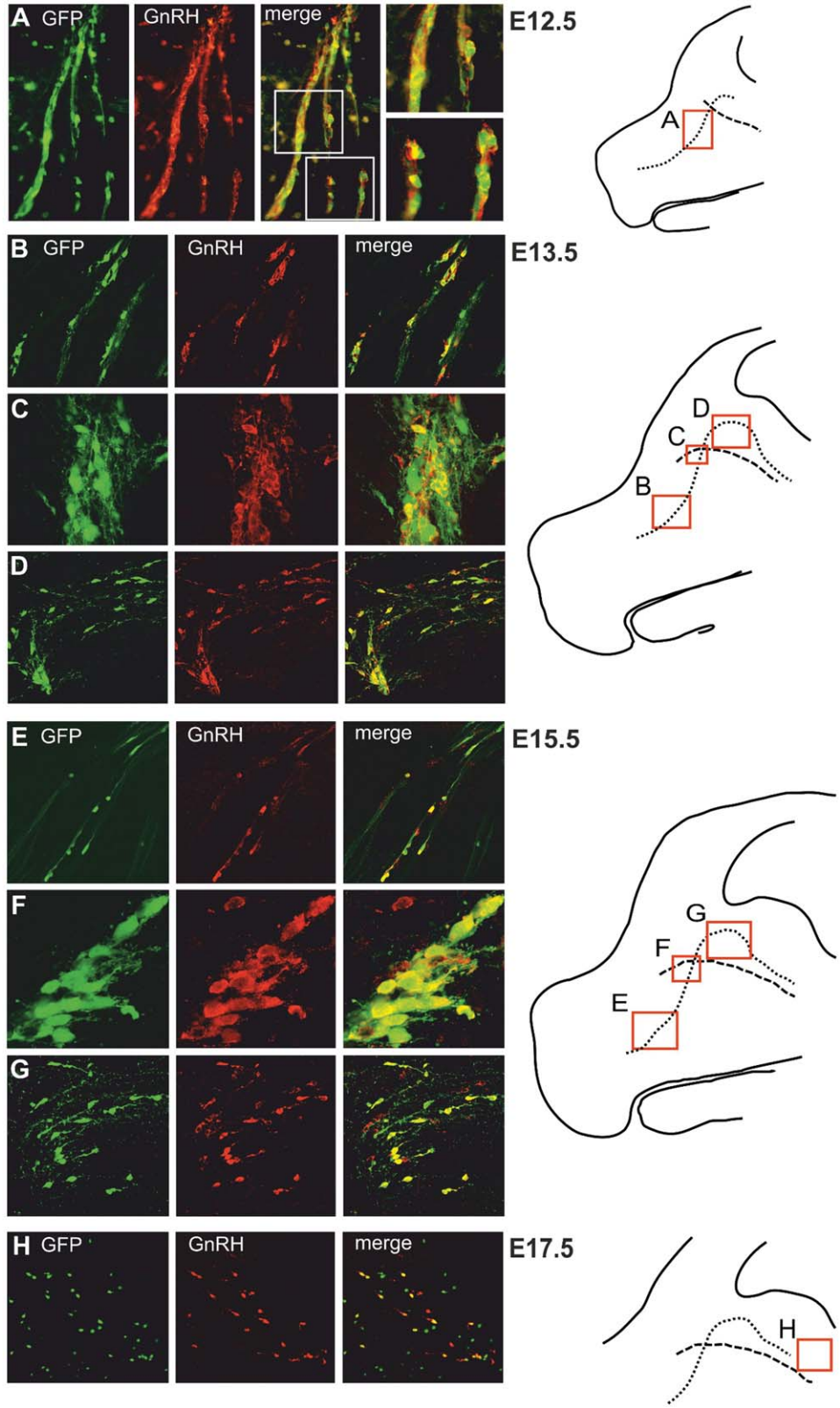

Figure 5 Migratory GnRH neurons in GAD65_1e-6e/gfp mouse embryos coexpress GFP. (A–H) Transgenic mouse line GAD65_1e-6e/gfp was derived with a DNA construct that contains GFP fused into the initiation codon of GAD65 found in exon 1 (Supporting Information Fig. S1; see details in Methods). Sagittal sections from E12.5–E17.5 embryos were stained with anti-GnRH antibody (red fluorescence) and visualized also for GFP (green fluorescence). The images overlaid in Axiovision 4.8 software demonstrate an extensive overlapping between the two stains in the selected areas along the migratory route (marked A–H) as shown schematically on the scaled drawings for each embryonic stage.

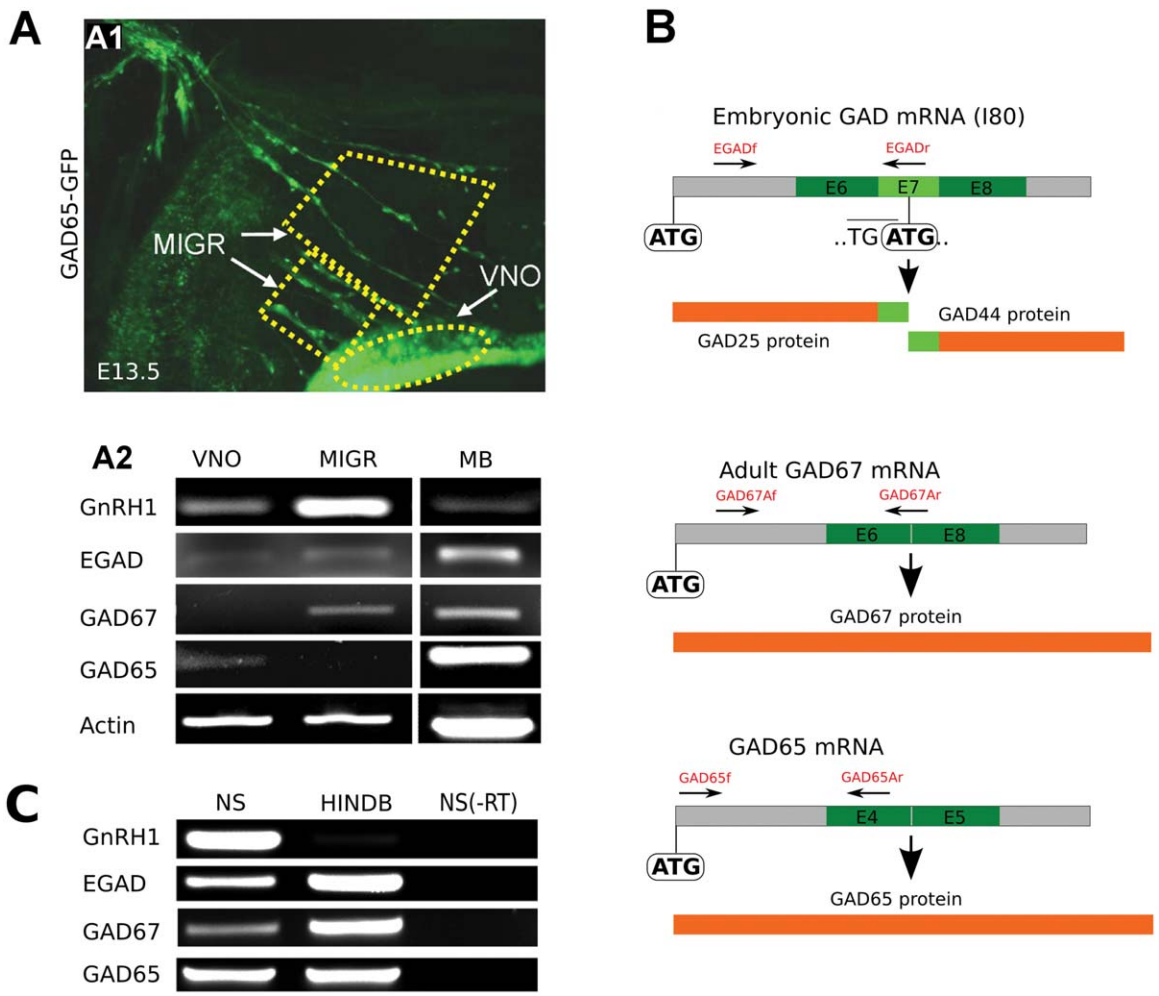

Figure 6 Dynamic regulation of different GAD forms during migration of GFP+/GnRH+ neurons in E13.5 GAD65_1e-6e/gfp embryos. (A1) Tissue punches were isolated under fluorescent microscope from the VNO (circled in A1) and GFP+ cells of the GnRH+ migratory route (MIGR; boxed areas in A1) were dissected from lightly fixed frozen sagittal sections from E13.5 GAD65_1e-6e/gfp embryos. (A2) RT-PCR performed with RNA isolated from punches taken from the VNO and migratory GnRH/GFP+ cells (MIGR) as shown in (A1) and E13.5 mouse brain (MB). GAD65 is amplified exclusively from the VNO, but not in (MIGR); GAD67 is detected in migratory GFP+/GnRH+ cells (MIGR), but not in the VNO, while EGAD and GnRH is detected in both with a higher expression in MIGR. Actin was amplified as control. (B) Schematic presentation of the mouse GAD protein forms encoded by different mRNAs: *Gad1* codes for three mRNAs (GAD67, I-80, and I-86) and protein forms (GAD67, GAD25, and GAD44) through alternative splicing and coupled-translation (Szabo et al., 1994; Varju et al., 2001); here only the bicistronic I–80 (termed for simplicity EGAD) coding for GAD25 and GAD44 is shown. *Gad2* encodes a single GAD65 mRNA and protein. The initiation codons for all GAD forms are indicated a well as the positions of the primers used for RT-PCR . (C) RT-PCR performed with RNA isolated from unfixed nasal septum (NS) and hindbrain (HINDB) of E13.5 GAD65_1e-6e/gfp embryos. GnRH is expressed in the NS, but not HINDB, while all GAD forms are expressed in NS and HINDB, respectively being more abundant in the latter, especially GAD67. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

plate to group at the NFJ [Fig. 5(F, G)]. Upon entering the FB, GFP+/GnRH+ neurons detach from the VNN and disperse, thereafter in a fan-like shape within the basal FB [Fig. 5(D, G)]. At E17.5 and later stages more single-labeled neurons expressing either GnRH or GFP were observed migrating in the ventral FB (Fig. 5H and Supporting Information Fig. S5A–C). At P11, the overlap between the two markers was

partial *albeit* both GFP+ and GnRH+ profiles had the characteristic migratory features of GnRH neurons (Supporting Information Fig. S5A–C). At P21, the most advanced stage studied, processes double labeled for GFP/GnRH were visualized in the median eminence (Supporting Information Fig. S5D–F), thus GAD65_1e-6e/gfp transgenic mouse line affords an excellent model system to analyze the GnRH neuronal migration at different developmental stages and throughout the entire migratory route without interference from other GFP+ presumed GABAergic neurons of the ventral FB.

GFP+ cells of GAD65_1e-6e/gfp mouse embryos migrate on peripherin+ axons in the FNM, (shown for E13.5 in Supporting Information Fig. S6) as described previously for GnRH+ neurons (Fueshko and Wray, 1994), providing additional evidence for their GnRH identity. Similarly, studies in transgenic mouse lines carrying lacZ regulated by the GAD67 promoter (Katarova et al., 1998) also showed a robust expression of βgal in the GnRH neurons migrating along peripherin+ fibers (Katarova et al., 1998).

Using GABA IHC, we checked for the presence of GABA in the frontonasal region of E13.5 GAD65_1e-6e/gfp transgenic embryos. A strong GABA signal was detected in a subregion in the ventral aspect of the VNO (Supporting Information Fig. S7A–C, E–F) and migratory GFP+ neurons, which were previously shown to express GnRH and GAD67 (see Figs. 4 and 5). A small fraction of GFP+ cells along the migratory route were devoid of staining as the ratio of double- and single-labeled cells was visibly increased at the NFJ compared to the VNO and FNM (Supporting Information Figs. S7H–J and S7D–G, respectively).

Dynamic Regulation of GAD During GnRH Neuronal Migration

Previously, we have shown that at least four different GADs are expressed from *Gad1* and *Gad2* loci, as schematically demonstrated in Figure 6(B). A single GAD65 form is encoded by *Gad2* and three major splice variants and protein forms are synthesized from the *Gad1* locus: GAD67 protein produced from the full-length mRNA and two truncated GAD25 and GAD44 forms, produced by alternative splicing and coupled translation of the bicistronic I-80/I-86 message referred to here collectively as EGAD (Szabo et al., 1994; Varju et al., 2001) [Fig. 6(B)].

The dynamic expression of GAD forms during migration in GnRH neurons was further evaluated by RT-PCR using total RNA prepared from cell biopsies isolated from different parts of the GnRH migratory pathway using lightly fixed tissue sections from E13.5 GAD65_1e-6e/gfp embryos as demonstrated in Figure 6(A1) and primers designed for specific amplification of each GAD form (Supporting Information Table S2 and see Materials and Varju et al., 2002 for details). As shown previously, there is >90% overlapping between GFP and GnRH labels along the migratory route in this transgenic mouse line (Fig. 5). GnRH amplified at considerably higher levels in migratory GFP+ cells compared to VNO. A faint GAD65-specific band was only present in the VNO preparation, but not in the migratory neuronal fraction in contrast to GAD67, which was detected in migratory GFP+ neurons, but not in the VNO, in full agreement with our ISH results. We failed to obtain specific amplification of GAD65 in migratory neurons even when using high cycle number or nested oligonucleotide primers (data not shown). EGAD was present at similar (low) levels in both VNO and migratory cells. All GAD mRNAs were clearly detected in the control sample from E13.5 mouse brain [Fig. 6(A2)].

We also performed RT-PCR on RNA prepared from freshly dissected unfixed nasal septum and hindbrain of E13.5 GAD65_1e-6e/gfp embryos [Fig. 6(C)]. Hindbrain was used as control being devoid of GnRH expression. GAD65, EGAD, and GAD67 were all detected in the nasal septum, in agreement with our ISH data (see Figs. 2 and 4), although at a much lower level compared to hindbrain from the same-age embryo. As expected, GnRH was only amplified from the septum, but not hindbrain [Fig. 6(C)]. Furthermore, GAD67 was much less abundant than EGAD or GAD65 in the septal RNA preparations in sharp contrast to hindbrain samples [Fig. 6(C)].

The expression of GAD forms at both mRNA and protein levels was further studied in two immortalized neuronal cell lines corresponding to the NFJ migratory (line GN11) and postmigratory (line GT1–7) stages GnRH neurons, respectively (Maggi et al., 2000; Fig. 7). Tissue biopsies from the respective regions could not be used because of contaminations from near-by local GABAergic neurons. Compared to GN11, GT1–7 is much more aggregated and differentiated [Fig. 7(A1–A2)]. Both lines stained for GnRH [Fig. 7(A1–A2)]. The affinity-purified embryonic GAD44-specific antibody stained the cytoplasm of GN11 cells [Fig 7(A3)], while in the more differentiated GT1–7, this form was enriched in the perikarya and proximal cellular extensions [Fig. 7(A4)]. The GAD67-specific serum #6799 produced more even distribution over the cell bodies [Fig. 7(A5–A6)]. Interestingly, the GABA-specific antibody

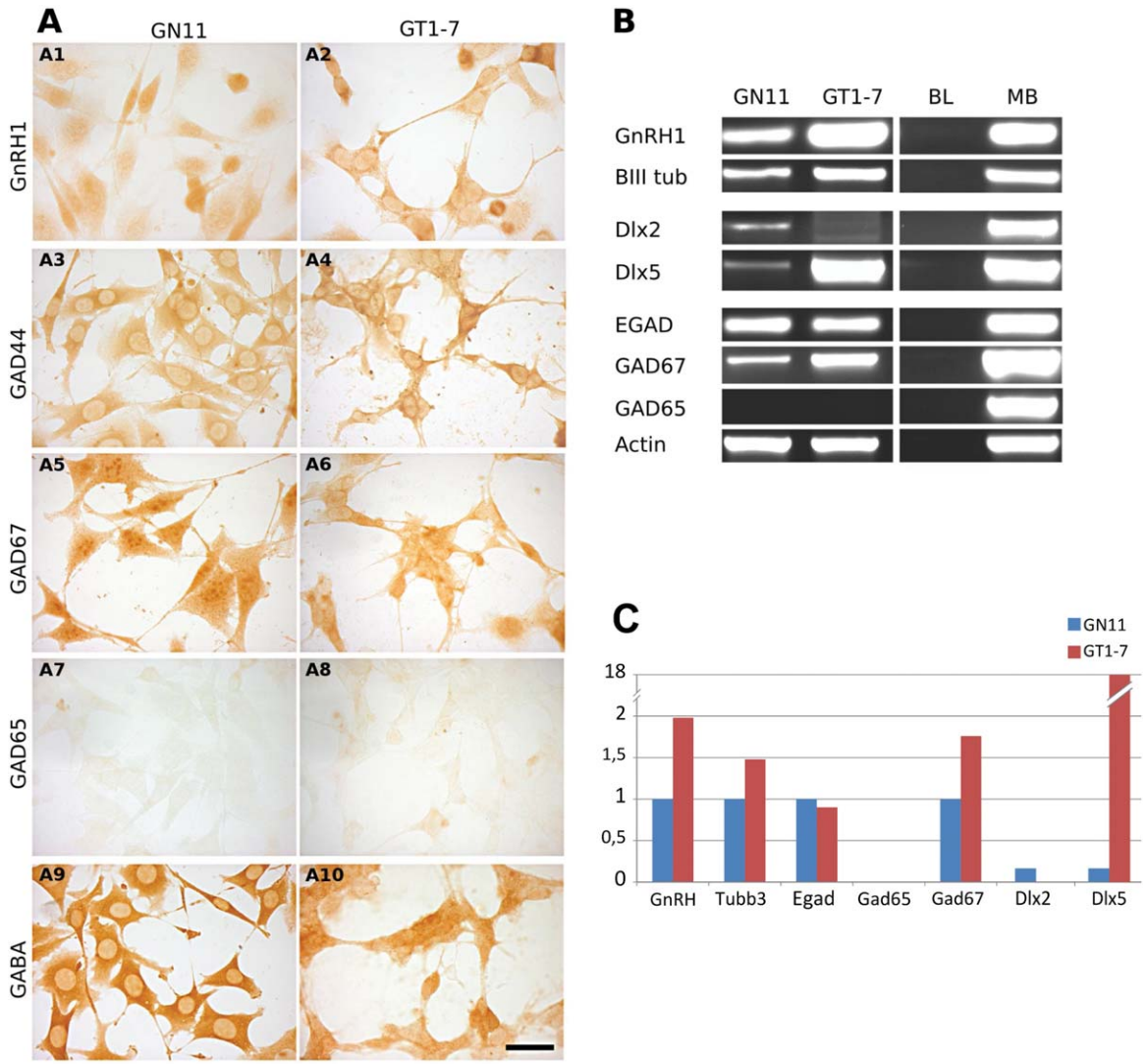

Figure 7 Differential regulation of different GAD forms, GnRH and Dlx2&5 in cultured GN11 and GT1–7 cells. (A) The immortalized GnRH-secreting neuronal cell lines GT11 (early migratory) and GN1–7 (late, postmigratory) were stained with anti-GnRH antibody (A1–A2) and GAD form-specific antibodies as follows: GAD44 (affinity purified; A3–A4); GAD67 (serum #6799; A5–A6); GAD65 (GAD-6; A7–A8); and with an anti-GABA antibody (A9–A10). Note the absence of staining for GAD65 in both GN11 and GT1–7 (A7–A8). (B) RT-PCR using total RNA isolated from GN11 and GT1–7 cell lines and specific primers for GnRH, GAD forms, Dlx1&2, and βIII-tubulin as indicated in Supporting Information Table 2S; actin was amplified as control; (MB) RNA from newborn mouse cerebellum. (C) Bar graphs representing the relative amplification of the genes shown in (B) normalized to actin. Note the lack of expression of Dlx2 in GT1–7 and GAD65 in both cell lines. Concurrent upregulation of GnRH, GAD67, βIII-tubulin, and Dlx5 in the more differentiated GT1–7 in comparison to GN11. EGAD is amplified to a similar level in both cell lines, while Dlx2 is detected at low levels only in GN11, but not in GT1–7. Scale bar in A: 10 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

consistently produced stronger signals in the perikarya of GN11 and weaker and more diffuse staining in GT1–7 [Fig. 7(A9–A10)]. We failed to detect GAD65 in either GN11 or GT1–7 using two different antibodies [Fig. 7(A7–A8)]. We next performed RT-PCR using total RNA from GT1–7 and GN11 and specific primers (Supporting Information Table S2) for GAD, GnRH, as well as Dlx2&5—upstream

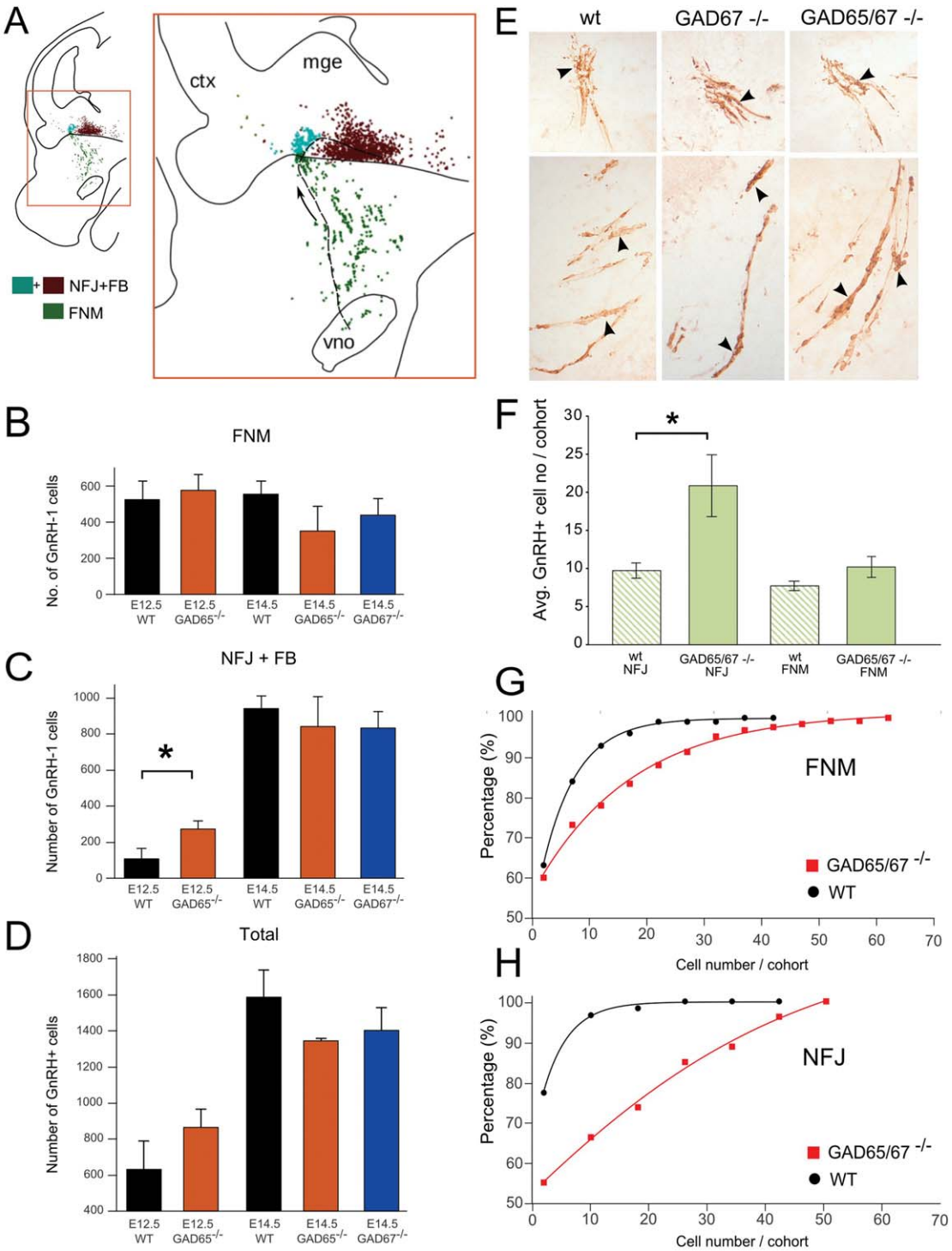

Figure 8 Altered migration of the GnRH+ neurons in mutant mice carrying deletions of GAD65, GAD67, and both. (A) Neurolucida 3D reconstruction of the GnRH migratory pathway using serial sections in the sagittal plane of E13.5 WT embryos stained with a-GnRH antibody. Ctx, cortex; mge, medial ganglionic eminence; vno, vomeronasal organ. Each dot represents a single GnRH+ neuron within a compartment labeled as NFJ, nasal–forebrain junction; FB, forebrain, FNM; frontonasal mesenchyme. (B–D and F) Bar graphs showing the number of GnRH+ neurons in the FNM, the NFJ+FB region and the total number of GnRH+ neurons at E12.5 or E14.5 WT, GAD65−/− and GAD67−/− embryos. A significant difference in numbers was found between the E12.5 WT and GAD65−/− embryos at the NFJ (C, F). (E) Serial sections from E13.5 WT, GAD67−/− and GAD65/GAD67−/− KO embryos were stained for GnRH and immunoreactive cells counted in three different regions of WT and KO embryos: FNM and NFJ+FB as shown in bar charts (A–C). (G) Cumulative frequency of GnRH cell cohorts in GAD65/67 double KO vs. WT E13.5 embryos. The graph demonstrates the size of GnRH+ cell cohorts (the number of cells within a migrating group) in a cumulative distribution (as described in Methods section. Note that in the GAD65/GAD67−/− double KO mice the number as well as occurrence of larger GnRH+ cell cohorts was more frequent compared to WT embryos both in the FNM and in the NFJ. Arrowheads in (E) point to cell GnRH+ cell cohorts in E13.5 WT and GAD65/67−/− embryos. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regulators of GAD (Stuhmer et al., 2002; Givens et al., 2005) and the neuronal-specific βIII-tubulin [Fig. 7(B,C)].

Both cell lines expressed GnRH, but the expression level was much higher in the more differentiated GT1–7 that also correlated with a stronger expression of the neuronal marker βIII-tubulin and a striking 18-fold increase of Dlx5 expression compared to GN11 [Fig. 7(B, C)]. Dlx2 was only weakly amplified from GN11 and undetectable in GT1–7 [Fig. 7(B, C)]. GAD65 failed to be amplified in either GN11 or GT1–7 even after 35 cycles and/or using nested primers [Fig. 7(B, C); data not shown].

EGAD (coamplified I80 and I86) was considerably more abundant than GAD67 in GN11, while the more differentiated postmigratory GT1–7 expressed similar levels of the two GAD variants [Fig. 7(B, C)].

Altered Migration of GnRH Neurons in Knockout GAD Embryos

The GnRH neuronal migration in the absence of one or both GAD forms was assessed in GAD65 or GAD67 KO and WT embryos. To this end, we performed GnRH IHC using tissue sections from E12.5 and E14.5 GAD65KO (Kubo et al., 2009), GAD67-GFP KI (Tamamaki et al., 2003) GAD65/67 double KO, and WT mouse embryos (Fig. 8). The distribution of GnRH immunoreactive cells along the migratory route was analyzed on consecutive series of sagittal sections. GnRH cells were classified into: (1) cells migrating in the region between the VNO and lamina cribrosa (FNM); (2) between the lamina cribrosa and the site of entrance into the ventral FB: the NFJ, and (3) basal FB area. The neurolucida reconstruction of the entire GnRH migratory route in the E13.5 embryo shown in Figure 8(A) demonstrates that the GnRH+ cells migrate initially on branches of the VNN in the FNM formed by VRN residing in the VNO, cumulate eventually at the NFJ to disperse again in the ventral FB on way to their final destination in the hypothalamic preoptic area. A smaller group of cells migrate dorsally to the AOB as previously described (Yoshida et al., 1999).

The GnRH+ cells present in FNM and NFJ+FB areas were counted in serial sections obtained from different KO and WT animals and processed in parallel for GnRH IHC, averaged and presented as bar charts in Figure 8(B–D). The number of GnRH+ neurons migrating in the FNM of E12.5 GAD65 KO embryos was slightly elevated compared to WT [575 ± 50 in the KO vs. 525 ± 58 in the WT; Fig. 8(B)]. Significantly increased numbers were estimated in the NFJ+FB compartment (274 ± 26 for GAD65 KO; $n = 3$) compared to WT [109 ± 32; $n = 3$, Student's t-test $p < 0.05$; Fig. 8(C)]. The total number of GnRH+ neurons in the E12.5 GAD65 KO embryos was also increased compared to WT [Fig. 8(D)]. In addition, we encountered increased numbers of early migrating GnRH neurons in the region of the NSE adjacent to the VNO of E12.5 GAD65 KO embryos (Supporting Information Fig. S7).

At E14.5, there was a moderate decrease of GnRH+ neurons in the FNM of both GAD65 KO (353 ± 153) and GAD67 KO (439 ± 92) compared to control (554 ± 73) embryos, the effect being more pronounced in GAD65 KO [Fig. 8(B)]. The number of GnRH cells at the NFJ+FB at this stage was reduced by 13–14% [841 ± 164 in GAD65 KO; 834 ± 89 in GAD67 KO versus 939 ± 69 in WT; Fig. 8(C)]. The difference was more pronounced in the FNM compared to NFJ+FB compartments [Fig. 8(B, C)]. The total number of GnRH+ neurons in WT exceeded by 20–25% the respective numbers in GAD67-GFP KI or GAD65 KO embryos [Fig. 8(D)].

Next, we compared the migration of GnRH+ neurons in E13.5 GAD67 and double GAD65/67 KO mutant, which contains very low levels of residual GABA (Ji et al., 1999) with WT embryos. GnRH IHC in both GAD67 and GAD65/67 KO embryos demonstrated that the GnRH+ neurons were visibly assembled in larger clusters compared to WT throughout the whole migratory route [Fig. 8(E)]. This effect was visibly more pronounced in the double GAD KO, therefore, we chose to perform quantitative cell number analysis of the GnRH+ cell cohorts on consecutive series of frozen sagittal sections from GAD65/67 KO and WT E13.5 mouse embryos ($n = 3$) as described in Methods section. The number of GnRH+ cells in each cohort of migrating GnRH+ neurons for both control and KO embryos was estimated, cell cohorts were ranked by their sizes, then the cumulative frequencies were calculated and plotted for both FNM and NFJ [Fig. 8(G, H)]. The plots showed a significant shift in the distribution of cohort size toward the bigger values in the GAD65/67 KO embryos compared to the WT. This difference was already apparent in the FNM, where roughly 75% of the GnRH+ cell cohorts in the GAD65/67 KO contained $\leq$10 cells/cohort compared to 90% for the WT [Fig. 8(G)]. In addition, the GnRH+ cell clusters in the FNM of the GAD65/67 double KO appeared to migrate on fewer and thicker axonal bundles [Fig. 8(E)]. Even larger GnRH+ cell cohorts were formed at the NFJ of GAD65/67 KO embryos, where 65% of the cells were found in cohorts of $\leq$10 cells/cohort, while 35% of the cohorts belong to much larger complexes [Fig. 8(H)]. In

comparison, over 97% of cohorts in the WT were found in the first group [Fig. 8(H)], in support of the notion for an enhanced aggregation of the migratory GnRH+ in the absence of GAD/GABA. Furthermore, we found that the average number of GnRH neurons per cohort at the NFJ of GAD65/67 KO embryos was significantly higher compared to WT [20.86 ± 4.08 vs. 9.72 ± 1.02, respectively; *t*-test, $p < 0.05$; Fig. 8(F)].

DISCUSSION

In the CNS of rodents, the two genes responsible for the synthesis of the neurotransmitter GABA—*Gad1* and *Gad2* are coexpressed from the earliest studied embryonic stages through the adult *albeit* are differentially regulated in the GABAergic neurons (Feldblum et al., 1993; Katarova et al., 2000; Varju et al., 2001). It has been proposed that the two GAD forms—GAD65 and GAD67—synthesize different intracellular GABA pools (Martin and Barke, 1998; Varju et al., 2001), the existence of which has gained support from recent experiments (Hablitz et al., 2009). However, the subcellular functional segregation of the two GADs in the CNS has been difficult to tackle because of their coexpression and existence of partial functional redundancy as well (Martin and Barke, 1998). Expression of a single GAD form is more common in the peripheral nervous system (Fukushima et al., 2009; Nowak et al., 2011) and in different non-neuronal cell types during embryonic development, the best-described example of which is the eye lens (Kwakowsky et al., 2007).

Previous work in the developing mouse olfactory system has been largely focused on the role of GABA in the migration of GnRH+ neurons, a subpopulation of which accumulates the neurotransmitter (Heger et al., 2003; Tobet and Schwarting, 2006; Lee et al., 2008; Wray, 2010). So far no detailed study exists on spatiotemporal expression and regulation of the existing GAD forms. Here, for the first time we show that the two *Gad* genes are differentially regulated in the developing olfactory region being expressed in a virtually nonoverlapping spatiotemporal fashion as schematically presented for the E13.5 embryo in Figure 9. This segregated expression is already present at the olfactory placode stage (E9.5). The olfactory placodes, which give rise to the vomeronasal, olfactory, and respiratory epithelia (Treloar et al., 2010) are the primary source of progenitors for three different neuronal lineages—the ORN, VRN, and GnRH neurons that are essential for feeding, sexual development and reproduction, and social interaction (Tucker et al., 2010). The pioneer ORN and VRN and the GnRH neurons are born and become postmitotic during the stages of "primary" neurogenesis (E9.5–E12.5); the VNO is also formed during this period (Kawauchi et al., 2004; Beites et al., 2005; Ikeda et al., 2007; Tucker et al., 2010). At these initial stages of peripheral olfactory development, the identity of the ORN, VRN, and GnRH neurons is thought to be established through complex E/M interactions mediated by morphogenetic factors like all-trans RA. *Fgf8* and *BMP4*, which define the expression levels and boundaries of a number of transcription factors like *Meis1*, *Sox2* and the neurogenic bHLH *Mash1* (*Ascl1*), *Ngn1*, and *NeuroD* (Rawson et al., 2010; Tucker et al., 2010).

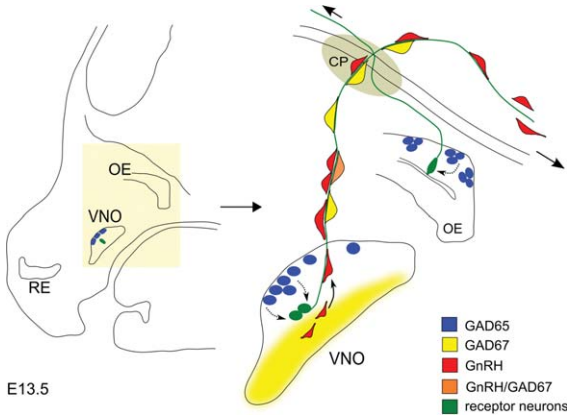

Figure 9 Schematic presentation of GAD65, GAD67, and GnRH expression in the nasal region of the E13.5 embryo. GAD65 is confined to the proliferative basal/apical layer(s) of the OE and VNE. Based on their position and properties, GAD65+ cells correspond to ORN/VRN progenitors. The receptor neurons of the VNO (in green) reside in the middle zone of the epithelium sending axons in the VNN. GAD67 transcribed from the *Gad1* gene is expressed in a region of the ventral VNO and a population of GnRH neurons. Since the expression of GAD67 in samples collected from tissue biopsy of migrating GnRH+ neurons or whole septum is low, GAD44 is the predominant form expressed by the GAD+ cells in the nasal compartment. GnRH+ (red), GAD67+(yellow), and GnRH+/GAD67+ (orange) neurons migrate towards the basal forebrain along the VNN with a major branch turning caudally toward the ventral forebrain (nervus terminalis). Receptor neurons of the OE (green) with their bodies positioned in the middle zone project to the main olfactory bulb do not express GAD or GABA. GAD65+ (but GABA−) ORN/VRN progenitors (blue) occupy predominantly the basal layer of the OE/VNE. The remote portion of the RE is devoid of GAD expression. The color code for different cell lineages is indicated. OE, olfactory epithelium; RE, respiratory epithelium; VNO, vomeronasal organ; CP, cribriform plate.

Of the two *Gad* genes, only GAD67 is expressed in the olfactory placode (E9.5), while both genes are detectable in the olfactory pit (E10.5–E11.5) where their expression domains partially overlap, but not at the cellular level (data not shown). At E11.5–E12.5, the region of GAD67 expression encompassing a portion of the VNO/pit and adjacent RE essentially overlaps with the expression domain of *Fgf8* and *Dlx1&2* (Merlo et al., 2007) (Figs. 4 and 9; Allen Brain Atlas). This expression pattern is faithfully reproduced by GFP in the GAD67-GFP KI embryos. Interestingly, GAD67 like *Fgf8* is expressed in the apical ectodermal ridges (Maddox and Condie, 2001), a region sharing similar morphogenetic mechanisms suggesting the involvement of GAD67 and GABA in E/M transitions (Hagiwara et al., 2003). In chicken, the GnRH phenotype is induced by *Fgf8* (Sabado et al., 2012) and the hypomorphic *Fgf8* mouse mutant has disrupted VNO morphogenesis and absence of GnRH neurons (Tucker et al., 2010). This strongly suggests that the RE/VNO domain of E11.5–E12.5 embryos marked by GAD67 (and *Fgf8*) expression overlaps with the initial pool of GnRH progenitors. In E12.5–E13.5 GAD65 KO, many GnRH+ neurons could be traced outmigrating from this site before joining the VNN axons. These observations serve as additional evidence in support of the notion that postmitotic GnRH neurons originally arise and migrate from the nonsensory RE (Wray, 2010; Forni and Wray, 2012) and that GAD67 can serve as an early identification marker for their progenitors. An independent and useful label for early GnRH neurons migrating from the NSE is afforded by GFP in GAD65_2e/gfp and GAD65_1e-6e/gfp transgenic embryos, in which a separate migratory stream emerging from the RE similar to that observed in the chicken and in the AP-2 KO mouse (Wray, 2010) was also detected.

In contrast to GAD67, which continues to be expressed in the GnRH lineage, GAD65 is progressively silenced after E10.5 and becomes virtually undetectable at the end of primary neurogenesis (E12.5). It is induced again at E13.5 exclusively in the sensory OE/VNE. At stages >E12.5, the ORNs are generated through a secondary wave of neurogenesis, whereby proliferative cells residing in the apical and basal layers of the pseudostratified OE give rise to either sustentacular cells or ORN/VRN, respectively, and this type of "established" neurogenesis is uniquely preserved throughout life (Beites et al., 2005; Ikeda et al., 2007) and references therein). Previous extensive studies have demonstrated that the ORNs differentiate from *Sox2*+ olfactory neural stem cells, which initially proliferate at the apical surface (Gokoffski et al., 2011; Rosenbaum et al., 2011) then move to and proliferate at the basal surface of the embryonic OE. Subsequently cells of the basal layer undergo a temporal differentiation program similar to that of other neuroepithelia of the CNS regulated intrinsically by a cascade of sequentially induced transcription factors—*Ascl1*, *Ngn1*, and *NeuroD* (Gokoffski et al., 2011; Rosenbaum et al., 2011).

Gad2 has binding sites for and shows a similar expression to *Mash1 (Ascl1)* in both apical and basal layers at earlier stages (E12.5–E14.5) of OE development and is restricted to the basal layer at later stages (E15.5–P0). Similar to *Mash1* and *Ngn1*, GAD65 was never detected in nascent or terminally differentiated ORNs. Interestingly, *Mash1* is known to determine the GABAergic cell fate, while *Ngn1* and *NeuroD* abrogate *Mash1* action in neuronal progenitors of the ganglionic eminence (Lee et al., 2008; Roybon et al., 2010). *Gad2* gene has a high-score binding site for the insulinoma-associated protein 1 (*Insm1*), a Zn-finger transcription factor downstream of *Mash1* that has been implicated in regulating cell cycle arrest in proliferating cortical (Lan and Breslin, 2009) and OE (Rosenbaum et al., 2011) progenitors prompting the suggestion that the smaller GAD form may play a role in the progressive exit from the cell cycle and transition to neuronal fate during olfactory sensory neuronal fate determination. This notion gains an additional support by previous findings showing that GABA acting on GABAAR inhibits the proliferation of PSA-NCAM+ neural progenitors of postnatal striatum (Nguyen et al., 2003) and/or cell-cycle reentry of neural stem cells in the subventricular zone (SVZ) (Liu et al., 2005).

The characteristic "patchy" expression of GAD65 in the basal VNE and OE, especially at E15.5 and later stages is highly reminiscent to that of *Mash1* and *Ngn1* and some guidance molecules involved in intercellular signaling and establishing the correct topographic projection map like *Slit2*/*Robo* (Cariboni et al., 2012), *Sema3a* (Cariboni et al., 2011), or *Eph5A*-ephrin (Gamble et al., 2005; Cariboni et al., 2012), although its exact role in the process is unknown. Interestingly, we failed to detect GABA in the GAD65+ cells of the SE suggesting they do not accumulate the neurotransmitter, but rather it may be released as soon as it is synthesized acting locally in an autocrine/paracrine fashion to modulate short-range intercellular interactions, through activating GABAR and intracellular Ca^{2+} signaling.

The two *Gad* genes are regulated in a spatially and temporally strictly segregated manner, in sharp contrast with the GABAergic neurons of the CNS, where

they co-localize from early developmental stages (Katarova et al., 2000). There is also a clear functional segregation between the two forms as GAD67 associates with migratory cells, while GAD65 with cells undergoing their final proliferative stages.

Similar to the developing CNS (Szabo et al., 1994) GAD67 and its embryonic splice variants EGAD are sequentially induced during GnRH neuronal migration and differentiation as only EGAD is expressed in the VNO, while GAD67 is induced and progressively upregulated in the GnRH at later migratory stages, a tendency also preserved in the immortalized cell lines GN11 and GT1–7. Notably, EGAD is the predominant GAD form in the GnRH of the nasal septum.

Interestingly, *Dlx*2&5, positive regulators of GAD are also subjected to migratory stage-specific dynamic regulation, which may suggest that GAD67 splice forms are regulated by different combinations of *Dlx1–6* during migration.

GABA is clearly detectable by IHC in migrating GnRH and the immortalized cell lines GN11 and GT1–7 showing a prominent cytoplasmic staining at the NFJ and GN11, which is weakened and more diffused at later stages and in GT1–7 concomitant with the increase of GAD67 expression. This indicates that the dynamic regulation of different GAD forms during GnRH migration is accompanied by changes in the intracellular accumulation and/or release of GABA.

It has been shown that GABA exerts an excitatory action and regulates the pulsatile GnRH release until puberty (Han et al., 2002; Heger et al., 2003). GnRH and GAD are oppositely regulated following dissociation of GnRH neurons from nervus terminalis such as GnRH is upregulated while GAD is gradually turned off at puberty (Heger et al., 2003). Concomitantly, GABA effect exerted through $GABA_AR$ on GnRH neurons switches from depolarizing to hyperpolarizing and inhibiting GnRH secretion around postnatal day 31 (Han et al., 2002).

An important aspect of the dynamic regulation of EGAD and GAD67 during GnRH migration are the distinct functions EGAD and GAD67 may exert through specific intracellular GABA pools (Szabo et al., 1994; Varju et al., 2001). The truncated GAD44 is regulated at the posttranscriptional level and its recombinant counterpart displays a high specific activity *in vitro* (Szabo et al., 1994). It is localized near the cellular membrane and proximal processes in GN11 and GT1–7 as in differentiating neurons (Varju et al., 2002) and its forced expression alters cell adhesion both *in vitro* and *in vivo* (data not shown). Conversely, GAD67 shows a more widespread distribution in cell bodies and distal neurites (Varju et al., 2002). We have suggested that GABA produced by GAD44 is released through a reversal of the membrane GABA transporter GAT-1 and acts as an autocrine/paracrine factor via nonsynaptic GABAR, while GAD67 synthesizes the synaptic vesicular GABA released at inhibitory synapses and/or growth cones (Szabo et al., 1994; Varju et al., 2001; Varju et al., 2002; Kanaani et al., 2010).

The migratory route of GnRH neurons consists of an early (septo-nasal) and late (NFJ and FB) phases (Bless et al., 2005), which differ profoundly in migratory features. The early nasal stage is a prototype of an axophilic migration dependent on GnRH/axonal interactions mediated by a variety of adhesion molecules, diffusable factors and guidance cues, both extracellular and membrane-bound (Tobet and Schwarting, 2006; Cariboni et al., 2007; Wray, 2010; Wierman et al., 2011; Messina and Giacobini, 2013) The initially slow saltatory migration of GnRH in the FNM characterized by predominant EGAD expression is followed by much faster movements and turning effects at the NFJ (Bless et al., 2005; Casoni et al., 2012), which correlate with upregulation of GAD67. As previously shown, overexpression of GAD67 using GnRH promoter does not substantially affect the speed of migration in the FNM, but rather alters the direction of migration in the FB causing straying of GnRH to noncanonical brain regions (Heger et al., 2003). Altogether these findings suggest that GAD67-produced GABA may act predominantly to promote directional navigation, while GABA synthesized by EGAD (GAD44) may modulate the speed of migration, thus the two forms cooperatively regulate the precision (speed and timing) of migration. This effect could be achieved through differential utilization of local $GABA_AR$-evoked Ca^{2+} signaling and remodeling of the cytoskeleton (Hutchins et al., 2013).

The availability of both GAD65 and GAD67 KO mouse strains (Condie et al., 1997; Kash et al., 1997; Tamamaki et al., 2003; Yamamoto et al., 2004; Kubo et al,2009) allows the direct testing of the proposed roles for different GADs in the GnRH neuronal migration. Previously, it has been shown that deletion of GAD67 results in an increased motility of GnRH through the FNM, moderate increase of GnRH numbers at the NFJ of E12.5 and significant increase in E14.5 GAD67 KO embryos (Lee et al., 2008). Our analysis showed increased GnRH numbers in the FNM and NFJ at E12.5, but decreased numbers at E14.5 in both GAD65 KO and GAD67-GFP KI embryos clearly indicating the complementary role of the two *Gad* genes in the regulation of GnRH migration. The discrepancies between our data and these of

Lee et al. (2008) concerning E14.5 embryo may be explained by differences in the strains used for the two studies and/or substantial variations among individual GAD67KO embryos (Lee et al., 2008).

The statistically significant increase in the number of migratory GnRH neurons in E12.5 (but not E14.5) embryos with a deletion of GAD65 is peculiar as this form is not expressed in the GnRH lineage or differentiated VRN whose axons provide the substrate for axophilic migration. It demonstrates the primarily inhibitory role of GAD65 on the differentiation and acquirement of migratory properties of the "early" GnRH pool delaminating from the pit during stages of "primary" neurogenesis. The appearance of numerous GnRH+ neurons on the territory of the NSE of GAD65 KO embryos is in support of this conclusion, *albeit* the underlying mechanisms are still unknown. It should be stressed, that the adult GAD65 mice of both sexes have a normal onset of puberty, but are infertile, although the underlying causes were not persued in this study. In agreement with previous data, GAD67 deletion has a small impact on the total number of migrating GnRH at this embryonic stage (Lee et al., 2008).

In E14.5 embryos, GnRH+ cell numbers from different parts of the migratory route as well as the total GnRH numbers of both GAD65 KO or GAD67-GFP KI are reduced, but not significantly indicating the existance of compensatory mechanisms (Lee et al., 2010).

Surprisingly, our studies in double GAD65/GAD67 KO mouse embryos revealed that the GnRH expression is not dramatically affected in these mice, as there is only a subtle change in migratory GnRH+ cell numbers compared to the respective single GAD KO mice (data not shown). Previous results in our laboratory have shown that GAD67-GFP KI and the GAD65/67 double KO express a novel alternatively spliced message and increased level of embryonic GAD44 protein (data not shown), which may be responsible for the synthesis of residual GABA in this mutant (Ji et al., 1999) and a partial rescue during early and mid-gestation embryonic stages, which have been the focus of this study.

The most apparent change in the double mutant is the abnormal GnRH cell aggregation especially at the NFJ. The effect was seen also in the GAD67-GFP KI mutants, but to a lesser extent, which clearly points to the contribution of GAD65-mediated GABA signaling, possibly through delayed effect on the VNN axons. The precise spatiotemporal migration of the GnRH neurons is critically dependent on their proper guidance by the VNN axons and mediated by a variety of cell signaling molecules like ephrins, semaphorins and their receptors, which similar to the GAD forms are differentially expressed and dynamically regulated along the GnRH migratory route (Cariboni et al., 2011; Messina and Giacobini, 2013) or cause abnormal clumping of GnRH neurons in the FNM at altered expression levels (Gamble et al., 2005) suggesting a possible interplay with the GABA signalling.

Excessive GnRH/GnRH aggregation may result from weakened interactions between leading/trailing processes of GnRH and VRN axons due to GAD67 deficiency under conditions of stronger GnRH adhesion at the cellular membrane in the presence of GAD44 that may be mediated by distinct intracellular GABA pools. Consistent with this is the observed increased aggregation at NFJ compared to the FNM region correlating with the increased contribution of GAD67 at later migratory stages. As previously shown, GABA can mediate changes in adhesion properties through direct binding of $GABA_AR$ to cell adhesion and/or cytoskeletal proteins (Wang and Olsen, 2000; Zhang et al., 2010). An increased cell adhesion/gap junction coupling through $GABA_AR$-evoked activation of intracellular Ca^{2+} signaling has recently been demonstrated for mouse ES cells (Schwirtlich et al., 2010).

In conclusion, our results demonstrate that GAD67 and GAD65 display a nonoverlapping expression and nonredundant roles in two cell lineages of distinct origin and fate: the migratory GnRH neurons originating in the NSE and ORN/VRN progenitors confined to the proliferative layers of the sensory OE/VNE (Fig. 9). This clear association of GAD65 and GAD67 in the developing olfactory region with a "proliferative" and "migratory" cellular responses may have a broader significance in and outside the CNS that should allow for a precise determination of the special roles of different GADs and downstream components in the GABAergic differentiation.

Finally, our data also suggest that GAD65, GAD67, and EGAD act through distinct subcellular GABA pools and may activate selectively downstream signaling cascades to conjointly contribute to the precise timing of migration, arrival and ultimately positioning of GnRH in the hypothalamus.

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