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CHARACTERIZATION OF EVOLUTIONARY CONSERVED SUBDIVISIONS IN THE EMBRYONIC ZEBRAFISH FOREBRAIN BASED ON GENE EXPRESSION PATTERNS

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ABSTRACT

Intersecting transverse and longitudinal transcription factor expression domains divide the embryonic brain into a grid of histogenetic fields with distinct positional identities. The topology of histogenetic fields has been highly conserved during vertebrate evolution and can therefore be used to reveal homologous relations of brain areas between vertebrates. In order to further establish zebrafish as a model system to study vertebrate brain development and related neurological disorders, homologous relationships of zebrafish and mammalian brain regions have to be clarified. For this purpose we developed a multi-color fluorescent in situ hybridization (FISH) protocol to distinguish abutting and overlapping gene expression patterns at high-resolution. We improved the sensitivity of peroxidase (POD) based FISH through the inclusion of the polymer dextran sulfate into hybridization and substrate reaction and the application of substituted phenol compounds as POD accelerators. The utilization of bench-made fluorogenic POD substrates further increased sensitivity and allowed for detection of up to three different transcripts simultaneously. This multi-color FISH method was used to construct a comprehensive map of regulatory gene expression domains of the embryonic zebrafish diencephalon and hypothalamus. Our analysis was consistent with a three-prosomeric organization of the zebrafish diencephalon. It also showed that intraprosomeric subdomains in the alar pretectum, thalamus and prethalamus have been conserved. The strikingly similar topology of hypothalamic gene expression domains of murine and fish orthologous transcription factors allowed identifying homologous progenitor domains. Using corticotropin-releasing hormone as a molecular differentiation marker we could further establish homology between the nucleus preopticus and the paraventricular nucleus as well as for the supramammillary band in fish and mammals. The detailed expression maps generated in this work also provide a predictive tool to aid functional studies on genetic pathways involved in specification of neuronal fields and cell types. The compilation of embryological zebrafish expression data in gene maps will facilitate comparisons across species and help to promote studies on vertebrate brain development and neurological disorders using zebrafish.

LIST OF PUBLICATIONS

I. **Lauter G**, Söll I, Hauptmann G
Multicolor fluorescent in situ hybridization to define abutting and overlapping gene expression in the embryonic zebrafish brain;
Neural Development 2011, 6:10

II. **Lauter G**, Söll I, Hauptmann G
Molecular characterization of prosomeric domains and subdomains of the embryonic zebrafish diencephalon;
Journal of Comparative Neurology, Submitted manuscript

III. **Lauter G**, Söll I, Hauptmann G
A molecular model of the developing zebrafish hypothalamus;
Manuscript

IV. Chandrasekar G, **Lauter G**, Hauptmann G
Distribution of corticotropin-releasing hormone in the developing zebrafish brain;
Journal of Comparative Neurology, 2007, 505(4), 337

FURTHER PUBLICATIONS

I. Hao L, Johnson R, **Lauter G**, Baillie D, Bürglin TR.
Comprehensive analysis of gene expression patterns of hedgehog-hog related genes; BMC Genomics. 2006; 7:280

II. Archer A, **Lauter G**, Hauptmann G, Mode A, Gustafsson JA.
Transcriptional activity and developmental expression of liver X receptor (lxr) in Zebrafish; Dev Dyn. 2008; 237(4):1090-8

III. **Lauter G**, Söll I, Hauptmann G
Two-color fluorescent in situ hybridization in the embryonic zebrafish brain using differential detection systems;
BMC Development; Submitted manuscript

Mit dem Wissen wächst der Zweifel.

Johann Wolfgang von Goethe

Für Eleonor,
mein Fels in der Brandung,

für Ebba und Thilo,
meine Freude,

für Margit und Friedrich Lauter,
meine glückliche Erinnerung.

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LIST OF ABBREVIATIONS

ABB	alar basal boundary
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3'-indolylphosphate
bHLH	basic Helix Loop Helix
CNS	central nervous system
CoP	commissural pretectum
FAM	5-(and-6)-carboxyfluorescein
FISH	fluorescent in situ hybridization
GABA	γ-aminobutyric acid
hpf	hours post fertilization
JcP	juxtacommissural pretectum
LC	locus coeruleus
NBT	nitroblue tetrazolium
NBT-DF	nitroblue tetrazolium – diformazan
NPO	nucleus preopticus
p1-6	prosomere 1-6
dpf	days post fertilization
PVN	paraventricular nucleus
PBS_T	phosphate buffered saline Tween
PcP	precommissural pretectum
PFA	para-formaldehyd
POD	peroxidase
SM	supramammillary band
SPV	supraoptic-paraventricular area
TAMRA	5-(and-6)-carboxytetramethylrhodamine
TM	tuberomammillary hypothalamus
TNT_T	Tris NaCl Tween
TSA	tyramide signal amplification
TU	tuberal hypothalamus
WISH	whole mount in situ hybridization
zli	zona limitans intrathalamica

1 INTRODUCTION

1.1 HISTORICAL CONCEPTS OF FOREBRAIN ORGANIZATION

During development of the vertebrate brain multiple morphogenetic processes cause the dramatic transformation from a planar neural plate into the complexly folded structure that constitutes the functional organ. This way, the developing central nervous system (CNS) becomes progressively subdivided into the major divisions of the brain. As a sign for early regionalization, a series of rostrocaudally arranged brain vesicles form (Nieuwenhyus, 1998). The appearance of prominent bulges has long been recognized with the first account dating back to Malpighi in the 17th century and the first systematic description to von Baer in 1828. Von Kupffer (1906) distinguished two phases of regionalization during which the neural primordium becomes subdivided into segmental units along the anteroposterior axis. Consequently, he termed the first phase primary neuromery, which deals with segmentation of the neural plate, and secondary neuromery, which deals with segmentation of the neural tube and can be followed by the appearance of brain vesicles (Bergquist, 1964; Kuhlenbeck, 1973; Nieuwenhyus, 1998; von Kupffer, 1906). Beginning with formation of the two-vesicle-stage, the neural tube shows a bipartition into the archencephalon anteriorly and the deuterencephalon posteriorly (Fig.1). The boundary between the two coincides with transition from the underlying prechordal plate to the notochord. Upon reaching the three-vesicle-stage the well-known subdivisions of fore- (prosencephalon), mid- (mesencephalon) and hindbrain (rhombencephalon) have formed. This stage arises by the forming isthmus, thereby dividing the deuterencephalon into mid- and hindbrain. The former archencephalon is now called prosencephalon. The five-vesicle-stage is characterized by further subdivisions of the rhombencephalon into met- and

myelencephalon and of the prosencephalon into tel- and diencephalon (Bergquist, 1964; Källén, 1953).

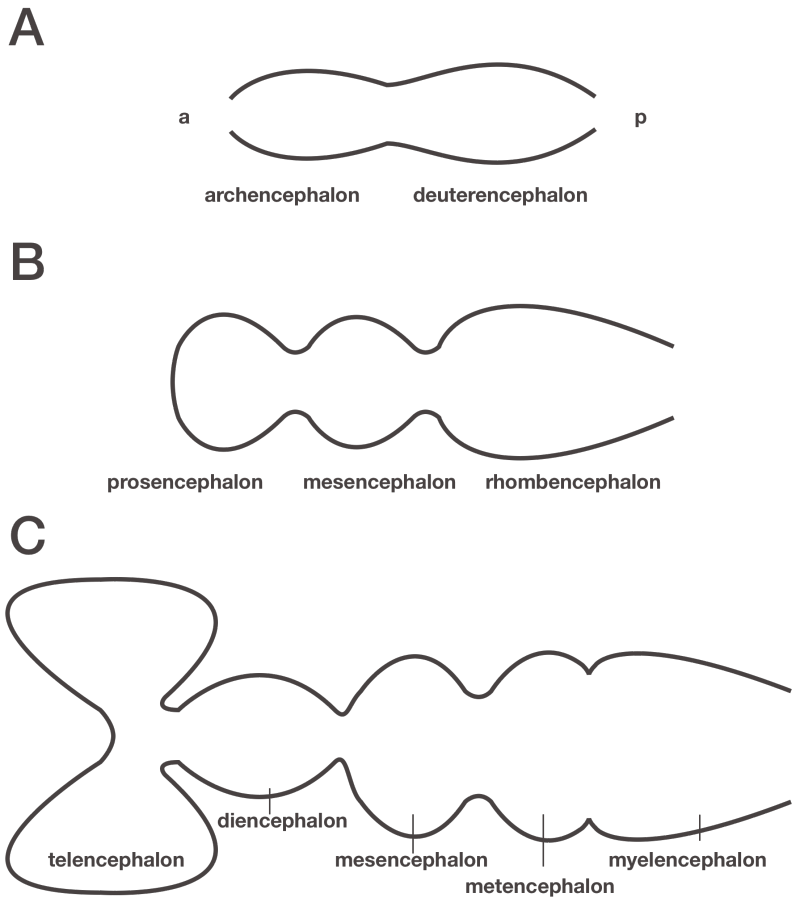

Fig.1 Development of the main brain divisions.
A) two-vesicle stage; **B)** three-vesicle stage; **C)** five-vesicle stage;
(a = anterior; p = posterior; adapted from (Nieuwenhyus, 1998)

Despite von Kupffer using the term "neuromery" to describe the appearance of the five brain vesicles as signs of segmentation, this term has generally been reserved to describe finer, segmental subdivisions, so-called neuromeres, within each of the major brain regions (Mueller and Wullimann, 2005; Nieuwenhyus, 1998). Hence, transverse subdivisions of the pros-, mes- and rhombencephalon have been named prosomeres, mesomeres and rhombomeres, respectively (van Meek 1907; 1909; Pombal et al., 2009). In the rhombencephalon a neuromeric organization has been observed very early

(von Baer, 1848). The existence of 7-8 rhombomeres has been corroborated since then and criteria for the identification of segments have been put forward (Kiecker and Lumsden, 2005). Whether these criteria can be or should be applied to define segments also in the prosencephalon is still up for debate (see section 1.2).

However, back in pre-molecular times the transient appearance of ring-shaped bulges was questioned to be a sign for true segmentation, as was segmentation generally questioned to be the underlying principle of brain organization. The prevailing conception at the beginning of the 20th century was that the brain is organized into longitudinal zones (Nieuwenhuys, 2009b; 1998; Northcutt, 1995). This concept can be seen as an extrapolation of spinal cord organization into longitudinal, functional columns to other brain parts. The most prominent advocates of this columnar model were C. J. Herrick and Kuhlenbeck. According to the columnar model neuromeres are only transient structures in the early embryo, which are soon replaced by functional columns. Spinal cord and hindbrain are composed of a dorsal roof plate, paired lateral plates and a ventral floor plate. Anteriorly, the lateral plates increasingly thicken and become the alar and basal plates separated horizontally by the sulcus limitans. The rostral limit of the sulcus limitans has been perceived differently. His (His, 1893) postulated that the sulcus limitans reaches into the preoptic area whereas Herrick (Herrick, 1848) saw it terminate in the rostral midbrain. Hence, Herrick assumed that the midbrain comprised alar and basal plates, whereas the forebrain forms exclusively from the alar plate. Based on observations in postnatal specimen Herrick identified epithalamus, dorsal thalamus, ventral thalamus and hypothalamus as four longitudinal columns that reflect the basic columnar organization of the diencephalon (Herrick, 1910). His analysis was based on the appearance of ventricular sulci, which were supposed to run in parallel to the forebrain axis and to bound functional, longitudinal

zones. This columnar brain organization was propagated by Kuhlenbeck and many others and has been very influential to the present day.

However, the columnar model is built on the assumption of a straight brain long-axis as a reference line, what is a crude simplification and neglects the actual curvature of the forebrain axis. The Swedish neuroanatomists Bergquist and Källén based their work on embryonic studies and arrived at conclusions that torpedoed the authority of the columnar model. Taking the actual curvature of the neuraxis into account, the former longitudinal zones turned out to represent transverse subunits arguing for a segmental organization (Bergquist and Källén, 1954; Nieuwenhuys, 2009b; 1998). Bergquist and Källén found neuromeric bulges to occur in all vertebrates at a certain stage of development and result from transversely arranged zones of high mitotic activity (Bergquist, 1952; Bergquist and Källén, 1954; Källén, 1952; 1953). During subsequent development, transversely oriented zones intersect with longitudinal proliferation zones to partition the forebrain into a grid of areas with individual centers of high mitotic activity (Fig.2). They termed these areas Grundgebiete or migration areas, as each area is also the site of future differentiation and migration. When Bergquist and Källén extended their studies to all major vertebrate groups and all brain parts, they found a surprising degree of conservation in the topography of migration areas. Their idea was that conserved migration areas should be the basis for sound homologisation, in the sense that they are the same by origin and not by function (Nieuwenhyus, 1998). The description of the conserved network of migration areas is also a description of a common vertebrate Bauplan of the brain and clearly in line with a neuromeric organization of the forebrain. Based on the recognition of migration areas Bergquist and Källén defined five transverse bands in the prosencephalon lying perpendicular to a curved brain long-axis. Two longitudinal proliferation zones, columnae dorsolateralis

and ventrolateralis, cross the transverse bands and divide the prosencephalon into seven dorsal areas and five ventral areas (Fig.2) (reviewed in Nieuwenhyus, 1998).

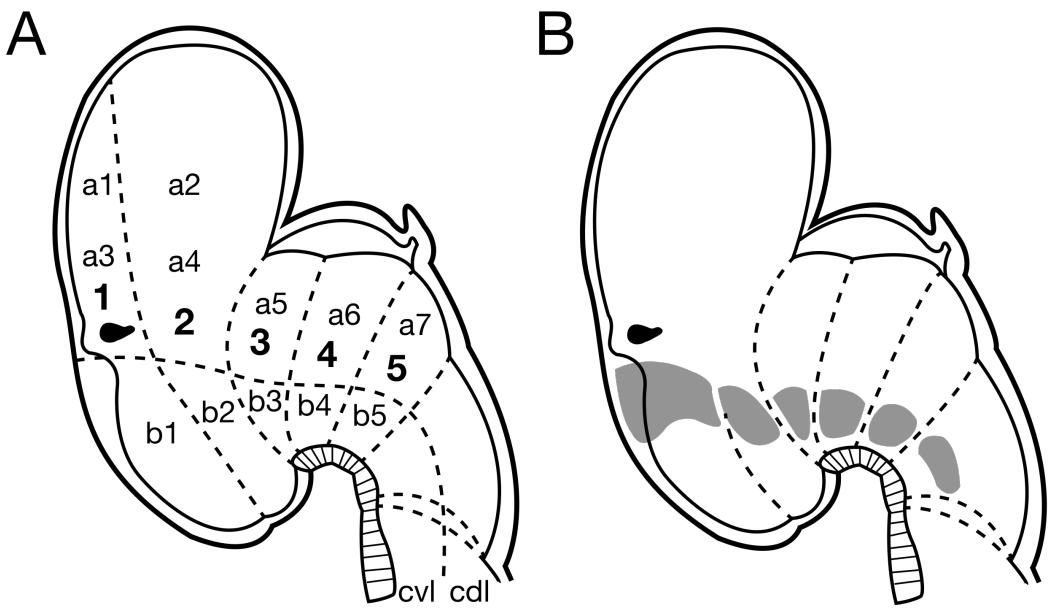

Fig.2 Neuromeric forebrain organization

A) Model by Bergquist and Källén showing the topology of transverse bands (1-5) longitudinal columns (cdl: columna dorsolateralis, cvl: columna ventrolateralis) and different migration areas (a1: area ventralis telencephali, a2: a. dorsalis telencephali, a3: a. optica, a4: a. rostralis thalami, a5: a. medialis thalami, a6: a. caudalis thalami, a7: a. commissurae posterioris, b1: a. rostralis hypothalami, b2: a. intermedia hypothalami, b3: a. caudalis hypothalami, b4: a. tuberculi posterioris, b5: a. fasciculi longitudinalis medialis.

B) Early neuronal differentiation zones

Early neuronal differentiation zones (gray) occur in the centre of forming neuromeres as revealed by the presence of acetylcholinesterase.

(Anterior is oriented to the left; adapted from Bergquist and Källén 1954; Puelles et al. 1987)

1.2 BRAIN SEGMENTATION

The work of Bergquist and Källén did not receive the credit it deserved regarding the results, accuracy and efforts it shows. This is due to the fact that for many the existence of proliferation zones remained somewhat elusive, as they appear only transiently and are difficult to identify on histochemically stained brain sections (Nieuwenhuys, 2009b; Nieuwenhyus, 1998; Puelles et al., 1987; Striedter, 2005). The columnar interpretation of the diencephalon, on the other hand, can easily be recognized from late developmental stages onward. A paradigm shift commenced when new techniques for gene and protein visualization gave fresh impetus to the concept of neuromeric brain organization. Supported by several lines of evidence, a segmental organization of the hindbrain has now gained general acceptance. The chick and zebrafish hindbrains show a reiterative architecture with reticulospinal and motor neurons positioned in rhombomere centers and commissural axons crossing at the level of rhombomere boundaries (Lumsden and Keynes, 1989; Trevarrow et al., 1990). Single cell labeling after boundary formation demonstrates that rhombomeres r2-r6 represent lineage restriction boundaries (Fraser et al., 1990). Rhombomere boundary cells are marked by selective expression of *radical fringe* and the sustained activation of the Notch signaling pathway in zebrafish (Cheng et al., 2004). Furthermore, a segmental organization of the hindbrain is supported by nested expression of *Drosophila Homeobox* (*Hox*) gene orthologues, which coincide with rhombomere boundaries. Rhombomeric expression was first reported for members of the *Hox-2* cluster in mouse (Wilkinson et al., 1989), but has since also been shown for members of the *Hox-1, -3* and *-4* cluster (Wilkinson, 1993). These findings show the segmental nature of rhombomeres that represent developmental compartments (reviewed in Kiecker and Lumsden, 2005).

Whether or not the entire brain shows a segmental organization is a question of ongoing debate and often arises from differing definitions for segmentation. Based on their findings in the hindbrain Keynes and Lumsden defined four criteria to identify segmental subunits in the brain (Fraser et al., 1990; Guthrie, 1995; Keynes and Lumsden, 1990). First, segmental units show a reiterative architecture in proliferation, neurogenesis and axonal projection. Second, segmental boundaries show reduced proliferation and express distinct molecular markers. Third, segmental units correspond to gene expression domains. Fourth, segmental boundaries are also lineage restriction boundaries that prevent cell mixing. The criterion of clonal restriction is considered most decisive, as it also defines developmental compartments reminiscent to those found in *Drosophila*. According to Keynes and Lumsden only the hindbrain fulfills all criteria and is therefore the only truly segmented brain part.

Puelles and Rubenstein, on the other hand, argue for a segmental organization of the entire brain and do not agree on the necessity of lineage restriction as a criterion to define segmental units (not at least, since lineage restriction is a property acquired secondarily to anteroposterior patterning) (Puelles and Rubenstein, 1993; 2003). Segmental units rather have to be molecular distinct and comprise a complete set of dorsoventral longitudinal zones from floor to roof.

1.3 THE PROSOMERIC MODEL

Nevertheless, evidence supporting a neuromeric organization of the forebrain has also accumulated with modern molecular techniques. By using an acetylcholinesterase assay Puelles and colleagues showed that early differentiating neurons appear in distinct successive clusters in the center of developing neuromeres, corroborating early transverse proliferation zones as described by Bergquist and Källén (Puelles et al., 1987). Homologues of *hox* genes that control segment identity in *Drososphila* larvae

and similarly in the vertebrate hindbrain are not expressed in the forebrain. However, other transcription factors, including many homeobox genes like orthologues of *Drosophila empty spiracle* (*emx*) and *orthodenticle* (*otx*) genes and members of the *Lim*, *Pax* and *Nkx* gene families are expressed in the vertebrate forebrain and govern its development. The expression boundaries of numerous regulator genes coincide and fall onto common transverse and longitudinal boundaries. In this way, the embryonic forebrain is subdivided into a chessboard-like network of expression domains. This has let to the formulation of a prosomeric model that describes the neuromeric organization of the entire forebrain based on the expression of numerous developmental regulator genes (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). The prosomeric model has not only been of great value as a useful framework to understand topological relations between different brain parts and species, but also makes the complex neuroanatomy of the forebrain comprehensible from a developmental perspective. In doing so, it rests on several assumptions that are in opposition to former forebrain models, most notably the columnar model (Fig.3). One key point is the definition of the brain's long-axis that takes the curvature at the cephalic flexure into account and serves as an internal reference. This axis is at right angle to the proposed brain axis in the columnar model and demands a redefinition of directional descriptions like dorsal-ventral and rostral-caudal. Consequently, the dorsal-ventrally arranged diencephalic longitudinal domains of the columnar model actually represent anterior-posterior subdivision lying perpendicular to the long-axis. This affects also the position of the telencephalon, which becomes located dorsoanterior instead of representing the anterior limit of the brain (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003).

In the prosomeric model longitudinal zones present in the hindbrain are supposed to extend throughout the entire brain and run in parallel to the curved long axis. The actual embryonic zones are, from dorsal to ventral, roof, alar, basal and floor plate. The alar and basal plates run in between the medial roof and floor plates as pairwise arranged bands. At the rostral limit of the brain, alar and basal plate fuse with its respective counterpart from the other side. The alar-basal plate boundary defines at the same time the course of the brain long-axis, which ends rostrally close to the area of the optic chiasm (Pombal et al., 2009; Puelles et al., 2004; Rubenstein et al., 1998).

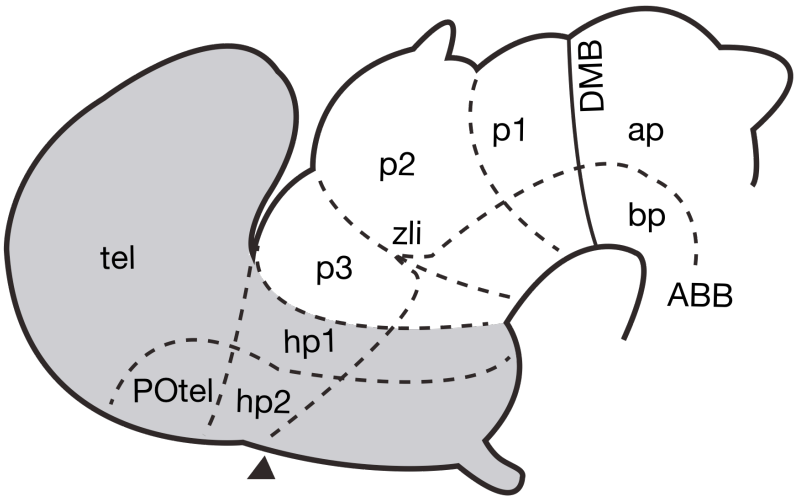

Fig.3 The prosomeric model
Revised prosomeric model showing the distinction between secondary prosencephalon (gray hp1-2) and the diencephalon proper (p1-p3). All prosomeres comprise a complete set of longitudinal floor, basal (bp), alar (bp) and roof plates (roof and floor are not shown). The alar-basal boundary (ABB) outlines the course of the bend longitudinal brain axis. Further abbreviations: DMB: diencephalic-mesencephalic boundary; hp: hypothalamic prosomere; tel: telencephalon; POtel: preoptic telencephalon; zli: zona limitans intrathalamica. Black triangle indicates the anterior end of the brain.
(adapted from Puelles 2010; http://developingmouse.brainmap.org/docs/ReferenceAtlas.pdf

Along the anteroposterior axis, the prosomeric model subdivides the forebrain into the secondary prosencephalon and the diencephalon proper. Telencephalon, eye vesicles and hypothalamus constitute the secondary prosencephalon, which forms the rostral most part of the brain. In contrast to other forebrain models, the telencephalon is perceived as an exclusively alar derivative. Ventral and dorsal thalamus, plus pretectum form the diencephalon proper (Bulfone et al., 1993; Puelles et al., 1987; Puelles and Rubenstein, 1993). According to the original version of the prosomeric model the entire forebrain is subdivided into six prosomeres that lie perpendicular to the brain axis. Each prosomere forms a ring-like segment that spans the entire neural tube and comprises a complete set of longitudinal domains from roof to floor plate (Bulfone et al., 1993; Puelles and Rubenstein, 1993). In sharing the same basic longitudinal composition, individual neuromeres qualify as serial homologues, despite their differences in regional identity (Pombal et al., 2009). In the diencephalon three prosomeres can be distinguished, named p1-p3 from caudal to rostral. The pretectum corresponds to p1, dorsal thalamus and ventral thalamus correspond to p2 and p3, respectively. Since the terms dorsal and ventral thalamus originate from the columnar model and erroneously imply a dorsal-ventral structuring, dorsal and ventral thalamus were renamed to thalamus and prethalamus (Puelles and Rubenstein, 2003). Likewise, the secondary prosencephalon is composed of the three anterior prosomeres p4, p5 and p6 that share parts of the hypothalamus ventrally and the telencephalon dorsally.

It has been emphasized from the beginning that the prosomeric model represents a conceptual framework and as such is not static. A growing body of new data either confirms the existing model or makes adjustments necessary (Pombal et al., 2009; Puelles and Rubenstein, 2003; Rubenstein and Puelles, 1994). Therefore the prosomeric model has evolved through several rounds (Nieuwenhuys, 2009b; Pombal et al., 2009;

Puelles and Rubenstein, 2003). The definitions of the diencephalic prosomeres (p1-p3) have thereby undergone little change and proven to be consistent with new expression data. One notably change occurred in the prethalamus (p3), where the eminentia thalami has been grouped into p3 instead of p4 due its location dorsal to the prethalamus. The recognition of prosomeric divisions in the secondary prosencephalon has been hindered by the complex morphogenesis of the telencephalon. Dramatic deformations occur during telencephalic development and secondary patterning events generate subdivisions independent from those in the underlying hypothalamus (Puelles and Rubenstein, 2003). Consequently, the apparent boundaries cannot represent interprosomeric boundaries, as they do not intersect the entire neural tube, from roof to floor, perpendicular to the long-axis. Therefore the secondary prosencephalon was considered as a single, unsegmented entity with a rostro-caudal bipartititon of the hypothalamus and an undivided telencephalon (Pombal et al., 2009; Puelles and Rubenstein, 2003). However, the latest version of the prosomeric model revived a segmental organization. The intrahypothalamic boundary divides the hypothalamus and curves around the preoptic area, thereby separating it from the remaining subpallium. As its dorsal end coincides with the rostral limit of the roof plate, the intrahypothalamic boundary encompasses a complete set of longitudinal zones and formally qualifies as a true interprosomeric boundary (Pombal et al., 2009).

Interestingly, the latest version of the prosomeric model bears great resemblance with the model proposed by Bergquist and Källén (as reviewed and illustrated in (Nieuwenhuys, 2009b). In both models the forebrain parcelates into five segmental domains that are split-up into three units in the diencephalon and two in the secondary prosencephalon. The most striking difference however, lies in the course of the boundary in the secondary prosencephalon. This boundary separates subpallium and

pallium in Bergquist and Källén's model. The latest version of the prosomeric model assigns most of the pallium and subpallium to the same prosomere (except for the region of the preoptic area that is ascribed to the rostral most prosomere).

1.4 IMPLICATION OF THE PROSOMERIC MODEL

As mentioned before (see section 1.3) the prosomeric model has contributed substantially to clarify forebrain anatomic relationships of embryonic as well as adult brain subdivisions. This is in great part a consequence of the redefinition of the brain long-axis. Already at the time when the prosomeric model was first proposed the authors envisioned that it "should be generalizable to other vertebrate species" (Rubenstein and Puelles, 1994). Over the course of almost two decades comparative data from representatives of all major vertebrate groups like lamprey, zebrafish, frog, chicken, mouse and also turtle have accumulated (Bachy et al., 2001; Bulfone et al., 1993; Fernandez et al., 1998; Figdor and Stern, 1993; Hauptmann and Gerster, 2000b; Hauptmann et al., 2002; Pombal and Puelles, 1999; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). This set of data reveals a high degree of conservation in gene expression patterns between different vertebrate species. The prosomeric model makes topological relationships apparent and comparable by functioning as a reference framework (Puelles and Medina, 2002). Comparative gene expression data from all studied vertebrate species do by and large fit into the prosomeric model. Regionally restricted combinations of developmental regulators define the molecular identity of histogenetic fields. As the prosomeric model provides the foundation to compare and identify the same histogenetic field in different species, it lays the foundation for sound homologisation (including adult brain structures, see section 1.5). Since expression patterns of putative developmental regulators (i.e. transcription factors and signaling molecules) build the basis for the model, it also holds some predictive powers on

possible interactions, patterning and organizing mechanisms (Rubenstein and Puelles, 1994). Regardless of any segmental organization of the forebrain the prosomeric model serves as a template for the construction of comparative gene maps that have great practical value for studying nucleogenesis and terminal differentiation processes.

1.5 THE HISTOGENETIC FIELD CONCEPT

The complexity of the brain poses a problem to homologize adult brain parts on the basis of morphological landmarks unambiguously. The many existing contradictory models on the organization of the telencephalon in ray-finned fish give a most striking example (reviewed in (Nieuwenhuys, 2009a). In the brain model by Bergquist and Källén transverse oriented proliferation zones intersect with longitudinal zones to subdivide the embryonic brain into a network of so-called “Grundgebiete” with high proliferative activity at their centres (compare 1.1). The Grundgebiete are the future sites of neurogenesis characterized by increased radial migration and differentiation. Therefore the term migration areas has been used synonymously for the German word Grundgebiete (translatable with founding areas) (Bergquist and Källén, 1954; Nieuwenhyus, 1998). Bergquist and Källén recognized that evolutionary conserved migration areas in the embryonic brain offer a way to homologize adult brain parts (Bergquist and Källén, 1954). As summarized in the concept of field homology, adult brain structures can be considered homologues when the embryonic fields from which they are derived are homologous (Puelles and Medina, 2002). The morphologically defined migration areas are reminiscent of the histogenetic fields introduced by Puelles and Rubenstein (1993; 2003). This is not surprising, since prosomeric subdivisions are defined by regionally restricted transcription factor expression. The transcription factor code active at a certain locus represents the specification state of that particular site and directs future development. Therefore, histogenetic fields visualize the underlying

genetic mechanism of regionalization and later morphogenesis. Conserved histogenetic fields can be identified and compared long before proliferation patterns and other morphological features become apparent (Morona et al., 2011; Puelles et al., 2004; Puelles and Medina, 2002).

1.6 GENOARCHITECTONICS

The pattern of evolutionary conserved embryonic neural progenitor domains reveals a glimpse on the underlying organization plan of the brain that is common to all vertebrates. This uniformity is somewhat surprising taking into account that vertebrates are adapted to very different environments and display an astonishing divergence in adult brain anatomy and functional capacities. Following primary patterning events that establish field identity, secondary histogenetic processes like proliferation, migration, differentiation, synaptogenesis, apoptosis and others begin to unroll and shape the neural wall along the ventricular-pial dimension. The more or less initially planar histogenetic field thus expands into a three dimensional radial domain (Puelles et al., 2004). Species-specific variations in histogenetic and morphogentic processes are thought to account for the observed diversity of adult vertebrate brains (Puelles, 2001; Puelles and Medina, 2002). Subtle variations in these processes can have great effects on the resulting shape of the neural wall. The late-equals-large-hypothesis describes how size increase in the cerebral cortex might be explained by a prolonged proliferation phase prior to neurogenesis (Chenn and Walsh, 2002; Smart et al., 2002). However, also early variations in patterning have been described to cause shape and size variations in progenitor domains, which subsequently manifest themselves in differences of adult brain parts (Sylvester et al., 2010)

A prominent feature during differentiation of the hypothalamus and diencephalon is the aggregation of neuronal somata into delineable clusters, or so-called brain nuclei (Lim and Golden, 2007; Nieuwenhyus, 1998). The appearance of brain nuclei is the result of differentiation processes that act in the ventricular-pial dimension within radial domains. After exit from the cell-cycle in the proliferating zone at the ventricle, postmitotic neuroblasts become progressively specified along their radial migration until they reach their terminal differentiation state. The whole differentiation process can be visualized stepwise with the help of gene markers (Mueller and Wullimann, 2005). The use of gene and protein expression patterns as morphological markers to describe the spatiotemporal sequence of development form a particular progenitor domain until the appearance of brain nuclei has recently been summarized under the term genoarchitectonics (Ferran et al., 2009; Morona et al., 2011). Central for this approach is the concept that the identity of a particular brain nucleus is determined by the identity of the histogenetic field from which it is derived (see Bergquist and Källén, 1954). With help of the prosomeric model combinations of developmental regulators can be selected as markers for major histogenetic field. Characteristically early regional markers tend to have a sheet-like expression that extends uniformly over one or several prosomeres and at the same time across the entire neural wall (ventricular and mantle zone). Prominent examples are members of the *Pax*-gene family like *Pax6,-3,-7* but also *Otx1/2* and *Gbx2* (Bulfone et al., 1993; Ferran et al., 2007; Puelles and Rubenstein, 1993; Simeone et al., 1992). The ventricular proliferation zone of a particular histogenetic field can be distinguished by PCNA (proliferating cell nuclear antigen) expression (Wullimann and Knipp, 2000; Wullimann and Puelles, 1999). During subsequent development major forebrain divisions are progressively sub-regionalized. The pretectum serves as an illustrating example for sub-regionalization. The alar pretectal area (marked by *Pax-3* expression) parcelates into three subregions named

pre-, juxta- and commissural pretectal domain (PcP, JcP and CoP) that can be selectively visualized by *Dbx1, Ebf1* vs. *Six3, Lhx1, Tal2* vs. *Pax7, Gsh1* (Ferran et al., 2009; Ferran et al., 2007; Morona et al., 2011; and this work). Another example is the bipartition of the thalamus into a rostral and caudal thalamic domain, marked by selective expression of *Emx2, Ascl1* versus *Neurog1* (Mueller and Wullimann, 2003; Scholpp et al., 2009; Vue et al., 2007; Wullimann and Mueller, 2002). In case of the thalamus it also becomes apparent that regional specification and neuronal differentiation are linked and under tight spatial and temporal control. The sub-regionalization marked by *Ascl1* and *Neurog1* reflects at the same time a fate choice between two neuronal subtypes. Both genes belong to the basic-Helix-Loop-Helix (bHLH) family of transcription factors that are prominent markers of neurogenesis and responsible for GABAergic and glutamatergic neuronal subtype specification, respectively (Bertrand et al., 2002; Fode et al., 2000; Vue et al., 2007). Another member of the bHLH family, *her6*, functions in the switch between the two alternatives fates and determines spatial patterning at the same time (Scholpp et al., 2009). Differentiated neurons of brain nuclei can be characterized by their morphology, connectivity to other sites as well as by the set of transmitters they express. Noteworthy in this respect are neuropeptides, which are often preferred differentiation markers as they are expressed at high levels in discrete cells of brain nuclei (Chandrasekar et al., 2007). At the same time are neuropeptides tightly linked to physiological functions of particular nuclei. For example are AgRP (agouti-related protein) and NPY (neuropeptide Y) both expressed in the arcuate nucleus where they are involved in the endocrine regulation of food-intake (Schwartz et al., 2000). Although it should be mentioned that most neuropeptides are not restricted to a single nucleus, but are abundant at other brain sites and involved in multiple functions.

The genoarchitectonic approach can be applied in multiple ways. It can be used as a molecular tool to dissect tempero-spatial events from specification of field identity to subregionalization, proliferation and neurogenesis to gradual differentiation events including neuronal subtype specification and expression of particular neuropeptides (Mueller and Wullimann, 2005). However, depending on the problem a detailed visualization of each differentiation step is often neither intended nor necessary. Since transcription factor codes determine regional field identity, there is a strong causal correlation between their expression and later derived mature nuclei (Lim and Golden, 2007; Puelles et al., 2004; Puelles and Medina, 2002). This is further underscored by the persisting expression of early regional markers during later development that are restricted or excluded from nuclei. Therefore regional markers have been very successfully used to delineate brain nuclei in development (Ferran et al., 2009; Ferran et al., 2008; Hashimoto-Torii et al., 2003; Lim and Golden, 2007; Morona et al., 2011; Nakagawa and O'Leary, 2001). Although the correlation of appearing brain nuclei with regional markers represents a strict morphological description, functional assumptions can be extrapolated (Ferran et al., 2008). It is apparent that a detailed morphological description by gene expression patterns aids future functional studies what has been demonstrated in numerous studies (Flames et al., 2007; Hashimoto-Torii et al., 2003; Nakagawa and O'Leary, 2001; Scholpp et al., 2009; Vue et al., 2009).

1.7 ZEBRAFISH AS A VERTEBRATE MODEL SYSTEM

Zebrafish offers several advantages that stand behind a growing interest in using it as a vertebrate model system. Great accessibility and transparency of embryos during development together with forward and reverse genetic tools, a growing collection of available mutant and transgenic stocks, easy handling and cost-efficiency have to be mentioned in this respect. Various questions in development, behaviour, disease and

pharmacology are now studied in zebrafish. For example has the zebrafish system been used as a model to study cancer and metastasis (Amatruda and Patton, 2008; Lee et al., 2009; Stoletov and Klemke, 2008), cardiovascular and lymphatic systems (Hogan et al., 2009; Jensen et al., 2009; Langenau and Zon, 2005) and drug discovery (Rihel et al., 2010; Strähle and Grabher, 2010). The conserved architecture of the central nervous system has made zebrafish a prime model to study neurodegenerative diseases like schizophrenia, Parkinson and Alzheimer (Panula et al., 2010). In order to claim that findings in the zebrafish brain can be extrapolated to other vertebrates and are of general validity, it is crucial to clarify homologous relationships of brain regions and neuronal systems. The prosomeric model has proven to function as an excellent framework to ensure comparability. However, although there is considerable amount of data from amniotes the resource of comparative data from anamniotes is less comfortable. In teleosts a prosomeric organization of the diencephalon is supported by the identification of partitioned proliferation zones together with neurogenic and proneural gene expression patterns (Mueller and Wullimann, 2005; Mueller and Wullimann, 2002; Mueller and Wullimann, 2003; Wullimann and Mueller, 2004; Wullimann and Puelles, 1999; Wullimann et al., 1999). The visualization of longitudinal and transverse gene expression domains in zebrafish with help of chromogenic two-color WISH further supports a similar prosomeric organization of the fish diencephalon as in other vertebrates (Hauptmann and Gerster, 2000b; Hauptmann et al., 2002).

The compiled gene expression analysis presented in this work was conducted to clarify homologous relations between zebrafish and other vertebrates within the context of the prosomeric model. Taking advantage of our high-resolution visualization technique, we wanted to see to what extent also intraprosomeric subdomains have been conserved during evolution. We hope this comparative analysis will help the zebrafish system on

its way to become an even more useful model to study vertebrate brain development and causally related diseases.

2 AIMS

The main objective of the work presented in this thesis was to establish a detailed genetic map of the embryonic zebrafish forebrain. The derived gene expression map is meant to enable the identification of homologous embryonic fields between fish and other vertebrates. As an analytical tool, a detailed gene map will also aid future functional studies by predicting causal mechanisms between developmental regulator genes and differentiation markers. This comparative analysis will help promote the zebrafish system as a model to study general vertebrate brain development and related diseases.

The specific aims were:

- To develop a detection and visualization method that allows comparing a high number of forebrain specific regulatory gene expression domains at high resolution.
- To identify regulator marker genes that can be used to reveal evolutionary conserved subdivisions in the zebrafish forebrain.
- To delimitate inter- and intraprosomeric boundaries and territories in the alar and basal plate of the zebrafish diencephalon.
- To identify homologues fields and derived nuclei in the zebrafish hypothalamus.
- To characterize the distribution of *corticotropin-releasing hormone* positive cells in correlation to known regional markers in the developing zebrafish brain.

3 METHODS

3.1 MOLECULAR CLONING METHODS

For amplification and cloning of cDNAs standard methods in molecular biology were used that are well established and have been extensively described elsewhere (Sambrook and Russel, 2001).

3.2 EMBRYO COLLECTION AND PREPARATION

Zebrafish were raised and maintained under standard conditions in a Schwarz Aquarienbau (Göttingen, Germany) fish facility. Embryos were obtained by natural matings of pairs of wild-type fish. Embryos were staged in hours post fertilization (hpf) according to Kimmel et al. (1995). To suppress pigmentation in embryos older than 24h, phenylthiourea was applied to a final concentration of 0.03%. Fertilized eggs were allowed to develop at 28.5°C until fixation in 4% para-formaldehyd (PFA) at the desired developmental stage. Embryos were fixed for 24h at 4°C, subsequently transferred to methanol and kept at -20°C. For further permeabilization embryos were incubated in a 2% peroxide solution in methanol for 20 min. After stepwise rehydration, embryos were digested with proteinase K as described by Hautpmann and Gerster (2000a) and transferred into hybridization buffer.

3.3 PROBE PREPARATION

RNA probes were generated by *in vitro* transcription and labelled either with digoxigenin-11-UTP (Roche), fluorescein-12-UTP (Roche) or dinitrophenyl-11-UTP (PerkinElmer) as described by Hauptmann and Gerster (1994; 2000a). To recover labelled RNA transcripts, E.Z.N.A RNA probe purification columns (Omega Bio-Tek R6249-02) were used as described by the manufacturer.

3.4 IN SITU HYBRIDIZATION

For two-color whole mount in situ hybridization (WISH) differently hapten labelled RNA probes were simultaneously hybridized at 60°C overnight (min 15h). Each probe was sequentially detected by an antibody conjugated to alkaline phosphatase (AP) and directed against the adequate hapten of the RNA probe. Embryos were first blocked in 8% normal sheep serum in PBS_T for 1h with gentle agitation and then incubated with the respective antibody at 4°C overnight without agitation. All antibodies were diluted in blocking solution. Using FastRed or NBT/BCIP as chromogenic substrates, each probe was detected in a different color (Fig.4). Prior to the second detection round AP activity was inactivated by incubation in glycine-hydrochloric acid pH2.2 for 10 min. For multi-color fluorescent in situ hybridization (FISH) each probe was detected sequentially by an antibody coupled with horse-radish peroxidase (POD) and directed against the adequate hapten of the RNA probe. Embryos were first blocked in 8% normal sheep serum in phosphate buffered saline plus 0.1% Tween-20 (PBS_T) for 1h with gentle agitation and then incubated with the corresponding antibody at 4°C overnight without agitation. All antibodies were diluted in blocking solution. The tyramide signal amplification (TSA) system was used for fluorogenic visualization. TSA utilizes POD activity to covalently link fluorescent-tyramide dyes to tyrosine moieties of proteins present close to the site of catalysis (Fig. 4). TSA reaction buffer was always prepared freshly and contained 100mM borate pH 8.5, 2% dextran sulfate, 0.1% Tween-20 and 0.003% H_2O_2. Tyramide reagents were synthesized by coupling amine reactive succinimidyl esters to tyramine (Hopman et al. 19(Hopman et al., 1998)98). The following bench-made tyramide reagents were used: 5-(and-6)-carboxyfluorescein tyramide (FAM-tyramide), tetramethylrhodamine (TAMRA-tyramide) and DyLight633-tyramide. Each tyramide reagent was diluted 1:250 in TSA

reaction buffer (except for DyLight633-tyramides, which were diluted 1:167). The TSA reaction was allowed to run maximal 30 min protected from light without agitation. Thereafter, samples were rinsed thoroughly. When more than one probe was detected, POD activity was inactivated by incubating the embryos with glycine-hydrochloric acid pH2.0 for 10 min prior to the next round of detection.

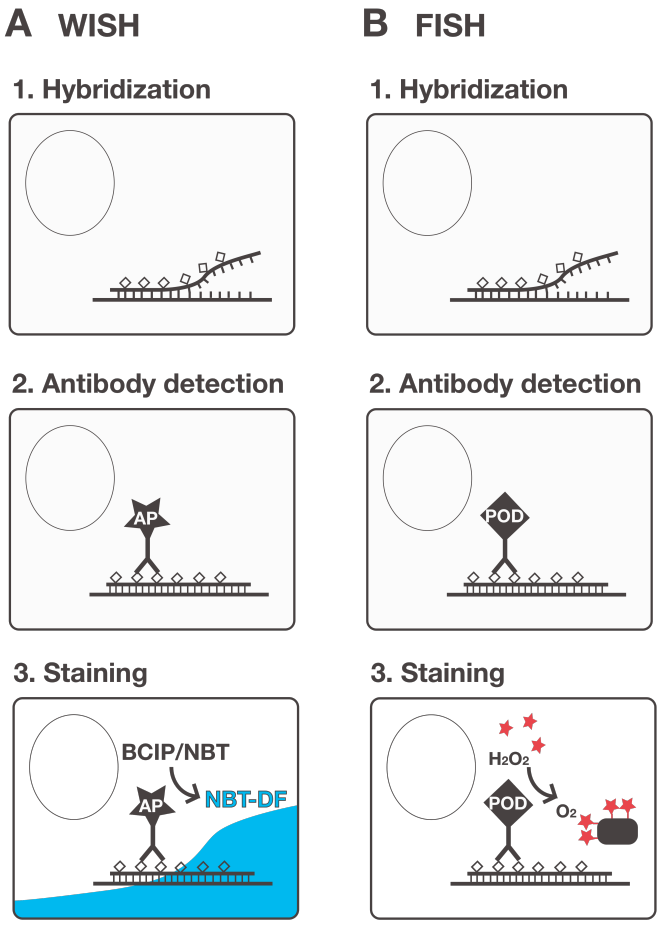

Fig.4: Principles of WISH and FISH
1.A,B) A hapten-labelled antisense RNA probe is hybridized to the mRNA of interest. **2.A)** Labelled RNA probe is detected by anti-hapten antibody conjugated to alkaline phosphatase (AP). **2.B)** Labelled RNA probe is detected by anti-hapten antibody conjugated to peroxidase (POD). **3A)** During AP-based detection in WISH color-less NBT/BCIP is converted into the blue-purple precipitate NBT-DF, which accumulates in the cytoplasm. **3B)** During POD-based detection in FISH fluorophore-labelled tyramides are covalently linked to tyrosine residues of nearby proteins (black box), resulting in high spatial resolution.

3.5 IMAGING

Stained embryos were gradually transferred into 75% glycerol in PBS_T for a better refraction index and for storage. Chromogenic stained embryos were imaged with a 20x air objective under a Zeiss Axioplan2 microscope equipped with a Zeiss Axiocam color-camera. Fluorescent samples were imaged on an inverted Zeiss LSM510 confocal microscope using a 488 and a 633 nm laser line for excitation. To avoid any undesired movement of specimen during recording, embryos were embedded in 75% glycerol in 100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20 (TNT_W) plus 1% low melting agarose. Usually z-stacks of whole mount embryos were recorded with optimal settings for optic sectioning as suggested by the system (pinhole, number of slices, distance between slices). The maximum distanced covered in z-stacks was around 100 μm, so that in sagittal orientations the half of the embryo closest to the objective was imaged. Routinely each staining was recorded from two different perspectives (in most cases sagittal and dorsal). In two-color FISH experiments we used the combination of fluorescein-tyramide (excitation at 488nm) with the DyLight633-tyramide (excitation at 633nm) as substrates, because of their long spectral separation. To test for any residual bleed-through in two-color experiments we performed single color FISH with selected probes recorded in all detection channels. None of the two substrates produced any detectable bleed-through (see Lauter et al., 2011).

3.6 IMAGE PROCESSING

The field of digital image processing is advancing with rapid pace and offers numerous tools to extract desired information in a comprehensible and presentable way from raw data. At the same time this creates a lot of pit falls and the danger for manipulation. In principle, the best way to improve image quality (reliable signal representation) is to

improve the applied staining method. Therefore image-processing tools have been applied in a conservative way in this work, meaning as little as possible and as much as necessary. In particular, we tried to use the entire intensity spectrum during recording and to avoid any overexposure in the sites we were interested. For image processing ImageJ software was exclusively used. During processing the cut-off was set before contrast was enhanced. Next, a Laplacian of Gaussian filter was applied that proved to combine optimal filter effects, as it removes background noise and functions as an efficient edge detector at the same time. For better contrast visualization different channels were false-colored with cyan and magenta look-up-tables. Overlays were generated with help of the ImageJ plugin Color functions/Color merge. For better perception the difference operator of this plugin was used to visualize overlap in yellow (cyan and magenta normally mix to white).

4 RESULTS AND DISCUSSION

4.1 MULTI-COLOR FLUORESCENT IN SITU HYBRIDIZATION (FISH)

In order to generate a detailed map of gene expression patterns, a method was required that enabled comparing a multitude of forebrain-specific regulatory gene expression sites at high resolution. We therefore developed an optimized protocol for multi-color fluorescent in situ hybridization in whole mount zebrafish embryos based on the tyramide signal amplification (TSA) system. Because of the known limitations of conventional POD based FISH, we tried to increase signal intensity and ratios by several means.

Application of the viscosity increasing polymer dextran sulfate during hybridization and TSA reaction resulted in increased sensitivity and signal-to-noise ratios. The effectiveness of dextran sulfate can be explained by an increase in effective probe/tyramide concentration caused by molecular crowding (Ellis, 2001). To further increase efficiency of the fluorogenic TSA reaction, we tested the effect of peroxidase rate enhancers (Stout, 1985). Addition of the substituted phenol compounds 4-iodophenol and vanillin resulted in significantly increased signal-to-noise ratios. This effect was dose-dependent with 4-iodophenol being the most potent rate enhancer. To match fluorogenic TSA substrates to our microscope setup we generated fluorophore-labeled tyramides by conjugating amine reactive succinimidyl esters to tyramine (Hopman et al., 1998). In addition to a substantial cost reduction, we could also show that bench-made tyramide substrates have a higher sensitivity than commercially available substrates. Differences in sensitivity between commercial and bench-made tyramide reagents have also been reported previously (Vize et al., 2009). The dramatic

increase in sensitivity and signal-to-noise ratios required effective inactivation of POD activity between detection rounds. We assessed alternative methods and found that peroxide treatment only partially inactivated POD activity, despite being commonly used. Glycine-hydrochlorid acid treatment, on the other hand, resulted in complete inactivation of POD activity. These results are in accordance with findings by Liu and colleagues that quantitatively assessed the efficacy of a number of peroxidase inhibitors (2006). The implemented improvements allowed visualizing transcript distributions of up to three different genes simultaneously. For this purpose up to three different RNA labelled probes were hybridized together and subsequently detected in up to three rounds of antibody POD based detection using different fluorogenic tyramides as substrates.

4.2 REGIONALIZATION OF THE EMBRYONIC ZEBRAFISH DIENCEPHALON

In order to address to what extent topological relationships of histogenetic fields have been conserved between anamniotes and amniotes, we aimed to determine inter- and intraprosomeric subdomains in the embryonic zebrafish diencephalon. First, we identified zebrafish orthologues of putative forebrain specific regulatory genes with expression in the diencephalon as diagnostic markers. Using our multi-color FISH protocol we then compared expression patterns of a multitude of markers to generate a high-resolution gene map of evolutionary conserved domains. The study is focused on 1dpf embryos, as this stage represents the onset of the vertebrate phylotypic stage and the vertebrate bauplan is established (Kimmel et al., 1995).

We show that the combinatorial expression of *pax3a* and *pax6a* delimitates the alar pretectal territory in zebrafish. This is consistent with findings in other vertebrates showing that the anterior limit of *Pax3* expression marks the course of the thalamo-

pretectal boundary and the posterior limit of *Pax6* expression marks the diencephalic-mesencephalic boundary (Derobert et al., 2002; Ferran et al., 2008; Ferran et al., 2007; Hauptmann and Gerster, 2000b; Hauptmann et al., 2002; Murakami et al., 2001). Ventrally, the alar pretectum reaches to the alar-basal boundary (ABB), which coincides with the ventral *pax6a* expression limit. The pretectal area defined in this way matches alar p1 (Ferran et al., 2007; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). Furthermore we could show a tripartition of the pretectum into a rostral-, intermediate- and caudal-pretectal subdomain selectively marked by the respective expression of *dbx1a*, *six3a* and *pax7a*. These results suggest that in addition to major prosomeric subdivisions also intraprosomeric divisions have been conserved from fish to mammals. Corresponding domains have been identified in frog, chicken and mouse named PcP, JcP and CoP (Ferran et al., 2009; Ferran et al., 2008; Ferran et al., 2007; Morona et al., 2011; Redies et al., 2000; Yoon et al., 2000). We found that epithalamus and thalamus are molecularly distinct territories, as they are characterized by different molecular specification codes (*pax3a, six3a, lhx9* versus *dbx1a, emx2, olig3*). In the thalamus we define a bipartition into a rostral thalamic and caudal thalamic progenitor domain marked by expression of *emx2*, *nkx2.2* and *pax6a, dbx1a*, respectively. The bipartition of the thalamus is in agreement with studies in mouse and fish that showed how the respective subregions translated into alternative neuronal subtypes (Scholpp et al., 2009; Vue et al., 2007; Vue et al., 2009). Based on the expression of *dbx1a*, *dlx2a* and *pax6a* we show a tripartition of alar p3 into slim posterior (*dbx1a*) and broad anterior prethalamic domains (*dlx2a* and *pax6a*) and a dorsally located eminentia prethalami (*pax6a* alone). In the posterior diencephalon we identified a longitudinal domain located in between alar *pax6a* and basal *shha* expression selectively marked by the expression of *nkx2.2a.* An identical arrangement of longitudinal bands has been found in amniotes and ascribed a parabasal identity

(Ferran et al., 2007; Garcia-Calero et al., 2006; Gimeno and Martinez, 2007; Puelles, 2000). The parabasal band represented by *Nkx2.2* expression is thought to "approximate" the course of the alar-basal boundary.

The dependence of *nkx2.2a* expression on *shha* argues for a basal ascription of the *nkx* domain, so that the ABB is set in between dorsoventral expression limits of *pax6a* and *nkx2.2a*. In the rostral diencephalon (anterior to the zli) *nkx2.2a* partially reached into the alar plate preventing the use of *nkx2.2a* to delineate the ABB in this brain part. We found that members of the *nkx6* gene family (*nkx6.1* and *nkx6.2*) delimitate inter-prosomeric boundaries in basal p1. The rostral limit of *foxa2* expression demarcates the entire p2-p3 boundary, as it coincides with the rostral limit of the zli in the alar plate and abuts anterior *dbx1a* expression in p3 (Hauptmann and Gerster, 2000b; Rubenstein et al., 1998). We show a bipartition of basal p3 into a posterior domain (marked by *dbx1a*) and an anterior domain marked by the combined expression of *nkx2.1a* and *lhx5*. This bipartition corresponds to previously defined transverse expression domains d5 and d6 (Hauptmann and Gerster, 2000b; Hauptmann et al., 2002).

In summary, we could determine a high-resolution gene map for the embryonic zebrafish diencephalon that reveals great consistency in prosomeric domains and boundaries between fish and other vertebrates. The degree of conservation reached to the level of intraprosomeric domains demonstrating the universality of the vertebrate brain bauplan at an additional level.

4.3 A MOLECULAR MODEL OF THE EMBRYONIC ZEBRAFISH HYPOTHALAMUS

The hypothalamus is the master regulator of neuroendocrine and metabolic systems that ensure homeostasis. Dysregulation of neuroendocrine cell-type specification during hypothalamic development may therefore be tightly linked to the occurrence of

neuroendocrine disorders. We attempted to construct a model of conserved hypothalamic histogenetic fields to clarify topological and homologous relationships between fish and mammals in order to provide a sound basis for comparative studies.

Using our multi-color FISH protocol (Lauter et al., 2011) we directly compared expression domains of hypothalamic markers in zebrafish. To facilitate a direct comparison of early histogenetic fields, we focused our study on an early developmental stage (28-32hpf) before differentiated morphological features are recognizable.

We found that *nkx2.1a* expression marked the entire basal hypothalamus, as it abutted alar *dlx2a* expression dorsally and *dbx1a* in the posterior tuberculum caudally. A corresponding topology has been described for the murine hypothalamus (Shimogori et al., 2010). Likewise, within the basal hypothalamus several major subdomains were identified that have also been described in the murine hypothalamus based on similar expression patterns. In particular, we could distinguish a rostral area marked by *six3a* expression, which corresponds to the tuberal hypothalamus (TU) (previous retrochiasmatic) (Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003; Rubenstein and Puelles, 1994). Caudally, this region was followed by the basal extension of *dlx2a* expression marking the tuberomammillary region (TM) (previous tuberal) (Bulfone et al., 1993; Eisenstat et al., 1999; Puelles and Rubenstein, 1993). The combined expression of *sim1* and *otp1* was interpreted as corresponding to the supramammillary band (SM), since it delimited the posterior basal hypothalamus terminating jointly with the caudal expression boundary of *nkx2.1a* (Puelles et al., 2004; Puelles and Rubenstein, 2003). The primordium of the mammillary and retromammillary region, as marked by the combined expression of *emx2* and *lef1*, was located basal to the TM and SM. Shimogori and colleagues made use of nuclei-specific expression markers to delineate histogenetic fields from which distinct hypothalamic

nuclei are derived (2010). We show here the presence of distinct hypothalamic nuclei in the zerbrafish hypothalamus. In correspondence to the mammalian hypothalamus zebrafish *pomca* and *ff1b* orthologues identified the precursors of the arcuate and ventromedial hypothalamic nucleus, respectively.

Also the topology of gene expression domains in the alar hypothalamus was correlating to that in other vertebrates. A telencephalic and a hypothalamic longitudinal *dlx2a* expression domain were clearly discernable and served as a reference to describe topological relations (Bulfone et al., 1993; Eisenstat et al., 1999; Hauptmann and Gerster, 2000b; Hauptmann et al., 2002; Puelles and Rubenstein, 1993; Shimogori et al., 2010). In mouse, the diencephalic *Dlx2* expression demarcates the so-called suprachiasmatic band, which continues rostrally from the suprachiasmatic area, over the hypothalamic cell cord and posterior entopeduncular area to the prethalamus caudally (Bulfone et al., 1993). In zebrafish, the corresponding domain was separated from the telencephalic *dlx2a* expression domain by a *dlx2a*-negative stripe and designated optoeminential band. The conserved expression of *lhx1a,-6a,-9* and *lhx5a* confirmed the arrangement of alar hypothalamic subdivisions into a suprachiasmatic and optoeminential band, respectively (Abellan et al., 2010; Shimogori et al., 2010). Within the optoeminential band combined expression of *sim1* and *otp1* marked a subregion located directly rostral to the eminentia prethalami that corresponds to the supraopto-paraventricular area (SPV) in other vertebrates (Bulfone et al., 1993; Fan et al., 1996; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003).

In summary, we showed that the topology of hypothalamic subdivisions has been highly conserved from fish to mammals and likely represent homologous histogenetic fields.

4.4 CORTICOTROPIN-RELEASING HORMONE IN THE EMBRYONIC ZEBRAFISH

Abnormal development of neuroendocrine, hypothalamic nuclei is tightly linked to the occurrence of hormonal disorders. In order to enable comparative studies in zebrafish, we wanted to resolve homologous relations between fish and mammalian hypothalamic brain nuclei.

For this purpose we used corticotropin-releasing hormone (*crh*) as a molecular differentiation marker, since it has been highly conserved as a central stress mediator of neuroendocrine and behavioural responses. We identified a cDNA clone encoding for full-length CRH precursor protein and used it in WISH to determine the distribution of *crh* expressing cells during embryonic zebrafish development. We found several prominent *crh* expression sites located within the hypothalamus. In order to determine the position of *crh* cell clusters within the framework of the prosomeric model, we compared *crh* expression in relation to the forebrain-specific markers *shha*, *dlx2a* and *pax6a*. Within the preoptic area we found a cluster of *crh* positive cells located anterior to *pax6a* expression in the prethalamus and dorsal to *dlx2a* expression in the alar hypothalamus. This region corresponds in fish to the area of the nucleus preopticus (NPO) for which the presence of *crh* positive cells has also been reported in other fish (Ando et al., 1999; Morley et al., 1991; Okawara et al., 1992). In mammals, this region corresponds to paraventricular nucleus (PVN), which is located within the supraoptic-paraventricular area (SPV) (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003). The distinct expression of the transcription factors *Otp* and *Sim1* within this area in fish and mammals further supports a correspondence of the NPO with the PVN. Carried on to the functional level, both genes play a role in the specification of CRH cells during development in fish and mouse (Acampora et al., 1999; Keith et al., 2001; Lohr et al., 2009; Michaud et al., 2000; Michaud et al., 1998).

To confirm a homologous relation between NPO and SVP, we further looked at the expression of isotocin (it) and vasotocin (vt), which are orthologues of mammalian oxytocin and vasopressin and known neuroendocrine cell type markers of the PVN (Brownstein et al., 1980). Co-distribution of *crh* with *it* and *vt* strongly suggests that the fish NPO is homologous to the mammalian PVN consistent with a central hypophysiotropic role in vertebrates.

We identified another prominent cluster of *crh* expressing cells located ventral to *pax6a* in the prethalamus and caudal to the basal hypothalamic extension of *dlx2a*. In the context of the prosomeric model this area maps to the caudal hypothalamus and more specifically to the region of the supramammillary band (SM) (see section 4.3). Expression of the *otp1* and *sim1* is characteristically confined to the SM and has been shown to direct specification of catecholaminergic cell types (Löhr et al., 2009; Puelles et al., 2004; Puelles and Rubenstein, 2003; Ryu et al., 2007). Consistently, we found *tyrosine hydroxylase* (*th*), a marker for catecholaminergic neurons (Holzschuh et al., 2001), in close association with *crh* expressing cells in that region. Location, and co-distribution of *crh* cells with gene regulators and differentiation markers suggest a homologous relation between mouse and fish supramammillary hypothalamus.

Co-distribution of *crh* and *th* was also seen in the basal region of rhombomere 1 within the area of the locus coeruleus (LC). This is consistent with the interaction of CRH and major monoaminergic systems in mediation of behavioural responses to stress (Curtis et al., 1997; Valentino and Foote, 1987; Valentino and Foote, 1988; Valentino et al., 1991). *crh* positive cells were detected in further brain regions (telencephalon, thalamus, epiphysis, midbrain tegmentum, rostral hindbrain and neural retina) suggesting additional non-hypophysiotropic functions for CRH.

In summary, we showed homology between fish and mammalian SPV and SM, supported by topological, genetic and functional arguments.

5 CONCLUSIONS AND PERSPECTIVES

We have presented here a novel and optimized multicolor FISH method that enables the comparison of forebrain specific gene expression patterns at cellular resolution. We have used this technique to generate a molecular map of the embryonic zebrafish diencephalon and hypothalamus. In addition we identified homologues neuroendocrine nuclei in zebrafish and mouse by mapping neuropeptide differentiation markers on to our models of diencephalic and hypothalamic subdivisions.

A future challenge will now be to use our FISH method to generate similar detailed molecular maps of the zebrafish subpallium and pallium. Topological relationships between fish and mammalian pallium have been difficult to resolve, because of their different modes of morphogenesis. This long lasting problem has also been called "… the central enigma of the forebrain …" by Nieuwenhyus (2009a). However, our FISH technique has the potential to solve this problem, especially when combined with 3D imaging techniques.

Gene expression maps of conserved regulatory genes in the fish and mammalian forebrain will provide a helpful tool to clarify homology of forebrain subdivisions, derived nuclei and neuronal systems. One prime focus will be to establish homology between brain structures associated with the occurrence of human neurological disorders. A subsequent step will be to use our gene maps as predictive tools to elucidate genetic pathways required for the formation of disease related subdivisions, nuclei, neuronal subtypes and circuits.

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