Specific aim 1
Characterize the expression of different GAD mRNAs in the nasal region of E9.5-E15.5 embryos in normal or transgenic mice by in situ hybridization (ISH), quantitative RT-PCR and IHC of the four known GADs- GAD65, GAD67, GAD44 and GAD25 using form-specific antibodies.

Expression of different GAD forms in the nasal region of mid-gestation embryos (E9.5-E10.5)
The earliest step in the development of the nasal region is the formation of the olfactory placode- a thickening on the lateral aspects of the frontonasal prominence at around embryonic day 9.5-10 in the mouse. Our studies show that both GAD65 and GAD67 are expressed in the olfactory placode and its subsequent derivative- the nasal pit (Fig. 1- arrow). To study the expression of GAD in the FNM we used both wild-type and transgenic/knock-in mice, which express the marker gene green fluorescent protein (GFP) under the control of either GAD67 (GAD67GFPKI-knock-in line) or GAD65 (GAD65GFP transgenic line) promoter. When we compared the expression pattern obtained by immunostaining (anti-GAD67 antibody) and GFP fluorescence of GAD67GFP knock-in (KI) and GAD65GFP transgenic mouse embryos stage E9-E10, it was apparent, that most, if not all of the GAD67 expression is represented by the embryonic GAD, since the GAD67 antibody also cross-reacts with the embryonic GAD forms and the GAD67KI animals do not express (or express at very low levels) in this region. Our previous findings demonstrated the presence mostly of the embryonic GAD forms in the GABAergic migratory lineages in the OE and FNM (OTKA grant #). The present results expand our previous knowledge in two important ways, which should contribute profoundly to our understanding of the role of GAD and GABA in the differentiation of the GABAergic cells of the FNM: they demonstrate the predominant expression of embryonic GAD to that of GAD67 and more importantly- the presence of GAD65, which so far has not been reported. The failure to detect GAD65 by IHC or in situ hybridization in the OE-derived migratory GABAergic cells so far may be due to low levels of the signal. The above data was confirmed by our independent Western blot studies of micro-dissected FNM of E11.5-E13.5. We similarly found that the embryonic GAD25, GAD44 and the adult GAD65 are the predominant GAD forms during these stages. The unexpected finding that
the two predominant forms expressed in the migratory GABAergic cells of the FNM are GAD65 and embryonic GAD44 (25) strongly suggests that these GADs are involved in the early migration and/or differentiation of the GABAergic cells of the frontonasal process, including the embryonic LHRH. The significance of this finding is still unknown, but it would require a revision of the currently existing view about the specific roles of the different GAD forms, namely, that GAD65 is predominantly involved in synaptic transmission compared to the “cytoplasmic” GAD67. The sub-cellular distribution and specific intracellular role(s) of embryonic GADs and GAD65 in the early GABAergic cells is still obscure.

GAD65 partially overlaps with the GAD67 (eGAD)+ cells of the migratory mass (MM)

Next, we compared the expression of two transgenic lines derived with the GAD67 (GAD67lacZ) and GAD65 (GAD65GFP) promoters, respectively at stages E12.5 (Fig.2, similar results were found for stages E11.5 through E13.5). We found that GAD65 gene is expressed in only a subset of GAD67+ cells, which mark the migratory mass (MM)- a ganglion-like structure on the ventral side of the telencephalon of E11.5-E14.5 mouse embryo, which has been implicated in the induction of the olfactory bulb and was found to express GAD44, peripherin, OMP (olfactory marker protein) and GABA (OTKA grant proposal). Staining with anti-LHRH antibody revealed that the GFP-positive cells of the MM in GAD65GFP mice represents LHRH+ neurons migrating in the MM. In fact, all of the so far derived GAD65GFP transgenic mice were found to express in the embryonic LHRH lineage in an essentially overlapping fashion. This expression may be regulated by region-specific transcription factors like Olf-1, which has a binding site on the promoter region of GAD65. For most of our studies we used two transgenic lines, referred to as GAD65GFP#5 and GAD65GFP#30.

**Fig.2.** X-gal and GFP expression in the MM of E12.5 transgenic GAD67lacZ and GAD65GFP embryos
Fig. 3. GFP+ FNM migratory cells of the GAD65GFP transgenic embryos are essentially LHRH+: sagittal sections from E13.5 mouse embryos line #30 stained with anti-LHRH antibody. a, b, c, d-GFP fluorescence; a’, b’, d’- stained with a rabbit anti-LHRH antibody; a’’, b’’, and d’’- composite images. c- the migratory route of the LHRH neurons from the olfactory epithelium through the fronto-nasal mesenchyme into the forebrain towards the pre-optic area.

At E13.5 the GFP (GAD65) and LHRH largely overlap throughout the migration of LHRH+ neurons and in the POA. Later on, this co-localization is less extensive in the POA and at P21 it was only found in fiber tracts in the area of Nucleus arcuatus (NA, Fig. 4)

Fig.4. GFP and LHRH fluorescence overlapping in the brain of E17.5 embryos and young post-natals POA: preoptic area, NA- nucleus arcuatus

The adult LHRH neurons are considered non-GABAergic, although it is yet unknown when they stop synthesizing GAD and GABA.
In summary:

- Two partially overlapping migratory GABAergic lineages could be identifies in the frontonasal mesenchyme of mouse embryos: GAD67+/eGAD+ and e-GAD+/GAD65+/eGAD+/LHRH+.
- The neurons of the migratory mass (MM), a transient population implicated in the induction of the olfactory bulb is mostly eGAD+ (OMP+), only a subset of the cells are GAD65+/eGAD+/LHRH+.
- During embryonic life, the LHRH neurons continue to express GAD65 even after settling into the POA. The expression decreases gradually in the first postnatal month.

In collaboration with the laboratory of M. Brilliant (University of Arizona), we studied the dynamic expression of GABA and GAD-GFP during palate development, which is closely linked to the development of the olfactory-oral-respiratory epithelium. This study clearly indicated, that the source of GABA in this region is non-neuronal and is, in fact synthesized in the epithelium cells by the same GAD forms characterized in the brain. However, unlike the olfactory epithelium, the oral-respiratory-palatal epithelium expresses mostly adult GADs and never gives rise to neurons. The palate-cleft phenotype of GAD67 knock-out (knock-in) mouse lines is therefore due to a failure of the GABA signaling operating in the palatal epithelium. This work was published in *Dev Biol*.

Two additional collaborations related to the main topic of this OTKA grant was initiated with the Department of Prof. M. Shipley (University of Baltimore) and Dr. Lopez-Bendito (Institute of Neuroscience, Alicante, Spain) and resulted in two publications.

Finally, the transgenic mice we have derived have been used and are currently used in more than 30 laboratories in Europe and USA.

We also studied the expression of the GAD forms and other components of the GABAergic signaling during *in vitro* differentiation using two different model systems- the neuronal-like stem cells NE-7C and embryonic stem (ES) or carcinoma (EC) cells. Some of these results have been published, another part (concerning the ES cell differentiation) has been reported at several prestigious international meetings and are currently in the process of submission for publication. Briefly, the sequential order of induction of different GAD forms during differentiation follows a strict order, which is similar in ES (EC) cells and in the mouse embryos (including lens and nasal process) and differs in the neuronal stem cell-like cells NE-7C., which may represent a later (committed) stage of differentiation.

**Specific aim 2**

Determine the role of the embryonic GADs compared to that of GAD67 on the migration and/or differentiation of LHRH neurons in transgenic mice by selectively suppressing all GAD67-related transcripts. Study the role of embryonic GAD44 compared to that of GAD25 or GAD67 in transgenic mice expressing embryonic GAD cDNAs I-80, I-86 or GAD67 under control of LHRH promoter (Year 2, 3 and 4).

Regarding the second aim we encountered numerous problems, which led to the modification of the original plan. Originally, we planned to use the LHRH promoter to drive specific expression in the respective lineage of the FNM.
We used a cassette, containing the entire gene coding for the LHRH gene to generate expression plasmids containing the coding sequence of GAD67, I-80 and I-86 fused to GFP. In another approach, we used an existing plasmid constructed by Daniel Spirgel, containing portions of the 5'-upstream and 3'-downstream regions of the LHRH gene and the marker gene GFP, in which we have fused the coding sequence of GAD. GAD fusions have been tested in cell culture to verify the continuous open reading frame. Both types of constructs were used in the generation of transgenic mice. However, the expression of GFP from both constructs was very low and quickly inactivated, thus this approach proved to be not worth pursuing.

Therefore, we were forced to modify the original plan and use another approach to perturb the intracellular ratio of different GAD forms. We generated transgenic mice over-expressing GAD67 and embryonic GAD25/GAD44 in the lens using the widely-used lens fiber cell-specific αA-crystalline promoter. The expression profile of GAD forms in the wild-type mouse lens epithelium and lens fiber cells is strikingly similar to the one in the OE and migrating LHRH cells, namely these cells express all four GAD forms. The crystalline promoter-containing expression cassettes coding for GAD clearly interfere with the patterning of the lens inducing lens-retina and lens-cornea fusions and retina foldings. Our data indicate that the over-expression of GAD67 and GAD44 forms produce clearly distinct effects, which supports the idea that the different GAD forms have distinct roles within the same cell and can influence both proliferation and differentiation in a divergent way (interestingly of both the lens epithelial and retinal cells) via GABA acting through a paracrine mechanism. These results have been reported at several international meetings and are currently under preparation for submission.

Regarding the role of different GAD forms in the migration/differentiation of LHRH+ cells, we chose an alternative approach, in which we used the GAD65/GAD67 knock-out mice, provided by us by Y. Yanagawa (department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, Maebashi, Japan). The double heterozygous GAD65 and GAD67 knock-out mice were bred on C57Bl/6 background and single GAD65 knock-out and GAD67 knock-in (KI) lines were isolated. Subsequently, we bred the GAD65GFP line#5 into the KO lines, which resulted in model mouse lines lacking either GAD65 or GAD67 and expressing GFP in the migratory LHRH cells (and several other regions of the brain).

E11.5-E14.5 embryos from both genotypes were sectioned and the GFP expression in the migratory route of the LHRH+/GFP+ cells (the nasal region, septum and the POA) studied compared to control GAD65GFP#5 embryos.

We found that in the GAD65 KO mice, there are more cells migrating in the forebrain region and less numerous in the FNM, which would indicate that the absence of GAD65 speeds up the migration of the LHRH+ neurons into the brain. In addition, we found more cells in the region of the accessory olfactory bulb, where a minor population of LHRH+ neurons migrates and more randomly-spread population in the septum. This finding is greatly reminiscent to the findings reported for the LHRH population in the Dlx1/2 (transcription factors regulating GAD expression) knock-out mice (Givens et al., JBC
 Concerning GAD67 KO, this line is a neonatal-lethal and can be used with certain limitations in this study. These limitations are further enhanced by our unexpected finding that the embryonic GAD44 (derived also from the GAD67 gene) is still expressed in both GAD65 and GAD67 KO animals and may substitute functionally for GAD67, so the measured effect(s) would solely correlate with the absence of the adult GAD65 and GAD67, but not embryonic GAD. We are currently evaluating these results. Interestingly, we have found that the GAD65 KO mice, which are viable have impaired breeding capacity.

In conclusion, the work outlined in Aim 2 clearly indicates, that GAD65 acting through GABA can significantly influence the speed and direction of migration of LHRH+ neurons, which has a significant functional impact on the reproduction of the animal.

ABSTRACTS

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