4. Discussion

4.1 Murine *Tshzs* functionally overlap during spinal cord development

The zinc finger protein *Teashirt* (*Tsh*) was first identified in Drosophila, where it was characterized as a homeotic factor essential for the specification of segmental identities in the trunk (Fasano et al., 1991; Röder et al., 1992; De Zulueta et al., 1994). In addition, it was shown that *Drosophila Tsh* (*Dtsh*) is required for the transcriptional repression of *Ultrabithorax* in the midgut mesoderm (Waltzer et al., 2001). The interactions of *Dtsh* with *Hox* genes and with the *Wingless/Armadillo* signaling pathway (homologous to the vertebrate *Wnt/β-catenin*) have been described (De Zulueta et al., 1994; Gallet et al., 1998, Gallet et al., 1999).

In vertebrates, three *Tsh* homologues were identified, and their sequence and expression was characterized (mouse *Tshz 1/2/3*)(Caubit et al., 2000; Manfroid et al., 2004 and this work), but little is known about their function. The amino acid sequences of Teashirts from *Drosophila* and mouse are moderately conserved (35%), and homologies are essentially restricted to the region of the three atypical zinc finger motifs and the acidic domain. Moreover, murine *Teashirts* contain two classic zinc fingers and a homeodomain that are not found in the fly homologues. Ectopic expression of all three murine *Teashirts* in Drosophila was however reported to produce effects similar to those generated by *Dtsh* (Manfroid et al., 2004), e.g. to induce head to trunk transformation in different head segments. This, together with the finding that murine *Tshzs* act as transcriptional repressors, suggested a functional conservation between *Drosophila* and vertebrate homologues.

Recently, the first functional description for murine *Tshz1* in embryonic development was published (Core et al., 2007); *Tshz1* was found to be essential for the correct formation of components of the middle ear, including the tympanic ring and the malleus, and of the soft palate. *Tshz1* mutants exhibit *Hox*-like vertebral malformations and homeotic transformations in the cervical and thoracic regions, suggesting interaction between *Tshz1* and *Hox* genes in development of the bone. However, the functional relevance of murine *Teashirts* in the development of the

nervous system remains unexplored. A possible function in the central nervous system is suggested by the enriched expression of the three *Teashirt* genes in the spinal cord from E10.5 to adulthood (See fig.3.5) and in the developing forebrain (Caubit et al., 2005). Moreover, one of the Xenopus homologues to Drosophila *Teashirt* participates in the specification of the hindbrain and of the mid/hindbrain boundary (Koebernick et al., 2006). This demonstrated for the first time a role of the vertebrate Teashirts in the developing brain.

Here, I describe the beginning of a functional characterization of *Tshz1* in the developing mouse central nervous system. For this, I generated a strain of *Tshz1* mutant mice, in which the coding sequence was disrupted by insertion of a GFP reporter cassette. Aa starting point of my analysis I chose the dorsal spinal cord. The choice was done because of the relevant accumulation of *Tshz1* transcript in this tissue throughout development, and the interaction between *Hox* and *Teashirt* genes suggested by previous studies. *Hox* genes are also expressed in the dorsal spinal cord and have important functions in the maturation of spinal neurons (Graham et al., 1991; Goddard et al., 1996; Tiret et al. 1998; Lin and Carpenter 2003; Choe at al., 2006). I used genome-wide expression analysis of *Tshz1* mutant and wild type dorsal spinal cords to screen for pronounced changes in gene expression caused by the loss of *Tshz1*. This screening can be useful as a preliminary approach because it gives a broad overview of regulation and interactions of *Tshz1* with other genes and can therefore provide molecular insights about its function. The result of this analysis revealed that loss of *Tshz1* does not lead to a major molecular phenotype in the dorsal spinal cord. This could be due to a functional redundancy of *Tshz1* with the other two *Teashirt* genes, which are coexpressed during development and could therefore compensate the phenotype in the *Tshz1* mutant. Alternatively, the heterogeneity of dorsal neurons might be too large to detect gene expression changes in a small neuronal subpopulation by such an approach. Finally, the time point chosen for the analysis might be too early, and it might be advisable to repeat this analysis at later stages.

However, the genome-wide expression analysis revealed that in the dorsal spinal cord *Tshz1* functions as an autorepressor, and *Tshz1* transcripts were substantially up-regulated in the mutant mice. This is in agreement with the findings that Teashirts

function as transcriptional repressors (Manfroid et al., 2004), and that *Drosophila Teashirt* acts as an auto-regulator (Robertson et al., 2004).

4.2 *Tshz1* is expressed in the dLGE during embryonic life

The telencephalon has two major subdivisions: the pallium and the subpallium. The pallium consists primarily of laminar glutamatergic cortical structures. Progenitors of the pallium express dorsal molecular markers such as Pax6, Tbr2, Ngn2. The subpallium consists of the GABAergic basal ganglia, and its precursors express ventral molecular markers such as Gsh2 and Mash1. The pallial-subpallial boundary, where Pax6 and Mash1 are co-expressed, is called the dorso-lateral ganglionic eminence (dLGE). It contains progenitors that express a unique combination of transcription factors, like *Gsh2, Mash1, Arx, Er81*, that are implicated in OB neurogenesis. In *Gsh2* mutant mice, the dLGE loses its molecular characteristics and is respecified into a ventral pallium-like structure. As a consequence, the GABAergic interneurons in the olfactory bulb are severely reduced in number, showing that this area of the LGE is the major region responsible for the generation of olfactory bulb interneurons during embryonic life (Yun et al., 2001). Here I show that at E14.5 *Tshz1* expression, as revealed by GFP immunoreactivity from the *Tshz1*^{GFP} allele, is localized in the dLGE, exactly at the boundary between pallium (visualized through *Pax6* immunostaining) and subpallium (visualized with *Mash1* immunostaining) (Fig. 3.9 A, B). Yun et al. (2001) used high *Pax6* expression as one of the defining features of the dLGE. I demonstrate that some of these dLGE derived *Pax6*+ cells co-express *Tshz1* (Fig. 3.8 A, A´; Fig. 3.9 B, B´). The partial co-expression of *Pax6* and *Tshz1* supports the notion that the dLGE is a heterogeneous domain consisting of several different subpopulations of progenitors. Similar conclusions were drawn by the observation of *Sp8-Pax6* partial co-expression in the same region (Waclaw et al., 2006).

Recently, on the basis of the expression of *Er81* and *Islet1*, two distinct pools of progenitors were defined within the LGE (Toresson et al., 2000; Stenman et al., 2003; Wichterle et al., 2001): an *Isl1*+ population giving rise to striatal interneurons and an *Er81*+ population, located in the dLGE, contributing to the OB. Co-staining of GFP

and *Islet1* showed that *Tshz1* expressing cells are excluded from the *Islet1* expression domain. However, *Tshz1* and *Er81* immunoreactivities localize to the same region, the dorsal most part of the LGE, but these two factors are not co-expressed in the same cells. The immunoreactivities of *Tshz1* and *Er81* indicate that *Tshz1* is expressed in a domain, identified by *Er81* expression, that generates olfactory interneurons. The presence in the dLGE of two subsets of cells, one *Tshz1+* and the other *Er81+,* is a further indication of the heterogeneity of this progenitor zone, already suggested by Waclaw and colleagues. All transcription factors implicated in olfactory bulb neurogenesis, such as *Dlx5, Sp8, Arx,* are expressed in the dLGE in a way similar to *Er81* and *Tshz1*. Therefore, the *Tshz1* expression pattern is in accordance with a role of *Tshz1* in the development of one or more populations of olfactory bulb interneurons.

4.3. *Tshz1* expression is maintained in the postnatal SVZ

When development proceeds, the neuroepithelial germinal zone regresses and a relative small proportion of neural stem cells persist in the SVZ of the adult and postnatal forebrain. As the majority of olfactory interneurons are generated during the first weeks of life, the postnatal SVZ is an active germinative zone, and previous studies suggest that it derives from the embryonic dLGE and MGE (Stenman et al., 2003). *Tshz1* immunoreactivity is maintained in the P0 SVZ, together with the other transcription factors implicated in both embryonic and postnatal olfactory neurogenesis, such as *Dlx1/2, Er81* and *Gsh2*.

4.4. *Tshz1+* progenitors populate the bulb from E14.5 to adulthood

Tshz1 is strongly expressed in the OB from E14.5 to adulthood. At E14.5, *Tshz1* expression is segregated to the outer region of the bulb and overlaps with GABA immunoreactivity. At this stage, only few *Tshz1+* cells express GABA, but their numbers increase as development proceeds. My analysis shows that at E14.5 *Tshz1* is expressed by a stream of cells extending from the dLGE to the caudal extremity of the OB primordium as well as in cells that distribute at the periphery of the bulb (Fig. 3.11). The stream of *Tshz1+* cells extended next to the pallial ventricular marker *Pax6*

without overlapping with it (fig. 3.12). However, a series of coronal sections showed that *Tshz1*+ cells overlap with *Er81* expression in the sub-ventricular zone all along the stream (fig. 3.12). Using fate mapping and expression analysis of several markers, Long et al. (2007) proposed that the embryonic rostral migratory stream consists primarily of sub-ventricular zone cells expressing subpallial-type transcription factors (such as *Er81* and *Sp8*) and *GAD67*. Taken together, these results and observations suggest that *Tshz1*+ stream of cells, elongating at E14.5 from the lateral ganglionic eminence to the bulb primordium along the sub-ventricular zone of the lateral ventricle, belongs to the rostral migratory stream. Further studies (for instance fate mapping) are advisable to support the idea that the *Tshz1*+ cells located in the bulb have telencephalic origin. Progenitors of olfactory bulb cells do not only migrate from the telencephalon, but are also generated within the bulb. In embryonic and early postnatal life, local olfactory bulb progenitors are capable of giving rise to interneurons, but the local progenitor domain is depleted during adulthood. Vergano-Vera and colleagues (2006) showed with transplantation experiments that locally born olfactory bulb precursors contribute to the pool of GABAergic interneurons. Thus, a part of the *Tshz1*+ population in the embryonic olfactory bulb could be generated from local progenitors, even though a number of *Tshz1*+ cells could derive from the dLGE.

At P0, *Tshz1* is weakly expressed close to the center of the bulb, where the neuroblasts locally generated or derived from the RMS are located; however, it is strongly expressed by the outer ring of cells of the granule cell layer, where *Tshz1* immunoreactivity totally overlaps with GABA, meaning that *Tshz1* is expressed by a subpopulation of granule cells that is already integrated in the local circuits. BrdU labeling/chase experiments showed that this granule cell subpopulation originates at around E12.0, and by P0 had time to migrate radially to the external granule cell layer. During postnatal life the *Tshz1*+ granule cells increase in number, and by adulthood *Tshz1* is homogeneously expressed through the entire granule cell layer. Periglomerular interneurons expressing *Tshz1* are rare at P0 but increase during postnatal life, and in the adult olfactory bulb *Tshz1* is expressed by the entire Calretinin+ subpopulation of periglomerular neurons. Thus, *Tshz1*+ cells continue to be produced during postnatal life and adulthood and not only during embryonic

development. During adulthood, as the local OB germinative zone is depleted, new *Tshz1*+ cells can only be produced by the subventricular zone and populate the bulb through the rostral migratory stream.

4.5 *Tshz1* is essential for the maturation of granule cells

The functional relevance of *Tshz1* for the development of the OB could be analyzed only during embryonic life because its mutation leads to lethality at birth.
Tshz1 loss leads to impaired granule cell maturation and radial distribution, indicating that these two processes might rely on common molecular mechanisms. The failure of granule cell maturation is associated with a loss of GABA and TH expression in granule cells. Surprisingly, markers like Calretinin and Doublecortin are not lost. However, the definitive evidence for the loss of granule cell maturation in the absence of *Tshz1* is the loss of NeuN expression in the GFP expressing cells, as NeuN is a marker expressed exclusively by neurons that completed the maturation process. A clustering of Calretinin expressing neurons caused by cell autonomous mechanisms is also observed in mice with a mutation in the gene encoding the trancription factor Sp8. Sp8 is essential for the formation of Calretinin+ periglomerular neurons and GABAergic granule cells, and its expression resembles that of *Tshz1* in the adult OB.

Loss of *Tshz1* results in loss of the outer ring of the granule cell layer at P0, which includes GAD67+/GABA+ and TH+ granule cells. It is interesting to observe the selectivity of the defect that is restricted to the GABA+ and TH+ interneurons of the granule cell layer, while GABA and TH expressions remain unchanged in the glomerular layer. In accordance, during embryonic life, unlike in adulthood, *Tshz1* is hardly expressed by periglomerular interneurons. A differential level of expression in the embryonic and adult periglomerular neurons is not described for any of the other transcription factors implicated in olfactory bulb neurogenesis, such as *Dlx1/2, Dlx5, Sp8, Arx*. When these genes are deleted, GABAergic olfactory bulb embryonic neurogenesis is impaired without distinction between granule and periglomerular neurons. Thus, *Tshz1* is the first transcription factor described that during embryogenesis has a selective role for the correct establishment of GABAergic interneurons of the granule cell layer.

Mutations of most transcription factors with a known role in OB neurogenesis lead to hypotrophic bulb due to deficit in neurogenesis, failure of RMS migration (*Dlx5, Sp8*), or to a disorganization of the layering caused by a reduction of projections from the olfactory epithelium (*Fez, Arx*)(Long et al., 2003; Waclaw et al., 2006; Hirata et al., 2006; Yoshihara et al., 2005). Neither of these is the case for *Tshz1* mutant OB, which have normal size compared to the control and normal innervation. Only granule cells are affected by the mutation. Thus, the functional analysis of *Tshz1* provides specific insights on the mechanism of maturation of granule cells. The loss of granule cells in *Tshz1* mutants could be due either to reduced production of progenitors in the dLGE, to a reduced tangential migration to the bulb, or to a failure of maturation or radial migration within the OB. The localization of GFP+ cells in the $Tshz1^{GFP/GFP}$ OB helps addressing the question, as GFP expression in the mutant is misplaced, but not reduced. This suggests that, in the absence of *Tshz1*, the granule cell progenitors are formed and reach the bulb, but fail to mature and integrate in the local circuits. The hypothesis was supported by BrdU labeling experiments which showed that the *Tshz1*+/GABA+ cells forming the outer granule cell layer at P0 undergo their last cell division at around E12.0. In the control, these cells reach the outer granule cell layer through radial distribution from the core to the periphery of the bulb (Fig.3.19), in agreement with the results described by Tucker and colleagues (2006). However, in the absence of *Tshz1*, supernumerary BrdU+/GFP+ cells are observed that aggregate in the core of the bulb instead of integrating in the correct layer (Fig.3.20).

In the *Tshz1* mutant, a subpopulation of the granule cell layer, located at the core of the bulb and expressing markers such as Er81 and GAD65, is expanded to the layer normally occupied by mature, *Tshz1*+ granule cells (see *GAD65* expression in the mutant, Fig3.17 E, F). This could mean that in normal conditions the outer layer of granule cells inhibits the expression of marker genes in the neurons of the inner layer.

The functional analysis of *Tshz1* established a molecular distinction between the outer and inner granule cell layer. In early perinatal life, immature granule cells constitute the inner core of the layer. Also in the adult OB, the molecular properties of superficial versus deep granule cell layer are different and for example, grafting

experiments showed that *Pax6* contributes selectively to the formation of the superficial granule cells (Kohwi et al., 2005), supporting the notion that superficial and deep granule cells depend on different molecular programs for their generation (Orona et al., 1983). Previous studies (Lemasson et al., 2005) showed that early born interneurons are specifically targeted to the external edge of the granule cell layer, while the newly born neurons are positioned deeper. Moreover, early-generated neurons survive till adulthood, while younger interneurons are replaced within a few weeks. It will be interesting to analyze conditional *Tshz1* mutant if maturation of the superficial granule cell layer will be selectively affected in adult mice.

4.6 Sema3c and radial migration.

Genome-wide analysis of gene expression in P0 olfactory bulb from control and mutant mice revealed that *Tshz1* is directly or indirectly activating the expression of the guidance molecule Sema3C and of its putative signaling mediator Cypin, which could provide a molecular mechanism for *Tshz1* function in the radial migration of granule cells in the olfactory bulb. Clarifying guidance cues and mechanisms underlying interneuron migration is a question of general interest, because the olfactory system is often considered a useful model to study neuronal migration in the CNS.

To date, most research was focused on the mechanisms regulating tangential migration, which is the main type of migration in the developing forebrain, used by the interneuron precursors migrating from the LGE to the olfactory bulb and from the MGE to the cortex. Tangential migration is guided by a combination of repulsive, motogenic and chemoattractive cues. For instance, the proteins SLIT1 and 2 and their receptors ROBO1, 2 and 3 are expressed by the cells surrounding the SVZ and are able to repulse the newly generated cells from the SVZ, pushing them toward the OB and modulating their polarity during the process (Wu et al., 1999). In SLIT1 deficient mice, clusters of SVZ-derived neuroblasts migrate caudally into the corpus callosum, instead of moving to the RMS (Nguyen-Ba-Charvet et al., 2004). Other guidance cues important for the maintenance of the neuroblasts migration are the tyrosine kinase

receptor ErbB4 and its ligands (NRG1 and NRG2) and Eph tyrosine kinase receptors and their Ephrin ligands.

The radial migration within the OB is probably also regulated by a combination of several guidance cues, although the dynamics are slightly different from that of tangential migration. Radially migrating cells use radial glia as a scaffold for their movement, while the neuroblasts moving along the RMS slide along each other in neuronal chains, providing each other a substrate for motility. Thus, both mechanisms can be regulated by cues that act by cell-cell contact. For example, cells that migrate in the RMS express high levels of the polysialylated neural cell-adhesion molecule (PSA-NCAM), which is required to maintain a permissive milieu for their migration (Rousselot et al., 1995). After the interneuron precursors reach the bulb, however, they need to change orientation and switch from tangential to radial migration. To achieve this, different types of signals are necessary: initiation signals, allowing the neuroblasts to be released from the RMS to cease chain migration and to start to migrate individually along the glia, and maintenance signals, guiding the direction and orientation of the migration from the center to the periphery of the bulb. The first type of signal is given by Reelin, which is able to change adhesive interactions from tangential/chain to radial/individual migration in the postnatal OB (Hack et al., 2002). In *Reeler* mutant mice, neuronal precursors fail to migrate individually and accumulate in the OB; as a consequence, in the internal part of the granule cell layer the calretinin+ cells are irregularly organized with a tendency to form clusters. Lack of *Tshz1* provokes a similar (although more evident) clustering phenotype. However, Reelin expression remains intact in the *Tshz1* mutant, showing that loss of Reelin is not responsible for the radial migration impairment in mutant mice. Reelin was shown to lack guidance properties, and it was suggested to need collaboration of some other molecules to correct the migration of the neuron precursors (Hack et al., 2002). This implied the presence of maintenance signals given by guidance cues that were not clarified by previous studies.

Sema3C is a secreted member of the Semaphorin gene family; class 3 semaphorins are secreted glycoproteins that contain an approximately 500 amino acid N-terminal semaphorin domain, a C2 type immunoglobulin domain, and a highly basic C-

terminal tail. The first identified vertebrate semaphorin, semaphorin 3A, is a secreted protein purified on the basis of its ability to repel dorsal root ganglion axons in culture (Luo et al., 1993). In semaphorin 3A mutant mice, axons fail to avoid territories that normally express semaphorin 3A (Taniguchi et al., 1997). Semaphorin3 molecules have roles not only in axon guidance but also in regulation of neuronal migration from the subpallium to the cortex during embryogenesis. In particular, Sema3a and Sema3f exert chemorepellent activity in the striatal mantle, sorting the neurons that migrate to the striatum from those that migrate to the cortex (Marin et al., 2001). Semaphorins do not only act as repellent, and Sema3c can act as either a repellent or an attractant for axons growing in culture. For example, the growth cones of sympathetic neurons are repelled by Sema3c (Koppel et al., 1997), whereas growth cones of rat cortical axons are attracted towards a source of Sema3c (Bagnard et al., 1998). Phenotypes of Sema3c mutants in the nervous system were not described. High-affinity receptors for semaphorins have been identified. They include the plexins, which are a family of large transmembrane molecules that are conserved from invertebrates to humans, and the neuropilins (NP1 and NP2), that are found only in vertebrates (Tamagnone and Comoglio, 2000). The functional receptor for secreted class 3 semaphorins is a complex, including both neuropilins and plexins (Tamagnone et al., 1999; Takahashi et al., 1999; Rohm et al., 2000). Although neuropilins plasmatic domain is short and dispensable for the signal transduction, the interaction plexin-semaphorin3 requires the presence of neuropilins.

In the olfactory bulb at P0, Sema3c is expressed in the outer ring of the granule cell layer, immediately abutting the reelin expressing mitral cells, while this expression is lost in the *Tshz1* mutant. Similarly to Reelin, Sema3c could provide a cue for the initiation or the maintenance of the radial migration, representing the molecular key to the function of *Tshz1* in regulating the distribution of immature granule cells. How would Sema3c, which is expressed by external granule cells in the periphery of the olfactory bulb, traverse the entire granule cell layer to attract neuronal precursors from the center of the structure? As other guidance molecules, Sema3c might passively diffuse through the granule cell layer creating a concentration gradient that induces a biological response. Alternatively, Sema3c could be secreted by the radially migrating interneurons themselves in an autocrine fashion, as was shown to happen

for migrating cells of the developing vascular system (Serini et al., 2003). In this study, Serini and co-workers showed that, during vascular development, migrating endothelial cells generate autocrine signals of class 3 semaphorins, which are able to block integrin adhesion receptors. Spatio-temporal control of integrin functions is important for the dynamic adhesion of the migrating cells to the extra cellular matrix. It is interesting to note that the expression of a second molecule, cypin, is dramatically decreased in *Tshz1* mutants. Firestein and colleagues first isolated this protein from the rat brain in 1999, and found that it shares high homology with CRMP. CRMP mediates intracellular responses to Semaphorin3 (Goshima et al., 1995). We can therefore hypothesize that the Cypin+ granule cells that are lost in the *Tshz1* mutant are able to respond to the Semaphorin signaling. This hypothesis is supported by the observation that proteins of the PlexinA family, which were shown to serve as co-receptors for the Semaphorin3 signaling, are differentially expressed throughout the granule cell layer during development. In particular, PlexinA1 is expressed in the outer granule cell layer in a similar way to Tshz1, Cypin and Sema3c (Murakami et al., 2001). This suggests that the Tshz1-dependent, Cypin+/PlexinA1+ outer ring of the granule cell layer is able in normal conditions to respond to the attractive cues provided by Sema3c. Consequently to the loss of *Tshz1,* both Sema3c and Cypin are not correctly expressed, and this could explain the inability of the immature granule cells to move toward the periphery of the bulb. Taken together, these results and observations support the idea that *Tshz1* regulates the radial distribution of granule cells by modulating the transmission of the guidance cues provided by Sema3c.

4.7 Outlook

Genesis and migration of olfactory bulb neurons are considered a good model to provide insights into the general mechanisms of cell maturation during forebrain development. Despite the advances in understanding the mechanisms underlying embryonic olfactory neurogenesis, many aspects remain to be clarified. A number of transcription factors play a role in this process, including Gsh2, Dlx5/6, Pax6, Sp8, Arx. None of these, however, determines the fate of a single interneuron population. Mutations of these genes affect development of granule and periglomerular interneurons and, in some cases, also of projection neurons. In this work, I show that *Tshz1* has a specific role in development of granule cells. Loss of *Tshz1* leads to an

arrest of granule cell maturation in the embryo, but does not affect the periglomerular interneurons, suggesting a specific molecular mechanism underlying the embryonic maturation of this particular GABAergic interneuron subpopulation. Moreover, I show that *Tshz1* is essential for the correct radial distribution of granule cells within the bulb. Thus, my functional analysis of *Tshz1* helps in elucidating the mechanisms underlying the last part of olfactory interneuron maturation.