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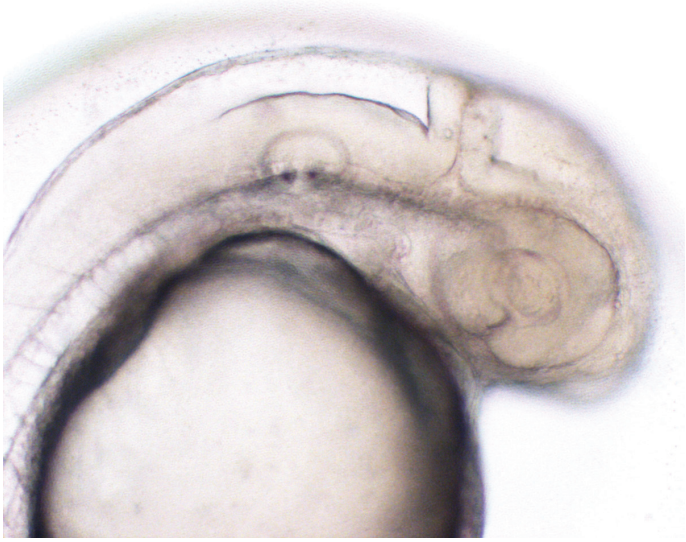

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Neuroscience Center, and Department of Biosciences,
Faculty of Biological and Envrionmental Sciences,
Brain & Mind Doctoral Program
University of Helsinki

HMGB1 (AMPHOTERIN) AND AMIGO1 IN BRAIN DEVELOPMENT

Xiang Zhao

ACADEMIC DISSERTATION

To be presented for public criticism, with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in the auditorium B105 at Viikki, Cultivator II, Viikinkaari 4, Helsinki on 22nd Jan 2016, at 12 noon.

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Supervised by Professor Heikki Rauvala, MD, PhD
Neuroscience Center,
Univerisity of Helsinki, Finland

Professor Pertti Panula, MD, PhD
Neuroscience Center and Institute of Biomedicine
University of Helsinki, Finland

Reviewed by Docent Jari Rossi, PhD
Institute of Biomedicine
University of Helsinki, Finland

Docent Jan Kaslin, PhD
Australian Regenerative Medicine Institute,
Monash University, Australia

Custos Professor Juha Voipio, PhD
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Opponent Professor Corinne Houart, PhD
MRC Centre for Developmental Neurobiology,
King's College London, United Kingdom

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For Mother and Father

致亲爱的父亲和母亲

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LIST OF ORIGINAL PUBLICATIONS

This study is based on the following original publications, which are referred to by Roman numerals:

I **Zhao, X.**, Kuja-Panula, J., Rouhiainen, A., Chen, Y. C., Panula, P., and Rauvala, H. (2011) High mobility group box-1 (HMGB1; amphoterin) is required for zebrafish brain development. The Journal of biological chemistry 286, 23200-23213

II **Zhao, X.**, Kuja-Panula, J., Sundvik, M., Chen, Y. C., Aho, V., Peltola, M. A., Porkka-Heiskanen, T., Panula, P., and Rauvala, H. (2014) Amigo adhesion protein regulates development of neural circuits in zebrafish brain. The Journal of biological chemistry 289, 19958-19975

III **Zhao, X.**, Rouhiainen, A., Panula, P., Rauvala, H. (2015) HMGB1 depletion impairs mouse brain development. **Manuscript in preparation**

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I: Zhao designed and conducted most of the experiments, analyzed the data and participated in writing the manuscript.
II: Zhao designed and conducted most of the experiments, analyzed the data and participated in writing the manuscript.
III: Zhao designed and conducted most of the experiments, analyzed the data and wrote the manuscript

ABBREVIATIONS

5-HT	5-hydroxytryptamine receptors
AC	anterior commissure
AMIGO	amphoterin-induced gene and open reading frame
ANB	anterior neural boundary
ANR	anterior neural ridge
Aβ	β-amyloid peptide
BMP	bone morphogenetic protein
CA	Cornu Ammonis
cAMP	Cyclic adenosine monophosphate
CCL	chemokine ligand
CNS	central nervous system
CR	Cajal-Retzius
CRISPR	Clustered regularly-interspaced short palindromic repeats
CXCL	chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
DA	Dopaminergic neurons
DAMP	damage-associated molecular pattern
DBH	dopamine beta-hydroxylase
DiV	diencephalic ventricle
dpf	days post fertilization (dpf)
drc	dorsal rostral cell cluster
DRG	dorsal root ganglion
DVDT	dorso-ventral diencephalic tract
ec	neurons of the epiphyseal cluster
EdU	5-ethynyl-2´-deoxyuridine
EMV	extracelluar microvesicle
ENU	N-ethyl-N-nitrosourea
FACS	fluorescence-activated cell sorting
Fgf	fibroblast growth factor
FLIP	Fluorescence Loss In Photobleaching
FN	fibronectin domain
Foxg1	forkhead transcription factor 1
FRAP	Fluorescence Recovery After Photobleaching
GABA	γ-Aminobutyric acid
GFAP	glial fibrillary acidic protein
GST	glutathione S-transferase
HMGB1	high mobility group box-1; amphoterin
hpf	hour post fertilization
Hy	hypothalamus
IL	Interleukin
Ig	immunoglobulin
Kv	voltage-gated K^+ channel
LC	locus coeruleus
lcp	lateral catecholaminergic projections
LLC	long latency C-bend
LLF	lateral longitudinal fascicle
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAC1	macrophage antigen complex 1
MAG	myelin-associated glycoprotein
MBP	maltose-binding protein

MDO	mid-diencephalic organizer
MHB	midbrain hindbrain boundary
mlct	medial longitudinal catecholaminergic tract
MLF	medial longitudinal fasciculus
MO	morpholino oligonucleotide
Mo	medulla oblongata
NF-κB	nuclear factor-κB
ODD	ordered differential display
OMgp	oligodendrocyte myelin glycoprotein
PC	posterior cluster
PKR	protein kinase R
PR	posterior recessus
PT	posterior tuberculum
PVOa	paraventricular organ, anterior part
PVOi	paraventricular organ, intermediate part
PVOp	paraventricular organ, posterior part
RAGE	Receptor for Advanced Glycation End Products
RGC	retinal ganglion cell
ROS	reactive oxygen species production
SDF-1	Stromal cell Derived Factor-1
SGZ	subgranular zone
Shh	sonic hedgehog
SLC	short latency C-bend
SOT	supra-optic tract
SRF	superior reticular formation
SVZ	subventricular zone
TALENs	transcription activator-like effector nucleases
TeO	optic tectum
TH	tyrosine hydroxylase
TILLING	Targeting Induced Local Lesions IN Genomes
TLR	Toll-like receptor 4
TNF-α	tumor necrosis factor-α
TPC	tract of the posterior commissure
TPOC	tract of the post-optic commissure
TREM1	Triggering Receptor Expressed On Myeloid Cells 1
Trk	tyrosine kinase receptor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VC	ventral commissure
vcc	ventral caudal cell cluster
VLT	ventral longitudinal tract
vrc	ventral rostral cell cluster
Wnt	wingless-integrated
ZFNs	zinc finger nucleases

ABSTRACT

Vertebrate brain is one of the most complex and mysterious objects for biological research. Embryonic brain development involves stereotypic brain structure formation, and a vast number of precise intercellular connections are established for the generation of the highly complex circuitry of the brain. This work aims at explaining the roles of HMGB1 (high mobility group box-1; amphoterin) and AMIGO1 (amphoterin-induced gene and open reading frame) in modulating vertebrate brain development.

This study has found that HMGB1 is essential for the neurogenesis and differentiation occurring at the developmental stage when forebrain structures are forming. Severe defects in the forebrain and a gross deterioration of the catecholaminergic system is seen in *hmgb1* knockdown zebrafish morphants produced by injection of morpholino oligonucleotides (MO). The morphant is also deficient in survival and proliferation of neural progenitors. Similar central nervous system (CNS) developmental defects have been observed in Hmgb1 knockout mouse embryos, in which embryonic brain cells demonstrate much lower proliferating and differentiating activities compared to wild type animals. An associated alteration in chemokine CXCL12/CXCR4 machinery expression in HMGB1 null mice suggests that the immune and nervous systems crosstalk during development.

hmgb1 knockdown zebrafish and knockout mice have confirmed that AMIGO1 expression is regulated by HMGB1. Zebrafish has three orthologs of the AMIGO protein family. Amigo1 has been confirmed to be the predominant family member expressed during nervous system development in zebrafish. In this study, the knockdown of *amigo1* expression using MO is demonstrated to impair the formation of fasciculated tracts in early fibre scaffolds of brain and the development of aminergic system. The same defect can be induced by mRNA-mediated expression of the Amigo1 ectodomain that inhibits adhesion mediated by the full-length protein. In addition to its role in the formation of fasciculated tracts, Amigo1 binds to the potassium channel Kv2.1 and regulates its expression during development. At the behavioural level, *amigo1* knockdown morphants show enhanced locomotor activity and attenuated escape response. The phenotype can be rescued by overexpressing Kv2.1 in *amigo1* knockdown morphant.

We suggest that the mechanism of Amigo in regulating neural circuit development involves homophilic interactions within the developing fibre tracts and regulation of the Kv2.1 potassium channel to form functional neural circuitry that controls locomotion.

Combing the results from both zebrafish and mouse, HMGB1 and AMIGO1 show a conserved expression pattern. HMGB1 and AMIGO1 are both crucial for embryonic brain development and formation of neural connections.

REVIEW OF THE LITERATURE

1. HMGB1 AS THE MEMBER OF HMG PROTEINS

1.1 HMG SUPERFAMILY OF PROTEINS

'High mobility group' （HMG）proteins, the most abundant and ubiquitous non-histone chromatin binding proteins in eukaryotic cells (Bustin, 1999; Thomas and Travers, 2001), were first discovered and classified as acid-extractable components of chromatin that had high electrophoretic mobility (Goodwin et al., 1973; Hock et al., 2007). Subsequent studies revealed that canonical HMG proteins are widespread in most organisms and comprise a large and diverse superfamily. HMG proteins are normally characterized as architectural chromatin proteins. Chromatin structural changes are usually mediated by the binding of HMG proteins to transcription factors or nucleosomes in a non-sequence-specific manner (Bianchi and Agresti, 2005; Hock et al., 2007; Thomas and Travers, 2001). Similar to core histones, HMG proteins regulate gene expression dynamically via interaction with nucleosomes, transcription factors, and histone H1. HMG proteins also exhibit high mobility within the cell nucleus by roaming the nuclear space randomly in an energy-independent way (Harrer et al., 2004; Phair and Misteli, 2000; Scaffidi et al., 2002). Photobleaching techniques (FRAP and FLIP) clearly showed that HMGs move within the nuclei freely without preferred pattern or chromatin binding region (Catez and Hock, 2010; Scaffidi et al., 2002) . The HMG families participate in many common biological processes, such as in embryonic development, regulation of transcription, and modulation of DNA repair. Aberrant expression of many HMG genes leads to developmental abnormalities and is the underlying cause of many diseases, including cancer. There are three families of HMG proteins, HMGA, HMGB, and HMGN, classified with systematic reference to the characteristic functional sequence motif they contain (Bustin, 2001).

1.1.1 HMGA PROTEINS

HMGA proteins consist of four members, each containing several 'AT-hooks', which is a characteristic domain of this protein family (Reeves and Nissen, 1990). These 'AT-hooks' are functional motifs binding to AT-rich DNA stretches in the minor groove. HMGAs are usually very small proteins (~ 10 kDa) able to induce conformational changes of chromatin to promote subsequent recruitment of additional components at DNA binding sites (Reeves, 2001). HMGA proteins have an acidic C-terminal tail important for protein-protein interactions and post-translational modification (Bianchi and Agresti, 2005; Bustin, 2001; Fedele et al., 2001).

HMGAs are abundantly expressed in the mammalian embryo and are expressed at low levels in differentiated cells (Caron et al., 2005; Hock et al., 2007; Sgarra et al., 2004). The developmentally regulated expression of HMGA proteins suggests a role in differentiation processes. HMGAs are involved in the transcriptional regulation of many genes as they are key factors within enhanceosomes. Enhanceosomes are complex assemblages of transcription factors and cofactors on nucleosome-free control regions of genes (Agresti and Bianchi, 2003; Bianchi and Agresti, 2005; Martinez Hoyos et al., 2004). The faulty expression of many genes in tumourigenesis is a direct result of HMGA overexpression and binding with a versatile partner (Fedele et al., 2001; Fedele et al., 2006; Martinez Hoyos et al., 2004).

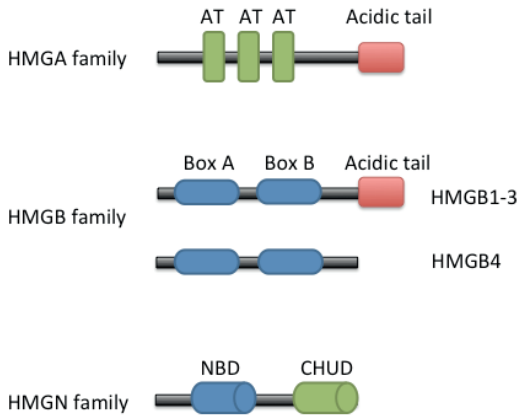

Figure 1. Schematic map of HMGs main structural features. All HMGAs contain three AT hooks (green) and an acidic C-terminal part (Red). HMGB 1-3 contains two HMG boxes (blue) and an extended acidic C-terminus (Red). HMGB4 also contains two HMG boxes, but lacks the acidic tail. The HMGN proteins are characterized by a positively charged nucleosomal binding domain (NBD, blue) and a negatively charged C-terminal region named chromatin unfolding domain (CHUD, green).

1.1.2 HMGN proteins

HMGN family proteins (HMGN1-5) are characterized by a positively charged domain, the highly conserved nucleosome binding domain(Hock et al., 1998), and by a negatively charged acidic C-terminal, the chromatin unfolding domain (Trieschmann et al., 1995) . HMGNs are the only non-histone proteins known to specifically bind inside nucleosomes. HMGNs unfold higher-order chromatin structure and enhance transcription, DNA repair, and replication (Bustin, 2001). HMGN proteins reduce the residence time of histone H1 on linker DNA, resulting in increase of chromatin accessibility and opportunity for regulatory factors to bind to their chromatin targets (Catez et al., 2002). HMGN proteins are found only in vertebrates, and HMGN expression patterns in Xenopus and mice show that the expression level of HMGN proteins is tightly linked to differentiation (Furusawa et al., 2006; Korner et al., 2003)

1.1.3 HMGB proteins

The HMGB protein family is the most abundantly expressed protein family among HMG chromosomal proteins and comprises 4 members in mammals (HMGB1-4). HMGB1 is the parent form of all HMG proteins that was originally isolated as a component of chromosomes from calf thymus (Goodwin et al., 1973). HMGB1-3 proteins have a molecular weight of ~25 kDa and contain two conserved HMG boxes, L-shaped DNA binding domains named HMG boxes A and B, and an acidic C-terminal tail (Thomas and Travers, 2001). HMGB4 protein has a molecular mass of ~21 kDa and also contains two HMG-boxes but lacks the acidic tail(Catena et al., 2009). All members of the HMG-box family possess a DNA-binding domain (the HMG-box) related to a motif originally identified in vertebrate HMGB proteins(Stros, 2010). HMGB proteins are major chromosomal binding proteins in the nucleus and participate in chromatin-modulating activities as architectural proteins (Bustin, 2001; Ueda and Yoshida, 2010). In the nucleus, HMGBs are the most abundant HMG proteins and regulate numerous activities, including transcription, replication, and repair (Bianchi and Agresti, 2005).

In summary, HMGA, HMGB, and HMGN protein families are distinguished by their unique protein domains, binding characters, relative reactions after DNA-binding, and different subsequences of cellular reactions. HMG families participate in many common biological processes such as embryonic development, regulation of transcription, and modulation of DNA repair.

1.2 EVOLUTION AND EXPRESSION OF HMGB PROTEINS

Recent studies into the evolution of HMGB proteins have shown that canonical HMGB proteins (chromatin HMGB proteins) appear to be present only in multicellular animals, from sponges onward, and appear to have arisen through the fusion of two different genes coding for the two boxes, respectively (Sessa and Bianchi, 2007). The organization of HMGB genes was conserved during Metazoan evolution by sharing similar intron-exon architectures of gene transcripts. Plants, fungi, and unicellular eukaryotes lack canonical HMGB proteins. Instead, they often have short proteins consisting of a single HMG box (Grasser et al., 2006; Pedersen and Grasser, 2010).

In mammals, there are four members of the HMGB family: HMGB1, HMGB2, HMGB3, and HMGB4 (Ueda and Yoshida, 2010). HMGB 1-3 share highly conserved primary structure (>80% amino acid identity) (Muller et al., 2001; Yanai et al., 2012). They all contain two homologous DNA binding domains, (HMG boxes A and B, ~75 amino acids in length each), and an acidic tail enriched with negatively charged glutamic and aspartic acid residues (Figure 1).

HMGB1 (which is identical to the neurite outgrowth-promoting protein called p30 or amphoterin or sulphoglucuronyl carbohydrate binding protein, SBP-1, in rat cerebral cortex and cerebellum) is the most abundant non-histone protein in the nucleus (approximately 1 molecule per 10–15 nucleosomes) (Chou et al., 2001; Daston and Ratner, 1991; Rauvala and Pihlaskari, 1987). HMGB1 expression is low in adult brain and liver, but high in lymphoid tissues and testis (Mosevitsky et al., 1989). HMGB1 is an evolutionarily highly conserved protein in mammals and amino acid sequences of all mammalian HMGB1 proteins are virtually identical (Sessa and Bianchi, 2007), implying a conserved biological role in distinct organisms. Recently it has been agreed that HMGB1 expression is ubiquitous and abundant in most adult mouse tissues (Andersson and Tracey, 2011). HMGB1 knockout mice even die within a few hours after birth due to inefficient activation of glucocorticoid receptor responsive genes and severe organ disorders (Calogero et al., 1999).

There are numerous reports indicating the developmental regulation of HMGB1 expression. HMGB1 is highly expressed in early brain structures and shows a dynamic pattern in the developing nervous system across invertebrate and vertebrate species (Fang et al., 2012). In mammals, it has been found that HMGB1 expression is widely spread from the beginning of development until adulthood. The level of HMGB1 mRNA transcripts in mice is dynamically regulated at different developmental stages. HMGB1 mRNA was first detected at the two-cell stage and increased until the morula stage with no further increase in the blastocyst stage (Spada et al., 1998). In mouse, specific silencing of HMGB1 mRNA (and the concomitant reduction in HMGB1 protein) using siRNA does not influence embryonic development until blastocyst stage, but decreases the number of cells in the blastocysts and increased apoptosis during later developmental stages (Cui et al., 2008). HMGB1 expression has been found in embryonic neural stem cells. It suggests that HMGB1 has an important role in neuronal proliferation and neurogenesis (Abraham et al., 2013). In mouse E14.5 embryos, HMGB1 expression has been observed in the cortical plate of the developing primary cortex and in the presumptive thalamic region (Guazzi et al., 2003). In embryonic mouse brain, HMGB1 expression boundary has been observed at the junction of the midbrain hindbrain boundary (MHB), with a pattern similar to brain-specific regulatory homeobox genes Otx2, Gbx2, Pax2/5, and En1/2 (Wang and Zoghbi, 2001). HMGB1 expression in the brain starts decreasing after the late developmental

stage but continues to be detectable in adult brain and at high levels in neural stem cells as well as in the granular cells of the dentate gyrus in the hippocampus (Guazzi S, et al., 2003). HMGB1 expression is possibly required for adult neurogenesis (Fang et al., 2014; Guazzi et al., 2003; Muller et al., 2004).

HMGB2 expression has been found to be prevalent during embryogenesis and becomes restricted mainly to lymphoid organs and testis in adult mice (Ronfani et al., 2001). HMGB2 showed redundant function with HMGB1 during early embryogenesis and HMGB2 is even more crucial than HMGB1 in cell proliferation (Abraham et al., 2013). Although no severe developmental defect is usually evident for HMGB2 knockout mice, HMGB1 and HMGB2 double null mice showed gross defects by interfering with the development of digit 5 in mouse (Itou et al., 2011). HMGB3 is mainly expressed in hematopoietic stem cells in the adult mice (Nemeth et al., 2005).

In contrast, HMGB3 expression is very low in developing embryos, and can only be detected at the transcriptional level by RT-PCR with the total RNA of whole embryos (Vaccari et al., 1998). HMGB3 deficient mice showed normal numbers of hematopoietic stem cells with too few common lymphoid and myeloid progenitors, but too many more mature progenitors (Nemeth et al., 2005).

HMGB4 is a novel member of the HMGB family lacking the acidic tail typically found in this family, and it is strongly and preferentially expressed in the adult mouse testis and weakly in the brain, but not in many other tissues (Catena et al., 2009). HMGB4 expression during early development is very restricted with extremely low expression levels in somatic cells. In E14 mouse, HMGB4 expression is only detected in brain and pancreas at low level. In adult mice, very low HMGB4 expression in kidney and brain can be detected (Rouhinainen A et al., unpublished results).

HMGB protein expression during development has also been reported in many other species. In zebrafish there are six members of the HMGB protein family attested by phylogenetic analysis, namely two co-orthologs for each mammalian HMGB1, HMGB2, and HMGB3 gene. All six *hmgb* genes are maternally expressed and, when expressed during embryonic development, show largely overlapping expression patterns (Moleri et al., 2011). In Xenopus eggs and Drosophila embryos, large amounts of HMGB-like proteins are expressed in early embryogenesis (Ner et al., 2001; Ner and Travers, 1994). In situ hybridization studies have shown clearly that HMGB1 mRNA accumulates in early brain structures of amphioxus, Xenopus and the lamprey (Guerin et al., 2009; Huang et al., 2005; Kinoshita et al., 1994). The conserved expression of HMGB proteins during development among species implicates the essential function of HMGB proteins.

1.3 INTRACELLULAR AND EXTRACELLULAR FUNCTIONS OF HMGB1

1.3.1 INTRACELLULAR HMGB1

HMGB1 is the most abundant nuclear non-histone protein, previously considered as an architectural protein supporting chromatin structure via nonspecific binding with DNA and facilitating DNA repair. In cell nuclei, HMGB1 serves as a transcription activator together with other nuclear transcription factors (Bianchi and Agresti, 2005; Celona et al., 2011; Gerlitz et al., 2009; Lange et al., 2008). The positively charged DNA-binding domains (A and B boxes) of HMGB1 contain nuclear-localization signals. The tail specifically interacts with the two boxes and influences their ability to bind the nuclear DNA (Knapp et al., 2004). Recent research findings have shown that HMGB1 is the activator of p53 by promoting p53 DNA binding (Jayaraman et al., 1998), and HMGB1 A box is basically responsible for the physical interaction with p53 (Bianchi and Beltrame, 2000; Livesey et al., 2012; Rowell et al., 2012). HMGB1 and p53 can form a complex within the nucleus and cytosol and regulate the balance between tumour cell death and survival (Livesey et al., 2012).

1.3.2 EXTRACELLULAR HMGB1

Our group has initially isolated HMGB1 as an extracellular protein and demonstrated that it binds to the cell surface and enhances neurite outgrowth in embryonic cortical neurons (Rauvala et al., 1988; Rauvala and Pihlaskari, 1987). From the recent studies of intracellular or extracellular HMGB1 we know HMGB1 can be released by different kinds of cells, including monocytes, leukocytes, astrocytes, and neurons (Andersson and Tracey, 2011; Andersson et al., 2000; Enokido et al., 2008; Harris et al., 2012; Meneghini et al., 2013; Wang et al., 1999). The secreted HMGB1 regulates cell migration in an autocrine/paracrine manner as it promotes both local proteolytic activation on the cell surface and intracellular signalling pathways required for reorganization of the actin cytoskeleton in motile cells (Rauvala and Rouhiainen, 2010). RAGE (Receptor for Advanced Glycation End Products) appears to be the major transmembrane receptor mediating HMGB1-dependent migration (Huttunen and Rauvala, 2004). HMGB1 can induce inflammation mainly through RAGE, Toll-like receptor 4 (TLR-4), and Toll-like receptor 9 in many diseases, such as rheumatoid arthritis, systemic lupus erythematosus, hepatic ischemia and reperfusion injury, and nephritis (Fang et al., 2012). The elevation of extracellular HMGB1 has been linked to diseases such as sepsis, arthritis, and cancer (Hock et al., 2007; Kang et al., 2013).

Immunohistological experiments have shown HMGB1 expression in the cytoplasm and at the cell surface (Merenmies et al., 1991; Rauvala et al., 1988), in addition to active shuttling back and forth between the nucleus and cytosol (Stros, 2010; Yang et al., 2013). HMGB1 is found as a cell-surface associated protein on activated platelets and early neurons involved in neurite outgrowth during development and nerve regeneration (Rauvala and Rouhiainen, 2010). Recent reports found that endogenous expression and release of HMGB1 to the cytoplasm are implicated in autophagy and in PKR/inflammasome activation (Lu et al., 2012; Tang et al., 2010). Extracellular HMGB1 has been found to play a crucial role in a variety of immune responses, acting as a prototypic alarm signal for activating immune cells (Bianchi and Manfredi, 2014).

In addition to DNA-binding in the nucleus, HMGB1 protein domains have also displayed extracellular functions. According to the current understanding, HMGB1 domains interact with cell-surface receptors through the basic N-terminal region. The N-terminal region of HMGB1 contains a consensus sequence of heparin-binding proteins (Cardin and Weintraub, 1989). The consensus sequence is contributed to the HMGB1 binding of proteoglycans at the same time (Huttunen and Rauvala, 2004). Importantly, the homologous B box of HMGB1 is recognized as the proinflammatory domain (Lotze and Tracey, 2005), which has been shown to mediate the cytokine activity of HMGB1 in neuroinflammation of the post ischemic brain (Kim et al., 2006). The RAGE-binding region is also located in the B box at the C-terminal area (Huttunen et al., 2002). RAGE ligation of HMGB1 regulates the cytoskeleton to promote migration in various cell types, and sustained activation of the RAGE receptor has been shown to result in chronic cellular activation and tissue injury, contributing to mechanisms of several common diseases where chronic inflammation is known to play a role (Rauvala and Rouhiainen, 2010). HMGB1 binds to RAGE with an affinity (K_d 5–10 nM) consistent with HMGB1 interactions with the cell surface. This has been initially identified in rat cortical neurons and the binding has also been found in immune cells (Hori et al., 1995; Lotze and Tracey, 2005). In addition to proinflammatory and migration-enhancing activities, the cell differentiating activity has also been ascribed to the B box (Sparatore et al., 2001). The HMGB1 homologous A box displays anti-inflammatory activity as an antagonist of B box proinflammatory activity (Li et al., 2003). The molecular basis of HMGB1 anti-inflammatory activity remains unclear. It has been found that the A box includes a sequence motif which is homologous to the Alzheimer's β-amyloid peptide (Aβ). It is speculated that HMGB1 contributes to the formation of amyloid fibrils *in vitro*, and binds avidly to Aβ (Kallijarvi et al., 2001).

Taken together, extracellular HMGB1 vastly affects cell migration and cell motility and acts as a cytokine that mediates the response to infection, injury, and inflammation (Lotze and Tracey, 2005). Extracellular and intracellular HMGB1 has compartment-specific functions.

1.4 HMGB1 IN IMMUNE AND NERVOUS SYSTEM

1.4.1 HMGB1 IN IMMUNE SYSTEM

HMGB1 is released by macrophages exposed to bacterial lipopolysaccharide (LPS) (Wang et al., 1999). This finding triggered the interest in previously unrecognized inflammatory activities and the crucial role of HMGB1 in immunology. Although HMGB1 itself has proinflammatory activity, a recent study showed that the activity can be enhanced by lipids binding to HMGB1 (Rouhiainen et al., 2007). HMGB1 appears to be the archetypal damage-associated molecular pattern (DAMP), which is a group of molecules is released by necrotic cells and alerts immune cells in response to danger (Bianchi, 2007; Scaffidi et al., 2002).

Recent reviews have thoroughly explained the roles of extracellular HMGB1 as an immune alarmin for stimulation of the innate immune system and as a key mediator of inflammation responses (Andersson and Tracey, 2011; Yang et al., 2013; Harris HE et al., 2012). Active secretion of HMGB1 from monocytes or macrophages occurs in response to inflammatory stimuli, such as LPS, or cytokines, for example TNF-α, IL-1β, or interferon (reviewed by Lotze and Tracey, 2005). For the translocation and secretion of HMGB1, recent data implicates that the JAK/STAT signalling pathway mediates the acetylation of the NLS (nuclear localization signal) of HMGB1 to facilitate its eventual exportation in most cells (Lu et al., 2014). The subsequent extracellular release is partly modulated by the double stranded RNA-activated protein kinase R (PKR)/ inflammasome-mediated pyroptosis-programmed necrotic cell death induced by caspase-1 (Lu et al., 2012). In addition to PKR-dependent pyroptosis, immune cells can release HMGB1 via non-classical vesicles and lysosomes (Gardella et al., 2002). The mechanisms under non-classical HMGB1 transportation, however, remain to be fully understood.

Secreted HMGB1 can combine with specific membrane receptors that regulate immune cell functions, including chemoattractant and inflammatory activities. *In vitro* tests have elucidated that HMGB1/RAGE signalling is important for chemotaxis, cell proliferation and differentiation of immune cells (reviewed by Andersson and Tracey, 2011). HMGB1 binding to RAGE could promote proliferation and differentiation of immune cells, and even regulate stem cell migration, homing, and development (reviewed by Rauvala and Rouhiainen, 2010). Furthermore, HMGB1/RAGE signalling could upregulate expression of cell-surface receptors including RAGE and TLR4, which is a crucial event in inflammation and might be relevant for migration control (Rouhiainen et al., 2013; Rouhiainen et al., 2004; Yang et al., 2007).
HMGB1- RAGE interactions lead to the activation of the ERK MAP kinase pathway, which is important in cell migration, tumour proliferation, invasion, and expression of matrix metalloproteinases (Taguchi et al., 2000). Conversely, blockade of HMGB1-RAGE signalling can inhibit HMGB1-mediated cell migration and suppress tumour growth and metastases in mice (Huttunen et al., 2002; Taguchi et al., 2000). HMGB1-RAGE interactions have important roles in the migration and recruitment of inflammatory cells through HMGB1/RAGE/Dia/GTPase/cytoskeleton axis or integrin activation (Rauvala and Rouhiainen, 2010; Rong et al., 2004a). HMGB1/RAGE signalling increases the nuclear transcription of NF-κB and the transcription of cytokine and chemokine genes, including TNF, IL- 6, CCL3, CCL4, and chemokine factor CXCL12 (Penzo et al., 2010).

HMGB1 binds to Toll-like receptors (TLR-2, 4, 9) to affect immune responses during infections and sterile inflammation (Takeda et al., 2003; Yang et al., 2010). HMGB1 recognition by the TLR2 and TLR4 receptor *in vitro* was firstly found in macrophage activation (Park et al., 2004). There is evidence to show that HMGB1/TLR4 signalling is strictly required for activation of cytokine release in macrophages, and TLR2 has no direct interaction with the process (Yang et al., 2010). HMGB1 alone may not be able to cause TLR activation together with a burst of secretion of inflammatory mediators, but complexes of HMGB1 with DNA/lipids are able to cause an inflammatory reaction and tissue destruction, in a manner that depends both on RAGE and Toll-like receptors (Rouhiainen et al., 2007; Tian et al., 2007). Recent evidence has shown the assembly of TLR/RAGE on cell membrane (Sorci et al., 2011), and HMGB1 has been implied to bind TLR/RAGE complex and cooperate in cytosolic signalling (Sakaguchi et al., 2011).

Post-translation modification of HMGB1 (including reversible and terminal cysteine oxidation, acetylation, methylation, ADP ribosylation, glycation, and phosphorylation) is crucial for its nuclear transportation and interaction with different membrane receptors (Andersson and Tracey, 2011; Bianchi and Manfredi, 2014; Yang et al., 2013). It has been shown that intracellular HMGB1 is largely in the reduced state as for the strongly reducing redox potential inside the cell (Tsung et al., 2014; Yang et al., 2013). When three cysteines of HMGB1 (at positions 23, 45, and 106) all are in the reduced (thiol) state, HMGB1 forms a heterocomplex with homeostatic chemokine CXCL12 (alias is Stromal cell Derived Factor-1 or SDF1) and its canonical receptor CXCR4. The heterocomplex acts as a potent chemoattractant for promoting the migration of monocytes and fibroblasts (Schiraldi et al., 2012). CXCL12 is a potent chemoattractant for lymphocytes and monocytes and it mediates engraftment of haematopoietic stem cells (Bleul et al., 1996; Kim and Broxmeyer, 1998; Peled et al., 1999). This function of CXCL12 has been considered to be important for the development of the immune system as well as in inflammatory processes (Baggiolini, 1998; Luster, 1998). CXCR4 is expressed on lymphocytes, hematopoietic stem cells, endothelial and epithelial cells, and cancer cells (Guyon, 2014). The HMGB1-CXCL12-CXCR4 heterocomplex may also more generally influence the migration of all motile cells expressing CXCR4 (Tsung et al., 2014).

In addition to its interaction with RAGE, TLRs and CXCL12/CXCR4 complex, HMGB1 has been shown to bind to thrombospondin, syndecan, TREM1, and MAC1 (Andersson and Tracey, 2011). The functions of these HMGB1-binding proteins and their regulation in the pathogenesis of infection and inflammation are unclear and require further validation.

1.4.2 HMGB1 IN THE NERVOUS SYSTEM

The high expression of HMGB1 in developing CNS has been shown in several vertebrate and invertebrate species. Two HMGB1 paralogous genes, *hmgb1a* and *hmgb1b*, have been characterized in zebrafish (Moleri et al., 2011). Whole mount *in situ* hybridization has demonstrated that *hmgb1a* and *hmgb1b* mRNA are highly expressed in the rostral CNS from 24 hour post fertilization (hpf) to 3 days post fertilization (dpf) (Moleri et al., 2011). HMGB1 is detected in lamprey embryos during forebrain development (Guerin et al., 2009). In amphioxus, HMGB1 is expressed in cerebral vesicles, neural tube, and notochord during early development (Huang et al., 2005). In Xenopus, a HMGB1 homolog was upregulated in neuroectoderm-derived tissues throughout early development, and eventually down-regulated in all adult tissues examined except ovary (Kinoshita et al., 1994). All these studies suggest that HMGB1 contributes to early central nervous system development across different species.

Originally, extracellular HMGB1 has been found from developing rat brain by using neurite outgrowth in embryonic forebrain neurons as a readout in protein fractionation (Rauvala and Pihlaskari, 1987). In addition to HMGB1 function in neural injury and neuroinflammation, several reports described wide distribution of HMGB1 in neurons, microglia, and astrocytes in mammalian central nervous system (Enokido et al., 2008; Gao et al., 2011; Rauvala et al., 1988). In the peripheral nervous system, HMGB1 is detected in neurons and Schwann cells (Daston and Ratner, 1991). In mouse embryos, HMGB1 shows a dynamic expression pattern from the beginning of development (Guazzi et al., 2003). Different from previous presumptions of chromosomal protein, HMGB1 expression is not ubiquitous during early embryonic phases. At E14.5, HMGB1 is highly expressed in the cortical plate of the developing primary cortex and in the presumptive thalamic region, and low levels of extracellular HMGB1 can also be detected (Guazzi et al., 2003). In E14 brain, HMGB1 expression is restricted to newly formed neurons of the cortical plate, while in E16 brain, HMGB1 expression is more expanded in most neurons of the cortical plate. In E16 brain, non-nuclear HMGB1 expression is found in subplate, ventricular and intermediate zones. After E18, HMGB1 expression is decreased dramatically and high levels can only be detected in the areas of adult neurogenesis, such as the olfactory bulb, ventricular forebrain, hippocampal dentate gyrus, and the granular layer of the cerebellar cortex (Guazzi et al., 2003). Until now, the signalling pathway involved in HMGB1 regulation during brain development is obscure. There is much evidence showing that HMGB1 is involved in the Wnt/beta-catenin signalling pathway regulating neuronal fate specification and formation of primary brain structures (Itou et al., 2011; Jiang and Sternberg, 1999). It remains unclear whether HMGB1 affected Wnt signalling through regulating transcription factor expression in nuclear, or via the extracellular interaction with receptor proteins. Interestingly, Hmgb1 and Hmgb2 showed redundant function in maintaining a proper level of Shh expression by enhancing effects on Wnt/b-catenin signalling (Itou et al., 2011). Further investigation into HMGB1 signalling through other developmental transcription factors during CNS development is clearly required.

In neuronal cells, HMGB1 is adopted for regulating cell migration through binding to RAGE (Rauvala and Rouhiainen, 2010). It has been confirmed that HMGB1 plays an essential role in microglial activation and HMGB1 is released from cells after a variety of injuries, such as spinal cord and brain injuries (Kawabata et al., 2010; Kim et al., 2006). Extracellular HMGB1 expression correlates with apoptosis and neuronal degeneration (Kawabata et al., 2010). HMGB1 guided neurite outgrowth in cortical neurons and in neuroblastoma cells is the initial form of migration response found for HMGB1 (Rauvala et al., 1988). HMGB1/ RAGE signalling has also been proven to be crucial for the neurite outgrowth, which facilitates peripheral nerve regeneration via recruitment of both inflammatory and axonal outgrowth pathways *in vivo* after sciatic nerve lesion (Rong et al., 2004b). In rat spinal cord injuries, HMGB1 expression appeared earlier than that of tumour necrosis factor-α, interleukin (IL)-1β, and IL-6, in both macrophages and neurons. The translocation of HMGB-1 from nucleus to cytoplasm in some neurons can be observed at an early stage after neural injury. Up-regulation and co-operation of HMGB-1, RAGE, and TLRs was observed after injury at the same time (Chen et al., 2011). Extracellular HMGB1 promotes neurite outgrowth and cell migration, and displays a high propensity to be a neuroinflammatory factor after CNS injury (Fages et al., 2000; Rauvala and Pihlaskari, 1987).

A recent finding shows that HMGB1 has a specific cytoplasmic expression pattern in adult neurons. HMGB1 mRNA is transported constitutively into axons of adult DRG neurons, where HMGB1 mRNA is under translational control and promotes axonal outgrowth (Merianda et al., 2015). After applying the preconditioning nerve injury, only the levels of the HMGB1 protein in axons are increased without a corresponding increase in the HMGB1 mRNA (Merianda et al., 2015). The regulation mechanism of HMGB1 translation in axons after injury is unclear at present.

As the newly identified binding partner of HMGB1, CXCL12/CXCR4 is not only expressed in immune cells but also expressed in neuronal cells in developing brain. CXCL12 is one of the few chemokines found in neurons and is expressed constitutively in the central nervous system (Guyon, 2014; Zhu and Murakami, 2012). Similar to HMGB1 expression in embryonic and adult mouse brain, CXCL12 and CXCR4 proteins were found co-expressed in multiple brain regions such as cerebral cortex, hypothalamus, and cerebellum (Banisadr et al., 2002; Ragozzino, 2002).

Phylogenic analyses have shown that the ancestral role of CXCL12 and other chemokines might be within the central nervous system (CNS) but not within the immune system (Huising et al., 2003). It was found that chemokine CXCL12 and its receptor CXCR4 regulate cerebellar granule cell development, which is the first evidence suggesting that chemokines are required for the proper development of the mammalian nervous system (Ma et al., 1998; Zou et al., 1998). Both CXCL12 and CXCR4 are very widely expressed in the developing embryo. Both CXCL12 and CXCR4 null mice displayed impaired embryonic development of the cerebellum (Ma et al., 1998; Zou et al., 1998). A recent study elucidating the role of CXCL12/CXCR4 in CNS development showed its important role in guiding neuronal migration and neurogenesis (Lu et al., 2002). In rat hippocampal neurons, CXCL12/CXCR4 signalling mediates distinct modifications in the voltage-gated K^+ (Kv) channel Kv2.1, and the modification can directly result in CXCR4-dependent regulation of neuronal survival and death (Shepherd et al., 2012). Mice deficient in either CXCL12 or CXCR4 exhibit disordered neuronal migration in the cerebellum, dentate gyrus, and dorsal root ganglia (Bagri et al., 2002; Belmadani et al., 2005; Ma et al., 1998). Cajal-Retzius (CR) cells originate in the developing brain in multiple sites within the neocortex and hippocampus from the beginning, and experience migration through the marginal zone. Further data confirmed that meningeal expression of CXCL12 controls positioning and migration of (CR) cells via CXCR4 signalling (Borrell and Marin, 2006). Mice with CXCR4 mutations have Cajal-Retzius cells displaced to deeper cortical layers (Paredes et al., 2006). CR cells also control radial migration and laminar positioning of pyramidal neurons of the cortical plate (Meyer, 2010). CXCL12/CXCR4 seems to have a more general role in modulating neuronal migration in early cortical patterning. Numerous reports interpreted CXCL12/CXCR4 signalling to control cortical interneuron migration by focusing the cells within migratory streams and controlling their position within the cortical plate (Li and Ransohoff, 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003; Tiveron et al., 2006). CXCL12/CXCR4 chemotaxis in neuronal migration has been suggested to depend only on the ability of CXCR4 coupled G protein to inhibit the cAMP pathway, because CXCL12 alone cannot lead to chemotaxis required for the migration (Zhu and Murakami, 2012).

CXCL12/CXCR4 is critical for controlling the migration of neurons by functioning as a guidance cue during brain development. As the guidance of migrating neurons and that of growth cones of extending axons share similar signalling and chemical guidance cues (Guan and Rao, 2003), many results have indicated that CXCL12/ CXCR4 signals regulate axon guidance (Chalasani et al., 2003; Chalasani et al., 2007; Kreibich et al., 2004; Lieberam et al., 2005). *In vitro* tests first showed that the growth cones of rat cerebellar granule neurons could turn either away or toward the source of a steep gradient of CXCL12, depending on the intracellular cyclic nucleotide levels (Xiang et al., 2002). A dual functionality of CXCL12 on axons was also demonstrated by using a dissociated mouse cerebellar granule cell culture, where CXCL12 promoted axon elongation at low concentration but inhibited it at high concentration (Arakawa et al., 2003). Similar to the *in vivo* rodent results, zebrafish tests confirmed that ectopically expressed Cxcl12 appears to aberrantly attract retinal ganglion cell axons on their way to the optic stalk (Li et al., 2005). In zebrafish, the ubiquitously expressed Cxcl12 works equally effectively as endogenous Cxcl12 in guiding olfactory sensory axons (Miyasaka et al., 2007). CXCL12/CXCR4 seems to play a chemoattractant role in

RGC (retinal ganglion cell) axon pathfinding, by showing anti-repellent effect *in vivo* counteracting Robo/Slit signalling during development (Chalasani et al., 2007; Xu et al., 2010). Intriguingly, axonal guidance of CXCL12 *in vivo* seems to solely depend on a CXCR4-mediated elevation of cAMP levels, which contrasts the previous identified CXCR4 inhibition of the cAMP pathway (Lysko et al., 2011). It implies the complexity of CXCL12/CXCR4 signalling in CNS, which awaits further validation.

It is already known that HMGB1-CXCL12/CXCR4 complex promotes recruitment of inflammatory cells (Schiraldi et al., 2012; Venereau et al., 2012), and contributes to the migration of innate immune cells together with the HMGB1/RAGE signalling pathway (Campana et al., 2009; Dumitriu et al., 2007). Direct evidence is lacking regarding whether HMGB1 regulates neuronal cell migration and axon guidance together with CXCL12/CXCR4 signals. CXCL12 and CXCR4 are also highly expressed in immune-like competent cells such as astrocytes and microglia in rodent brain (Banisadr et al., 2005; Cho and Miller, 2002). This suggests that CXCL12/CXCR4 and HMGB1 functions in the developing CNS may be similarly correlated as in the innate immune system, which would reveal a novel crosstalk of immune-nervous system interaction.

1.4.3 HMGB1 IN APOPTOSIS, NECROSIS AND AUTOPHAGY

Apoptosis has been intensively studied and widely appreciated as a major mechanism of programmed cell death, employed not only upon cell damage or stress, but also during normal development and morphogenesis. Apoptosis has been classically contrasted to pathological necrosis. For a long time, apoptosis was thought to represent a diametrically "opposite" mode of unordered and passive cellular explosion in response to acute and overwhelming trauma. It has been recognized that apoptosis allows cells to actively recruit a defensive or a reparative response to regions that have sustained damage or invasion (Zong and Thompson, 2006). Conversely, autophagy has been considered to be a process of "programmed cell survival", and it is important in tumour development and response to therapy (Livesey et al., 2012). Through mediating the lysosomal degradation pathway, autophagy recycles cellular proteins and organelles to promote cell survival. At present, several findings accumulated suggesting that apoptosis, necrosis and autophagy are often regulated by similar pathways, the mechanisms of which share common cue molecules and involve a similar machinery at similar subcellular sites and organelles (Nikoletopoulou et al., 2013). They all are employed by cells in a complementary fashion to facilitate cellular destruction.

HMGB1 has been identified as a critical regulator of apoptosis, necrosis, and autophagy during different pathological and physiological processes. Previously, HMGB1 was considered not to be secreted by apoptotic cells, but passively released from necrotic cells or actively secreted by immune cells for stimulation of inflammatory responses (Lotze and Tracey, 2005; Scaffidi et al., 2002). The necrosis-induced release of HMGB1 results in chemoattractive binding with CXCL12/CXCR4, which guides the recruitment of leukocyte to the site of tissue damage to clear cellular debris and protect against possible infection that often follows trauma (Bianchi and Manfredi, 2014; Dumitriu et al., 2005; Schiraldi et al., 2012). In addition, HMGB1 is predominantly released by necrotic cells and mediates recruitment of monocytes and immature dendritic cells via RAGE to inform neighbour cells that tissue repair might be required (Vogel et al., 2015).

In contrast to necrosis, apoptotic cells release substantially less HMGB1 (Bell et al., 2006). The chromatin of apoptotic cells sequesters HMGB1 to prevent inflammation (Scaffidi et al., 2002). During apoptosis, generalized under-acetylation of histones enhances the affinity of chromatin for nuclear HMGB1, leading to minimal or no HMGB1 release. Subsequent studies have demonstrated that apoptotic cells can also release HMGB1 at a later stage. It has been shown that nuclear DNA and

histones are released during apoptosis, and they are well-known binding partners of HMGB1 in the nucleus. It has been shown that macrophage engulfment of apoptotic cells is associated with induction of active HMGB1 release with binding DNA (Bell et al., 2006). The mechanism for HMGB1 release undergoing apoptosis partly involves caspase-3/7-mediated mitochondrial ROS (reactive oxygen species production) (Kang et al., 2013; Kazama et al., 2008). The consequently generated tolerogenic signals can suppress the immune activity rather than deliver a proinflammatory signal (Kazama et al., 2008). This mechanism is one possible explanation as to why apoptosis fails to provoke an inflammatory reaction. Previous studies showed that reduced HMGB1 inhibits both intrinsic and extrinsic programmed cell death/apoptosis in a caspase-dependent way in cancer cells (Kang et al., 2013), but oxidized HMGB1 induces apoptosis (Tang et al., 2010). Conditional HMGB1 knockout in fibroblasts inhibits antimetabolite drug-induced apoptosis (Krynetskaia et al., 2008) and HMGB1 has the ability to induce apoptosis in macrophage-derived dendritic cells (Kusume et al., 2009). Taken together, HMGB1 plays distinct roles in apoptosis depending on cell types and environment.

Conversely, HMGB1 can increase autophagy critically (Kang et al., 2013). Endogenous HMGB1 can promote autophagy in both transcription-dependent and independent ways (Tang et al., 2010; Tang et al., 2011). During upregulation of autophagy, HMGB1 binds to Beclin 1 in cytoplasm activated by Beclin 1-PtdIns3KC3 complex (Kang et al., 2011). Additionally, exogenous HMGB1 promotes autophagy in tumour cells through interactions with RAGE (Kang et al., 2010).

The interplay of HMGB1 between apoptosis and autophagy regulates cell death and determines cell fate in anticancer therapy. A number of studies have de monstrated that suppression of HMGB1 expression by RNAi increases the anticancer activity of cytotoxic agents, whereas overexpression of HMGB1 by gene transfection increases drug resistance. HMGB1 and p53 are capable of physical interaction, and a region of inducible structure in the p53 transactivation domain (residues 38–61) is the essential element for binding to the A box (residues 7–74) (Rowell et al., 2012). p53 and associated molecular pathways are the most commonly mutated regulators of signalling mechanisms in human cancers, regulating apoptosis, autophagy, metabolism, and persistence in hypoxic environments (Green and Kroemer, 2009). DNA damage occurring during apoptosis promotes interactions between p53 and HMGB1 in the nucleus and cytoplasm. Usually, decreased expression of p53 increases cytoplasmic HMGB1, which causes the increase of autophagy and decrease of apoptosis. Conversely, loss of HMGB1 increases cytoplasmic p53 and apoptosis, which decreases autophagy (Livesey et al., 2012). These findings provide new insights into HMGB1-p53 signalling in apoptosis and autophagy.

In the nervous system, neuronal injuries and degeneration result in increased HMGB1 translocation and release from neuronal cells (Kawabata et al., 2010). HMGB1 promotes apoptosis of motor neurons in a spinal cord ischemia model through RAGE mediation signals (Muhammad et al., 2008). Decrease of the HMGB1 in this model resulted better survival of motor neurons in the spinal cord (Huang et al., 2011). Recent studies have suggested that HMGB1 is a nuclear factor for neurodegenerative diseases (Fang et al., 2012). In transgenic mouse models of Huntington's disease, HMGB levels are generally reduced in striatal neurons and co-localized with mutant huntington proteins in nuclear inclusion bodies (Goula et al., 2009). HMGB1 was found to be decreased in neurons of aged brain and in parallel with increased DNA double-strand breaks, suggesting that a reduction of nuclear HMGB1 is relevant for the accumulation of naturally occurring DNA damage leading to neuronal degeneration in the aged brain (Enokido et al., 2008). Abnormal accumulation of alpha-synuclein filaments in Lewy bodies is a neuropathological hallmark of Parkinson's disease. HMGB1 shows preferential binding with aggregated alpha-synuclein and is present in alpha-synuclein filament-containing Lewy bodies isolated from brain tissue affected with dementia with

Lewy bodies or Parkinson's disease(Lindersson et al., 2004). HMGB1 is released from activated microglia and/or degenerating neurons, binds to microglial Mac1 (macrophage antigen complex 1) and activated nuclear factor-κB pathway and NAPDH oxidase expression, leading to induction of chronic progressive neuroinflammation and dopaminergic neurodegeneration. Neutralization of HMGB1 and genetic ablation of Mac1 and gp91 (phox) (the catalytic submit of NADPH oxidase) blocked the progressive neurodegeneration (Gao et al., 2011). These findings imply HMGB1's promoting roles during neural degeneration in the chronic phase of the disease.

2. AMIGO PROTEINS AS A NEW FAMILY OF LRRIG PROTEINS

The genes encoding the AMIGO family were initially identified based on ordered differential display (ODD) (Matz et al., 1997) analysis of neurons (Kuja-Panula et al., 2003). Amphoterin-induced gene and ORF (AMIGO) was a gene induced by growing E18 rat hippocampal neurons in the presence of amphoterin (or high-mobility group box 1 protein, HMGB1). Together with two other cloned homologues, named AMIGO2 and AMIGO3, the three AMIGOs form a novel family of type I transmembrane proteins with six LRRs (leucine-rich repeat) and a single immunoglobin (Ig)C_2-like immunoglobulin (Ig) located next to the transmembrane segment. Transcript level analyses indicate that AMIGO1 is almost exclusively expressed in the nervous system. The expression of AMIGO2 and AMIGO3 is more widespread but is also brain-enriched (Ahmed et al., 2013; Laeremans et al., 2013). In the brain, AMIGO is specifically detected in neuronal cell soma and neurites (Kuja-Panula et al., 2003). Compared to the other LRR proteins, the AMIGO protein family has not been yet extensively studied, and except in mouse no other results from different animal models have been reported.

2.1 LRRIG PROTEINS AND THEIR FUNCTIONS IN NERVOUS SYSTEM

The combination of leucine-rich repeat (LRR) and immunoglobulin-like (IG) domains was originally reported in the domain architecture of the Trk-neurotrophin receptor protein (Bothwell, 1995; Chao, 2003; Huang and Reichardt, 2003). There are 36 LRRIG proteins which have been divided into 13 subgroups according to phylogenetic analysis (Homma et al., 2009); Fig 2). They are (1) LINGO, LRR and Ig domain-containing, Nogo Receptor-interacting proteins; (2) NGL, netrin-G ligand; (3) SALM, synaptic adhesion-like molecules; (4) NLRR, neuronal leucine-rich repeat protein; (5) Pal, membrane glycoprotein membrane glycoprotein; (6) ISLR, immunoglobulin superfamily containing leucine-rich repeat; (7) LRIG, leucine rich repeats and immunoglobulin-like domains proteins; (8) GPR124 and GPR125, G protein-coupled receptor; (9) Adlican, adhesion protein with leucine-rich repeats and Immunoglobulin domains related to perlecan; (10) Peroxidasin and PXDNL; (11) Trk neurotrophin receptor; (12) unnamed protein AAI11068; and (13) AMIGO. As shown in Fig 2, LRRIG proteins have been classified according to their protein domain structure. All LRRIG proteins contain multiple LRR domains, whose numbers of LRR domains range from 5 to 15. In contrast, both AMIGO and Trk proteins contain 6 LRR domains. Ig domains are always present in the carboxyl side to the LRR domain. LRIG, Adlican, Peroxidasin, Trk, and AAI11068 have multiple Ig domains, the others have only one Ig domain. SALM, NLRR, and Pal subgroups have a single fibronectin (FN) domain, which is always located to the carboxyl side of the Ig domain. A degenerated FN domain is present in the three members of the NGL subgroup and in ISLR2 protein. NGL, SALM, and GPR have the PDZ domain in the carboxyl end, whereas Trk has a kinase domain in the intracellular region (de Wit et al., 2011; Homma et al., 2009).

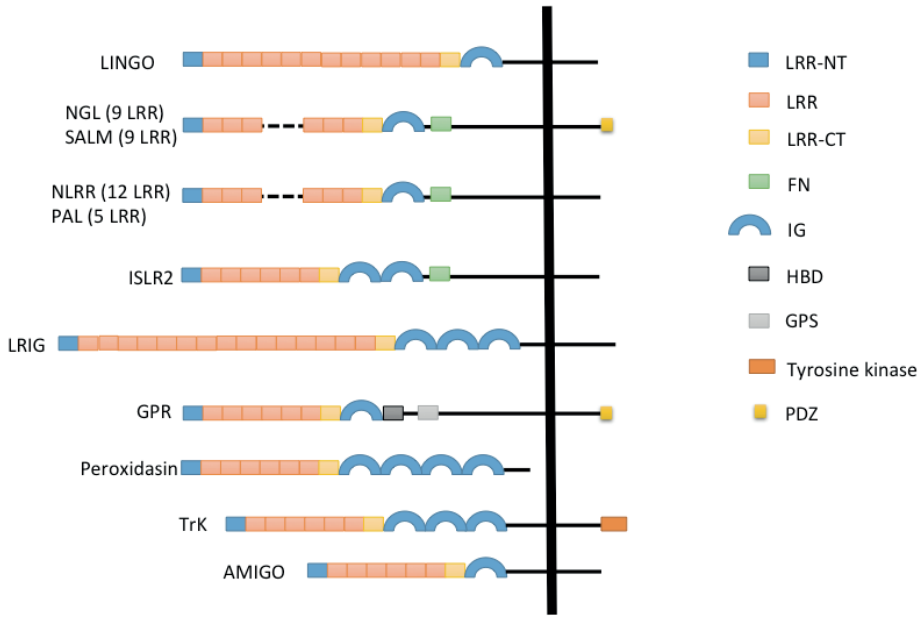

Figure 2. Domain architecture of the identified subgroups of human LRRIG proteins. All LRRIG proteins contain amino terminal-flanking LRR domain (LRR-NT, blue box), carboxyl terminal-flanking LRR domain (LRR-CT, bright yellow box) followed by one or more immunoglobulin domain (IG, blue arc). NGL, SALM, NLRR, Pal and ISLR2, they contain fibronectin domain (FN, green box) after the IG domains. At the carboxyl terminal of the NGL, SALM, and GPR subgroups are PDZ domains (PDZ, smaller dark yellow boxes). The dark grey and light grey boxes in the GPR subgroup are hormone binding domain (HBD) and GPR proteolysis site (GPS), respectively. The tyrosine kinase domain in the Trk receptor is denoted brown. Peroxidasin is a secreted protein, which does not contain the transmembrane domain. Vertical black denotes the cell membrane.

Leucine-rich repeats (LRR) are protein-protein interaction motifs and are found in a large number of proteins with diverse functions and cellular locations (Kobe and Kajava, 2001). The Ig domain found in many neural recognition protein molecules is responsible for heterophilic or homophilic molecular interactions in cell adhesion molecules (CAM) (Maness and Schachner, 2007).

Several plasma membrane-localized LRR proteins show exclusive brain-enriched expression in rodent, implying their specific functions in the CNS (Chen et al., 2006). Trk neurotrophin receptors are proto-typical proteins of the LRRIG family (Bothwell, 1995). Trk receptors regulate neuronal cell survival through an accessory receptor p75, and are involved in the differentiation and process outgrowth of neuronal cells during nervous system development (Chao, 2003; Huang and Reichardt, 2003). Neural signalling mediated by Trk proteins is also important for synaptic plasticity in the adult nervous system (Bothwell, 1995; Hennigan et al., 2007).

Recent data suggests that the Trk receptors have physical interaction with other LRR proteins including AMIGO1 and AMIGO2 that can modulate the outcomes of Trk signalling. Linx (alias Islr2) was recently identified as a LRRIG protein physically interacting with TrkA and modulates its activity to control axonal extension and targeting (Mandai et al., 2009).

Recently, dozens of proteins with either LRR or Ig domains have been identified, and have been

shown to play important roles in neuronal process outgrowth and synapse formation (Chen et al., 2006). Several LRR and Ig domain family members, LINGO-1, Lrrc4b, AMIGO1, and LRIG1, are expressed in non-overlapping subsets of sensory and motor neurons (Mandai et al., 2009). LINGO-1 is a key inhibitor of central myelination expressed in neurons and oligodendrocytes (Mi et al., 2004). Inhibition of LINGO-1 in cultured oligodendrocytes induced elevated oligodendrocyte differentiation and myelination of axons (Mi et al., 2004). In contrast, NGL-1 interacts with netrin-G1 through its LRR region and is most abundantly expressed in the striatum and the cerebral cortex, and promotes thalamocortical axons outgrowth and mediates axonal signalling via membrane-bound netrins (Lin et al., 2003).

The LRR domain-containing protein Slit and its receptor Robo have been intensively studied as one of the most crucial ligand-receptor pairs regulating axon guidance (de Wit et al., 2011; Wong et al., 2002). Slit2 was originally found in drosophila (Seeger et al., 1993). Robo/Slit signalling is a classical axonal guidance mechanism with established roles in axonal branching, dendritic development, and cell migration (Dickson and Gilestro, 2006). Slit2 signals through Robo1 to antagonize the action of the CNS midline attractant Netrin and to prevent ipsilateral axons from crossing the midline during development (Kidd et al., 1999; Li et al., 1999). Slit2 protein also shows certain similarity with AMIGO proteins (Kuja-Panula et al., 2003).

Some LRR domain-containing proteins play a role in the CNS growth-inhibition signalling axis and the inhibition of injured CNS neuron regeneration (Yiu and He, 2006), such as the myelin-associated inhibitors Nogo, myelin-associated glycoprotein (MAG) and the oligodendrocyte myelin glycoprotein (OMgp). They all bind to the LRR domain-containing neuronal cell surface Nogo-66 receptor (NgR) (Hunt et al., 2002). The OMgp/Nogo/MAG-NgR axis inhibits adult regeneration of injured CNS neurons (Filbin, 2003).

Not surprisingly, LRR proteins might also be cell adhesion molecules responsible for synapse formation. LRRTMs (leucine-rich repeat transmembrane proteins) and Slitrks (Slit and Trk-like family) have emerged as important synapse organizers, which control synapse formation, maturation, refinement, and/or elimination (Ko, 2012). The mechanism involved still requires further validation. Altogether, LRRIG proteins are essentially involved in CNS development and have crucial functions in neural plasticity.

2.2 EXPRESSION OF THE AMIGO FAMILY PROTEINS

AMIGO, AMIGO2, and AMIGO3 are mainly expressed in brain regions in adult mice, however AMIGO3 has a more widespread distribution also being found in liver, kidney, and spleen (Kuja-Panula et al., 2003). AMIGO1 and AMIGO2 mRNA are expressed in neurons and astrocytes, as well as oligodendrocytes, during adulthood (Chen et al., 2012; Kuja-Panula et al., 2003).

During embryonic development, AMIGO1 mRNA is only detected in the anterior regions of the developing CNS (Homma et al., 2009; Kuja-Panula et al., 2003). In E10 mouse embryos, AMIGO1 is largely expressed in the developing CNS. Whole mount *in situ* hybridization showed Amigo1 mRNA was in post-mitotic neurons in the telencephalon, mesencephalon, rhombencephalon, and nasal placode. All ganglia of the cranial and spinal nerve were stained by RNA probe of Amigo1. Amigo1 staining was also observed in the inner mesenchyme cells in the branchial arches and limb bud (Homma et al., 2009). AMIGO2 mRNA expression is primarily observed only in a small number of post-mitotic cells in the telencephalon and mesencephalon. In non-neuronal tissue, AMIGO2 expression was only observed in the mesonephros (Homma et al., 2009). Neither AMIGO1 nor AMIGO2 expression are observed in the spinal cord. No expression of AMIGO3 mRNA was detected throughout the embryonic stage (Homma et al., 2009).

In adult brain, AMIGO1 is specifically detected in axonal fibres and neuron tracts (Chen et al., 2012; Peltola et al., 2011). AMIGO1 is also present in both astrocytes and oligodendroglia. AMIGO2 was independently identified as Alivin-1 (Ali1) by differential display screening for genes involved in depolarization and NMDA-dependent survival of cerebellar granule neurons (Ono et al., 2003). A detailed comparison between the sub-regional mRNA expression patterns of AMIGO2 and Pcp4 (Purkinje cell protein 4), a known molecular marker of hippocampal CA2 (Cornu Ammonis 2), revealed a prominent AMIGO2 mRNA expression level in both the CA2 and the CA3a (Cornu Ammonis 3a) subregion of the dorsal and ventral hippocampus in adult brain (Laeremans et al., 2013). Expression of alivin-1/AMIGO-2 appears to promote depolarization-dependent survival of cerebellar granule neurons, and possibly also hippocampal neurons and the granule cells of the dentate gyrus (Ono T et al., 2003). AMIGO2 is also identified as a gene DEGA, which was reported to be differentially expressed in human gastric adenocarcinomas, and may have a role in promoting the invasion of cancer cells (Rabenau et al., 2004). AMIGO2 shares similarity to other cell adhesion molecules of the Ig family in the brain such as NCAM and L1, which have known pro-survival activities as mentioned earlier (Ditlevsen et al., 2007; Loers et al., 2005).

Recent reports suggest that AMIGO3 has a role in neuronal injury models. It has been noticed that AMIGO3 expression was inhibited at the site of neuromuscular junction after injury (Lain et al., 2009). In contrast, AMIGO3 expression is increased in the retina immediately after optic nerve crush. It has been speculated that AMIGO3 may replace LINGO-1 in the NgR1-p75/TROY inhibitory signalling complex to mediate myelin-induced inhibition of axon growth acutely in the CNS (Ahmed et al., 2013). AMIGO3 level is also significantly higher than LINGO1 in dorsal column lesions and reducing in later injury in models of dorsal root ganglion neuron (DRGN) axon regeneration (Ahmed et al., 2013). Taken together, AMIGO proteins are widely expressed in rodent CNS, however, very few studies have addressed their *in vivo* functions and developmental roles. It would be useful to use other animal models for studying the *in vivo* functions of AMIGO proteins.

2.3 AMIGO1 FUNCTION IN NEURAL DEVELOPMENT AND DISORDERS

The AMIGO subfamily contains AMIGO1, AMIGO2, and AMIGO3. AMIGO1 is a neurite-outgrowth promoting factor isolated by ordered differential display via HMGB1 induction in rat hippocampal neuronal cell culture. Amigo1 is implicated in cell adhesion events that control axon extension and fasciculation of axon bundles by promoting neurites extension via homophilic binding through its extracellular part (Kuja-Panula et al., 2003). The in vitro test showed that the ectodomain part of AMIGO can promote attachment and neurite outgrowth of hippocampal neurons.

In developing DRG sensory neurons, AMIGO1 is expressed in nearly all $TrkA^+$ neurons as well as in a subset of medium and large diameter $TrkA^-$ neurons (Mandai et al., 2009). This indicates that the binding and/or coexpression of AMIGO1 with other LRRIG family members may control development of select populations of motor and sensory neurons by modulating the functions of Ret, Trks, or other Trk receptor tyrosine kinases during distinct stages of axonal extension, guidance, branching, and target innervation.

Furthermore, recent findings of the co-expression of AMIGO1 and Kv2.1 in cerebral neurons indicate that AMIGO1 contributes to the fundamental properties of neuronal channels. AMIGO1 protein is coimmunoprecipitated and coexpressed with the Kv2.1 potassium channel in hippocampal neurons. AMIGO1 and Kv2.1 have been found to co-localize in dendrites and axon initial segment, both *in vitro* and *in vivo*, where they may modulate action potential frequency and back propagation (Peltola et al., 2011). AMIGO1 knockout neurons show altered electrophysiological properties, and KV2.1 (KCNB1) is identified as a susceptibility gene for schizophrenia spectrum disorders in

humans (Consortium, 2014; Peltola et al., 2015). Thus, AMIGO1 /KV2.1 complex provides a potential new drug target for several diseases concerning neuronal excitability, such as epilepsy, stroke, and psychiatric disorders (Peltola et al., 2011 & 2015). These studies confirm the important role of AMIGO1 in neuronal plasticity, including its function in neurite outgrowth and neuronal excitability.

2.4 HOMOPHILIC AND HETEROPHILIC BINDING OF AMIGO1

LRR proteins interact with a wide diversity of partners by utilizing their LRR domains to recognize an extremely diverse repertoire of ligands (West et al., 2006). LRR domains have been used throughout evolution to regulate cell-cell interactions in neural circuit development, from axon pathfinding and target selection to synapse formation (de Wit et al., 2011). However, the extracellular binding partners for many LRR proteins remain to be discovered and the downstream signalling mechanisms are unidentified for almost all LRR proteins. As a newly identified LRR protein family, homophilic and heterophilic binding of AMIGO proteins have been recently investigated and described.

2.4.1 HOMOPHILIC BINDING OF AMIGO1

Coimmunoprecipitation and bead assays first showed that AMIGO1 proteins are homophilic adhesion molecules. Homophilic binding of AMIGO contributes to neurite fasciculation (Kuja-Panula et al., 2003). By further analysing the crystal structure of AMIGOs, AMIGO1 is found to be the first membrane associated LRR protein known to function as a dimer (Kajander et al., 2011). The only extensive contacts made between AMIGO1 monomers are through their LRR domains, that is, only one type of dimer is observed in the crystal structures. Dimerization appears to be required for transport to the cell membrane, implying that the same interface might be used for trans-dimerization of AMIGO1. Homophilic binding and highly identical domain organization of AMIGO2 and AMIGO3 have also been suggested (Kajander et al., 2011).

2.4.2 HETEROPHILIC BINDING OF AMIGO

In addition to homophilic binding with themselves, coimmunoprecipitation has also demonstrated heterophilic binding between the members of the AMIGO protein family (Kuja-Panula et al., 2003). The function of this heterophilic binding remains to be elucidated. In vitro recombinant AMIGO1 expression demonstrates heterophilic binding with TrkA, TrkB, and Ret proteins (Mandai et al., 2009). Recent coimmunoprecipitation studies suggest that AMIGO1 binds Kv2.1 and AMIGO1 knockout mice showed reduced Kv2.1 expression in hippocampal neurons (Peltola MA et al., 2011 & 2015). Surprisingly, enhanced CXCL12/CXCR4 signalling results in the increase of Kv2.1 regulated by p38 MAPK (Shepherd et al., 2012). Importantly, AMIGO1 and CXCL12/CXCR4 expression can both be induced by HMGB1 in mouse embryonic neurons or fibroblasts (Penzo et al., 2010). Together with the above, it suggests the regulation mechanism of AMIGO1 and Kv2.1 expression might be tightly relevant to HMGB1-receptor pathways together with their heterophilic binding.

2.5 LRR PROTEINS IN NEURODEVELOPMENTAL DISORDERS

LRRIG proteins have crucial roles in the establishment of neural circuits and functional connections among neurons (de Wit and Ghosh, 2014). Most of the LRRIG mRNAs can be detected from early developmental stages (Homma et al., 2009). The fundamental role of LRR proteins during the

development of neural circuits is displayed by the neurological disorders of the LRR/LRRIG knockout mice.

Most recent reports confirm that AMIGO1 null mice showed Schizophrenia-related defects (Peltola et al., 2015). AMIGO null mice showed similar hyperactive behaviour as Kv2.1 deficient mice (Peltola et al., 2015; Speca et al., 2014). Mice lacking LGI1 die of severe epileptic seizures, a phenotype that could be rescued by transgenic neuron-specific expression of LGI1 (Fukata et al., 2010). Polymorphisms in the transmembrane LRR genes, LRRTM3 and LRRN3 (leucine-rich repeat neuronal 3), are associated with autism spectrum disorders (Sousa et al., 2010). LRRTM3 was also identified as a candidate gene for late-onset Alzheimer's disease (Majercak et al., 2006). Activation of Toll-like receptors (TLRs, transmembrane LRR proteins) has been implicated in neurodegenerative diseases such as multiple sclerosis, stroke, and Alzheimer's disease (Kielian, 2009; Okun et al., 2009). In human, the gene encoding the transmembrane LRR protein Slitrk1 (Slit and Trk-like family member 1), was identified as a candidate gene for Tourette's syndrome (TS) on the basis of rare sequence variants found in TS patients, including a frameshift mutation that results in a truncation of the protein (Abelson et al., 2005). Slitrk5 mutant mice show excessive self-grooming and increased anxiety-like behaviour (Shmelkov et al., 2010), characteristics of obsessive-compulsive-like behaviour.

The mechanism by which LRR/LRRIG regulates neurodevelopmental disorders remains unknown, however. Many LRRIG gene mutations show very subtle effects and many patients still carry a functional copy of the gene (de Wit and Ghosh, 2014).

3. ZEBRAFISH IN DEVELOPMENTAL NEUROBIOLOGY

The main challenge of modern neuroscience is to elucidate the biological mechanism underlying the formation, organization, and evolution of the functional human brain. During the centuries, developmental neuroscientists have adopted a variety of animal models including the mouse (Mus musculus), the fruit fly (Drosophila melanogaster), the zebrafish (Danio rerio), xenopus (laevis tadpoles), and the worm (Caenorhabditis elegans), among others. With respect to the commonalities of mammalian brain phenotype and development, mouse has been appreciated as most popular model because of the valuable comparative knowledge of human brain. However, it is difficult to study early developmental processes in the mouse because they occur in utero. On the other hand, Drosophila melanogaster and Caenorhabditis elegans have the priority of the large scale of mutagenesis screening. Combining with the rather simple development and integrative action of the nervous system in these models, they help to understand most conserved mechanisms of developmental neurobiology. However, these models cannot be utilized to address the development and function of vertebrate-specific features, such as neural crest cells and organogenesis (Dooley and Zon, 2000).

3.1 ZEBRAFISH AS A VERTERBRATE MODEL

The zebrafish has emerged as an excellent model organism to study vertebrate biology. It has been selected as a genetic model system about 30 years ago by George Streisinger and colleagues (Streisinger et al., 1981). External development and optical clarity during embryogenesis allow for visual analyses of early developmental processes at the cellular level. High fecundity and short generation times of zebrafish facilitates both forward and reverse genetic analyses. All these marked zebrafish to become a popular model in developmental biology. Since then, many important techniques have been established and publicly available resources have been systematically accumulated (e.g., www.zfin.org).

The adult zebrafish brain is only about 4.5 mm long and between 0.4 and 2 mm in diameter, and major nuclei/brain regions are conserved: arcuate nucleus, hippocampus, amygdala, locus coeruleus, *etc.* (Rinkwitz et al., 2011). The larval brain at five days post fertilization (dpf) is less than 500 μm thick and 1.5 mm long, making virtually all neurons accessible to confocal and/or multiphoton microscopy *in vivo* (Friedrich et al., 2010; Wullimann and Puelles, 1999). Although the zebrafish is phylogenetically distant from humans, its CNS development is essentially conserved with mammalians (Canestro et al., 2007).

For a long time, targeted genome modification has heavily relied on large-scale traditional forward genetic screens, such as ENU (N-ethyl-N-nitrosourea) mutagenesis derived TILLING (Targeting Induced Local Lesions IN Genomes) strategy and pseudo-typed retrovirus mediated insertional mutagenesis. Over the past decade, zebrafish research has undergone fast development through the introduction of molecular technologies such as antisense oligonucleotides (morpholinos) for early knockdown of gene function (Eisen and Smith, 2008; Nasevicius and Ekker, 2000). Recently, programmable site-specific nucleases have enabled targeted gene disruption in the zebrafish. Engineered endonucleases, including ZFNs (zinc finger nucleases) and TALENs (transcription activator-like effector nucleases), provide new and efficient strategies to directly generate site specific indel mutations by inducing double strand breaks in target genes (Cade et al., 2012; Huang et al., 2012). The success of programmable bacterial nuclease Cas9 application in the zebrafish to introduce heritable lesions genome at high frequency made zebrafish a crucial and efficient model system to investigate vertebrate development (Hwang et al., 2013). With the completion of the zebrafish genome sequencing project, it is now theoretically and technologically possible to generate mutations in all zebrafish genes to evaluate their functions systematically. In addition, emerging field of optogenetics with advancing optical methods such as high-speed and high-resolution microscopy, as well as new manipulative tools can be applied to zebrafish embryos. Thus, a unique combination of genetics, embryology, and state-of-the art optical techniques makes the zebrafish a unique vertebrate model organism to study neurogenesis.

3.2 ZEBRAFISH NEURAL PATTERNING

Zebrafish brain conforms to basic vertebrate brain organization. The gross architecture of many brain areas, e.g., retina, olfactory bulb, cerebellum, and spinal cord, is similar to that of other vertebrate classes (Friedrich et al., 2010). Like in other vertebrates, zebrafish brain is subdivided into fore-, mid-, and hindbrain as that of any other vertebrate (Rinkwitz et al., 2011). The early subdivision of forebrain into telencephalon, eyes, hypothalamus, and diencephalon is conserved among vertebrates (Wilson and Houart, 2004). Zebrafish early development stages have been subdivided into segmentation period (10-24 h), pharyngula period (24-48 h), hatching period (48-72 h) and early larval period after fertilization and main morphological features of developing CNS have already been described in great detail (Kimmel et al., 1995; Ross et al., 1992).

Zebrafish also shares the similar principal neurotransmitter systems in neural circuitry with mammals. Most neurotransmitters found in mammals are largely conserved in zebrafish, such as amino acids (Glutamate, GABA, Glycine) (Higashijima et al., 2004; Hoppmann et al., 2008), monoamines (dopamine, histamine, serotonin, norepinephrin, epinephrin, melatonin) (Cahill, 1996; Kaslin and Panula, 2001; Kastenhuber et al., 2010; Lillesaar et al., 2007; Yamamoto et al., 2010), acetylcholine (Mueller et al., 2004), and others. Due to their opacity, zebrafish larval brain can be easily observed and thus neurotransmitter systems can be followed thoroughly during development. Based on embryo transparency and convenient genetic manipulation of zebrafish, zebrafish is the model of choice for research on neural-circuit formation and function.

One of the most critical events of CNS development is neurogenesis. Neurogenesis consists of the induction and proliferation of neural progenitor cells and their subsequent differentiation into functional mature neurons or glia cells in the developing central nervous system. The zebrafish has been used in numerous studies on the various aspects of neurogenesis as a vertebrate model organism. In contrast with mammals, teleosts like the zebrafish exhibit a much greater proliferative potential (Grandel et al., 2006). Up to 16 different proliferating regions were detected in discrete areas of the brain of adult zebrafish, including the regions equivalent to the mammalian subventricular zone (SVZ) and subgranular zone (SGZ) (Schmidt et al., 2013). Neural development and CNS patterning is dynamically determined by neurogenesis during embryogenesis.

The first step in the development of the vertebrate nervous system is the specification of the neuroectoderm. This process is called 'neural induction' and is initiated during early embryonic development. Fate maps produced by Kimmel's lab first showed that the position of neural territory of neurogenic pluripotent stem cells is at the beginning of gastrulation in zebrafish embryos (Kimmel et al., 1990). In most cases, vertebrate neural induction and patterning depends on complex interactions between extrinsic signalling factors, such as members of the bone morphogenetic protein (BMP), wingless-integrated (Wnt), and fibroblast growth factor (Fgf) families (Streit et al., 2000; Wilson et al., 2001). Ventral secretion of BMPs (BMP2, 4, and 7) blocks neural induction by inducing an epidermal fate in Xenopus and Drosophila (Sasai and De Robertis, 1997). BMP antagonists, Noggin and Chordin, are produced in the early dorsal pre-organizer region, which later forms the Spemann organizer, corresponding to the shield organizer in fish (Sasai and De Robertis, 1997). All these secreted proteins act permissively for the establishment of the neural fate in the dorsal ectoderm and allow the formation of the neural plate (Lumsden and Krumlauf, 1996). Fgfs from the blastoderm margin are needed to induce a complete neural fate (Lamb and Harland, 1995). Fgf signalling can induce a posterior neural ectoderm fate without any correlation with BMP inhibition (Londin et al., 2005). However, low levels of BMP are required during late blastula to early gastrula stage in zebrafish, which is crucial for the establishment of the telencephalon initiated by BMP.

Together with the inhibition of BMP signalling and activation of Fgf signalling, it has been shown that SoxB1 members are important for maintaining the pool of neural stem cells in early gastrulation stages in the zebrafish embryo. In zebrafish, SoxB1 family protein expression depends on the early Fgf signalling from the blastoderm margin, and SoxB1 regulates expression of early BMPs, such as BMP2 and 7 (Okuda et al., 2010; Rentzsch et al., 2004). Sox2 is one of the most important factors of SoxB1 members required for the maintenance of neural progenitor properties and functions in the developing CNS. In mouse, neural stem cell maintenance in developing neocortex requires Sox2-dependent regulation of Sonic hedgehog (Shh) (Favaro et al., 2009). Sox2 activates repressors of neuronal differentiation, such as hesx1 and her3, which are required for zebrafish forebrain development (Favaro et al., 2009; Okuda et al., 2010). So far, sox 1, 2, 3, and 19 of the SoxB1 members have been characterized in zebrafish. They are induced and redundantly required to specify neural ectodermal fate during blastula stages (Okuda et al., 2010).

One of the most fascinating problems in developmental biology is how the CNS is patterned along the anterior-posterior (AP) axis, especially in the neuroectoderm. During neural induction, progenitor cells showed an anterior neural plate fate from the beginning with subsequent signals imparting posterior pattern (Rinkwitz et al., 2011; Schmidt et al., 2013). In zebrafish, the postulated posteriorising signals are Wnt, Fgf, and Nodal proteins, as well as retinoic acid (Wilson et al., 2002; Wilson and Rubenstein, 2000).

Wnt proteins have been identified as posteriorizing factors by overexpression of Wnt induced β-catenin (McGrew et al., 1995). In zebrafish, wnt8 is expressed in lateral marginal cells of gastrula-stage embryos and is excluded from dorsal margin cells (Ho et al., 1999; Kelly et al., 1995). Recent studies have shown that Wnt8 expressed from lateral mesendodermal precursors determines the location of MHB boundary, which is important for acquisition of hindbrain fate (Rhinn et al., 2005). Consistent with this, Wnt8 is required for induction of the posterior expression of gbx1 in hindbrain and establishment of the posterior border of otx2 expression (Rhinn et al., 2009). The anterior neural boundary (ANB) acts as an organizer to pattern the anterior neural plate by the release of Wnt antagonists to establish a Wnt-gradient (Houart et al., 2002; Houart et al., 1998). The graded Wnt activity acts to initiate AP patterning within the neural plate. Other signalling factors such as the canonical Wnt proteins Wnt1, Wnt3, Wnt3a, and Wnt10b, are broadly expressed throughout the forebrain and midbrain, until their expressions become restricted to the mid-diencephalic organizer (MDO), the midbrain-hindbrain boundary (MHB), and the dorsal midline (Lekven et al., 2003). Wnts do not elicit inductive effects comparable to those of the principal signals Shh and Fgf8. However, Wnt signalling is generally needed for maintenance of local organizing centres (Mattes et al., 2012). Thus, Wnt signalling activity is a common theme upstream of many local brain organizers. Local organizing centres within the neural tube are colocalized with boundaries between the forebrain and midbrain mediating specific positional information along the AP axis from anterior to posterior (Kiecker and Lumsden, 2005).

In vertebrates, dorsoventral neural tube pattern is also influenced by signalling centres (Tanabe and Jessell, 1996). Non-neural ectoderm, which is adjacent to prospective dorsal neuroectoderm, secretes Bmps to promote formation of neural crest and dorsal neural tube fates. Axial mesoderm or notochord, adjacent to prospective ventral neuroectoderm, secretes Shh, which promotes development of ventral neural tube fates. Dorsoventral neural tube patterning also requires graded signalling that originates in embryonic midline structures, notochord, and floor plate. Prevailing models propose that mesodermal notochord is the source of Shh, which acts as a vertical signal to induce overlying neuroectoderm to differentiate as floor plate (Tanabe and Jessell, 1996).

During CNS development, the forebrain is most intensively investigated for its importance in higher-order brain functions. It is imperative to understand the genetic circuitry of forebrain development as many human neuropsychiatric and neurodevelopmental disorders are due to genetic forebrain defects (Nord et al., 2015). Zebrafish is a newly emerged model for studying forebrain development and the early subdivision of zebrafish forebrain into telencephalon, eyes, hypothalamus, and diencephalon is conserved among vertebrates (Wilson and Houart, 2004). Previous result shows that the forkhead transcription factor (Foxg1), previously named BF-1, is one of the first transcription factors expressed in the neural plate telencephalic territory of developing rat brain (Tao and Lai, 1992). Foxg1 is expressed in the zebrafish telencephalon from embryo to adulthood in zebrafish similar to the expression found in mammals (Danesin and Houart, 2012). Foxg1 is essential to patterning of the telencephalon and neuron survival in the adult cerebral cortex. The zebrafish neural tube displays graded Foxg1 expression: high expression drives ventral fate downstream of the Shh/Fgf pathways and low expression restricts the dorsal signalling centre. Thus, Foxg1 controls the size of the future cortex. Foxg1 integrates Shh and Wnt signalling activities in telencephalic progenitors, which also depends on Fgf8 expression at the anterior neural ridge (ANR) (Danesin et al., 2009) (Fig 3). A more recent finding showed that CXCR4a controls morphogenetic separation of eye-field and telencephalic cells during neurulation by taking part in the BMP signalling pathway during late blastula to early gastrula stage in the anterior neural ectoderm of the zebrafish embryo (Bielen and Houart, 2012). Regulation of genes involved in zebrafish telencephalic development is very similar to in mammals. The genomic regions around many of these genes are conserved among all sequenced vertebrate genomes (Canestro et al., 2007). For example, zebrafish cis-regulatory

sequences can be used to reveal cortical migration of GABAergic neurons in the mouse (Stuhmer et al., 2002). Although the zebrafish forebrain is significantly different from the mammalian forebrain, comparative genomic analysis and experimental testing of vertebrate regulatory elements is able to reveal conserved neuronal gene regulation during brain development.

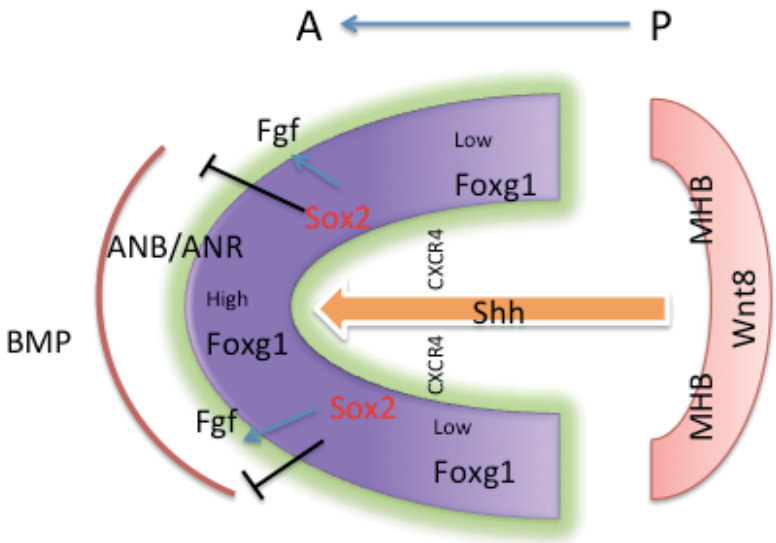

Figure 3. Schematic map of the zebrafish neural plate, posterior (P) to anterior (A) is indicated by the arrow. Posteriorising signals of the Wnt family released from the MHB and midbrain territory would inhibit Foxg1 expression and repress telencephalic fate. Foxg1 induction in the presumptive telencephalon relies on Fgf signalling from the anterior neural ridge (ANR), which is dependent on earlier secretion of Wnt antagonists of the sFRP family at the anterior neural border (ANB). Shh, secreted by the underlying prechordal plate (in green) contributes to induction of high Foxg1 in the prospective ventral telencephalon (medial-anterior part). The graded Foxg1 expression is resulted subsequently for maintaining the forebrain size. Sox2 in the anterior neural plate activates Fgf signals and inhibits BMP expressed in blastoderm margin from early gastrulation stage. CXCR4 expressed along the midline of ventricular area regulates telencephalic cells differentiation during the stage.

3.3 EMBRYONIC AXONAL TRACT DEVELOPMENT IN ZEBRAFISH

Highly complex circuitry of the CNS originates from the embryonic formation of axon tracts (Wilson et al., 1990). The early axon tracts in the CNS are stereotypically organized into sets of longitudinal tracts connected by commissures in many different animals, including nematodes, insects, amphibians, and fish (Chitnis and Kuwada, 1990). The commonality of the initial framework of simple pattern of tracts may indicate that different animals share similar strategies for the formation of tracts (Chitnis and Kuwadai, 1990).

In zebrafish, clear landmarks of axonogenesis during early development stages are stated. The first axons to navigate the zebrafish brain emerge from the vcc (ventral caudal cell cluster) at approximately 16 hpf (Chitnis and Kuwada, 1990; Ross et al., 1992). By 24 hpf, a bilaterally symmetrical, stereotypical set of five axon tracts and four commissures have formed (Hjorth and Key, 2002) (Fig 4). Acetylated tubulin and HNK1 labelling of the earliest stages of axonogenesis in the zebrafish brain indicated that many neurons project axons in a defined direction to connect cell

clusters (Wilson et al., 1990).

In 1 dpf zebrafish brain, a bilaterally symmetrical set of five axon tracts and four commissures have formed in anterior forebrain area. All axons extend caudally from vcc for pioneering the medial longitudinal fasciculus (MLF) development. MLF merges into the larger ventral longitudinal tract (VLT) connecting midbrain and hindbrain (Wilson et al., 1990; Chitnis and Kuwada, 1990; Ross et al., 1992). The pioneer axons of the vcc do not grow rostrally from the beginning. After 2dpf this cluster projects axons rostrally into the tract of the post-optic commissure (TPOC) (Ross et al., 1992). By 18 hpf the first axons in the forebrain emerge from the vrc (Ventral rostral cell cluster) and grow caudally to pioneer the TPOC (Chitnis and Kuwada, 1990; Ross et al., 1992). The most ventral of these fascicles appears to remain tightly fasciculated as it merges into the VLT (Chitnis and Kuwada, 1990). Some neurons of the vrc project axons rostrally, pioneering the post-optic commissure (POC). POC cross the rostral surface of the diencephalon and course into the contralateral TPOC. Neurons of the drc (Dorsal rostral cell cluster) begin to extend axons in two directions to pioneer separate tracts by 18 hpf. The supra-optic tract (SOT) is formed by axons of the drc projecting ventrally from the telencephalon, passing caudally to the optic stalk, and then growing into the region of the vrc and TPOC (Chitnis and Kuwada, 1990; Ross et al., 1992). At this point, axons of the SOT project either caudally or rostrally among POC and TPOC axons. The anterior commissure (AC) is the second tract pioneered by axons of the drc. Axons of the AC cross the rostral surface of the telencephalon in a thick, tight fascicle (Wilson et al., 1990). Several main axon tracts in early zebrafish embryos are shown in the following schematic map (Fig 4).

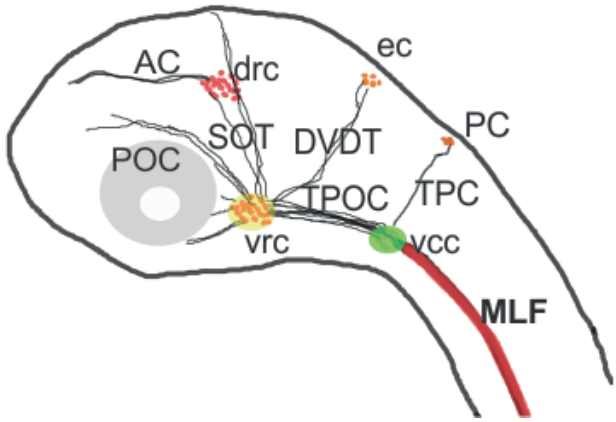

Figure 4. The schematic map shows the main axonal tracts in uninjected 28-hpf larvae. AC, anterior commissure; drc, dorso-rostral cluster; DVDT, dorso-ventral diencephalic tract; MLF, medial longitudinal fasciculus; ec, Neurons of the epiphyseal cluster; PC, posterior cluster; POC, post-optic commissure; SOT, supra-optic tract; TPC, tract of the posterior commissure; TPOC, tract of the postoptic commissure; vcc, ventro-caudal cluster; vrc, ventro-rostral cluster.

At approximately 20 hpf the dorsoventral diencephalic tract (DVDT) is pioneered by a single axon growing ventrally from neurons of the epiphyseal cluster (Chitnis and Kuwada, 1990; Wilson and Easter, 1991; Ross et al., 1992). This axon courses ventrally until it encounters axons of the TPOC growing caudally (Wilson et al., 1990). In the midbrain, neurons project axons ventrally to pioneer

the tract of the posterior commissure (TPC) at approximately 20 hpf (Chitnis and Kuwada, 1990; Wilson et al., 1990). At 20 hpf the ventral-growing axons from the vcc starts to crossing the midline to form the ventral commissure (VC) of the midbrain (Fig 4, Chitnis and Kuwada, 1990; Wilson et al., 1990).

The medial longitudinal fascicle (MLF) and the dorsal longitudinal fascicle (DLF) are the two main longitudinal axon tracts in the embryonic hindbrain (Hjorth and Key, 2002). The rostral portion of the hindbrain MLF is pioneered by midbrain descending axons, and the caudal portion is projected by caudal hindbrain interneurons (Mendelson, 1986). The DLF is pioneered by trigeminal sensory axons and ascending axons from the spinal cord Rohon-Beard primary sensory neurons (Mendelson, 1986). The Mauthner neuron is the first reticulospinal neuron in the hindbrain to extend an axon. Beginning at approximately 21 hpf, this axon courses ventrally and crosses the midline, after which it turns caudally and projects among contralateral MLF (Metcalfe et al., 1986). The Mauthner neuron has been characterized for its crucial locomotor function in zebrafish fast escaping startle response, which implies the complex function of neural circuits could be originated from simple neuronal organization (Fetcho et al., 2008). During the formation of this collection of tracts and the associated extensive development, primary axons play a crucial role in guidance. Most of the additional axons in early CNS are added into pre-existent tracts rather than pioneered new ones (Hjorth and Key, 2002). Most molecular determinants involved in commissural pathway formation have been characterized, but the regulation mechanisms specifying the development of longitudinal axon tracts in the vertebrate nervous system are largely unknown.

3.4 AMINERGIC SYSTEM DEVELOPMENT IN ZEBRAFISH

Neurotransmitters are substances that relay, amplify, and modulate electrical signals between neurons and other cells. Neurotransmitter systems, such as dopaminergic, serotoninergic, cholinergic, glutamatergic, purinergic, histaminergic, glycinergic, and GABAergic systems are conserved in zebrafish. The aminergic neurotransmitters (dopamine, noradrenaline, adrenaline, serotonin, and histamine) are known to mediate many important brain functions. Abnormalities of aminergic neurotransmitter expression and distribution have been implicated in distinct human central nervous system (CNS) diseases (Belmaker and Agam, 2008; Murray et al., 2008). Aminergic systems are fully developed after 3 days post-fertilization (dpf) (Kaslin and Panula, 2001; Rink and Wullimann, 2001), at a time when the larvae have become free swimming with locomotor activity (Rink and Wullimann, 2002). The aminergic neurotransmitter systems have been studied and documented in both zebrafish embryos and adults (Kaslin and Panula, 2001; McLean and Fetcho, 2004a). Dopaminergic and serotonergic systems share high similarities between teleosts and mammals, making zebrafish a feasible model for evaluating the general properties of both systems (Flinn et al., 2008; Panula et al., 2006).

3.4.1 ZEBRAFISH DOPAMINERGIC SYSTEM

Although the dopaminergic neuron population is less than 1% of the total neuronal population of the brain, it has important effects on modifications of synaptic plasticity in the brain (Arias-Carrion et al., 2010). Dysfunction in the dopaminergic system is associated with a variety of neuropathologies, such as Parkinson's disease, Tourette syndrome, and schizophrenia (Missale et al., 1998).

Zebrafish dopaminergic systems show a general pattern that shares many important features with mammals (Panula et al., 2006). Dopaminergic neurons (DA) have been extensively analysed in the adult zebrafish brain primarily for revealing their development and organization by TH (tyrosine hydroxylase) immunohistochemistry (Kaslin and Panula, 2001; Ma, 2003; Rink and Wullimann, 2001). Zebrafish TH-immunoreactive (TH-ir) catecholaminergic (CA) neurons were found in all

forebrain divisions. CA (TH-ir) neurons can be considered as putatively dopaminergic due to the lack of dopamine beta-hydroxylase immunoreactivity (DBH-ir) (Panula et al., 2006).

In the telencephalon, DA neurons are in the olfactory bulb (OB) and in dorsal, central, and ventral nuclei of the ventral telencephalic area (Fig5; Kaslin and Panula, 2001; Rink and Wullimann, 2001). In the diencephalon, DA neurons are detected in the anterior and posterior parts of the preoptic area (Po), in the suprachiasmatic nucleus (SC), in the periventricular pretectum (Pr) and ventral thalamus, in the nucleus of the posterior tuberculum (PTN) and paraventricular organ (PVO), and in the caudal hypothalamus (Hy) (Fig 5; Kaslin and Panula, 2001; Ma, 2003; Rink and Wullimann, 2001).

The formation and distribution of DA groups in the early zebrafish brain has also been studied by TH immunohistochemistry (McLean and Fetcho, 2004a; Rink and Wullimann, 2002; Sallinen et al., 2009b). The earliest postmitotic precursors of ventral diencephalic DA neurons are born before 16 hpf, suggesting that these neurons directly derive from neural plate cells. Most of the DA cell groups described in the adult brain could are already detectable in 3dpf embryos (Rink and Wullimann, 2002).

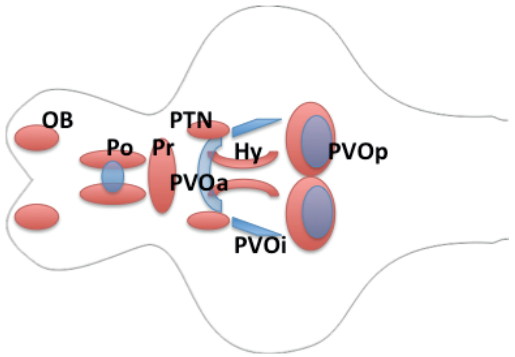

Figure 5. A schematic dorsal view of *th1* and *th2* cell populations in the zebrafish brain at the stage of 7dpf. The red areas illustrate groups of *th1* expressing cells. The blue areas are groups of *th2* expressing cells. Abbreviations: Hy, hypothalamus; OB, olfactory bulb; Po, preoptic area; Pr, pretectum; PTN, posterior tuberculum; PVOa, anterior part of the paraventricular organ; PVOi, intermediate part of the paraventricular organ; PVOp, posterior part of the paraventricular organ.

There is another tyrosine hydroxylase gene (*th2*) that has been recently identified in teleosts. *th1* and *th2* are the functional non-allelic genes in the duplicated zebrafish genome, as *th2* is regulated differentially at the transcriptional and post-translational levels from Th1 (Candy and Collet, 2005). Th2 is expressed in four main clusters restricted to the diencephalon (Fig 5). The most rostral group is detected in the preoptic region, whereas the other three hypothalamic groups were found to be lining the lateral and posterior recesses of PVO (Chen et al., 2009; Filippi et al., 2010; Yamamoto et al., 2010). During embryonic and early larval stages, Th2 expression is much weaker compared to Th1 (Chen et al., 2009). Th2 expression partly overlaps with Th1 in the preoptic region and PVO, but is mostly exclusive in the hypothalamic groups (Fig 5; Filippi et al., 2010; Yamamoto et al., 2010). Interestingly, neurons immunoreactive for Th2 and 5-HT were distinct, too (Semenova et al., 2014). Apart from the potential role of Th2 in the synthesis of dopamine during adulthood (Yamamoto et al., 2010), little is known about TH2 function and relevant mechanism in zebrafish brain yet.

3.4.2 DEVELOPMENTAL REGULATION OF DOPAMINERGIC SYSTEM

As the most crucial developmental transcription factors among species, Shh (sonic hedgehog) and Fgf8 (fibroblast growth factor 8) have been analysed during DA development in zebrafish to determine their function in the induction of mesencephalic DA neurons in mammals (Holzschuh et al., 2003). There is a hypothesis that the combined signalling action of the secreted Fgf8 and Shh on the adjacent brain tissue induces dopaminergic neurons. The induced dopaminergic neurons would reach their adult midbrain and forebrain locations by subsequent migration (Wullimann and Mueller, 2004). However, zebrafish Shh mutants or its co-receptor smooth-ended mutants fail to display major defects in formation of ventral diencephalic DA groups but lack late forming pretectal and amacrine DA neurons (Schweitzer et al., 2012). All DA groups form normally in zebrafish Fgf8 mutants, though with a slight delay.
Early zebrafish TH cells are tightly contacted by Pax6 expressing cells. Similar to mouse, dopaminergic cells directly derive from Pax6-positive cells (Andrews et al., 2003; Mastick and Andrews, 2001; Vitalis et al., 2000). Therefore, inductive action of Pax6 cells on zebrafish dopaminergic cells has been suggested (Rink and Wullimann, 2001 & 2002). A recent report showed a role of canonical Wnt signalling on DA development in zebrafish (Russek-Blum et al., 2008). Ubiquitous overexpression of the Wnt signalling antagonist dkk1 resulted in an increase in the number of ventral diencephalic DA neurons likely via modulation of a Wnt8b/Lef1 signalling cascade. It was concluded that canonical Wnt signalling selectively limits the initial pool of DA progenitors during early forebrain patterning (Russek-Blum et al., 2008). Further genetic analyses of developmental transcriptional factors would be required for understanding the regulation mechanism of zebrafish dopaminergic system development.

3.4.3 ZEBRAFISH SEROTONINERGIC SYSTEM

Serotonin (5-HT) is another neurotransmitter important in modulating brain physiology and behavior. 5-HT has impacts on numerous behaviours and physiological functions, including mood, sleep, aggressiveness, fear, appetite, vascular function, pain, and reproduction (Jacobs and Azmitia, 1992; Lucki, 1998; Parsey, 2010). Serotonin has a fundamental role during CNS development and influences plasticity in the vertebrate CNS (Cote et al., 2007; Daubert and Condron, 2010). Importantly, the dysfunction of serotonergic neurons during development or adulthood has been implicated in several psychiatric diseases, including depression, drug addiction, and schizophrenia (Lucki, 1998; Sallinen et al., 2009a).

In zebrafish, serotonin (5-HT) containing neurons are all found around the posterior recessus (PR) of the caudal hypothalamus. In the adult zebrafish brain, serotonin immunoreactive neurons are mainly located in the three populations of the posterior tuberculum/ hypothalamus, pretectal area, and anterior raphe nucleus (Lillesaar, 2011). Serotoninergic neurons and fibres show a complementary non-overlapping manner with other aminergic system (Kaslin and Panula, 2001), 5-HT-ir, and Th-ir neurons in PVO are in close proximity from the beginning of development. The only colocalization of TH-ir and 5-HT-ir can be detected in some early diencephalic neurons, which corresponded to the adult 5-HT-ir anterior periventricular cell population (Sallinen et al., 2009a).

3.4.4 AXONOGENESIS OF AMINERGIC SYSTEMS

The axonogenesis of aminergic systems during zebrafish early developmental stages between 1-3 dpf has been analysed by anti-TH and anti-5-HT immunohistochemistry (Kastenhuber et al., 2010; McLean and Fetcho, 2004a; Sallinen et al., 2009b). The almost complete axonal scaffold of DA circuits described in the adult zebrafish (Ma, 2003; Kaslin and Panula, 2001) is already present in 3-day-old larvae (McLean and Fetcho, 2004a; Kastenhuber et al., 2010).

The first TH-ir neurons in the zebrafish appear between 16 and 24 hpf in the ventral diencephalon and start projecting axons (Holzschuh et al., 2001; Kastenhuber et al., 2010; McLean and Fetcho, 2004b), which is almost at the same time of zebrafish general axonogenesis (Chitnis and Kuwada, 1990). These TH-ir axons project longitudinally and grow along the midline symmetrically to form the medial longitudinal catecholaminergic tract (mlct) (Fig 6, McLean and Fetcho, 2004b; Kastenhuber et al., 2010). The TH-ir mlct projects towards the spinal cord in the vicinity of the medial longitudinal fascicle (MLF) and lateral longitudinal fascicle (LLF). It is hypothesized that TH-ir mlct axons in the spinal cord have a potential function in spinal motor control as for its adjacency and connetions to the dendritic processes of primary and secondary motor neurons (McLean and Fetcho, 2004b). Recently it has been reported that the development of these far ranging DA projections from diencephalon is necessary for normal locomotor development (Lambert et al., 2012).

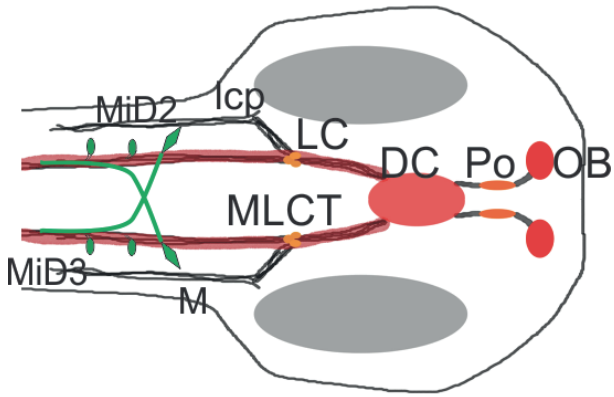

Figure 6. The schematic map of catecholaminergic tracts (Red) and the axonal projections of main reticulospinal interneurons (Green) in 3dpf larvae. The axonal projections of Mauthner neurons do not show any immunoreactivity to anti-Th antibodies, and are located ventral to the MLCT. Abbreviations, DC, diencephalic dopaminergic clusters; LC, locus coeruleus; lcp, lateral catecholaminergic projections; M, Mauthner neuron; MiD2cm, middle dorsal 2 contralateral MLF interneuron; MiD3cm, middle dorsal 3 contralateral MLF interneuron; MO, medulla oblongata; OB, olfactory bulb; Po, preoptic region.

In telencephalon and preoptic area, Th-ir and 5-HT ir fibres partially co-localize in the anterior commissure and postoptic commissure and the lateral margins of the diencephalon (Mclean and Fetcho, 2004b). In the ventral diencephalon, TH- ir cells are in close proximity to the 5-HT-ir cells, but they are not co-localized. However, there were no caudally projecting 5-HT axons apparently projecting from this population as mlct (Mclean and Fetcho, 2004b). By 32 hpf, 5-HT reactivity was detectable in cells within the spinal cord and by 2 dpf they had differentiated sufficiently to distinguish faint, short projecting processes in the ventrolateral cord (Mclean and Fetcho, 2004b). Even by this stage there are no clear descending processes emerging from the serotonergic population. In the brainstem of 5dpf larvae, TH-ir and 5-HT-ir axons are closely apposed to the dendritic processes of the nucleus of the medial longitudinal fascicle (nMLF), in addition to the ventral dendrites of the Mauthner neuron and its serial homologs MiD2cm and MiD3cm (Fig 6). Mauthner neuron and its serial homologs are main reticulospinal interneurons regulating startle responses in zebrafish (Burgess and Granato, 2007; Kimmel et al., 1980). Thus, dopaminergic and serotoninergic axonal networks might have a dual role in modulating motor output in larval zebrafish (Gabriel et al., 2009).

In contrast to the extensively analyzed aminergic projections, the cellular and molecular mechanisms underlying axonal pathfinding of DA tracts are less well understood. The mechanism of axon guidance that has been studied thus far is only in the formation of the mlct. During mlct pathfinding, the DA neurons in the ventral diencephalon co-express the two axon guidance receptors, Robo2 and DCC, at the ventral midline. Slits and Netrins are the ligands for the Robo2 and DCC receptors, respectively (Kastenhuber et al., 2009). The DCC/Netrin system primarily mediates axon attraction, whereas Robo2/Slit signalling mediates repulsion (Rajasekharan and Kennedy, 2009; Ypsilanti et al., 2010). As for axon guidance, heparan sulfate proteoglycans are able to bind with Robo2 and DCC for lateral positioning of the mlct (Kastenhuber et al., 2009). The full understanding of aminergic system development and wiring of the projections requires further investigation on more guidance cues and factors involved in neural circuit development.

3.5 ZEBRAFISH AS A TOOL FOR GENE KNOCKDOWN AND KNOCKOUT

Sequencing of the zebrafish genome revealed high genomic conservation between zebrafish and humans. Due to the presence of 70% gene orthologs between zebrafish and human genome, many zebrafish mutants can be tested as disease models for the relevance in human (Howe et al., 2013). The Human Genome Project is producing enormous amounts of sequencing information but lacks functional information for many of the identified genes (Amsterdam and Hopkins, 2006). The analysis of zebrafish mutagenesis generated by forward and reverse genetic approaches can serve as a functional complement for it.

Most forward genetic screens that isolate zebrafish mutants with developmental phenotypes have been conducted using ENU (ethylnitrosourea) as a mutagen. Many mutants carry phenotypes reminding of human disease states, and they provide a powerful approach for gaining insight to the corresponding pathophysiology (Dooley and Zon, 2000; Amsterdam and Hopkins, 2006). However, zebrafish has undergone an additional genome duplication event during teleost evolution. Such forward genetic screens showed rather low effectiveness and difficulty in the isolation of mutations that demonstrate a phenotype for every single gene due to the potential existence of functional redundancy of genes (Eisen and Smith, 2008). Reverse genetic screening of specific gene mutants of zebrafish, based on gene knockdown and knockout methods, is the most common approach currently being employed.

3.5.1 KNOCKDOWN INDUCED BY MORPHOLINO OLIGONUCELOTIDES

Morpholino Oligonucelotides (MOs) anti-sense knockdown technology became the most popular tool for zebrafish research after it was invented 20 years ago (Bill et al., 2009). MOs are synthetic oligonucleotides similar to DNA and RNA oligonucleotides using the morpholine ring to replace the ribose ring in nucleotides. Conventional antisense RNA and the subsequently developed RNAi technology failed in studying gene functions in zebrafish development, as antisense RNA has been shown to have widespread sequence nonspecific side-effects (Oates et al., 2000). MOs have been developed as a way to inhibit the translation of RNA transcripts *in vivo* (Partridge et al., 1996; Summerton and Weller, 1997).

MOs have a neutrally charged backbone which has high binding affinity for RNA. MOs are microinjected into the yolks of 1–8-cell-staged zebrafish embryos to induce steric hindrance of proper transcript processing or translation (Bill et al., 2009). Compared to DNA and RNA, MOs are more resistant to nucleases with less non-specific interaction with other components of the cell. After delivering into embryos, MOs are very stable, and present rather low toxicity *in vivo*. In most cases, knockdown effects induced by MOs can be observed within 3 days. The effective inhibition can last

up to one week after the injection depending on the specificity and dosage of MOs introduced (Bill et al., 2009).

Two major types of MOs are usually designed for splice blocking and translational blocking of pre-mRNA processing via inhibition of the splicesome components or hindering ribosome assembly. The level of splice blocking caused by knockdown should be assessed by RT-PCR to identify the quality and quantity of any new transcripts as well as knockdown of the wild-type mRNA. The translational blocking knockdown efficiency detection requires an antibody to the protein of interest (Nasevicius and Ekker, 2000). Splicing MOs are usually used to corroborate data obtained with translational blocking by comparing the morphant phenotypes caused by both MOs (Eisen and Smith, 2008).

There is a standard protocol for designing and choosing suitable MOs according to genomic sequence of target genes (www.gene-tools.com). The MO should normally be about 25 bases in length with about 50% GC content, and little or no secondary structure. Translational blocking MOs should be designed to be complementary to sequence between the 5' cap and about 25 bases 3' of the AUG translation start site. There is a sharp decrease in the efficacy of MOs that are positioned any more 3' downstream of the translation start site than this area.

3.5.2 MONITORING MO SPECIFICITY IN GENE TARGETING

In most cases when applying MOs to zebrafish embryos, targeting a gene with uncertain functions, it is hard to know what kind of specific morphant defects will be caused by MO-introduced knockdown. Translation blocking MOs interfere only with translation or with pre-mRNA splicing, but do not cause destruction of mRNA. If there is no optimal commercial antibody, it is difficult to determine the inhibition effects caused by them in general or in specific regions, because the inhibition effects by translation blocking MOs cannot be determined exactly by the RNA transcription level. Although splicing MO knockdown effects can be quantified by using RT-PCR, sometimes the reduction in mRNA levels does not necessarily result in the same reduction in protein levels. A more insidious problem of MOs is the "off-site target" effect. The morphant phenotype may be only partially the result of, or have nothing to do with, the target. In a large-scale screen utilizing translational blocking MOs, reproducible phenotypes resembling those MO off-targeting have been observed (Ekker and Larson, 2001; Kok et al., 2015).

The most reproducible phenotype is cell death. Many MOs tend to activate the ectopic upregulation of the p53 apoptosis pathway, and therefore lead to non-specific phenotypes (Robu et al., 2007). The mechanism of p53 activation by MOs is unknown. The coinjection of one effective anti-p53 MO with targeted gene MOs, or performing all the experiments on a p53-mutant background can overcome the off-targeting cell death effects. Many MOs also show p53 irrelevant pseudophenotypes such as small head and eyes, curled body, etc. (Law and Sargent, 2014).

Given the potential problems described above, several control experiments should be included when using MOs for quantifying efficiency, and for screening typical phenotype caused by target-gene inhibiton. One possible solution is to design mismatch MO, which is a standard five-nucleotide mispaired MO (5Mis-MO) comparing to antisense MO. Not only a control for the injection process, 5Mis-MO is used for the introduction of significant amounts of exogenous oligonucleotides, and for the specificity of a particular MO. Another possible solution is to use two or more sequence different antisense MOs for one target gene independently. The specific knockdown of target gene can be observed by the similar phenotype of the mutants in different MO injections. The best control is to attempt to 'rescue' the phenotype by the injection of synthetic mRNA encoding the protein of the targeted gene. The synthetic mRNA should not have the 5' UTR region that was targeted by translation-blocking MOs. The specific knockdown morphant phenotype can be confirmed by the

rescue effects caused by mRNA coinjected with MOs.

3.5.3 CORRELATION OF KNOCKDOWN AND KNOCKOUT

Recent development of new genome engineering techniques, such as TAL effector nucleases (TALENs) and the programmable bacterial nuclease Cas9, have been applied successfully to introduce heritable lesions in the zebrafish genome at high frequency (Hwang et al., 2013). This allows the rapid generation of zebrafish mutations and has revived concerns over the lack of specificity of MO-induced knockdown. Controversially, many morphant phenotypes are not recapitulated in mutant embryos. Comparison of a total of 98 published morphant defects with the Sanger Zebrafish Mutation Project revealed that approximately 70% of morphant phenotypes were not observed in mutant embryos, after taking into account the maternal contribution. Many MO knockdown morphant phenotypes may be due to off-target effects (Kok et al., 2015). The Zebrafish Mutation Project supports the previous report well, which suggested a high degree of redundancy built into the zebrafish genome resulting in few phenotypes in a characterization of nearly 1,000 zebrafish mutant lines (Kettleborough et al., 2013).

More recent reports found that some mutant alleles fail to exhibit any phenotype, which may be due to the genomic compensation effects (Rossi et al., 2015; Stainier et al., 2015). Such genomic compensation may also occur in some MO experiments (Eisen and Smith, 2008). However, a recent study showed that the genomic compensation happened more frequently in the knockout mutants than in the knockdown morphants (Rossi et al., 2015). The use of MOs is essential for down-regulating maternal and zygotic gene expression to recognize the related phenotype, when mutants generated by programmable site-specific nucleases have shown no defect or phenotypes (Kok et al., 2015). In most cases, the MO knockdown and gene knockout mutants can complement each other and increase the confidence of results.

AIMS OF THE STUDY

To date, there is no published data on HMGB1 function in nervous system development *in vivo*, possibly due to the severe neonatal syndrome evident in knockout mice. This work aims to reveal the role of HMGB1 and AMIGO1 in embryonic CNS development using mouse and zebrafish knockout/knockdown models. The specific aims of the study are:

1. To characterize HMGB1 function and mechanism in brain development by using the HMGB1 knockout mouse embryos and HMGB1 knockdown zebrafish. The HMGB1 knockout/knockdown strains are expected to aid resolution of *in vivo* functions of HMGB1 in the developing CNS, such as: regulation of cell proliferation, migration, axon guidance, and neuronal differentiation.

2. To determine the role of AMIGO1 in brain development. Generation of AMIGO1 knockdown zebrafish morphants should reveal the *in vivo* function of AMIGO1 in neurite outgrowth and neuronal tract development.

3. To explore the regulation of AMIGO1 expression by HMGB1 *in vivo*. It is already known that AMIGO1 expression can be induced by HMGB1 in the cultured neuronal cells, but there is little evidence from *in vivo* studies. Both HMGB1 knockdown and knockout models are employed here to show if HMGB1 is required for AMIGO1 expression in developing CNS, and how the regulation mechanism relates to their functions in CNS development.

4. To analyze the behavioral defect of AMIGO1 knockdown morphants, and find out if interactions of Amigo1 with Kv2.1 play a role in the amigo1 morphant phenotypes. Together with AMIGO1 knockout mouse data, the functions of AMIGO1 homophilic and heterophilic binding in neural development are explored.

5. As for the crucial function and clear signaling pathway of HMGB1 in immune system, we are addressing the question if HMGB1 shares signaling pathways between immune cells and neuronal cells via similar membrane receptors to regulate CNS development.

MATERIALS AND METHODS

Methods used in this study are listed in Table 1. Detailed information about material and methods can be found in the original publications (I, II) and manuscript (III).

Table 1. Overview of methods used in this study

Method	Publication
Antibody production	II
Behavioural Assays	II
Biostatistics and Bioinformatics	I, II, III
BrDU staining	III
Cell culture and transfection	I, II
Co-immunoprecipitation	II
Confocal Imaging	I, II, III
DNA constructs	I, II
EdU staining	I, II
Fluorescence-Activated Cell Sorting	III
Immunocytochemistry	I, II, III
Morpholino oligonucleotide and mRNA Injections	I, II
Neuronal cell culture	III
Quantitative RT-PCR (qRT-PCR)	I, II, III
Recombinant protein production	I, II
Tunel Staining	I, II
Western Blotting	I, II, III
Whole Mount *in Situ* Hybridization	I, II, III
Zebrafish tissue preparation	I, II

RESULTS

1. HMGB1 EXPRESSION IN DEVELOPING CNS (I)

1.1 CLONING AND DETECTION OF ZEBRAFISH HMGB1 ORTHOLOGS

HMGB1 shares high similarity across species. The full-length zebrafish *hmgb1* cDNA was cloned using mRNA from zebrafish larvae and primers designed according to the putative homologous sequence found in the Zv9 database (http://www.ensembl.org/Danio_rerio). The zebrafish *hmgb1* gene has the same exon-intron organization as compared to the mammalian Hmgb1. The zebrafish Hmgb1 protein also has two homologous HMG boxes (HMG boxes A and B), followed by the acidic tail consisting of only glutamate and aspartate residues.

The deduced zebrafish Hmgb1 amino acid sequence is slightly shorter (205 amino acids) than the mammalian one (214 amino acids). Zebrafish Hmgb1 displays 86% and 78% similarity to the human or mouse HMGB1 and HMGB2 sequences respectively. The similarity between zebrafish Hmgb1 and Hmgb2 is 74%. Compared to chicken and Xenopus tropicalis HMGB1, the similarity is 79%.

The antibodies against a synthetic peptide corresponding to a highly conserved area immediately before and at the beginning of the B box of the mammalian HMGB1 (Parkkinen et al., 1993) bound to the zebrafish Hmgb1 in western blotting. This was demonstrated by *in vitro* HMGB1 expression in 293T cells using fusion plasmid clones containing the mouse and zebrafish HMGB1 cDNA sequence.

1.2 HMGB1 EXPRESSION PATTERN IN ZEBRAFISH EMBRYOS

Hmgb1 is widely expressed in embryonic nervous system. *hmgb1* mRNA is ubiquitously expressed in blastula, gastrula, and segmentation stages until 14 hpf. After this, Hmgb1expression is mainly in brain and other parts of the nervous system until 5 dpf.

To gain further insight into Hmgb1 expression in zebrafish embryos, whole mount immunostaining experiments of 28 hpf (hours post fertilization) larvae were performed using antibodies against Hmgb1. The 28 hpf larvae have already completed primary neurogenesis and are entering into the stage of secondary neurogenesis. Hmgb1 is found to be prominently expressed in the forebrain, in particular in rostral telencephalon and in telencephalon close to the ventricular wall, in pretectum and at the anterior part of the diencephalic ventricular wall. The areas where Hmgb1 is expressed are actively proliferating zones, which contain the pools of brain stem cells/neuronal progenitor cells during the primary and secondary neurogenesis. In addition to the forebrain, Hmgb1 expression is found symmetrically along the brain midline in the spinal cord and in the notochord.

2. AMIGOS IN DEVELOPING ZEBRAFISH EMBRYOS (II)

2.1 CLONING OF ZEBRAFISH AMIGO ORTHOLOGS

Three putative *amigo* transcripts are found in the zebrafish Zv9 database (Ensembl, search *Danio rerio*) according to the characteristic LRR and Ig domains. The full-length cDNAs were cloned by using mRNA from zebrafish larvae and primers designed according to the putative homologous sequences. Comparison of the deduced amino acid sequences of the zebrafish Amigos to the human, rodent, chicken, medaka, and Xenopus proteins shows that Amigo1 has the highest degree of homology when compared with the orthologs expressed in different species. Zebrafish Amigo1

shares over 50% identity in its amino acid sequence to AMIGO1 in all other species. Another two zebrafish Amigo-like proteins display similarity to AMIGO3 and are designated as Amigo3a and Amigo3b. No Amigo2-specific ortholog has been found in teleost species. RT-PCR results showed, however, that Amigo1 CNS-enriched expression pattern is evolutionary conserved in most regions of adult zebrafish brain as in the rodent brain.

2.2 DYNAMIC EXPRESSION PATTERN OF AMIGO1 DURING ZEBRAFISH EARLY DEVELOPMENT STAGE

In order to quantify the level of Amigo1 expression/transcription in larval zebrafish, the primers for qRT-PCR of *amigo1, amigo3a,* and *amigo3b* transcripts were designed and used for expression analysis in larvae at the developmental stages 1-8 dpf. The qRT-PCR results revealed that all three amigos start to be expressed from 1dpf. Amigo1 expression is the highest of the three *amigo* transcripts. Amigo1 expression was increasing exponentially during the first week of development. Expression of Amigo3a and Amigo3b started to increase from 3 dpf and were always lower than expression of Amigo1. Expression of Amigo3b was found to be the lowest of the three transcripts.

Amigo1 protein distribution and expression pattern in developing nervous system were detected by immunohistochemistry methods. The specific antibodies were generated against the zebrafish Amigo1 extracellular portion. Amigo1 ectodomain-GST (glutathione S-transferase) fusion plasmid clone was constructed and transformed for large scale *in vitro* expression. The affinity purified fusion protein was used as antigen for immunization. Amigo3a-GST fusion plasmid clone was constructed and expressed at the same time for identifying the specificity of Amigo1 antibodies. Western blotting data showed that the affinity-purified Amigo1 antibodies can specifically stain the *in vitro*-expressed Amigo ectodomain-MBP (maltose-binding protein) fusion protein and the endogenously-expressed Amigo protein bands. It shows no staining with Amigo3a-GST fusion protein in western blotting.

Whole-mount immunostaining shows that Amigo1 is highly expressed along the diencephalic ventricle (DiV) of the 28hpf larval forebrain; a region undergoing active neurogenesis during development. Furthermore, the anterior diencephalon and upper layer of posterior tuberculum (PT), from which the anterior thalamic nuclei derive later on, are also intensively stained with anti-Amigo antibodies. In 28 hpf larvae, Amigo1 was not only found to be expressed in cells but also in the early developing fibre tracts. Amigo1 was detected at the growth-cones of the developing tracts by double immunostaining with Anti-HNK1 antibodies. Amigo1 is also highly expressed in medial longitudinal fascicle (MLF) and the post-optic commissure (POC), which are the early developing fibre scaffolds of zebrafish.

In 5dpf larvae, zebrafish brain structure is almost the same as during adulthood. Amigo1 is widely distributed in most nuclei in telencephalon, commissures in diencephalon and Ppv of thalamus in 5dpf larvae. Amigo1 is also strongly stained in the dorsal layer of optic tectum (TeO) and in the tracts along SRF (superior reticular formation) in the hindbrain along the midline.

2.3 COLOCALIZATION OF AMIGO1 WITH HMGB1/ KV2.1 IN ZEBRAFISH BRAIN (I,II)

Double-immunostaining of 28hpf larvae with the anti-HMGB1 and anti-Amigo1 antibodies revealed partial co-expression of Hmgb1 and Amigo1 in developing forebrain and hindbrain ventricle, and also in the notochord. Under a high-resolution view of confocal imaging, HMGB1/AMIGO1 double-immunostaining in diencephalon revealed a patchy detection of both proteins, which is partially

colocalized at the plasma membrane. Hmgb1 was more widely spread than Amigo1 in the developing brain.

Amigo1 was recently reported to be an auxiliary partner of Kv2.1 in rodent neuronal cells. In adult zebrafish brain, whole-mount immunostaining shows that Amigo1 expression almost overlaps with Kv2.1 in most cells located at thalamus, gray zone of optic tectum, diencephalon ventricle layers, and cerebelli. Both proteins are highly expressed in the diencephalic region of the brain. Compared to adult, whole mount immunostaining showed that Kv2.1 expression is lower in 28hpf larvae than Amigo1. During the early development stage, very weak Kv2.1 staining can be observed in the forebrain. Kv2.1 expression was found to colocalize together with Amigo1 in midbrain and adjacent to hindbrain ventricle, but the expression was much lower than Amigo1 expression in these regions.

3. HMGB1 AND AMIGO KNOCKDOWN EFFECTS IN DEVELOPING EMBRYOS (I, II & III)

3.1 HMGB1 KNOCKDOWN DEFECTS IN ZEBRAFISH (I)

Three different MOs were designed to interfere in *Hmgb1* expression. The first one (MO1) was designed to inhibit HMGB1 translation by recognizing the sequence from the starting code of the HMGB1 transcript. The other two oligonucleotides (MO2 and MO3) were designed to introduce partial deletion of HMGB1 transcript by targeting extron-intron gaps. Uninjected larvae and larvae injected with a 5-mispaired MO (5misMO) were used as controls.

3.1.1 MORPHOLOGICAL DEFECTS CAUSED BY KNOCKING DOWN HMGB1

The *hmgb1* antisense MO injected zebrafish showed developmental defects from early stages of development (1-2 dpf). The phenotypic changes in Hmgb1 knockdown morphants can be observed clearly in 30 hpf larvae. Closer examination of the MO1 injected larvae during an early developmental stage (30 hpf) displayed a disordered pattern of prosencephalon due to perturbed formation of both diencephalon and telencephalon. The diencephalon and telencephalon displayed distorted morphology making it difficult to discern these brain structures, and the midbrain-hindbrain boundary formation was interrupted. In 3dpf larvae, the morphologic defects of Hmgb1 morphants are characterized by smaller size, smaller brain width, shorter rhombomeres and shorter distance between the eyes. They develop curling tails and shorter trunks compared to the controls. All *hmgb1* antisense MO injected morphants are immobile, and they stay alive until about 5 dpf. As MO1 can cause over 90% inhibition of *in vivo* Hmgb1 expression by blocking the translation, it shows higher efficiency in Hmgb1 knockdown than MO2 and MO3, which was confirmed by western blot analysis. The 5 misMO injected larvae did not display any relevant morphological changes. Coinjection of *hmgb1* mRNA significantly rescued the morphological defects in the *hmgb1* MO injected larvae.

To characterize the regional role of HMGB1 in brain development, the expression of transcription factors that have been implicated in regional development of brain was studied. As the classical markers of early developing neuromeres, Pax6, Pax2a, and Krox20 expression in the Hmgb1 knockdown morphants were investigated. The whole mount *in situ* hybridization of each RNA probe in zebrafish larvae showed that the most anterior Pax6a-expressing cell group in telencephalon of Hmgb1 knockdown morphants was absent, which could be restored by coinjection of *hmgb1* cRNA in the embryos. In contrast to the telencephalic area, Pax6a expression appeared normal in more posterior areas of the central nervous system. At the same time, Pax2a or Krox20 expressions in the midbrain and hindbrain areas were unchanged in Hmgb1 knockdown larvae. These results are in

agreement with the morphological findings, suggesting that forebrain development is especially vulnerable to the down-regulation of Hmgb1 expression.

3.1.2 DEFECTS OF CA SYSTEM DEVELOPMENT IN HMGB1 KNOCKDOWN MORPHANTS

Catecholaminergic (CA), mostly dopaminergic, neuronal networks form an important and well-studied structure in early zebrafish forebrain. The earliest Th1 (tyrosine hydroxylase1)-immunoreactive neurons are detected in the basal forebrain just before 24 hpf in close proximity to the nucleus of the post-optic commissural tract, also known as the ventro-rostral (the hypothalamus) cluster. The onset of Th1 and dopamine transporter mRNA expression is found in the same area at 18 hpf and onwards. The catecholaminergic networks of the early postembryonic zebrafish brain are essentially fully formed at 5 dpf; they have been carefully mapped and can be accurately detected using immunostaining of Th1.

Immunostaining of Th1-positive networks revealed prominent changes in the 5dpf Hmgb1 knockdown morphants. In MO-injected larval brain, the staining of Th1-positive neuron clusters in the telencephalon and the anterior basal diencephalon almost disappeared. Furthermore, the HMGB1 morphants demonstrated significantly fewer Th1 cells in the hypothalamus. In hindbrain, Hmgb1 knockdown caused a clear disordered distribution of Th1-reactive axon fibres along the midline. The phenotype was essentially rescued in MO1and *hmgb1* mRNA-coinjected larvae, with only slight changes compared to wild-type controls.

3.1.3 APOPTOSIS AND PROLIFERATION DEFECTS OF HMGB1 KNOCKDOWN IN DEVELOPING BRAIN

Neurogenesis and proliferation are the most important processes during early development. In fast developing stages, a subtle change of the neural progenitor cells might cause strong defects later on. HMGB1 has been previously demonstrated to enhance survival of cultured embryonic cells (Huttunen et al., 2000). From Hmgb1 expression detection we already know that Hmgb1 is largely expressed in the active proliferating zone of larval forebrain. This work confirms that the abundant expression of Hmgb1 in those progenitor cells is required for survival and proliferation in embryonic forebrain.

In order to confirm whether Hmgb1 is involved in the neurogenesis in developing zebrafish brain, whole mount TUNEL staining of apoptotic cells and EdU (5-ethynyl-2′-deoxyuridine) staining of proliferating cells was carried out with 28hpf larvae and 3dpf larvae respectively. Normally the programed cell death is not yet commenced in 28hpf larvae, only few apoptotic cells can be detected in normal developing larval brain. The *hmgb1* knockdown morphants showed a significantly elevated population of apoptotic cells in forebrain and hindbrain compared to normal and 5mis MO injected larvae. Coinjection of *hmgb1* mRNA together with antisense MO was essentially able to reverse the effect, indicating that increased apoptosis was specifically caused by inhibition of Hmgb1 expression.

Not surprisingly, 3dpf *hmgb1* knockdown morphants showed a significant decrease in proliferative activity in comparison to the other groups. EdU labelling revealed prominently stained cell layers in the active proliferating zones of normal developing zebrafish embryos, which was essentially absent in *hmgb1* knockdown larvae. After coinjection of *hmgb1* mRNA with MO, the staining of cell proliferation was largely rescued in the larvae. The ectopic expression of injected mRNA even elevated proliferative activity along the ventricle cell layers since the injected coding sequence lacks

regulatory elements. Hmgb1 thus appears to directly affect cell proliferation even in nervous system areas where it is not endogenously expressed. These results confirm that Hmgb1 is required for neurogenesis and proliferation in early developmental stages.

3.2 AMIGO1 KNOCKDOWN DEFECTS IN ZEBRAFISH (II)

3.2.1 MORPHOLOGICAL PHENOTYPE OF AMIGO KNOCKDOWN MORPHANTS

The strategy employed to knock down endogenous HMGB1 expression was also applied for Amigo1 knockdown in zebrafish larvae. As there are no exon-intron gaps in the *amigo1* transcript, two different specific *amigo1* antisense MOs were designed to block translation of endogenous Amigo1. The inhibition effects of both MOs were measured with western blotting and whole-mount immunostaining. In addition to 5mis MO control, *amigo3a* MO was designed for confirming specificity of anti-Amigo1 antibodies. Significant down-regulated Amigo1 expression was observed in *amigo1* MO1 and MO2 -injected larvae (over 70% inhibition comparing to uninjected and 5mis MO controls), but no typical morphological phenotype was found in Amigo1 knockdown morphants in contrast to the HMGB1 knockdown morphants. The whole mount *in situ* hybridization of transcription factors (*pax6a, pax2a* and *krox20*) did not suggest any defects either. The early Amigo1 knockdown larvae (1dpf or 2dpf) only showed a slightly smaller brain size compared to the controls.

3.2.2 APOPTOSIS AND PROLIFERATION CHANGES IN AMIGO KNOCKDOWN MORPHANTS

Immunostaining studies already suggested co-expression of Hmgb1 and Amigo1 in brain. AMIGO1 was previously cloned as a gene that is robustly induced by cell matrix-bound HMGB1 in neuronal cells *in vitro*. Western blotting experiments revealed that the expression of Amigo1 is indeed closely linked to Hmgb1 expression: in MO1-injected larvae, both proteins essentially disappear, and they both reappear when *hmgb1* mRNA is coinjected.

Knocking down Amigo1 failed to cause any clear morphological defects in developing embryos; nor did EdU and TUNEL staining show any apparent changes in the brain of Amigo1 MO1 or MO2 injected larvae compared with control groups. In the whole prosencephalon and mesencephalon of Amigo1 knockdown morphants, no significant changes were observed in the numbers of apoptotic cells.

When *amigo1* mRNA was coinjected with *hmgb1* MO, it partially rescued Hmgb1-knockdown effects on apoptosis and proliferation. This suggests that Amigo1 may be one of the downstream signalling factors involved in the brain development regulated through HMGB1.

3.2.3 IMPAIRED AXONAL TRACT DEVELOPMENT IN AMIGO1 KNOCKDOWN ZEBRAFISH

Amigo1 protein has been initially identified as a neurite outgrowth promoting factor that plays an important role in the fasciculation of neuronal tracts of cultured neurons. Given that Amigo1 is expressed in the early neuronal tracts (publication II), it was worth exploring the role of Amigo in early neuronal tract development in zebrafish larvae. As a commonly used marker for visualizing zebrafish primary neural tract (Metcalfe et al., 1990; Nordlander, 1989; Wilson et al., 1990), anti-HNK1 antibodies were adopted for specifically labelling neurons and axons in the early embryonic nervous system. The staining makes it possible to follow the development of early nervous system structures, as the initial axon scaffold of the 28hpf zebrafish brain is simple and easy to distinguish,

consisting of a few longitudinal tracts connected by commissures. We chose the zebrafish larvae at the stage of 28hpf for imaging analysis. Confocal stacking projections of the lateral side of larvae clearly showed disturbed fibre tract development in Amigo1 knockdown morphants. The medial longitudinal fascicle (MLF) and tract of the post-optic commissure (TPOC) were almost invisible in the *amigo1* MO1 morphants compared to the wild type or 5misMO-injected embryos. Coinjection of the full-length *amigo1* mRNA (Am1 mRNA) with MO displayed a prominent rescuing effect of the axonal scaffold development.

It is already reported that the AMIGO proteins display homophilic binding that appears important for fasciculation of neurites in cultures of rat brain neurons. Furthermore, the ectodomain of AMIGO in solution was shown to act as a dominant negative receptor and to inhibit AMIGO-mediated adhesion and fasciculation in neuronal cultures. Therefore we prepared an mRNA encoding the extracellular part of Amigo (coding for the six LLR domains and one Ig domain of Amigo) and injected it into fertilized embryos to provide an assay that does not depend on MOs. Expression of the dominant-negative Amigo ectodomain coding mRNA (Am1 EmRNA) in the injected larvae was distinguished by western blotting with anti-Amigo antibodies. As in the *amigo1* MO1 morphants, development of the early tracts was also severely disturbed in larvae injected with the Am1 EmRNA. In contrast to the Am1 EmRNA, larvae injected with the full length mRNA displayed no such inhibition but even displayed rescue of axonal growth.

Furthermore, we have studied the larvae coinjected with *amigo1* MO and *kv2.1* mRNA, which showed MLF defects as the *amigo1* MO1 morphants and the Am1 EmRNA-injected larvae. Although Kv2.1 expression had been downregulated in Amigo knockdown morphants, Kv2.1 is not directly required for early neuronal tract development in zebrafish.

The ventral caudal cell cluster (vcc) and the hindbrain cell cluster (hc) are important for the development of early ascending and descending long tracts. Both clusters were found to be correctly located in the knockdown morphants. Under higher magnification, the MO1 morphants displayed reduced HNK-1-staining of axons from the ventral caudal cell clusters compared with the other injection groups and the control ones. As the largest reticulospinal interneurons in zebrafish, the Mauthner neurons and their long axons contribute to early sensorimotor circuit formation. HNK-1 is also present in Mauthner neurons and the long growing trigeminal axons. Anti-3A10 antibodies were used for labelling of Mauthner neurons and their axonal projections selectively. Inhibition of axon projections was clearly observed in the Mauthner neurons of the Amigo knockdown morphants. Both anti-HNK-1 and anti-3A10 staining of the axons was partially rescued by coinjection with the *amigo1* mRNA.

As the first cytoskeletal elements to appear in axons, acetylated tubulin is a basic component of microtubules which has also been frequently used as a marker for labelling early developing axon tracts in zebrafish. The anti-acetylated tubulin antibodies have been reported to show a similar immunostaining pattern as the anti-HNK-1 antibodies. We therefore used western blotting to quantify expression of the HNK-1 epitope and acetylated tubulin as markers of the neuronal fibre tract development. Western blotting of 3 dpf larval samples showed that expression of both HNK-1 and acetylated tubulin is strongly reduced in the Amigo1 knockdown (MO1 and MO2) morphants. Coinjection of the full-length *amigo1* mRNA (Am1 mRNA) with MO1 or MO2 caused a significant rescue in expression. As in the MO-injected larvae, HNK-1 and acetylated tubulin were significantly decreased in the dominant-negative Amigo1 EmRNA-injected larvae.

In conclusion, the results clearly showed that Amigo is crucial for the development of early axonal scaffolds in zebrafish brain.

3.2.4 DEVELOPMENTAL DEFECTS OF NEUROTRANSMITTER SYSTEMS IN AMIGO1 KNOCKDOWN MORPHANTS

In order to follow the formation of neural circuits in Amigo knockdown morphants, more careful inspection of neurotransmitter system development was applied by using tyrosine hydroxylase1 (Th1) immunostaining in addition to Th2 as the marker to detect complete catecholaminergic (CA) networks, and 5-hydroxytryptamine (5-HT) as the marker to detect serotonergic neuronal networks. Both networks form the most important and well-characterized neurotransmitter systems in the early zebrafish brain. Amigo1 knockdown morphants showed specific developmental disorders of the aminergic systems during early embryonic stages.

3.2.4.1 CATECHOLAMINERGIC SYSTEM

The CA system is the major neuromodulatory system developing with far ranging projections in the brain, which is also important in the modulation of circuit activities in a broad range of behaviours. Notably, CA development in zebrafish starts quite early, and most of the CA groups and axon tracts found in adult brain can be already detected in 3 dpf larvae. The longitudinal axon tracts from dopaminergic neurons extend from diencephalon towards the spinal cord in the vicinity of the medial longitudinal fascicle (MLF; see above for the role of Amigo1 in MLF formation) and lateral longitudinal fascicle (LLF) but their formation may not depend on these early axonal scaffolds.

Based on the western blotting and immunohistochemistry results using Amigo1 antibodies, we knew that the inhibition of the endogenous Amigo1 expression caused by the *amigo1* antisense MO1 and MO2 was still remarkable in 3 dpf and even in 5 dpf larvae. Most of the CA groups and axon tracts found in adult brain can already be detected in 3 dpf larvae (Kastenhuber et al., 2010; McLean and Fetcho, 2004a; Rink and Wullimann, 2002). We thus explored the role of Amigo1 in the CA development using Th1 antibodies as markers in 3 dpf larvae and 5dpf larval brains.

From the confocal z-stacking sections of the 3 dpf whole larvae, Amigo1 knockdown morphants showed predominant defects in the formation of the medial longitudinal catecholaminergic tract (mlct). Lateral CA projections (lcp) in the hindbrain close to locus coeruleus (LC) displayed a disordered pattern. Coinjection of *amigo1* FcRNA (full-length coding) with MO1 or MO2 had a rescue effect on mlct and lcp development. Statistics of the CA development based on the mlct formation revealed a developmental defect in a high proportion of the *amigo* knockdown larvae at 3 dpf compared to the 5mis MO-injected or the uninjected larvae. The intensity analysis of Th1 antibody staining confirmed the dramatically decreased mlct development in the MO1 and MO2 morphants.

From the confocal z-stacking sections of the 5 dpf larval brain, the Amigo1 knockdown morphant defects of the mlct and lcp development could still be observed in the brains of the knockdown larvae. Th1-immunoreactive axons crossing the midline between caudal diencephalon and medulla oblongata (Mo), which connect the right and left lateral CA tracts, were absent in the *amigo1* knockdown morphants. This is probably due to defects in the development of the longitudinal fascicles (MLF and LLF). In the 5 dpf *amigo1* FcRNA-MO1-coinjected larvae, mlct and lcp developed normally but more Th1-immunorective fibres were developed compared to the uninjected larvae.

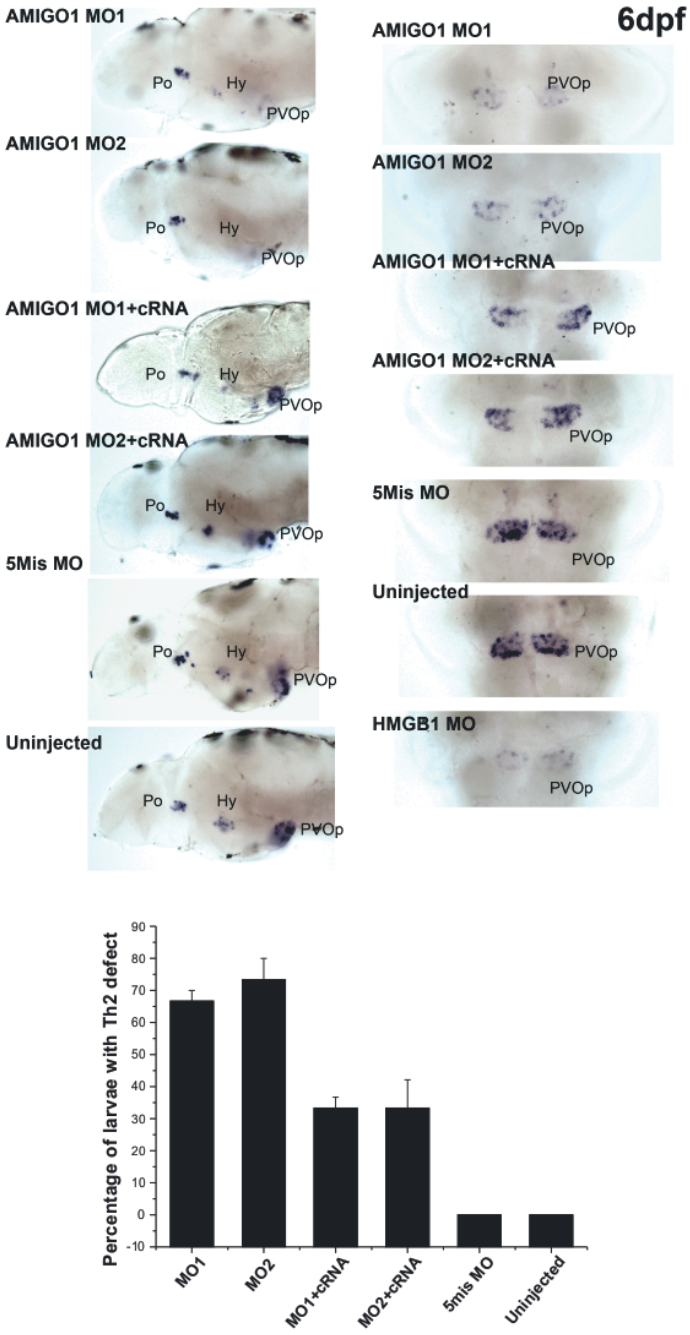

Figure 7. Th2 Whole mount in situ hybridization of 6dpf dissected larval brain. Th2 cells in the hypothalamic clusters were counted. The number of larvae with less than 50% of Th2 cells compared to uninjected controls was calculated. There are over 60% larvae in amigo1 MO1 and MO2 morphants that showed significantly

decreased Th2 cells. As the Amigo1 knockdown morphants, the *hmgb1* MO1 morphants also show similar defects of Th2 cells in PVOp. The experiment has been repeated for 4 times, and each time 10 larvae were selected randomly. Hy, Hypothalamus; Po, Preoptic area; PVOp, posterior part of paraventricular organ.

The whole mount *in situ* hybridization of 7dpf larval brains with *th2* cRNA probe showed that knocking down endogenous expression of HMGB1 would cause defects in development of most Th2 cell groups (Fig 7). In the HMGB1 knockdown morphant, the Th2 cell cluster in the preoptic area totally disappeared and the other three hypothalamic Th2 clusters also decreased substantially. In the Amigo1 knockdown morphant, the Th2 cell cluster defects were observed mainly in the hypothalamus (Fig 7). The results showed a different role for Amigo1 in forebrain development compared to Hmgb1.

3.2.4.2 SEROTONERGIC SYSTEM

Serotonin is another conserved amine expressed from early developmental stages in zebrafish brain. From 24 hpf, colocalization of 5-HT and Th1 can already be detected in diencephalic neurons (Sallinen et al., 2009a). Both 5-HT and Th1 immunoreactive cell clusters are found in the vicinity of the nucleus of the medial longitudinal fascicle (nMLF) from 5 dpf onwards (McLean and Fetcho, 2004b). Anti 5-HT whole mount immunostaining was applied in 5dpf larval brains to study development of the serotonergic system (Fig 8A&B). The knockdown brains showed attenuated staining of the serotonergic system that was partially rescued by injection of the *amigo1* cRNA.

The knockdown brains displayed decreased staining of 5-HT fibres originated from posterior tuberculum (PT). Staining of 5-HT positive fibres in postoptic commissure (POC) in telencephalon was also attenuated in the Amigo1 knockdown MO1 and MO2 morphants compared to the 5mis MO-injected or uninjected larvae (Fig 8A).

Compared to numerous TH positive dopaminergic cell groups in ventral diencephalon, there are only three periventricular 5-HT positive cell populations in 5 dpf larval brain (Fig 8C, PVOa, anterior part of the paraventricular organ; PVOi, intermediate part of paraventricular organ; PVOp, posterior part of paraventricular organ). These three 5-HT positive cell populations are located close to Th1 positive cells, but 5-HT and Th1 even rarely colocalize on this area (McLean and Fetcho, 2004a; McLean and Fetcho, 2004b; Sallinen et al., 2009a). In 5-HT antibody stained *amigo1* knockdown morphants, serotonergic cells and their fibres are clearly reduced. Remarkable decrease of anti-5-HT staining was observed in PVOa and PVOi, and some decrease was observed in PVOp. Coinjection of the *amigo1* FcRNA with MO1 or MO2 significantly rescued the changes in the 5-HT positive neuronal circuitry found in the Amigo1 knockdown morphants (Fig 8A&C).

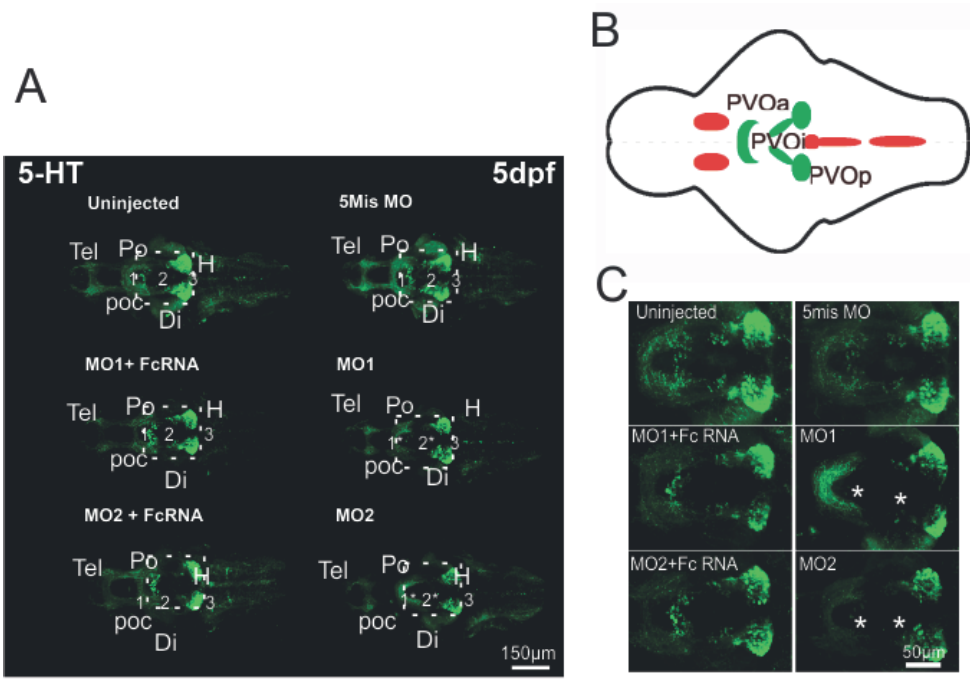

Figure 8. Immunostaining of the serotoninergic system in 5dpf larval brains. A, Whole mount immunostaining with anti-5-HT antibodies showing defects of 5-HT-immunoreactivity (5-HT-ir) neurons in 5dpf amigo1 morphants brain. MO1 and MO2 morphants show decreased 5-HT-ir innervations in the postoptic commissure (POC) in telencephalon (Tel). In AMIGO MO1 and MO2 morphants, the 5-HT ir cell cluster 1 and 2 close to preoptic (Po) area do not develop as in 5Mis MO or uninjected controls, labeled with asterisks. 1, 5-HT-ir cell in clusters PVOa; 2, 5-HT-ir cell clusters in PVOi; 3, 5-HT-ir cell clusters in PVOp.
B, Schematic map of 5-HT-ir cell groups in 5dpf larval brain. The three 5-HT-ir cell groups in hypothalamus are labeled in green. The 5-HT-ir cells in epiphysis (anterior) and ventral caudal raphe complex (posterior) are labeled in red. C. Anti-5-HT staining in the hypothalamic cell clusters (white rectangle area in panel A). Under higher resolution, amigo1 knockdown morphants show less 5-HT-immunoreactive (5-HT-ir) cells in diencephalon than the larvae in other groups. AMIGO MO1 and MO2 morphants show clear deficiency of PVOa and PVOi compared to the controls and FcRNA rescued larvae.
Abbreviations: Di, diencephalon; H, hypothalamus; poc, post-optic commissure; Po, preoptic region, PVOa, paraventricular organ, anterior part; PVOi, paraventricular organ, intermediate part; PVOp, paraventricular organ, posterior part; and Tel, telencephalon.

3.2.5 SENSORY-MOTOR DEFECTS IN AMIGO1 KNOCKDOWN ZEBRAFISH

Zebrafish aminergic systems have been reported to interact at all levels of the sensorimotor pathways involved in escaping and locomotor activities in recent decades (McLean and Fetcho, 2004b). Zebrafish Mauthner neurons, in particular, have been identified to be crucial for sensorimotor gating circuits. Hmgb1 knockdown morphant only survives one week post fertilization and most larvae are immobile, while Amigo1 knockdown morphants are more robustly developed as normal controls. Thus, locomotor activity and startle responses were tested for the Amigo1 knockdown morphants using established systems.

First, free swimming was assayed in 6-dpf larvae for determining the locomotor activity. Amigo1 MO1 and MO2 morphants both showed increased locomotor activity compared with control larvae.

The *amigo1* mRNA coinjected larvae showed no significant difference in locomotor activity. These results revealed that knocking down Amigo1 would result in hyperactivity, which might be related to the inhibition of serotonergic system development. Kv2.1 might also be involved in the neuromodulation of locomotor activity, as the coinjection of the *kv2.1* mRNA with Amigo1 MO1 or MO2 can also rescue the hyperactivity phenotype of the morphants.

Immunohistochemical analysis showed that Amigo1 is expressed in Mauthner neuron axons (early axonal tracts that are crucial for the functions of the sensorimotor *circuits*), and the MO experiments indicated that Amigo1 regulates development of connections from the Mauthner neurons *in vivo*. Based on previous reports, Amigo1 knockdown could therefore perturb startle motor activity. Therefore, the behavioural repertoire was explored further and the startle response of the Amigo1 knockdown morphants elicited by electrical stimuli was investigated (10 ms, 5 V). The Amigo1 MO1 and MO2 morphants showed significant defects in performing both short latency C-bend (SLC) and long latency (LLC) startle responses. In larvae coinjected with *amigo1* mRNA-MO1 or amigo1 mRNA-MO2, the SLC and LLC were rescued to the level found in the 5mis MO-injected larvae. Similar rescue effects were observed in the larvae coinjected with *kv2.1* mRNA. Taken together, these results demonstrate that transient knockdown of Amigo1 *in vivo* significantly affects the formation of functionally mature neuronal circuits that are necessary for driving essential behaviours in an intact vertebrate model organism. It might relate to both defects in the aminergic system development and down-regulation of Kv2.1 potassium channel expression.

3.3 DEFECTS OF BRAIN DEVELOPMENT IN HMGB1 KNOCKOUT MICE (III)

HMGB1 showed a rather similar temporal expression pattern in the mouse embryonic brain as in zebrafish larval brain. Its expression decreased dramatically in the brain after early developmental stages (Guazzi et al., 2003). Therefore, whether similar CNS developmental defects also happened in HMGB1 knockout mouse embryos as in the zebrafish larvae, was explored.

3.3.1 NEURONAL PROLIFERATION AND DIFFERENTIATION DEFECTS OF HMGB1 KNOCKOUT MOUSE

The proliferative activity in E16 mice brain was measured by BrdU staining at first. HMGB1 knockout embryos showed significantly decreased proliferation in the forebrain and telencephalon regions compared to heterozygous and wild type embryos.
The cortical neuronal cells of E16 embryonic brains were isolated and cultured *in vitro* for further quantification of apoptosis level and differentiation analysis. The cells from HMGB1 knockout mice showed abnormal high level of apoptosis starting from 2 days. After culturing for 7 days *in vitro*, the HMGB1 KO cells already lost proliferative activity and showed gross apoptosis. By adding exogenous recombinant HMGB1 protein (10 μg/ml) into the cell culture medium, HMGB1 KO cell apoptosis was dramatically reduced. HMGB1 knockout neuronal cultures also showed much lower proliferative activity than normal cells as demonstrated by immunostaining with anti-PCNA antibodies, which almost disappeared after 7 days. By staining with anti-GFAP and anti-NeuN antibodies, HMGB1 knockout neuronal cultures even showed significantly decreased differentiating level with much less glia-cell staining than normal cell cultures. GFAP staining of E16 sagittal sections shows that the HMGB1 KO mouse has very few differentiated glial cells in the cortical cortex. Fluorescence-activated cell sorting (FACS) results with E14.5 mouse cortical cells showed that HMGB1 KO mouse brains contain significantly lower proportions of oligodendrocytes and astrocytes than WT controls. The microglia population in HMGB1 KO mouse E14.5 brain is also about 10% smaller than WT controls.

3.3.2 NEURAL DEVELOPMENTAL DEFECTS OF HMGB1 KNOCKOUT

HMGB1 knockout mice show hypoplasia in telencephalon and diencephalon in haematoxylin stained E10 and E16 sagittal sections. HMGB1 knockout mice have smaller forebrain ventricle and shrunken telencephalon compared to wild type controls. Defects in embryonic forebrain development for HMGB1 knockout mice have been shown by via decreases in regional brain expression of transcription factors Foxg1, Tbr2, and Emx2 from *in situ* hybridization tests. Quantitative (q) RT-PCR also shows that HMGB1 knockout mice displayed aberrant expression of neurodevelopmental transcriptional factors. Comparing to wild type controls, HMGB1 knockout mice displayed significant decreases in Pax6, Shh, Foxg1, Fgf2, Emx2, BMP2, BMP4, and Tgfβ1. In contrast, HMGB1 knockout mice demonstrated a higher level of Wnt1 (80% more) and Wnt3 (70% more) compared to wild type controls.

3.4 MECHANISMS INVOLVED IN NEURODEVELOPMENTAL DISORDERS CAUSED BY INTERRUPTION OF HMGB1 AND AMIGO EXPRESSIONS (I, II, and III)

The mechanism underlying forebrain development is rather conserved between teleost and rodent. Even similar defects of forebrain developmental defects were observed in both HMGB1 knockdown zebrafish embryos and HMGB1 knockout mice embryos. It is worthwhile to employ both the zebrafish and the mouse model to study the common factors and possible signal pathways involved in the HMGB1 regulation of forebrain development.

It is well known that Wnt signalling directly regulates the anterior-posterior development of vertebrate and invertebrate CNS. Among numerous Wnt gene copies, Wnt8 signalling has been particularly connected to forebrain development in zebrafish. By qRT-PCR detection, zebrafish HMGB1 kncokdown morphant showed significantly up-regulated Wnt8a1, Wnt8a2, and Wnt8b expression, while Pax6a expression was down-regulated compared to normally-developed embryos. Coinjection of HMGB1 mRNA restored the Wnt8 and Pax6 expressions to normal levels. Furthermore, western blotting of β-catenin confirmed elevated canonical Wnt signalling in HMGB1 knockdown larvae. Similar changes were not found in Amigo knockdown morphants. Therefore, exogenous Amigo expression can partially rescue HMGB1 knockdown defects by elevating proliferative activity and decreasing apoptosis. Even the telencephalic TH-ir neuronal clusters that disappeared in HMGB1 knockdown morphants can be rescued by coinjection of Amigo mRNA. This implies that some other signalling pathway may also be involved in the HMGB1 regulation of forebrain development.

Kv2.1 has been found to be coexpressed with Amigo in neuronal cultures and in the zebrafish thalamic cell clusters. The *in vivo* expression of Kv2.1 seems to be related to a chemokine signalling pathway related to CXCL12/CXCR4 (Shepherd et al., 2012). Surprisingly, it has been recently reported that HMGB1 can form a heterocomplex with CXCL12 and signalling through CXCR4 *in vivo*. As a versatile chemokine factor, CXCL12 has multiple roles in the developing central nervous system, especially in neurogenesis and apoptosis. In HMGB1 knockout mice, qRT-PCR results showed that CXCL12 expression was significantly increased compared to wild type and heterozygote mice. *In situ* hybridization with CXCL12 RNA probe in E16 mice brain sections also showed that CXCL12 was elevated in the hypothalamus and dentate gyrus cells. Abnormal CXCL12 signalling might be a cue for increased apoptosis and decreased proliferation in the embryonic brain.

DISCUSSION

1. HMGB1 IN CNS DEVELOPMENT

HMGB1 is widely expressed in developing CNS. During early developmental stages of organogenesis, HMGB1 is detected in brain structures in many nonvertebrate species, such as amphioxus and Xenopus. In Drosophila a HMG-box containing protein DSP-1 (a co-repressor of the Dorsal protein) is also found to be widely expressed in the ventral nerve chord and brain (Mosrin-Huaman et al., 1998). HMGB1 is also highly expressed in the developing forebrain of the basal vertebrate lamprey.

In rodents, a high expression level of HMGB1 has been demonstrated in embryonic rat brain compared with the adult brain, and HMGB1 showed spatio-temporally regulated expression in the brain of prenatal mouse. In zebrafish, HMGB1 is essentially identified as a nervous system protein that is abundantly expressed in brain after organogenesis (ZFIN). However, the effects of HMGB1 on neural stem cells / neural progenitor cells and in brain development have remained to be explored.

1.1 HMGB1 FUNCTION IN FOREBRAIN DEVELOPMENT

This work has identified Hmgb1 as an essential factor for forebrain development in zebrafish. In knocking down experiments by antisense MO, efficient inhibition (~90% decrease) of Hmgb1 expression was achieved as shown by western blotting and immunohistological methods. Hmgb1 knockdown morphants showed typical morphological and regional marker expression defects with all three antisense MOs in the forebrain but not with the control MO (5mis MO). Both diencephalic and telencephalic forebrain structures appear vulnerable to the down-regulation of Hmgb1 expression. Staining for apoptosis and proliferation have demonstrated that Hmgb1 has an important function in the stem cell/precursor cell survival and proliferation, which are crucial for forebrain patterning. Compared with other parts of the brain, the forebrain may be more critically dependent on survival/proliferation enhancing factors than posterior parts of the nervous system, which leads to the situation that down-regulation of only one factor causes massive perturbation of forebrain structures. *hmgb1* mRNA coinjection clearly rescued the knockdown defects caused by MO. As previously described, HMGB1 is highly expressed in the central nervous system during early development in all species studied so far. The results of the work suggest that the role of HMGB1 in brain development is a conserved phenomenon in evolution.

1.1.1 MOLECULAR/CELLULAR MECHANISM OF HMGB1 IN CNS DEVELOPMENT

HMGB1 has gained much attention in the last couple of years as an important player in innate immune responses and as a modulating factor in several (auto) immune diseases within and outside of the nervous system. In addition to its role as an alarmin in immune cell regulation, HMGB1 was recently shown to participate in both extracellular and intracellular mechanisms of neurogenesis and apoptosis.

During early developmental stages, HMGB1 expression was found to be mainly located in the stem cell niche, in which the neuroprogenitor cells reside. Progenitor cells are regulated within these niches by soluble and membrane-bound molecules and by extracellular matrix. In HMGB1 knockout mouse embryos and Hmgb1 knockdown zebrafish larvae, neurogenesis and proliferation are clearly disturbed. We suppose that HMGB1 might participate in cell-to-cell signalling within the niches that regulate neural progenitors.

Although HMGB1 contains DNA-binding motifs and is primarily found to be located in the cell nuclei, a plethora of studies have shown that HMGB1 can also be actively released from cells upon many types of cell stimuli, such as cytokines/growth factors and cell contact with extracellular matrix (reviewed by Rauvala and Rouhiainen, 2010).

Hmgb1 has been found to be expressed together with Amigo1 in close vicinity on cell plasma membrane at the ventricle cell layer of 28 hpf zebrafish larval brain, where niches of stem/progenitor cell are found and many types of cytokines/ growth factors and matrix factors are expressed. Therefore Hmgb1 might also be secreted in this type of milieu in zebrafish embryonic brain.

A similar HMGB1 expression pattern is also observed in telencephalic ventricle cells in E18 mice (Guazzi et al., 2003). A previous *in vitro* study showed that HMGB1 can regulate neural cells and other cell types *in vitro* as a cell-matrix-associated molecule, which implies that HMGB1 may have a role as a factor surrounding neural progenitors and regulating them through transmembrane signaling (Hienola A, et al., 2006).

HMGB1 has several cell surface receptors, and further work is warranted to elucidate the relative importance of different HMGB1 membrane-receptors in neural progenitors. Regulation of many cell types by HMGB1 is generally suggested to depend, at least partially, on binding to RAGE. *In vitro* observations showed that HMGB1, RAGE, and HNK-1 were co-localized at the cell surface, and interestingly, RAGE combines with HNK-1 and HMGB1 to stimulate cell migration and neurite outgrowth, with RAGE being a receptor for HMGB1 in promoting neurite outgrowth (Chou et al., 2004). Although hypothetical protein structures displaying some domain structure similarity to RAGE can be found in the zebrafish genome database, no obvious RAGE homologues have been identified. Toll-like receptors have been identified as HMGB1 receptors in the immune system in several studies. Interestingly, Toll-like receptors were identified as proteins guiding neuronal development in Drosophila, and recently they were implicated in neurogenesis in mouse brain (Rolls et al., 2007). The role of toll-like receptors as HMGB1 receptors in brain development requires further elucidation.

However, HMGB1 may also have important intracellular functions in neural progenitor regulation due to its nuclear expression and DNA-binding characteristics. This possibility clearly warrants further studies.

1.1.2 MOLECULAR/CELLULAR MECHANISM OF HMGB1 IN DIFFERENTIATION

HMGB1 is not only crucial for neurogenesis, but is also required for gliogenesis in brain development. HMGB1 KO mice display early brain developmental defects with significantly decreased neuronal differentiation. Fluorescence-Activated Cell Sorting (FACS) analysis of E14 cerebral cortex, which is at the starting point of gliogenesis, clearly demonstrates that HMGB1 KO mice have significantly less labeling of oligodendrocytes and astrocytes than WT controls. Although the microglia population is very sparce at this stage, HMGB1 KO mice still show lower microglial staining than WT controls. This suggests that HMGB1 is not only crucial for neurogenesis, but also required for gliogenesis in brain development.

The mechanism involved in HMGB1 regulation of neuronal differentiation and gliogenesis during development is still unclear. It has been accepted that gliogenesis in developing CNS has been regulated by the JAK/STAT signaling pathway (Bonni et al., 1997). A recent study has demonstrated that JAK/STAT signalling has crucial function in regulating translocation of HMGB1 from nucleus to cytoplasm (Lu B, et al., 2013). In developing mouse brain, HMGB1 is mainly expressed in

cytoplasm (Guazzi S, et al., 2003). Thus, HMGB1 might be a downstream factor of the JAK/STAT signalling pathway in regulating neural differentiation and gliogenesis.

1.1.3 HMGB1 AND FACTORS KNOWN TO REGULATE BRAIN DEVELOPMENT

Hmgb1 knockdown larvae clearly show defects in forebrain development. As one important transcription factor identified to control early telencephalic DA cell differentiation and subdivision patterning, Pax6a expression was carefully checked in Hmgb1 knockdown zebrafish. Inhibition of HMGB1 from the beginning of development clearly reduced Pax6a expressing cells in the forebrain. This result confirms that the loss of telencephalic DA cell groups in Hmgb1 knockdown morphants is due to the interruption of Pax6a caused by the inhibition of Hmgb1 during development. In mammals, Pax6 is also crucial for neurogenesis during the development of the cortical cortex. The reduced Pax6 expression in Hmgb1 knockdown morphants should contribute to the increased apoptosis and decreased proliferation in developing CNS.

Wnt signalling is known to be up-regulated in Pax6-/- mutants and involved in the mechanism of Pax6 activity in mouse forebrain development (Muzio et al., 2002). The Hmgb1 knockdown larvae display enhanced Wnt signalling, which may therefore be due to downregulation of Pax6 activity.

Wnt signalling has a complex role from very early to late stages of nervous system development based on its role in anterior-posterior induction and differentiation. Of the numerous Wnt homologues, Wnt8 has been characterized as the key regulator in early brain development. Wnt8 has been shown to act as a posteriorising factor that is expressed in posterior parts of the central nervous system and is suggested to diffuse to anterior areas inhibiting its development. Furthermore, Wnt8 has been shown to restrict the number of catecholaminergic progenitors during neurogenesis in diencephalon. Wnt8b knockdown morphants/mutants showed specific increase of diencephalic dopaminergic cells (Russek-Blum et al., 2008). This implies that Wnt signalling modulates the initial pool of dopaminergic progenitors during early zebrafish development.

Wnt8 and β-catenin have been shown here to be dramatically up-regulated in Hmgb1 knockdown larvae. In contrast, Amigo1 essentially disappeared in the Hmgb1 knockdown larvae. Therefore, up-regulation of Wnt8 expression likely contributes to perturbed forebrain development in the Hmgb1 morphants.

Elevation of WNT/β-catenin signaling in the HMGB1 KO mouse has also been confirmed here. Together with the qRT-PCR array results of developmental transcription-factors expressions, in situ hybridization results clearly show the decrease of neurogenesis-factors expression in developing forebrain, such as Foxg1, Tbr2, and Emx2. HMGB1 plays a conserved role in regulating verterbrate forebrain development by tight interaction with key transcription factors and the Wnt/β-catenin signaling pathway during development. All these results reveal that the HMGB1 knockout defects characterized by hypoplasia of forebrain are mainly due to decreased neuronal proliferating and differentiation. The finding that HMGB1 signalling is associated with Wnt signalling potentiates novel views of the role that HMGB1 plays in development and disease.

1.2 AMIGO1 IS REGULATED BY HMGB1 DURING DEVELOPMENT

By using ordered differential-display analysis, a novel adhesion protein designated as AMIGO1 was found to be induced by HMGB1 in cultured neurons. Regulation of AMIGO1 expression was shown to depend on extracellular matrix-bound HMGB1 and transmembrane signalling in embryonic neural cells (Kuja-Panula et al., 2003).

This study confirms that AMIGO1 expression is regulated by HMGB1 in developing brain. Not only do HMGB1 knockdown larvae show a gross decrease of AMIGO1 expression in the developing CNS, but also HMGB1-knockout mouse also display reduced AMIGO1 expression in cortical neuronal cells. In rodent, AMIGO1 and AMIGO2 are expressed widely in neuronal cells (Chen et al., 2011). AMIGO2 has been independently confirmed to be crucial for neuronal survival (Ono et al., 2003). Furthermore, based on these in vitro studies, AMIGO1 has a similar cell survival enhancing effect on embryonic rat brain neurons as AMIGO2. Thus, the key question as regarding the regulation of AMIGO1 expression by HMGB1 is whether AMIGO1 should be included in the HMGB1-dependent survival/ proliferation mechanism.

Experiments in zebrafish using *amigo1* mRNA injection in the HMGB1 knockdown clearly demonstrate that Amigo1 is included in the signaling pathway through which Hmgb1 regulates neural progenitors. Increasing Amigo1 expression induced by exogenous Hmgb1 has also been observed in the rescueing experiment of Hmgb1 knockdown morphants by the coinjection of *hmgb1* mRNA.

Coinjected *amigo1* mRNA has significantly reduced apoptosis and increased proliferation in HMGB1 knockdown morphants. Furthermore, the telencephalic DA defects in Hmgb1 knockdown morphants have also been rescued by *amigo1* mRNA coinjection. However, evidence is still lacking regarding whether AMIGO1 is involved in the HMGB1 signaling with developmental transcription factor. In contrast to Hmgb1 knockdown morphant, there is no difference in Wnt8, Pax6a and Pax2a expression in Amigo1 knockdown morphant (publication II).

This finding provides further evidence for a role of HMGB1 as an extracellular factor that would mediate cell-to-cell communication within the niches that are required for survival/proliferation of neural progenitors. It also suggests that HMGB1 may regulate AMIGO1 expression in a more specific signalling pathway for regulating neuronal survival/ proliferation.

1.3 CONTRIBUTION OF HMGB1-CXCL12/CXCR4 CHEMOTAXIS TO BRAIN DEVELOPMENT

HMGB1 can specifically form a heterocomplex with the chemokine CXCL12, thereby promoting leukocyte recruitment to injured tissue via CXCR4 signals (Schiraldi et al., 2012; Venereau et al., 2012). Furthermore, HMGB1 is required for CXCL12-dependent migration of macrophages and dendritic cells (Campana et al., 2009). As one of the most abundant and broadly distributed chemokines, CXCL12 and its receptor CXCR4 are mainly known for the essential and unique role in homeostatic regulation of leukocyte traffic, haematopoiesis, organogenesis, cell differentiation, and tissue regeneration. However, the functions of CXCL12 and its receptor CXCR4 appear to extend far beyond the control of immune system traffic, including the regulation of cell migration, proliferation, survival, and adhesion (Guo et al., 2006). There is accumulating evidence pointing to wide involvement of CXCL12/CXCR4 in CNS development (Mithal et al., 2012; Zhu and Murakami, 2012). It has been found that the CXCL12-CXCR4 axis serves as an emergent salvage signal for initiating endogenous stem-cell-based tissue repair *in vivo* (Li et al., 2012). This study on the HMGB1-CXCL12/CXCR4 signalling pathway and its cognate receptors will be useful in isolating and developing therapeutic stem cells for patients with clinical neurodegeneration.

Strikingly, the CXCL12/CXCR4 signalling pathway was found to be important in zebrafish embryonic telencephalon and eye development, which is regulated by Wnt/beta-catenin signalling *in vivo* (Bielen and Houart, 2012). Wnt8b and beta-catenin were dramatically up-regulated in the

HMGB1 knockdown zebrafish embryos (publication I). These findings suggest that the HMGB1-CXCL12/CXCR4 axis found in the immune system may also regulate central nervous system development. Our data of the regulation mechanism of HMGB1 in CNS development suggests that HMGB1 might actively interact with the chemokine signalling pathway *in vivo*.

1.4 HMGB1 AS A BIDIRECTIONAL SIGNAL BETWEEN IMMUNE SYSTEM AND CENTRAL NERVOUS SYSTEM

Until a few decades ago, the brain was generally considered a functionally immune privileged site devoid of immune cells. On the basis of the evidence to date, it appears that the brain and the immune system have the capacity to establish a very sophisticated biochemical association, which has remarkable effects on the CNS (Boulanger et al., 2001). For example, an increasing number of reports show that EMV (extracelluar microvesicle) transportation is a revolutionary pathway for cell-to-cell communication and signal transduction. EMVs secreted by neural cells take part in modulating immune responses within the brain under physiological and pathological circumstances (Cossetti et al., 2012). It has been recently found that HMGB1 originated from monocytes and macrophages is expressed in extracelluar microvesicles (Pisetsky, 2014; Thomas and Salter, 2010), and recent works suggest that EMVs released by oligodendrocytes and microglia can serve as biomarkers for neurological disorders (Colombo et al., 2012). It is possible to speculate that HMGB1 is crucial for EMV stimulation and transportation in both neuronal cells and immune cells, and is directly regulating neuro-immune interplay. HMGB1-carrying EMV secreted by neuronal cells can be a novel biomarker in clinical analysis and a therapeutic target of neuroinflammation.

2. AMIGO PROTEINS IN ZEBRAFISH

LRRs (leucine-rich repeats) are highly versatile and adaptable proteins that participate in many biological processes and developmental events. Until now, over 140 LRR proteins have been distinguished in the human genome. AMIGO1 was recently found and cloned from mouse and rat. As a new member in the LRR protein family that is widely distributed in CNS*, in vivo* functions of AMIGO1 are not yet clearly described. As zebrafish Amigo1 shares high similarity to the mammalian ortholog, and the zebrafish's transparent embryo makes its CNS development easier to follow than that of mouse, zebrafish was employed to study Amigo function in neuronal circuit formation and development.

There is no evidence for an evolutionary relationship among LRR proteins yet, although they all share a characteristic LRR repeat structure. Similar to other LRRIG coding genes, Amigo1 exists widely in the genomes of vertebrate and invertebrate animals, but only mammals have three Amigo genes. Other animal species (Chicken, Xenopus, and teleost) only have one or two Amigo genes. No Amigo gene has been found in lower species. Amigo orthologous genes show higher similarity than paralogous genes. This means that Amigo proteins might be evolutionarily divergent.

Moreover, within the AMIGO family Amigo1 showed the highest similarity among species. Similar to many other eLRR proteins required for various aspects of embryonic and nervous system development, the parent form of the protein family (Amigo1) is the main family member with conserved function and the highest expression during early development in zebrafish and rodent.

2.1 AMIGO1 IN DEVELOPING ZEBRAFISH BRAIN

Zebrafish Amigo1 is already generally expressed in 5dpf larval brain. In 5dpf larvae, several neural circuits controlling locomotion and sensory gating have already been established. In addition,

zebrafish lacks an Amigo2 homolog which is expressed highly in rodent brain and exhibits a similar function as AMIGO1 in rodent neurons. Although AMIGO1 knockout mouse showed no morphological defects (Peltola et al., 2015), knockdown of *amigo1* expression in zebrafish embryos offer the chance to observe defects in neural circuit formation and development. This may be due to lower redundancy of zebrafish Amigo proteins compared to mice. In contrast to mouse, Amigo2 is absent in zebrafish and cannot compensate Amigo1 inhibition.

Amigo1 expression pattern in developing CNS has been investigated with affinity-purified antibodies by immunohistochemistry and western blotting. Whole mount immunostaining showed Amigo1 expression in the anterior dorsal part of the forebrain area in 28 hpf larvae, resembling the *in situ* results of Amigo1 RNA probe in the anterior brain area in E10 mouse embryos (Homma et al., 2009).

In 5 dpf zebrafish larvae, Amigo1 expression resembles the results obtained in rodents. In postnatal brain, AMIGO1 expression has been found to be elevated during maturation of adult circuitry and is found to be widely expressed in all areas of the CNS in most types of neurons and their fibers. The conserved dynamic expression pattern in vertebrate CNS suggests a conserved function of Amigo during embryonic development.

2.2 DEVELOPMENTAL DEFECTS OF AMIGO1 KNOCKDOWN MUTANT

Recent reviews have implied that a large number of LRR proteins have specific roles in various aspects of neural development in mammals based on *in vitro* results from the assays of neurite outgrowth, fasciculation, and/or synapse formation (de Wit et al., 2011; de Wit and Ghosh, 2014). Nonetheless, an increasing number of LRR proteins have been implicated in nervous system development or disease, this family of proteins has received far less attention than other better characterized families like the immunoglobulin and cadherin superfamilies. Except TLR genes (Beutler, et al., 2006) and small secreted proteoglycans (Ameye and Young, 2002), relatively few LRR genes have been studied *in vivo* and genetically in vertebrate. Although the CNS-enriched expression pattern of AMIGO1 has been identified in mouse, its role in CNS development has not yet been revealed.

2.2.1 AMIGO1 REGULATES LONGITUDINAL TRACT DEVELOPMENT

amigo1 antisense morpholino oligo nucleotides (MO1 and MO2) successfully inhibited Amigo1 protein expression in early zebrafish embryos. Endogenous Amigo1 expression measured by western blotting was decreased over 80% via MO microinjection. Very low Amigo1 expression can be detected in 28hpf Amigo1 knockdown morphants by whole mount immunostaining. Amigo1 and HNK-1 were clearly co-expressed in early axonal tracts in normal 28hpf larval brain. Amigo1 knockdown morphants displayed striking defects in the formation of early fibre scaffolds. Staining results clearly showed that Amigo1 is required for the formation of MLF descending out from VCC, which is one of the major early fibre scaffolds in developing brain. Amigo1 knockdown morphant larval MLF is much thinner than the robust fascicles observed in uninjected or 5mis MO injected 28hpf embryos.

The specific Amigo1 function in axonal tract development has been confirmed by the microinjection of mRNA encoding the ectodomain of Amigo1 as a dominant negative control to exclude unexpected off-target effects of the MOs. Previous *in vitro* experiments showed that Amigo1 ectodomain inhibits AMIGO-mediated adhesion and fasciculation of neurites in brain neurons. Similarly, an *in vivo* dominant-negative approach causes specific inhibition of MLF fasciculation in

zebrafish. Such a defect is significantly rescued by the coinjection of the full-length *amigo1* mRNA with the MOs. The results confirmed that Amigo1 protein is critical in neural tract development, especially to far ranging longitudinal tracts. The regulation mechanism of Amigo1 in neural tract development seems to be conserved from zebrafish to mammals.

In addition to the whole mount immunostaining of developing MLF and axonal scaffolds in Amigo1 knockdown morphants, the defective development of neuronal connections has been validated by the western blotting of larval SDS extracts using both anti-HNK1 and anti-acetylated tubulin antibodies as neural tract markers. Amigo1 knockdown larvae showed much weaker staining bands in the western blotting results than the control groups.

Furthermore, Amigo1 knockdown morphants have defects in the axonal outgrowth of a pair of giant motor neurons, Mauthner neurons, which has a single neuronal projection approached from hindbrain towards to spinal cord laterally to MLF. Mauthner neurons regulate C-start behavioural responses, which is a very quick startle or escape reflex that is employed by fish and amphibians (including larval frogs and toads). This suggests that Amigo1 knockdown in early developing CNS would cause a related behavioural phenotype in later developmental stages.

2.2.2 AMIGO1 IN AMINERGIC TRANSMITTER SYSTEM DEVELOPMENT

The zebrafish aminergic system has been systematically investigated in developing larvae. Antibodies to both tyrosine hydroxylase (TH) and 5-HT have identified fibres running longitudinally through the brainstem from diencephalon, as well as in a segmental distribution in the hindbrain.

Anti-TH antibodies identified a defect in the development of differentiating medial longitudinal catecholaminergic tract (mlct) in 3dpf Amigo1 knockdown larvae; 5-HT immunoreactive fibres also disappeared in the hindbrain of 5dpf Amigo knockdown morphants. These results also provide evidence that Amigo1 might be required for the basic generation of neuronal tracts during early development.

Slit2 is the LRR protein known as a conserved midline repellent in axon pathfinding during evolution, and Amigo1 protein shares a similar motif as Slit2. Similarly, Robo-slit proteins are reported to be important for the fasciculation of TPOC and the localization of the DA axonal tract in developing mlct in zebrafish larvae. In contrast with the role of Robo-slit proteins in repulsive axon guidance during development, Amigo1 seems to be more directly related to the formation and fasciculation of axonal tracts. In the Amigo full-length mRNA injected larvae, no dislocation of MLF or LLF is found.

Analysis of the Amigo1 knockdown morphants at later developmental stages shows no apparent defects in specific brain structures. By staining the catecholaminergic system with anti-TH antibodies in 3dpf and 5dpf larvae, there is no structural defect in Amigo1 knockdown morphants, which is clearly different from Hmgb1 morphants. It is consistent with the results of whole mount *in situ* with important brain regional markers, which showed that the Amigo1 knockdown morphants show no changes in expression of Pax2a, Pax6a, or Krox20.

Amigo1 knockdown morphants usually showed decreased brain length and width. The defects in Amigo1 knockdown morphant brain have been illustrated by the significantly reduced 5-HT ir cell clusters in diencephalon and Th2 cells clusters in hypothalamus. However, knocking down Amigo1 fails to cause much difference in the level of proliferation or apoptosis in developing zebrafish larvae shown by Tunel and Edu staining results. It seems possible that the defects in the early fiber scaffold

development generally disturb development of the aminergic connections, and there might be some specific effects of Amigo1 on the development of neurotransmitter-specific connections.

2.3 MECHANISM OF AMIGO1 IN THE DEVELOPMENT OF NEURAL CIRCUITRY: HOMOPHILIC OR HETEROPHILIC?

Defective tract development in Amigo1 knockdown morphants and in larvae injected with the mRNA encoding the Amigo1 ectodomain imply that tract development is specifically influenced. The number of TUNEL-positive cells showed no increase in the experiments, which agrees with the interpretation and suggests that defective tract development is not due to loss of neuronal cells.

The mechanism through which Amigo1 specifically affects tract development therefore remains to be elucidated. As the first and only identified LRR protein family in axonal guidance, the signalling pathway of slit proteins has been found to be related to its binding to Robo or netrin receptor proteins. No changes of Slit or Robo expression was seen in Amigo1 knockdown morphants (Zhao et al., unpublished results). It appears that the role of Amigo1 cannot be explained by Slit/Robo signalling, and the phenotypic changes in the Amigo1 morphants are different from those found in Slit/Robo studies.

Strikingly, the recent work shows that AMIGO1 crystallizes as a dimer through the binding with its extracelluar interface (Kajander et al., 2011), and AMIGO1 is also co-expressed and regulates the voltage-dependent current through Kv2.1 on neuronal cell membranes (Peltola et al., 2011). Therefore, there might be homophilic or heterophilic binding of AMIGO1 in neuronal regulation.

From previous *in vitro* tests, homophilic binding of Amigo1 seems to at least partially explain its remarkable role in the development of long tracts. Furthermore, inhibition of fasciculation by the Amigo1 ectodomain observed in the current study is similar to the fasciculation inhibition found in isolated neurons where homophilic interactions and heterophilic interactions were identified among the AMIGO protein family members.

In Drosophila, homophilic interactions within fiber tracts are well known for their essential roles in growth and axon guidance. Their roles in complex vertebrate tracts are much less clear, however. In zebrafish, it has been reported that the cohorts of identical axons could potentially use isotypic interactions to guide each other through multiple choice points in retinotectal development. It appears that this fundamental cellular mechanism, acting through pioneer-follower interactions and community effect (Pittman et al., 2008), is as important as guidance signals outside the tracts and may be used throughout the development of complex nervous systems in vertebrates. Further work on the Amigo family members in different neural systems is required to characterize Amigo-mediated homophilic interactions and cell signalling following such interactions. For example, Amigo-mediated homophilic interactions might affect development of neurotransmitter-specific phenotypes or expression of ion channels required in neural signalling.

AMIGO1 displays heterophilic binding to the Kv2.1 potassium channel in mouse brain. The two proteins show a patching colocalization on the plasma membrane. Furthermore, when the Kv2.1 clusters are dispersed at the plasma membrane upon the addition of various stimuli, such as glutamate, AMIGO1 is also dispersed together with Kv2.1 (Peltola et al., 2011). These findings indicate that AMIGO1 could be regarded as an auxiliary subunit of the Kv channel. From the functional viewpoint, AMIGO1 regulates the channel activity at voltage values close to those required to start action potentials and is therefore expected to play a role in neuronal signalling.

One should, thus, consider whether the heterophilic binding of Amigo1 to Kv2.1 plays a role in the *amigo1* morphant phenotypes found in the current study. Surprisingly, the *amigo1* morphants were found to express low levels of Kv2.1 in developing CNS. Expression of the channel in the amigo1 morphants could be restored by coinjection of the *kv2.1* mRNA and to a lesser extent by coinjection of the *amigo1* mRNA in the morpholino experiments. It thus appears clear that Amigo1 does not only bind to the channel and regulate its activity at the plasma membrane, but also strongly influences Kv2.1 expression *in vivo*. Further studies will be required to elucidate the biochemical basis of the regulation of Kv2.1 via Amigo1 in neuronal cells.

Because the expression of Kv2.1 is strongly inhibited in *amigo1* morphants, a rescue of the phenotypes was sought by restoring the Kv2.1 protein expression through coinjecting *kv2.1* mRNA with the morpholino oligonucleotides. *kv2.1* mRNA failed to rescue the embryonic tract development defects. In contrast, clear rescue effects were found in the behavioural phenotypes that obviously depend on functionally active neural circuitries. The anatomical connectivity therefore seems to remain in the morphants even when Kv2.1 is absent. Furthermore, Amigo1 and Kv2.1 were found to colocalize only in adult brain but not in early developing brain. However, they were found to be expressed in overlapping anatomical areas in both cases.

In free-swimming tests, increased locomotor activity has been recorded in Amigo1 knockdown zebrafish morphants. In Kv2.1 knockout mice, the deficiency of the Kv2.1 channel was also reported to lead to neuronal and behavioural hyperexcitability. General locomotor activity in the Kv2.1 knockout mice was found to be strongly enhanced, clearly resembling the enhanced activity in the amigo1 morphants in the current study. Because hyperactivity and defective startle responses in the Amigo1 knockdown morphants can be rescued by the *kv2.1* mRNA, it appears clear that defective Kv2.1 channel function strongly contributes to the behavioural phenotypes caused by knockdown of Amigo1 expression.

It is thus viable to propose that Amigo1 functions independently of Kv2.1 during early fiber tract development. Furthermore, for the construction of neural circuitry with adult-type normal functions, AMIGO1 regulates the expression and function of the Kv2.1 potassium channel of central neurons.

2.4 BEHAVIORAL PHENOTYPES IN AMIGO1 KNOCKDOWN MORPHANTS

As for its relatively simple central nervous system [~ 100,000 neurons at 7dpf (Sumbre and de Polavieja, 2014)] and transparency, zebrafish is an emerging model for correlating neuronal activity and sensory-motor behaviour. The disturbed development of neuronal connections and aminergic neurotransmitter system observed in the amigo1 knockdown morphants suggested a high possibility of behavioural alterations.

Unexpectedly, the amigo1 knockdowns were found to display increased locomotor activity as mentioned already. This study showed that this phenotype is specific because coinjection of the amigo1 mRNA with the morpholinos reduces the enhanced locomotion to the level seen in the misMO-injected and the uninjected larvae. Importantly, hyperactivity of Amigo1 knockdown morphants seems to be directly caused by down-regulated Kv2.1 expression, since the compensated *kv2.1* mRNA coinjection with Amigo1 MO could restore the locomotor activity of larvae to normal level. As a newly identified schizophrenia-associated genetic locus from human patients (Peltola et al., 2015), Kv2.1 might play a conserved role together with Amigo1 in regulating vertebrate motor activity. Amigo1 should be an ideal candidate gene for investigating the biological process and targeted gene therapy of schizophrenia.

In addition to spontaneous locomotor activity, escape behaviour has also been assessed in the amigo1 morphants. The amigo1 knockdowns have clear defects in both SLC response and LLC response. As shown in locomotor activity tests, the amigo1 mRNA coinjection with MO showed a clear rescue effect in both SLC and LLC. It has been reported that the SLC response is mainly mediated by Mauthner neurons, whereas the LLC response is not mediated by these neurons but depends more on the widely distributed neuronal connections in the CNS. Interrupted Mauthner neuron decussating axons after knocking down Amigo1 should therefore be the key factor contributing to the defective SLC response. A widespread escape network has been identified in the brain stem of zebrafish, however, containing several descending fiber pathways. The serotonergic networks and dopaminergic far-ranging tracts originated from diencephalic cells might also be responsible for such sensory gating motor functions, as Amigo1 morphants displayed defects on both tracts in the hindbrain. Maturation of this escape network to form functional connections from the brain to the motor neurons in spinal cord probably regulates the escape responses in zebrafish and is related to the association of Amigo1 to Kv2.1.

3. REVERSE GENETICS METHOD IN ZEBRAFISH

Reverse genetic approaches are popular to study *in vivo* functions of proteins in vertebrates. While not a true genetic approach, the introduction of antisense morpholino oligonucleotides (MOs) was initially greeted with excitement in the zebrafish community as a tool to interrogate gene function. The MOs are highly stable *in vivo*, and have a high affinity for RNA, displaying off-target binding to macromolecules (Summerton, 2007). MOs can be designed to block translation or splicing and their injection into one-cell stage zebrafish embryos can recapitulate known mutant phenotypes (Draper et al., 2001; Nasevicius and Ekker, 2000). Thus, MOs provide, in principle, an accessible and straightforward method for gene knockdown in the zebrafish embryo.

Given their ease of use in zebrafish, MOs have enabled widespread analysis of gene functions. However, MOs can induce p53-dependent apoptosis (Ekker and Larson, 2001; Robu et al., 2007) and off-target cell-type-specific changes in gene expression (Amoyel et al., 2005; Gerety and Wilkinson, 2011). While it is possible to alleviate off-target phenotypes by simultaneously reducing p53 levels (Robu et al., 2007), the mechanism of p53 activation is unknown.

Fortunately, definitive reverse genetic approaches in zebrafish recently have become available. In particular, programmable site-specific nucleases now enable targeted gene disruption in the zebrafish. Initial work utilized zinc finger nucleases (ZFNs; Doyon et al., 2008; Meng et al., 2008), and transcription activator-like effector nucleases (TALENs), which provide improved specificity over ZFNs, also have been developed (Cade et al., 2012). In both cases, mRNAs encoding ZFN or TALEN heterodimers were injected into one-cell stage embryos, where they bound to their target and induced a double strand break. Imprecise repair of this break by non-homologous end joining introduces small insertions or deletions that can lead to a frameshift when targeting coding sequence; in a proportion of the embryos, such lesions will occur within the germline. More recently, the programmable bacterial nuclease Cas9 has been applied successfully to introduce heritable lesions in the zebrafish genome at high frequency (Hwang et al., 2013). Together, these tools provide a robust means to generate zebrafish bearing targeted mutations in genes of interest.

A more troubling finding nowadays from zebrafish work is the discrepancy between morphant and mutant phenotypes. A high false-positive morphant phenotype rate has been noticed and reported, even when most of the morphant phenotype could be rescued by corresponding mRNA coinjection (Kok et al., 2015).

The zebrafish community has claimed that whenever possible, investigators should confirm a morphant phenotype by comparison to a mutant, if available. This is the most essential guideline to follow now. At the same time, it is also important to validate the characteristics of newly generated mutant lines in parallel to confirm their effect on candidate gene function.

Based on studies with mouse HMGB1 knockout mutants, HMGB1 function in forebrain development can be affirmed via similar structural defects of embryonic brain observed in both mouse and zebrafish. Although AMIGO1 knockout mouse shows no developmental defects as seen in zebrafish morphants, this might be because of the redundancy of AMIGO orthologous genes in difference species. Via fast developing specific nuclease technologies, most notably the CRISPR systems (Hwang et al., 2013), the application of this approach to generate Amigo mutants in the zebrafish model is warranted. It can aid understanding of Amigo function further even in mature/aging brain, which would greatly help in the validation of Amigo function and regulation in neurodevelopmental and neurological disorders.

CONCLUDING REMARKS

Vertebrate brain structure has been highly preserved throughout evolution. Many neuronal developmental disorders are due to genetic defects affecting brain development. In this thesis, HMGB1 and AMIGO1 functions in brain development have been examined by using zebrafish and mouse models. The main conclusions are as follows;

1. HMGB1 regulates AMIGO1 expression in the developing brain of zebrafish and mouse models. Both HMGB1 and AMIGO1 show conserved CNS enriched expression pattern during development. AMIGO1 is clearly involved in the molecular mechanism through which HMGB1 regulates neurogenesis and differentiation.

2. HMGB1 is intimately involved in the cellular and molecular mechanism of brain development in both zebrafish and mouse embryos. This phenomenon is likely to be conserved across many vertebrate and non-vertebrate species. HMGB1 has a crucial role in the regulation of survival, proliferation, and differentiation of neural progenitors. The transcription factor hierarchy affected by HMGB1 knockdown/knockout provides clues for further mechanistic studies on the role of HMGB1 in brain development.

3. In zebrafish developing CNS, Amigo1 is required for axonogenesis and neuronal tract development. The homophilic binding of Amigo1 within fiber tracts underlies its remarkable role in the development of the long tracts observed in the current study. Similar to mouse AMIGO1, zebrafish Amigo1 displays homophilic binding and heterophilic interactions with the Kv2.1 potassium channel protein. Amigo1 regulates the expression of Kv2.1 in zebrafish brain. Although both Kv2.1 and Amigo1 are involved in the modulation of sensorimotor functions, Amigo1 functions independently of Kv2.1 during early fibre-tract development.

4. HMGB1 regulates neuronal expression of CXCL12/CXCR4 signals in mouse developing brain. This study suggests that the HMGB1-CXCL12/CXCR4 chemotaxis identified in the immune system may also exist in the nervous system. The regulation of HMGB1 in the innate immune system and in the nervous system might share the same signalling pathway, and HMGB1 may be an important messenger for the crosstalk between immune system and nervous system.

As for the conserved function shown in vertebrate brain development, the crucial roles of HMGB1 and AMIGO1 in neurodevelopmental disorders are expecting to be revealed by the generation of specific zebrafish transgenic lines via targeted gene-engineering methods. Future work with conditional HMGB1 knockout mice is warranted to reveal the regulation-mechanism of HMGB1 shared between the immune system and the nervous system. Furthermore, it seems clear that HMGB1 and AMIGO1 should be considered in regenerative medicine as a factor that regulates neural progenitors and axonal plasticity during regeneration.

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赵翔
Xiang Zhao

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