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Brain derived neurotrophic factor (BDNF) expression in postnatal and adult zebrafish brain and related changes following mechanical injury

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ABSTRACT

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has emerged as an active mediator in many essential functions in the central nervous system of mammals. BDNF plays significant roles in neurogenesis, neuronal maturation and/or synaptic plasticity and is involved in cognitive functions such as learning and memory. Despite the vast literature present in mammals, studies devoted to BDNF in the brain of other animal models are scarse. Zebrafish is a teleost fish widely known for developmental genetic studies and is emerging as model for translational neuroscience research. In addition, its brain shows many sites of adult neurogenesis allowing higher regenerative properties after traumatic injuries. The topic of this dissertation is the study of the distribution of *bdnf* mRNAs in the larval and in adult zebrafish brain, and related changes of expression following mechanical injury of telencephalon.

In the brain of 7 days old larvae and mature female and male zebrafish in situ hybridization showed that *bdnf* mRNAs are widely expressed., mostly in the forebrain. Combining immunohistochemistry with in situ hybridization to characterize the phenotype of *bdnf*-expressing cells, showed that *bdnf* mRNAs are never expressed by radial glial cells or proliferating cells. By contrast, *bdnf* transcripts are expressed in cells with neuronal phenotype in all brain regions investigated.

Further experimental investigation was conducted in dorsal telencephalon of adult zebrafish after mechanic injury. This was obtained by a stab wound to mimic the cellular phenomena of adult traumatic brain injury, which is usually caused by an impact to the head that results in a mechanical insult to the brain. Particularly, the stab wound was made on telencephalon because it comprises the most studied neuronal stem cell and its dorso lateral zone is retained to be equivalent to the medial pallium (hippocampus) of mammals. This latter contains one of the two constitutive neurogenic niches of mammals. The zebrafish telencephalon, along the entire studied period from the lesion (1, 4, 7, 15 days) showed a general increase of *bdnf* expression, quantifiable both through BDNF levels in extracts and BDNF positive cell numbers. More specifically, *bdnf* expression was exclusively limited to neuronal populations, as suggested by co-localization of BDNF mRNA and the neuronal makers Huc/d and acetylated-tubulin.

In conclusion the study provides the first demonstration that the brain of zebrafish produces BDNF in neurons and the response concerning *bdnf* expression after telencephalic injury resulted substantial similar to that reported in mammals, indicating that BDNF is generally involved in the first response to brain damage. However, despite these comparable results, the regenerative properties of fish and mammals are dramatically different, suggesting the existence in fish of peculiar still unknown molecular programs, which might have specific roles after injury.

INTRODUCTION

Brain derived neurotrophic factor (BDNF) belongs to neurotrophins (NTs), a family of structurally and functionally related proteins. NTs regulate survival, proliferation and differentiation, of distinct, but often overlapping, neuronal populations during development, whereas in adult life they are involved in the maintenance, apoptosis and repair of neuronal phenotype and in the modulation of synaptic efficacy (for a review, see Skaper 2012, Ceni et al., 2014). NTs and their receptors sequences have been identified from many vertebrate species including fish, amphibians, reptiles, birds and mammals (Scott et al., 1983; UHrich et al., 1983; Ebendal et al., 1986; Meier et al., 1986; Wion er al., 1986; Sclby er al., 1987; Fahnestock and Bell, 1988: Whittemore et al., 1988; Schwarz et al., 1989; Kaisho er al., 1990; Herkerneier er al., 1991; Hallböök et al., 1991: Carriero et al., 1992; Götz et al., 1992, 1994; Lai et al., 1998; Lum et al., 2001; Hallböök *et al.*, 2006; Catania et al., 2007; Dalton et al., 2009; Teng et al., 2010; D'Angelo et al., 2012). Historically, the first family member identified was NGF (Levi-Montalcini *et al.*, 1954). It was discovered during experiments of transplanting mouse sarcoma tissue into a chick embryo. The transplanted tumor tissue induced hyperinnervation of internal chick organs, by releasing a diffusible agent that stimulated the growth and differentiation of developing nerve cells. Then, in 1982, BDNF was purified from pig brain, thanks to its survival-promoting action on a subpopulation of dorsal root ganglion neurons (Barde *et al.*, 1982). Few years later, the third member of the neurotrophin family, NT-3, was identified in mouse (Hohn *et al.*, 1990). NT-4 was isolated from Xenopus and viper (Hallböök *et a1*., 1991) first, and then, identified in both human and rat genomic DNA (Ip *et al.*, 1992), which represented the mammalian counterpart of Xenopus/viper NT-4. A fifth neurotrophin was then cloned from the human and named NT-5 (Berkemeier *et al.*, 1991; 1992). However, its chromosomal location and activity profile indicated that NT-5 was the ortholog of NT-4 (Berkemeier *et al.*, 1992). Finally, NT-6 was only cloned from *Xiphophorus maculatus*, a teleost species (Götz *et al.*, 1994), and is not expressed in other vertebrates. Later findings had suggested the existence of an additional fish neurotrophin, NT-7, cloned in the carp (Lai *et al.*, 1998) and in zebrafish (Nilsson *et al.*, 1998). NTs are synthesised as glycosilated precursor molecules called pro-neurotrophins (pro-NTs) of ~30–34 kDa, that undergo proteolytic cleavage in the endoplasmic reticulum and Golgi apparatus

by furin or proconvertase to produce C-terminally mature neurotrophins of ~13 kDa , and then extracellularly by the action of plasmin to generate mature NTs (Fig. 1). Plasmin is a serine protease enzyme that is formed from plasminogen by several enzymes including tissue plasminogen activator (tPA) and urokinase. Intact pro-NTs may also be released extracellularly and evoke direct biological activities that in general oppose that of the mature NT counterpart although there are exceptions. Pro-NTs may induce apoptosis whereas mature NTs are mainly associated with neuronal survival (Fig.1; for a review Khan and Smith 2015). The biological effects of mature NTs are mediated via two major receptor types, via neurotrophic tyrosine kinase (Trks) receptors and the pan neurotrophin receptor at 75 kDa (p75NTR). These transmembrane receptors are glycoproteins whose molecular masses range from 140 to 145 kDa. Each mature NT binds with high affinity to a specific Trk receptor. Specifically, NGF binds with high affinity to TrkA, BDNF and NT-4 bind with high affinity to TrkB, and NT-3 binds with high affinity to TrkC (Patapoutian and Reichardt 2001). Apart from activation of TrkC, NT-3 also activates TrkA and TrkB, albeit with lower affinities and all four mature NTs bind with similar affinity to the p75NTR (Fig.2).

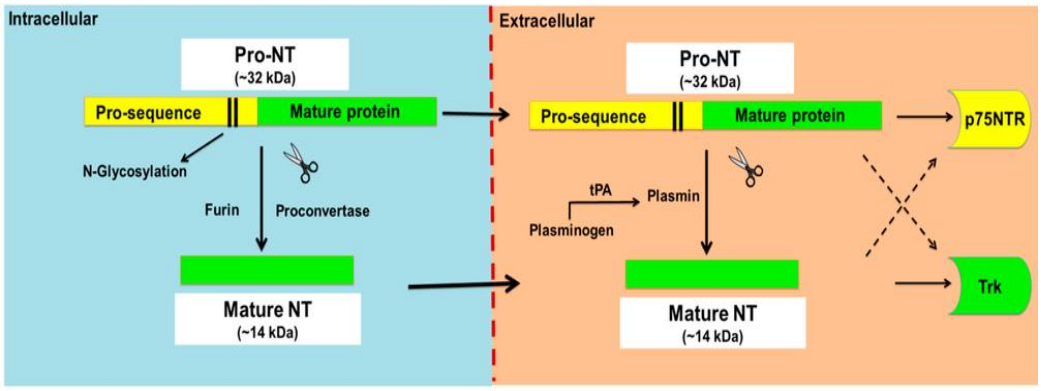


Fig 1 Proteolytic cleavage of pro-neurotrophins to mature neurotrophins.

By comparison, the biological actions of pro-NTs are relatively poorly understood. Intact pro-NTs are high affinity ligands at both the p75NTR and the structurally distinct co-receptor, sortilin, in contrast to mature NTs (for a review Khan and Smith 2015). Additionally, pro-NGF and pro-BDNF bind to TrkA (Masoudi et al., 2009) and TrkB respectively, albeit with lower affinity than to the p75NTR (Fayard et al., 2005). In addition the biological effects of neurotrophins require that signals be conveyed over long

distances. After neurotrophin-mediated activation, the receptors shuttle back from the axon terminals to the soma by retrograde transport in so-called signaling endosomes to regulate gene expression and exert their trophic responses. Endosomes are delivered to the cell body where signals are transduced to ensure neuronal survival and differentiation. Endosomes serve as a meeting point for the formation of signaling complexes for a wide variety of ligands and their receptors. Since the formation of transport vesicles would orient the intracellular domain of the receptors to face the cytoplasm, each receptor would be available for interactions with cytoplasmic proteins and produce many signaling possibilities (for a review see Chao, 2003).

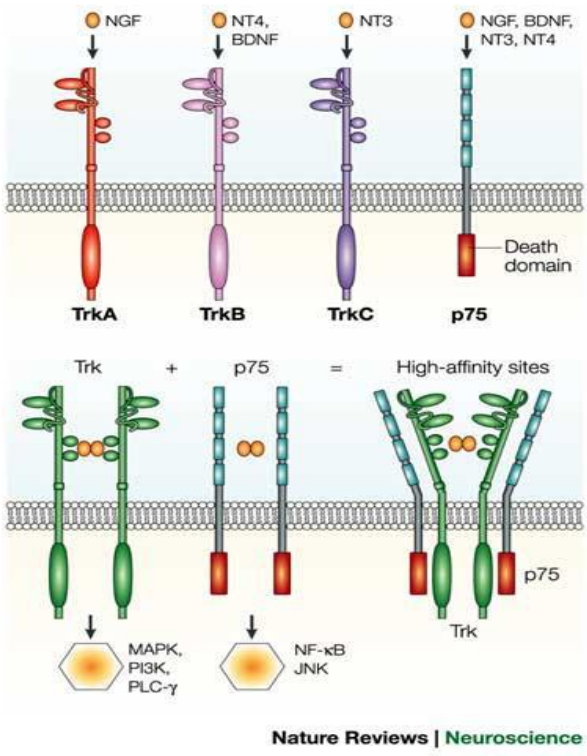


Fig 2. Neurotrophins bind selectively to specific Trk receptors, whereas all neurotrophins bind to p75. Neurotrophin binding to the p75 receptor mediates survival, cell migration and myelination through several signalling pathways. Interactions between Trk and p75 receptors can lead to changes in the binding affinity for neurotrophins. Abbreviations: **JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NT, neurotrophin; PI3K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase Cγ. (Chao et al., 2003)**

The retrograde trafficking process is well studied, and is becoming increasingly clear (Hirokawa *et al.*, 2009). Conversely, assumed mechanisms underlying anterograde Trk transport remain incompletely understood. Possible explanations on anterograde axonal transport involves heatshock proteins, such as HSP90, which bind to TrkB during axonal transport (Bernstein *et al.*, 2001), and APP, which may be a kinesin endosome receptor for a subset of transport vesicles (Kamal *et al.*, 2000). Because multiple kinesin isoforms are expressed in neurons, it could be hypothesized that different kinesins may be responsible for anterograde axonal transport of different types of endosomes, possibly facilitating their targeting to different destinations or uses, but as of yet, there is little direct evidence for this mechanism. However, recent evidences have indicated the involvement of sortilin as an important anterograde trafficking receptor for the Trk proteins (Vaegter *et al.*, 2010).

1.1 Brain derived neurotrophic factor (BDNF)

In 1982 BDNF was purified from pig brain, whereas provide its survival-promoting action on a subpopulation of dorsal root ganglion neurons (Barde *et al.*, 1982). BDNF is a basic dimeric 28-kDa protein of non-covalently linked 14-kDa subunits structurally related to NGF. Pro-BDNF, a 32-kDa precursor, undergoes cleavage to release mature 14-kDa BDNF protein as well as a minor truncated form of the precursor (28 kDa). BDNF gene consist of eight 5' untranslated exons and one protein coding 3' exon. Transcription of the gene results in BDNF transcripts containing one of the eight 5' exons spliced to the protein coding exon and in a transcript containing only 5' extended protein coding exon (Aid *et al.*, 2007). BDNF is synthesized as pro-BDNF, which is then cleaved proteolytically (N-terminal domain is removed) inside the neuron or after it is released, creating its final mature protein form (Amhed et al., 2015). Mature BDNF in the nervous system can promote, through TrkB receptor, neuronal survival, growth, differentiation and synaptic plasticity (for a review see Huang and Reichardt , 2001). Otherwise, the pro-BDNF binds to the p75 neurotrophin receptor (p75NTR) activating apoptotic pathways in peripheral neurons and glia.

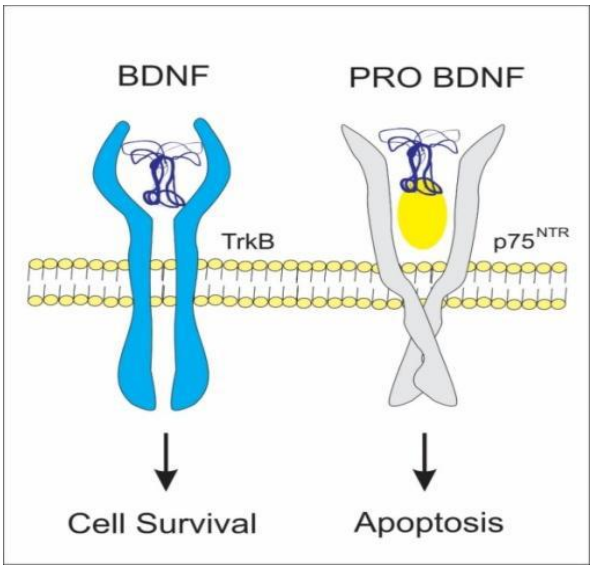


Fig. 3 BDNF and pro-BDNF receptor. BDNF binds to TrkB receptor and this interaction induces the activation of pathway signaling to culminate in cell survival. When the pro-BDNF binds p75NTR can lead to apoptosis. Abbreviations: BDNF, brain-derived neurotrophic factor; p75NTR, p75 neurotrophin receptor. (see review Budni et al., 2015).

1.2 Regulation of BDNF signaling

The Trk B receptor mediates the effect of BDNF and also (NT 4/5). There are several TrkB isoforms in the mammalian CNS. The full-length TrkB isoform is a typical tyrosine kinase in that homodimerization during ligand binding causes cross tyrosine phosphorylations in the intracellular Trk domains thereby initiating transduction of the BDNF signal. These phosphorylation events then trigger MAPK, PI3K or PLCγ intracellular cascades. Synaptic activity drives these signaling pathways that regulate the assembling of TrkB with synaptic proteins, gene transcription, protein translation and trafficking. The three pathways can, at least theoretically, work in parallel. Thus for most TrkB investigations, one Trk signaling pathway is generally identified as necessary for an outcome, but seldom do these experiments eliminate possible contributions, to the particular effect, of the other Trk signaling pathways. In a recent study has been showed the role of an adaptor the JIP3/TrkB interaction selectively drives TrkB anterograde transport in axons but not in dendrites of rat hippocampal neurons. Moreover,TrkB axonal transport mediated by JIP3 could regulate BDNF-induced Erk activation and axonal filopodia formation. JIP3-mediated TrkB

anterograde axonal transport in recruiting more TrkB into distal axons and facilitating BDNF-induced retrograde signaling and synapse modulation, which provides a novel mechanism of how the TrkB anterograde transport can be coupled to BDNF signaling in distal axons (Huang et al., 2011).

Phospholipase Cy (PLCy)

There are two tyrosine phosphorylation residues outside the kinase activation domain of TrkB. Phospho-Tyr785 recruits and activates PLCγ which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate, to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Huang and Reichardt, 2003). DAG activates protein kinase C (PKC), and IP3 releases Ca2+ from intracellular stores. In developing hippocampal neurons, focal application of BDNF results in fast calcium transients at postsynaptic sites (Lang et al., 2007). The BDNF-induced release of intracellular Ca2+ together with DAG activate the plasma membrane transient receptor potential canonical subfamily channel 3/6 (TRPC3/6) that contribute to BDNF-induced Ca2+ elevations at growth cones and synapses (Amaral and Pozzo-Miller, 2007). Some studies link the PLCγ pathways underlying BDNF induced Ca2+ transients directly to synaptic plasticity.

For example, in cultured cortical pyramidal neurons these transients translocate GluR1 subunit of α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPAR), but not N-methyl-D-aspartic acid receptors (NMDAR) subunits to synapses. The transients were due to release from intracellular stores requiring active IP3 receptors and a contribution from TRPCs (Nakata and Nakamura, 2007). The Ca2+ elevation triggered by PLCγ also increases Ca2+-sensitive adenyl cylclase (AC) activity that is necessary for the formation of synaptic PSD-95-TrkB complexes (Ji et al., 2005) and that also is involved in cyclic AMP responsive element binding (CREB)-dependent transcription (Shaywitz and Greenberg, 1999).

Mitogen activated protein kinase (MAPK)

The other TrkB phosphorylation site on Tyr515 recruits Shc to TrkB and phosphorylates it. In turn, Shc interacts with an adaptor protein Grb2 that recruits and activates the guanine nucleotide exchange factor SOS. SOS promotes the removal of GDP from Ras that can then bind GTP and become active. Ras activates the

downstream kinase B-raf, MEK and MAPK/Erk (Huang and Reichardt, 2003). MEK-MAPK/Erk signaling influences transcription events, such as the activation of the CREB transcription factor (Shaywitz and Greenberg, 1999). MAPK/Erk also regulates protein-synthesis dependent plasticity by increasing phosphorylation of eukaryotic initiation factor 4E (eIF4E), the 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (Klann and Dever, 2004). Recruitment of Shc to the Trk receptors also allows activation of the PI3K pathway by Ras, via Grb2 (Reichardt, 2006). Activation of PI3K changes the composition of inositol phospholipids in the inner leaflet of the plasma membrane. This results in the translocation of Akt/protein kinase B to the plasma membrane. Activated Akt is involved in a variety of functions such as cell survival, and protein translation. The TrkB-PI3K-AKT pathway activates translation via a cascade driving the mammalian target of rapamycin (mTOR), a major regulator of protein synthesis (Sarbassov et al., 2005). Akt accomplishes this by downregulating the tuberous sclerosis complex (TSC) protein, TSC2 that forms a complex with TSC1: a complex that otherwise inhibits Rheb a normal activator of mTOR. Active mTOR phosphorylates p70S6 kinase and 4E-BP1 permitting mRNA translation. The PI3K-Akt pathway also regulates the trafficking of synaptic proteins (Yoshii and Constantine-Paton, 2007). Thus, this pathway plays pivotal roles in a long-term maintenance of synaptic plasticity through translation and transport of synaptic proteins.

BDNF-TrkB truncated form

In addition to trkB Full Length (FL), the *Ntrk2* (trkB) locus encodes for several alternatively-spliced isoforms of the receptor (Middlemas et al., 1996), including trkB truncated (Tr), the predominant isoform expressed in the adult mammalian nervous system. The extracellular domain of trkB truncated is identical to the full-length isoform, which enables high-affinity BDNF binding (Middlemas et al., 1996). However, the 11 amino acid intracellular portion of trkB.Tr lacks the kinase activation domain necessary to activate classical signal transduction pathways. Since trkB Tr heterodimerization with trkB.FL inhibits trans-autophosphorylation of the trkB.FL kinase domain, studies support a model in which trkB.Tr functions to reduce BDNF signaling (Luikart et al., 2003). However, has been demonstrated evidence that trkB.Tr may signal independently. The primary function of TrkB.Tr could be the modulation of other cellular functions independent of the TrkB kinase receptor

(Baxter et al., 1997; Rose et al., 2003; Ohira et al., 2005; Carim-Todd et al., 2009). For example, TrkB.Tr has been reported to regulate astrocytic morphology by directly interacting with Rho GDP dissociation inhibitor 1 and modulate calcium release from intracellular stores in astrocytes (Rose et al., 2003; Ohira et al., 2005). TrkB.FL and TrkB.Tr expression are tightly regulated during development. Although TrkB.FL is the highest expressed isoform in early CNS development, TrkB.Tr is dramatically upregulated during postnatal brain development (Fryer et al., 1996).

1.3 Biological activities of BDNF

Neurotrophins represent strong candidates in regulating the continued plasticity of multiple brain systems long beyond the early organization that occurs during embryonic and perinatal period and adulthood. This is particularly true for BDNF, a key molecule critical in the control of neurogenesis, since it impacts cell proliferation within the neurogenic zone of mammalian brain (Bath and Lee, 2010), and contributes to the process of neuroblast migration (for a review see Bath and Lee, 2010). BDNF is a pivotal modulator of memory processing and storage. It is also critically involved in neurogenesis, differentiation, maintenance, and survival of many major neuronal types and synapses in the CNS and PNS (Poo MM., 2001). Behavioral evidence supports that BDNF is essential for at least certain forms of learning and memory. Hippocampus- dependent learning in the Morris water maze, contextual fear, and passive avoidance tests is associated with a rapid and transient increase in BDNF mRNA expression in the hippocampus (Yamada et al., 2002). Heterozygous BDNF mutant mice show a moderate but significant impairment of water maze learning without any effect on memory retention. Treatment with anti-BDNF antibodies causes impairment of memory in the water maze and passive avoidance tests (Hall et al., 2000). Growing evidence suggests that a decrease in BDNF levels could be associated with different neurodegenerative disease. In patients with Alzheimer's Disease, BDNF expression is severely decreased in the hippocampus and some cortical areas (particularly the temporal and frontal cortex). Most studies on BDNF expression have been done in postmortem brains of AD or aged patients, and show a reduction of BDNF levels compared with healthy subjects

(Huang et al., 2001). The BDNF level are also decreased in the parietal cortex and hippocampus, even in the pre-clinical stages of AD.

Further studies showed that BDNF has potent effects on survival and morphology of dopaminergic neurons and thus its loss could contribute to death of these cells in Parkinson's disease (PD). In particular, a pilot study in 1998 first hinted that reduced BDNF mRNA concentrations in substantia nigra might contribute to development of Parkinson's disease.

1.4 Neurotrophins during vertebrate evolution

Detailed phylogenetic analysis of the neurotrophin and Trk gene families have been performed either on mature aminoacid sequences and on nucleotide sequences of selected organisms at different evolutionary stages. These studies showed that neurotrophins are highly conserved in vertebrates evolution (Hallböök et al., 1991; van Kesteren et al., 1998; Hallböök et al., 1999; Lanave et al., 2007). The analysis supports the notion that neurotrophins evolved early in vertebrate history during the two rounds of genome duplication that characterized the origin and evolution of vertebrates (Hallböök *et al.*, 2006). Recent studies confirmed that *BDNF* gene is well conserved during vertebrate evolution, from fish to mammals (Heinrich and Pagtakhan, 2004; Aid et al., 2007; Tettamanti et al., 2010). BDNF gene is composed of multiple alternative exons: 10 in human, 8 in mouse, and 6 in lower vertebrates (Cohen-Cory et al., 2009). Multiple promoters can modulate the transcription of the *BDNF* gene (Aid et al., 2007; Tao et al., 2002; Rattiner et al., 2004; Kidane et al., 2009). BDNF cDNA underwent some degree of selective pressure in molecular evolution through the vertebrate phylogenesis, clustering together species belonging to the same class. An adaptive type of selective pressure seemed to be responsible for clustering of mammals apart from other classes. The mutants arose from point mutations as well as insertion/deletions modalities: these last are especially represented in teleosts. Differently conserved regions are recognized along the sequences, corresponding to different parts of the transcript.

1.5 Neurotrophins in fish

Fish show a high degree of evolutionary conservation compared to other vertebrates with respect to gene expression patterns in the developing brain and the establishment of early axonal pathways (reviewed by Kimmel, 1993). Neurotrophin genes are expressed in fish at the RNA level (Hashimoto and Heinrich, 1997), both in development and adulthood.

The primary structure of all fish neurotrophins has been deduced from cloned cDNAs or genes following cloning and nucleotide sequencing. To obtain the clones, two approaches have been taken. One approach was based on cross-hybridization of cloned neurotrophin probes from other species with the fish neurotrophin, and the second approach was based on PCR using primers that represent conserved regions of the neurotrophins (Heinrich and Lum 2000). The overall organization of all neurotrophin genes is similar: there is one major exon, enconding the whole precursor. Slight differences can be noticed among all neurotrophins. Particularly, BDNF-encoding exon possesses four upstream exons and a separate promoter. The mature BDNF transcripts possess two exons: the first is one of the four upstream exons and the second is the BDNF-encoding major exon (Hashimoto and Heinrich, 1997). NT-3 gene has a similar organization, characterized by two upstream exons possessing a separate promoter (Leingartner and Lindholm, 1994).

NGF transcripts contain up to four exons encoding a NGF precursor that is extended at the N-terminus. The upstream organization of the NT-4 gene is similar to that of the NT-3 gene. There are two promoters, each generating a different 5' end of the NT-3 transcripts. All transcripts therefore contain two exons. The second exon encodes the NT-4 precursor (Heinrich and Lum, 2000). The organization of the NT-6 presents 4 exons (Dethleffsen *et al.,* 2003).

The functional roles of neurotrophins in fish is still matter of debate. So far, hypothesis have been formulated. Actions may correlated to regulation of programmed neuron death and survival during the development and in the adulthood (Eisen *et al.,* 1990; Pike *et al.,* 1992). Neurotrophins, and particularly BDNF, may represent a strong candidate in regulating the continued plasticity of multiple brain systems long beyond the early organization that occurs during embryonic and perinatal period (Bath *et al.*, 2011)

2. Zebrafish

2.1 Characteristics

The zebrafish (Danio rerio) is a small tropical freshwater fish belonging to the family of Cyprinidae, which lives in rivers of northern India, northern Pakistan, Nepal, and Bhutan in South Asia. The characteristic stripes running along the body and the fins give its name to this species. In contrast to many other fish species, zebrafish adults are only approximately 3-5 cm long, so that they can be easily managed in large numbers in the laboratory (Kishi et al, 2003). Zebrafish have short generation times of approximately three to five months. After three to four months, zebrafish are sexually mature and can generate new offspring. A single female can lay up to 200 eggs per week (Stern and Zon, 2003). The development of the zebrafish is very similar to the embryogenesis in higher vertebrates, including humans, but, unlike mammals, zebrafish develop from a fertilised egg to an adult outside the female in a transparent egg. Moreover, the embryos themselves are transparent during the first few days of their lives (Wixon, 2000). The embryonic development of zebrafish is very rapid: in the first 24 hours after fertilisation, all major organs are developed and within three days the fish hatch and start looking for food. Recently the whole genome of zebrafish has been sequenced (Sanger Institute, http://www.sanger.ac.uk/Projects/D_rerio). It is even more complex than the human genome, as zebrafish have two more pairs of chromosomes than humans. This difference arose during evolution in teleosts, when the whole genome was duplicated. Many of these duplicated genes were lost again and only a small

proportion remains today. Functions of these duplicated genes changed in several cases (Hill et al, 2005).

2.2 Advantages of zebrafish as model organism

The principal physiological characteristics of zebrafish mentioned above, were initially the most important advantages that made this species a popular model to study the development of vertebrates. The tiny size of the larval and adult zebrafish greatly reduces costs as it enables the reduction of housing space and husbandry costs. Another advantage of this species is its high fecundity. One pair of adult fish is capable of laying ca. 200 eggs a day, and depending on the conditions of maintenance, this yield can be expected every 5–7 days. In addition, the rapid maturation of zebrafish also enables the performance of transgenerational studies Thus, with sexual maturation after around 100 days, zebrafish can be utilized in mutagenesis analyses (Hill et al, 2005). Zebrafish has become a popular model organism used in many different fields of research. In the past, it was a major vertebrate model especially in the developmental and genetic research (Hill et al., 2005), whereas now, the zebrafish gains also growing importance in other fields of biomedical and veterinary research.

2.3 Zebrafish as model in neurobiological studies

Zebrafish is an ideal model organism to study the molecular and cellular biology of the vertebrate brain. The use of “neurobiological models”, that are “simpler” than the mammalian brain, might facilitate research into central nervous system (CNS). Indeed, studies of comparative neurobiology from fish to mammals provided insight about the human nervous system and its role in health and pathological conditions. Fish models can lead to understanding of vertebrate neurology in general, and provide perspective by their fundamental evolutionary relationship with other vertebrates. Certain progressive neurological diseases are best understood in the context of the evolutionary states of the nervous system in which evolutionarily "higher" CNS functions are lost first, then sequentially "lower" evolutionary states,

with the order being reversed during recovery. Zebrafish possesses specific regions of the brain that are strikingly conserved in mammalian brains (Wullimann and Reichert, 1996; Mueller and Wullimann, 2009; Rink and Wullimann, 2004), and this allows zebrafish to be used as an excellent model for neurodegeneration. In fish, as in other vertebrates, two prominent external constrictions divide the early developing brain (neural tube) into the three neural vesicles giving rise to the basic regions of the adult brain: forebrain (or prosencephalon), midbrain (or mesencephalon), and hindbrain (or rhombencephalon). These vesicles represent the fundamental antero-posterior (i.e., rostrocaudal) subdivisions that later on may become subdivided into more or less evident transverse bands or neuromeres, which have defined relations with other structures of the head and the peripheral nervous system, being very useful in comparative studies. Several transgenic or mutant zebrafish lines are generated to model neurodegeneration in fish (Xia, 2010). Various techniques ranging from morpholino knockdown of specific disease related genes (Newman et al., 2009) to the use of neuronal promoters for driving mutant versions of different neuro-degeneration associated proteins (Bai et al., 2007), or generating mutants for loss of/function studies were used. These animals models provide important information of the pathophysiology of the disease progression and underlying molecular programs.

2.4 The central nervous system of teleost fish

The teleost brain is composed of three major regions: the forebrain, midbrain, and rhombencephalon or hindbrain. The forebrain can be further subdivided into the telencephalon and diencephalon. The midbrain is subdivided into a dorsal part, entirely occupied by lobes of optic tectum, and the ventrally located tegmentum. The hindbrain encompasses cerebellum and medulla oblongata, respectively dorsal and ventral positioned, and does possess structures that are present in the mammalian pontine tegmentum (the tegmental region dorsal to the pons *sensu stricto*). The above-mentioned structures can be identified in teleosts as well, e.g. the locus coeruleus and the principal trigeminal nucleus, and this region may be called the "pontine area." In teleosts, the latter area includes the lateral nucleus of valvula which receives telencephalic inputs and projects fibers to the cerebellum. The

nucleus may share a similar ontogenetic origin with the mammalian pontine nuclei, suggesting their homology (Yang *et al.*, 2004).

Telencephalon

The telencephalon represents the most rostral part of encephalon. It consists mainly of olphactory bulbs and telencephalon sensu strictu or telencephalic hemispheres. The olphactory bulbs are sessile and organized in layers, whose boundaries are not very distinct. From the most external layer, it can be recognized: the outer layer of primary olphactory afferent fibers, glomerular layer, layer of mitral cells or external and intercellular layer of granule cells. The olphactory bulbs receive a so-called primary olphactory input from sensory nerve cells in the sensory epithelium of the olphactory organ. The pathway is via the olphactory nerve. Both the bulbs are interconnected via the medial oplhactory tract and project predominantly to the ventral area of telencephalon and dorso-posterior telencephalon. The telencephalon is subdivided into a dorsal and ventral area, each with a number of cytoarchitectonically delineable subdivisions. In the dorsal area, it is possible to recognize dorso-lateral, dorso-dorsal, dorso-medial and dorso-central areas. Dorso-lateral and dorso-medial areas present a high density of small neurons, mainly organized in layers, whereas the dorso-central area consists of larger neurons. The dorso-posterior telencephalon is an important source of olphactory bulbs afferents fibers. The ventral area is divided into a ventro-dorsal, ventro-ventral, ventro-lateral areas, supracommissural and post-commissural parts. All these subdivisions are mainly characterized by small neurons and neuronal processes projecting back to olphactory bulbs or to midbrain regions.

Diencephalon

The diencephalon is located between the telencephalon and mesencephalon: the rostral boundary is the preoptic area and the caudal boundary is the pretectal region. The diencephalon proper is subdivided into epithalamus, dorsal and ventral thalamus, posterior tuberculum and hypothalamus zone. The preoptic area is regarded as the most rostral diencephalic subdivision, and includes the preoptic and suprachiasmatic nuclei. By cytomorphological criteria, the preoptic nucleus is split into a magnocellular and parvocellular portions. The epithalamus represents the dorsal part of diencephalon and comprises the habenular nuclei, the epiphysis and the dorsal

saccus. Habenular nuclei and epiphysis are interconnected structures, since input arise from one and reach the other. Moreover, epiphysis plays a key role in the synchronization of activity rhythms with the dark-light cycle.The ventral thalamus, located ventral to the dorsal thalamus only in its caudal extent, consists of an intermediate, ventromedial and ventrolateral nuclei. The posterior tuberculum consists of some periventricular nuclei and migrated anterior, lateral, medial and caudal preglomerular nuclei. The thalamic "complex" is the major sensory relay station of the brain, receiving all inputs (except olfaction) and connecting reciprocally with the overlying structures. Particularly, thalamic nuclei are characterized as a multimodal relay centre, relaying visual, somatosensory, acoustico-lateral and cerebellar input to the telencephalon. The dorsal thalamus of teleosts does not appear to serve as the major sensory relay center to the telencephalon (Northcutt, 2006); whereas accumulating evidences suggest that the preglomerular complex, located ventrolateral in the thalamus, serves as the major sensory relay station to the telencephalon (Northcutt, 2006). The hypothalamus, as the name implies, is located below the thalamus and represents the ventralmost diencephalic region. It is connected with the hypophysis by means the pituitary stalk, which contains hypothalamic and preoptic nerve fibers involved in neuroendocrine regulatory functions. In the teleostean hypothalamus, a periventricular zone and inferior lobe can be recognized. Finally, the pretectal region is located in front of the rostral margin of the optic tectum and includes both diencephalic and mesencephalic structures. Functionally, the pretectum may be characterized as a primarily visual region, receiving both retinal and tectal visual input.

Mesencephalon

The mesencephalon or midbrain consists of a dorsally located tectum, named optic tectum, a ventromedially located tegmentum and ventrolaterally located semicircular tori. This general subdivision reflects a diverse functional role of the structures. Indeed, the optic tectum is mainly involved in sensory functions, whereas the ventromedial and ventrolateral structures are predominantly involved in (pre)motor functions. The optic tectum is a paired structure, organized in layers. Anteriorly, at the medial tectal margin there is a paired structure, named longitudinal tori, one of the main source of visual projection to optic tectum. The optic tectum is not only

involved in visual inputs processing, but also in integration of visual signals with sensory information, and in generation and coordination of goal directed movements. Thus, it is considered as one of the main sensorimotor integration centers of the teleostean CNS. The tegmentum encompasses many motor structures, such as the oculomotor and trochlear nuclei, and continues into the medulla oblongata without any sharpen boundary.

Hindbrain or Rhombencephalon

The rhombencephalon is the most caudal part of the brain. Caudally it continues gradually in the spinal cord. It is composed in the dorsoanterior region of cerebellum, and ventrally of medulla oblongata. The cerebellum basically consists of three main regions: the valvula, the part protruding rostrally into the mesencephalic ventricle, the corpus, centrally located, and the caudal cerebellar region which consists of the caudal lobe and granular eminentiae. The primary role of the cerebellum is to coordinate movement, and it thus receives information from many sources while transmitting information primarily to motor cortical areas.

The medulla forms the fourth ventricle of the brain, and together with the midbrain, are often referred to as the "brainstem". The brainstem contains a conglomerate of cell groups that form a complex network termed the reticular formation, which is involved in higher order behaviors such as respiration, circulation, and wakefulness. The hindbrain also contributes 5 pairs of cranial nerves, numbers V through X. Cranial nerves can have both motor and sensory components, and those derived from the hindbrain are responsible for a wide variety of behaviors, including taste, hearing, balance, mastication, and some eye movements.

2.5 Adult neurogenesis

Vertebrate neurogenesis occurs not only during embryogenesis but also during adult stages (Alvarez-Buylla and Kirn, 1997, Doetsch and Scharff, 2001 and Zupanc, 2001). The terms of adult neurogenesis and plasticity, therefore, denote the overall ability of the brain to remodel its cellular composition and synaptic wiring on demand. In rodents and songbirds, adult neurogenesis is observed in highly restricted

spatial domains that generate new cells destined for distinct telencephalic regions. In rodents, these regions are the granular and peri-glomerular layers of the olfactory bulb that receive new interneurons (Altman, 1969, Luskin, 1993, Lois and Alvarez-Buylla, 1994 and Hack et al., 2005) and the dentate gyrus in the hippocampus where new granule neurons are produced during adulthood (Altman and Das, 1965, Cameron et al., 1993, Kaplan and Bell, 1984 and Seri et al., 2001). In songbirds, newborn neurons are added to diverse regions in the telencephalon. The best studied example is the hyperstriatum ventrale, pars caudalis HVC nucleus in canaries where interneurons and long projecting neurons are added throughout life and survive for several months (Kirn et al., 1991 and Nottebohm et al., 1994), implying growth of this particular brain nucleus at that time. In contrast, new cells in the hippocampus do not cause brain growth but rather indicate a constitutive, low turnover rate of neurons (Barnea and Nottebohm, 1996). In both vertebrate groups, proliferation zones that generate the new neurons are situated close to the forebrain ventricles. In birds, the ventricular zone directly contacts the forebrain ventricular lumen. In mammals, the subventricular/subependymal (SVZ) zone is located below the ependymal layer lining the forebrain ventricles, while the subgranular zone (SGZ), a derivative of the subventricular zone, has split off in adults. While in mammals and birds constitutive turnover of neurons has been emphasized, net brain growth occurs during adulthood in reptiles, amphibians and fish (reviewed in Font et al., 2001). The forebrains of lizards show an age- and bodyweight-dependent increase in neuronal numbers. In teleosts, the best studied brain parts are the retina and the optic tectum, which both grow from discrete proliferation zones near the margins of these structures (Johns, 1977, Raymond and Easter, 1983 and Marcus et al., 1999).

It has long been known that brains of adult teleost fish show widespread cell proliferation in all subdivisions along the rostrocaudal axis (Kirsche, 1967, Zupanc and Horschke, 1995, Zupanc et al., 2005 and Ekström et al., 2001). The generation of new cells may in fact account for the lifelong brain growth observed in different fish species (Brandstätter and Kotrschal, 1990). In the brain of adult zebrafish 16 distinct proliferative region domains along the whole anterior–posterior brain axis have been identified (Grandel *et al*. 2006; Kaslin et al., 2008) Fig.4.

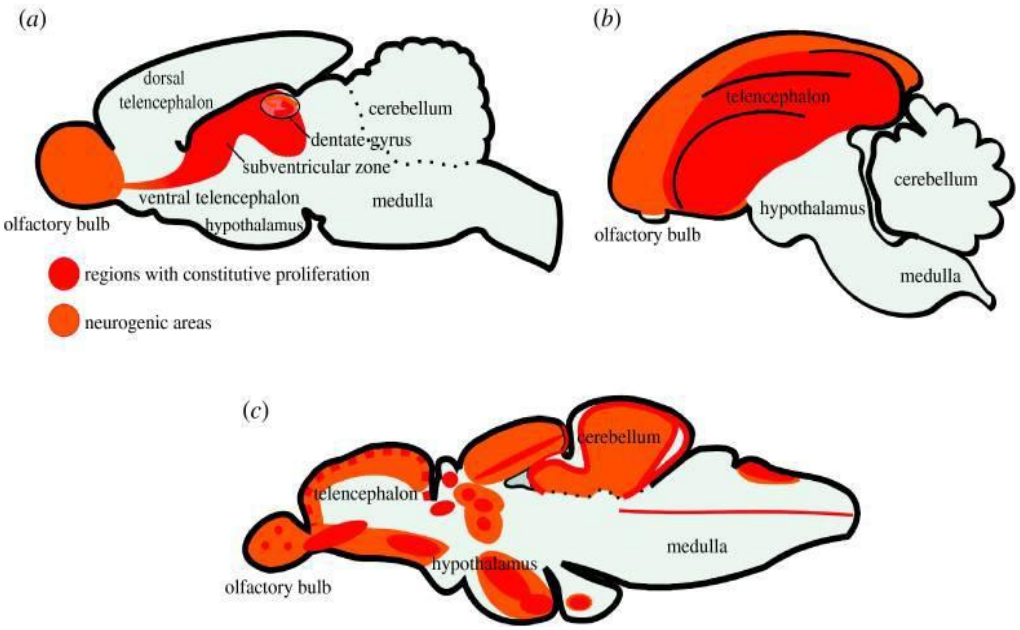


Fig 4 Parasagittal schematic overviews of the adult proliferation pattern and neurogenic regions in the brain of adult vertebrates. (*a*) Rodent (mouse), (*b*) Bird (songbird) and (*c*) Fish (zebrafish). (Kaslin et al., 2008)

Furthermore, these proliferative domains contained ventricularly positioned label-retaining cells, indicating that these cells are self-renewing neural precursors (NPCs). Interestingly, the progenitors residing in the adult zebrafish telencephalon seem to express a different set of transcription factors compared with their embryonic counterparts (Adolf *et al*. 2006). Most of the generated cells from these zones seem to migrate slowly and differentiate into neurons (Zupanc *et al*. 2005; Grandel *et al*. 2006).

The majority of these stem/progenitor cells are radial glial cells (RGCs). RGCs have also been observed in the developing mammalian telencephalon, where they act as embryonic neural stem cells. Mammalian radial glia disappear shortly after birth, giving rise to ependymal cells and astrocytes, some of which retain stem cell potential in the SVZ and SGZ. Thus, the adult zebrafish appears to have retained embryonic features. Indeed, the adult zebrafish radial glial cells express similar genes to the embryonic counterparts, such as the glial acidic fibrillar protein (GFAP), S100β and the brain lipid-binding protein (BLBP) (for a review Schmidt et al., 2013). With the expression of proliferation markers, such as PCNA, or by detecting incorporation of the thymidine analog BrdU, it is possible distinguish two classes of

radial glia cells: those which are in a quiescent state (Type I cells, BrdU−rdU−, PCNA−, GFAP+, β+, BLBP+) and slowly cycling cells (Type II cells, BrdU+, PCNA+, GFAP+, S100β+, BLBP+). Type II cells give rise to neuroblasts (Type III cells) that continue to proliferate, turn on neural marker genes, such as PSA-NCAM and the proneural gene ascl1, and eventually enter the rostral migratory stream or leave the periventricular zone to move deeper into the parenchyma. Proliferating Type II stem cells can divide symmetrically to self-renew or asymmetrically to generate Type III cells. The relative proportion of proliferating and resting stem cells can be shifted by interfering with Notch signaling. Activation of the Notch pathway drives stem cells into quiescence, suggesting that cross talk between cells controls the maintenance of the quiescent stem cell population (Fig.5). Hence, maintenance of neural stem cells in the adult appears to employ a similar mechanism as that seen in the embryo, where Notch-mediated lateral inhibition maintains a pool of embryonic neural stem cells for later production of neurons, such as the secondary motor neurons.

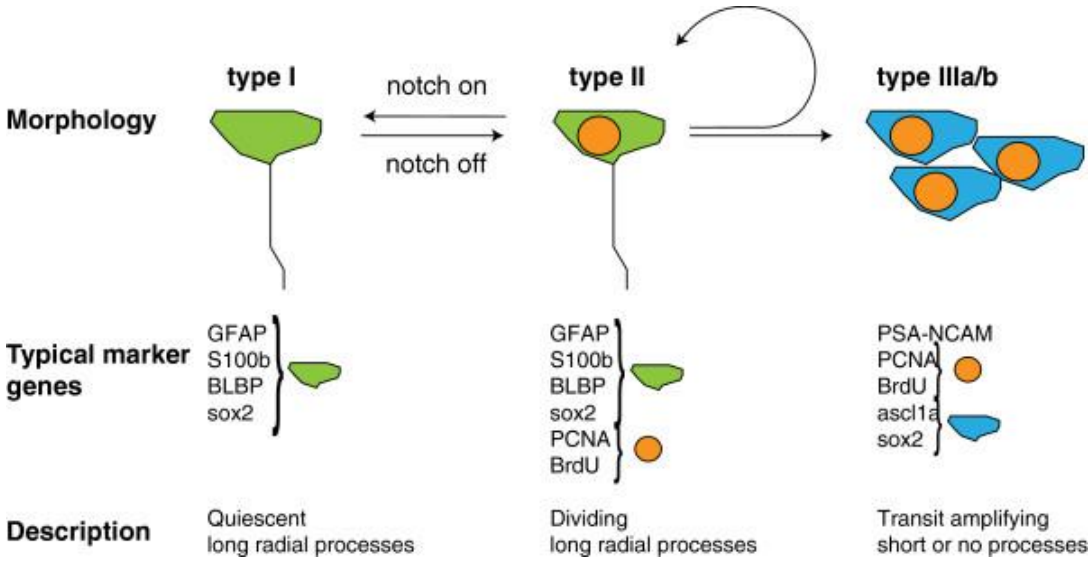


Fig 5 The major proliferative cell types in the telencephalon stem cell niche. The markers expressed by the three major cell types are indicated. Notch signaling transforms proliferating Type II radial glial cells into quiescent Type I radial glial cells. Exists heterogeneity in the cellular composition and marker expression of stem cell niches in the telencephalon.

2.6 Regeneration in mammals and non-mammalian vertebrate

Non-mammalian vertebrates have a remarkable capability to regenerate severe brain injuries such as stab wounds and ablation of whole brain parts, in striking contrast to mammals where neurons rarely if at all are replaced after injury. The cause for this remarkable plasticity is unknown but differences in the adult brain proliferation pattern are probably involved. Among non-mammalian vertebrates, fish and amphibians exhibit the most widespread adult neurogenesis and also the greatest capacity to regenerate injuries to the CNS. Compensatory proliferation and neurogenesis in response to injury of the CNS are the phenomena observed in all vertebrates including mammals. Different types of injuries to the CNS can induce ectopic proliferation and neurogenesis in proximity to the injury, as well as enhanced proliferation in constitutively active neurogenic domains followed by ectopic migration of neuronal precursors towards the site of injury. It should be noted that the origin and composition of cells contributing to the reconstitution are unclear in many studies. Currently, very little is known about the basics of compensatory neurogenesis and how it is possible to modulate and direct it. However, it is obvious that enhanced activation of inherent progenitors and recruitment of new neurons into the injury site could be a fundamental strategy in restorative therapies for brain injuries and neurodegenerative disease.

Mammals

It has long been thought that neurons generally are not replaced in the mammalian brain after injury or during the course of a disease. Interestingly, several recent studies have reported compensatory proliferation and neurogenesis in response to injury or disease in the mammalian brain (reviewed in Goldman 2005;Emsley *et al*. 2005; Zhang *et al*. 2005). General increases in proliferation and limited neurogenesishave been found in the constitutively neurogenic brain regions of patients with Alzheimer's and Huntington's disease (Curtis *et al*. 2003; Jin *et al*. 2004*a*,*b*). Chemically or electrically induced seizures stimulate proliferation and neurogenesis in the hippocampus of rodents (Bengzon *et al*. 1997; Parent *et al*. 1997, 2002*a*, 2006). Focal ischemia in rodents leads to enhanced proliferation in the SVZ and SGZ and ectopic recruitment of neuronal precursors into the injured brain

regions from the constitutively active neurogenic domains (Arvidsson *et al*. 2002; Nakatomi *et al*. 2002;Parent *et al*. 2002*b*; Jin *et al*. 2003; Zhang *et al*. 2004). Interestingly, compensatory neurogenesis from inherent progenitors takes place ectopically in the neocortex after selective ablation and apoptosis of cortical interneurons (Magavi *et al*. 2000) or long projecting corticospinal neurons (Chen *et al*. 2004). Other types of brain damage such as stab wounds, stroke and chronic injury by the accumulation of amyloid plaques also induce ectopic proliferation of various cell types such as microglia, astrocytes and endogenous precursors (Levine *et al*. 2001). However, normally these precursors fail to generate neurons and instead contribute to the generation of glial scarring. Some of these endogenous precursors show the potential to generate neurons *in vitro* but somehow fail to do so upon injury *in vivo*. Recently, it was shown that the basic helix–loop–helix transcription factor oligodendrocytes factor 2 (Olig2) is greatly upregulated in endogenous precursors after injury (Buffo *et al*. 2005). Interestingly, repression of Olig2 in proliferating precursors leads to the production of immature neurons indicating that Olig2 acts as a suppressive non-neurogenic signal *in vivo* and thus one of the first non-permissiveness signals that has been found to act *in vivo* in the mammalian brain (Buffo *et al*. 2005). These results clearly indicate that a limited compensatory neurogenesis can take place in the adult mammalian brain and, more importantly, that it may be possible to evoke endogenous neuronal repair in normally non-neurogenic brain regions. Although the compensatory proliferation and neurogenesis in mammals are very interesting and promising, the yields are so far modest and therapeutically insignificant.

Birds

Compensatory proliferation and neurogenesis as well as functional integration of interneurons and long projecting neurons are seen in the adult zebra finch after selective ablation of neurons (Scharff *et al*. 2000;Dawley *et al*. 2000). Three types of neurons, interneurons, HVC-archistriatum(RA) and HVC-X long projecting neurons, reside in the HVC of the adult zebra finch. Interneurons and long projecting HVC-RA neurons both show a low turnover rate, while the number of long projecting HVC-X neurons remains constant (Kirn *et al*. 1991, 1999; Scharff *et al*. 2000). Selective lesions of HVC-RA neurons induce deterioration of song. Shortly after the

lesioning, enhanced compensatory neurogenesis is seen in HVC-RA neurons and interneurons. New projections from HVC-RA neurons are formed and birds are eventually able to recover singing to variable degrees. The ability to produce song coincides with the time when the HVC-RA neuronal projections reach the RA area. This suggests that neuronal replacement can restore function and learned behaviour in birds (Scharff *et al*. 2000). Surprisingly, the HVC-X neurons are not regenerated after selective ablation indicating that only the neuronal types that normally display turnover are able to respond to the compensatory neurogenesis. The mechanism underlying this selective restoration is not yet known. Similarly, electrolytic lesions to the hypothalamus of adult doves induce compensatory neurogenesis and recruitment of new neurons into the lesion site (Cao *et al*. 2002).

Reptiles

The medial cortex of lizards shows a remarkable capacity to morphologically and functionally regenerate severe chemical and physical lesioning (reviewed in: Font *et al*. 2001; López-García *et al*. 2002; Romero-Aleman *et al*. 2004). Specific lesioning with the neurotoxin 3-acetylpyridine primarily affects the medial cortex and induces rapid degeneration of neurons. Already, degeneration is detectable only after 12 h of administration. The degeneration process progresses for 4–10 days, after which over 90% of the nuclei in the dorsomedial cortex display pyknotic in severely affected specimens (Font *et al*. 1997). One to two weeks after the lesion, there is a wave of compensatory neurogenesis directed towards the site of injury. The newly produced cells originate from the pool of ventricular progenitors that constitutively proliferate in the cortex of adult lizards. The compensatory neurogenesis morphologically and most probably functionally restores the damaged areas within 4–8 weeks after the lesion (Font *et al*. 1991; Molowny *et al*. 1995; Font *et al*. 1997). Compensatory gliogenesis and neurogenesis play a key role in the structural repair after physical lesioning of the cortex of adult lizards (Romero-Aleman *et al*. 2004). Interestingly, neuronal regeneration after injury is abolished in lizards during wintertime. The low temperature seems to prevent migration of newly generated neurons (Ramirez *et al*. 1997).

Amphibians

Regenerative studies in amphibians have mostly been performed in urodele newts and *Xenopus* and are mainly concentrated on regeneration of the peripheral nervous system, tail and limbs (reviewed in: Brockes & Kumar 2002; Ferretti 2004; Slack *et al*. 2004). In general, the regenerative capacity of urodele newts is much higher than that in other amphibians. However, both urodeles and anurans have the capability to regenerate severe injury to most brain parts during juvenile stages but not as adults (Srebro 1965; Filoni & Gibertini 1969, 1971). The urodele newt *Triturus cristatus carnifex* morphologically regenerates a lesion to the optic tectum or telencephalon after approximately three months. There seems to be a direct relationship in the compensatory response between the number of proliferating cells in the injured optic tectum and the extent of the lesion (Del Grande *et al*. 1990; Minelli *et al*. 1990). Interestingly, lesions to the telencephalon or the optic tectum induce a response in several proliferative zones indicating that signals mediated from the lesion site are able to elicit a broad proliferative response. The clawed frog *Xenopus laevis* can regenerate severe lesions to the telencephalon, optic tectum and cerebellum during larval stages but not after metamorphosis (Srebro 1965; Filoni & Gibertini 1969, 1971; Filoni *et al*. 1995). The reconstitution of ablated parts is rapid in juvenile frogs, for example the telencephalon is reconstituted already one month after lesion. Although morphological alterations are still seen, correct connections are formed from the regenerated telencephalon (Yoshino & Tochinai 2004). Recently, it was shown that juvenile frogs can regenerate severe telencephalic lesions both morphologically and functionally (Yoshino & Tochinai 2006). The differences in the decrease in regenerative capacity of various brain regions of larval and metamorphosed *X. laevis* are related to differences of the undifferentiated cell populations (Filoni *et al*. 1995). In the early-larval stages, the populations of proliferating cells are very widespread in the brain, while in late-larval stages and after metamorphosis the cells are discretely restricted to proliferation zones. The restriction of proliferating cells occurs later in the telencephalon than in the rhombencephalon and mesencephalon, and correlates with the regenerative capacity of the respective brain region (Filoni *et al*. 1995). However, more recently, it was shown that neither the lack of proliferative response nor an intrinsic deficiency in

organizational capacity is the main reason to why post-metamorphic frogs cannot regenerate severe telencephalic lesions. Instead, slow and imperfect sealing of the wound by ependymal cells seems to be a contributing factor for the poor regeneration capability (Yoshino & Tochinai 2004).

Fish

It has been shown that different teleost fish species have a tremendous capability for regeneration of the CNS, spinal cord and retina (Kirsche 1965; Zupanc 2001). This comprises regeneration after incisions in the brain or after removal of whole brain parts, as well as regeneration of the spinal cord after transection. It is also evident that the regeneration can take place throughout the whole length of the rostro-caudal brain axis. Neural regeneration has been demonstrated in many different teleost lineages suggesting that it is a common feature of teleost fish in general (Kirsche 1950, 1960, 1965; Richter 1965, 1969; Segaar 1965; Zupanc 2001). Different types of physical lesions have been carried out to test the regenerative abilities of post-embryonic teleost brains, ranging from stab wounds to removal of whole brain parts. Compensatory proliferation and morphological restoration are detected in the dorsal telencephalon of juvenile and adult guppies, *Poecilia reticulata* (*Lebistes reticulatus*) in response to a stab wound (Richter 1969). The stab wound first induces degeneration after which mitotic cells are detected in the constitutively active proliferation zones of the dorsal and ventral telencephalon. Later, the produced cells show a directional migration towards the lesion site and participate in the restorative process. Sprouting of capillaries into the injured area is also detected (Richter 1969). Interestingly, the regenerative capacity of the brain in the adult guppy is diminished. The reduction in regenerative capacity may be related to a reduction of proliferation in the adult brain (Richter 1969). Similarly, the architecture of the optic tectum is restored after a stab wound in juveniles of thecyprinid minnow, *Leucaspius delineatus* (Richter 1965). The necessity of proliferative zones for regenerative success in the teleost brain was elegantly demonstrated in a series of experiments by Kirsche (1960). Kirsche performed a series of experiments in which he removed parts or the whole lobes of optic tectum in juvenile and adult carps, *Carassius carassius*. Only when the proliferative zones remained, the regenerative process could take place. In the juvenile fish, he carried out four different types of experiments: first, unilateral ablation of the optic tectum, keeping the three

proliferative zones (dorsal, basal and caudal) intact. Second, unilateral ablation of the optic tectum and ablation of the rostral part of the dorsal proliferative zone. Third, unilateral ablation of the optic tectum together with the removal of the dorsal and caudal proliferative zones. Fourth, deletion of the whole optic tectum and removal of all proliferative zones. In the first case, the optic tectum regenerated, although the macro architecture did not recover to its original form. The regenerated tectum stayed smaller and the architecture of the layers was not completely restored due to the irregularly distributed proliferation zones. Nevertheless, new neurons and glia were produced and the fibre layers of the optic tectum were reconstituted, indicating that the micro arrangement was correctly restored. Increased removal of the proliferative zones lead to successively diminished regenerative capacity of the lesioned optic tect. When the front part of the dorsal proliferative zone was removed completely, there was no regeneration in that area. Furthermore, when all the proliferative areas were removed, regeneration did not take place at all. In the adult *C. carassius*, however, the first type of experiment also did not lead to any regenerative success, which Kirsche explains with an exhaustion of the proliferative zones. Taken together, these experiments illustrate the importance of the constitutively active proliferation zones in relation to successful histogenesis in the post-embryonic teleost brain. These experiments also illustrate that there are differences in the regenerative capacity between the juvenile and adult teleost brains. In a more recent study, the cerebellum of the weakly electric knifefish, *A.leptorhynchus*, regenerates completely within weeks after a stab wound (Zupanc 1999; reviewed in Zupanc 2001). There is normally a significant amount of constitutive proliferation in the cerebellum of adult *Apteronotus* (Zupanc & Horschke 1995; Zupanc & Ott 1999). The stab wound induces enhanced proliferation in both the constitutively active zones and in ectopic locations at the lesion site. There is a substantial increase of glial fibrillary acidic protein (GFAP)-positive radial fibres at the lesion site as well as the migration of newly produced cells from the proliferation zones (Clint & Zupanc 2001). At least some of the generated cells are differentiating into granule neurons that emit projections (Zupanc & Ott 1999).

2.7 Molecular mechanisms of neural repair in zebrafish

The process of regeneration definitely involves turning on "redevelopment." For instance, if a neuron will be generated, genes that govern the specification and differentiation of that particular subtype of neuron during development such as Delta-Notch signaling or pathways leading to subtype specification, axonogenesis, or synaptogenesis—become active again. However, in case of neuronal loss, be it acute or chronic, nonphysiological events that are normally not seen during development take place. These include stress response, inflammation, wound healing mechanisms, and other phenomena related to the breach of the homeostatic balance. In most cases, these phenomena were shown to be detrimental for the regenerative ability in mammals (Burda and Sofroniew 2014), and they have to be overcome for regeneration to succeed. On the other hand, zebrafish can regenerate even though experiencing such nonphysiological circumstances. Therefore, a plausible hypothesisis that the organisms that can regenerate might be use some "intermediary" molecular programs that link the initial events to the redevelopment of tissues. These intermediate programs could be specifically induced after neuronal loss and might be crucial to regenerative success as they might set the stage to alleviate the negative consequences of homeostatic compromise and to turn on the programs of redevelopment. A scientific challenge based on this hypothesis is to identify such putative intermediary genes and pathways in regenerating organisms. Thus, zebrafish serves as a promising animal model to this purpose.

Recent studies have so far shown that, during regeneration of the adult zebrafish tissues, genes that are not expressed during the development of the corresponding tissues can be induced (Kizil et al., 2014). Specifically in adult zebrafish brain, acute inflammation has been shown to contribute to activation of neural progenitor cells with radial glial identity. Leukotriene C4 (LTC4) was shown to emanate from immune cells that populate the brain tissue after lesion and activate an intracellular signal transduction in radial glial cells, where the cysteinyl leukotriene receptor 1 (cystlr1) is present (Kyritsis et al., 2012). Injection of LTC4 using cerebroventricular microinjection (CVMI) (Kizil et al., 2013) is sufficient to increase the proliferation of radial glial cells and subsequent regenerative neurogenesis by activating regeneration-specific molecular program involving the zinc finger transcription

factor gata3. This gene is interesting as it is not expressed during development and homeostatic adult telencephalons of the zebrafish brain, but is induced in the RGCs shortly after lesion. Knockdown experiments using CVMI and Gata3 antisense morpholinos showed that Gata3 does not partake in regulation of constitutive neurogenesis, but is specifically required for the injury-induced cell proliferation response of the ventricular neurogenic progenitor cells and subsequent reactive neurogenesis: two hallmarks of the regenerative response gata3 are injury induced inother regenerating organs of zebrafish and are functionally required for the proliferation of progenitor cells. Such a dynamic expression and biological relevance of gata3 suggests that this gene might be part of a molecular program that zebrafish might be using universally for regenerating its tissues. Additionally, gata3 has not been documented to be activated in mammalian brains upon injury or insult so far, suggesting that such genes like gata3 might underlie the disparity between the regenerative capacities of zebrafish and mammalian brains. Therefore, such molecular programs or novel epistatic interactions could be used as biomarkers of brain injury and regenerative response.

Another study identified the 7-pass transmembrane domain chemokine receptor Cxcr5 as a gene required for regenerative neurogenesis but not for increased proliferation of the radial glial cells (Kizil et al., 2012). Cxcr5 is expressed at low levels in the RGCs in homeostatic unlesioned adult zebrafish telencephalon and is predominantly absent in neurons. After a lesion, cxcr5 expression increases dramatically in periventricular neurons. Blocking this chemokine signaling by overexpressing a dominant negative version of the Cxcr5 receptor that lacks the transmembrane domains 5, 6, and 7, which renders the receptor incapable of eliciting an intracellular signaling cascade, does not result in any change in RGC proliferation in unlesioned or lesioned brains. However, the same genetic knockdown results in reduced number of newborn neurons only after lesion (Kizil et al., 2012). Similarly, morpholino-mediated knockdown of cxcr5 gene in adult zebrafish brain leads to similar reduction of regenerative neurogenesis. Conversely, when the full-length Cxcr5 is overexpressed, production of new neurons increased significantly only after lesion despite no change in RGC proliferation. These findings suggest that Cxcr5-mediated chemokine signaling might be specifically required for generation of

neurons after acute neuronal loss and might also serve as a biomarker for regenerative neurogenesis.

Alternatively, some molecular programs could be turned off or overridden during regeneration of adult zebrafish brain (Diotel et al., 2013). For instance, estradiol was shown to hamper proliferation of progenitor cells in the adult zebrafish brain under homeostatic conditions, while this regulation does not take place during regeneration. Since radial glial cells specifically express the aromatase that synthesizes estrogen (Pellegrini et al., 2007), certain physiological conditions might downregulate signaling pathways that are prevalent during homeostatic state. Collectively, an important but still partial list of molecular programs that allow the special regenerative response in the zebrafish brain was identified as described above. Interestingly, some of those programs are induced only during regenerative stage and are essential for production of newborn neurons. These findings suggest that regenerating organisms such as zebrafish could use special molecular programs to enable regenerative neurogenesis, and these programs might be responsible for different regenerative capacities of zebrafish and mammalian brains.

AIMS

The present research was aimed to investigate the role of BDNF in zebrafish brain. More specifically, since the exstensive adult neurogenesis and brain reparative properties of zebrafish, the investigation has been focused on BDNF involvement in regenerative processes of injuried telencephalon.

The study has been conceived in successive phases:

Firstly, the distribution of BDNF messengers in the whole brain of juvenile and adult zebrafish has been investigated by means of in situ hybridization (ISH).

Then, the study has been adressed to recognize the cell type synthesizing BDNF by employing double staining (ISH for BDNF mRNA /immunocytochemistry for cell markers).

Finally, the role of BDNF in regenerative processes has been studied in the dorsal telencephalon. Indeed, the zebrafish adult telencephalon is an excellent model system for comparative studies on adult neurogenesis, as it shares mammalian features while retaining a much broader capacity for neuronal production.

The following tasks have been investigated in the zebrafish telencephalon at different times from injury:

1) BDNF mRNA amount, evaluated in brain extracts by means of qPCR;

2) percentage of BDNF positive cells in the dorsal telencephalon, evaluated by means of ISH;

3) Characterization of BDNF expressing cells, by means of double staining (mRNA/cell marker).

3. MATERIALS AND METHODS

3.1 Animals and tissue processing

Animals were handled and sacrificed in agreement with the European Union regulation concerning the use and protection of experimental animals (Directive 86/609/EEC). Three to six months' animals were housed in the zebrafish facilities of the BIOSIT (INRA LPGP, Rennes) under standard conditions of photoperiod (14 hours light and 10 hours dark) and temperature (28°C). Zebrafish embryos were spawned and kept during 7 days in 100 ml glass bottles in an incubator. For sacrifice, sexually mature females and males were anesthetized in tricaine (MS-222) before spinal cord sectioning. The sex was then determined by direct examination of the gonads under a binocular stereoscopic microscope. After skull bone opening, zebrafish were fixed overnight at 4 °C in phosphate-buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde (PAF). The brain was then removed and fixed one more day in PSB-PAF. Seven days post-fertilization (pdf) larvae were anesthetized on ice and fixed overnight in the same conditions. Both larvae and adults brains were processed for paraffin embedding and microtome sections 7μm were mounted on poly-lysine slides.

3.2 Equipment

General lab equipment was used for the molecular and cell biology techniques, including balances, centrifuges, electrophoresis equipment, heating blocks and plates, hybridization and incubation ovens, micropipettes, PCR and gradient thermocyclers, pH meter, shakers, sterile hood, UV-transilluminator, vortexes and water baths. Brain sections observation was carried out with an Olympus Provis miroscope (AX70) equipped with a DP71 digital camera or with an Olympus confocal microscope (BX61WI) or a Zeiss Imager Z1 equipped with the Apotome module.

3.3 Solutions

Solutions were prepared with distillate water. Solutions were filtered or sterilized (0.2-0.45 μm pore diameter). Glassware was autoclaved. Standard stock solutions like, Tris-Hcl pH 8, Tris-base, PBS, SSC, Nacl, and culture media like LB and SOC.

3.4 RNA extraction

RNA extraction was performed on a pool of 10 whole brains. For brain repair experiments, 10 non-injured telencephali and injured at 1, 4, 7, 15 day were pooled before the extraction procedure. Brains and telencephali were sonicated (10 s, three times) in 1 mL Trizol Reagent (Gibco, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's protocol.

3.5 Quantitative real-time PCR

Reverse transcription was carried out by incubating 2 μg total RNA with 1 μg of random primer oligonucleotides, 2.5 mM dNTPs and 50 U MMLV-RT (Promega) in the appropriate buffer for 10 min at 65 °C and 60 min at 37 °C. Polymerase chain reaction(PCR) experiments were performed in an Cycler thermocycler coupled to the MyiQ detector (Bio-Rad. Hercules, CA, USA) using Q SYBR-Green Supermix (Bio-Rad) according to the manufacturer's protocol. The following primers were used:

GAPDH (Fw) 5-CGCTGGCATCTCCCTCAA-3
GAPDH (Rev) 5-TCAGCAACACGATGGCTGTAG-3

BDNF (Fw) 5-ATAGTAACGAACAGGATGG-3
BDNF (Rev) 5-GCTCAGTCATGGGAGTCC-3

PCNA (Fw) 5’-GCTGTACGACGAGTCAAC

PCNA (Rev) 5’-CTCTTTCACAGGCTGACTCTAC

Expression levels of GAPDH mRNA were used to normalize the expression levels of the other genes. Melting curve and PCR efficiency analyses were performed to confirm correct amplification.

3.6 Synthesis of the BDNF riboprobes for in situ hybridization

For BDNF riboprobes synthesis, has been used a pCMV-Sport 6.1 plasmid (Fig. 6) containing the full-length BDNF (1446 bp) cDNA I.M.A.G.E (Unigene DR.132862; Entrez Gene 58118).

BDNF Insert

TTAGCTGTGCTATGATTTCAGCACTTTGGACAGAGGCACCGGGAGTCGTTGAAC
GCTGCGGACTAACACGGAGTGAGTGGACTTCGCCTTTTGTGTTTTGCCAAAGCTA
GGGATTGTTTCACTTTTTCCTTCTTTTTTGCTCTCGGAGGGAGACCCGTGAGAAA
ACAAAACAAACAAACAAACGCGAGGAAAAGCATCAGTCGATAGCGGGGCTGCT
TACTTTGCGCTTGGAGTTTTGTCGCCACTTAGAGCATGTGAAAGTTGAAATCGTA
GAGCTGCTAGTGTACTCGTACTTTGCAATCTCCACTTGGTGCACAGCGTTAACTT
TGGGAAATGCAAGTGTTGGTCTTGGAGATCTAACCACTGATAGTGGTTGTCAGC
TCTTATAAAGTTCCAACAGGTTAGAAGAGTGATGACCATCCTGTTCGTTACTATG
GTTATTTCATACTTCAGTTGCATGAGAGCTGCGCCCATGAGAGAGATCCCGGGC
GTGCAGGGGGGCCACCGGGCCGAGGGCTACCTGGGCGCCGCTGCCGCTGCTGCC
GCTGCCGTCACATCGGGCAGCCGAGGCCACGGGACTCCACAGAGTGGGGGCGG
GCTGCCCTCGCTCACGGACACTTTCGAGCAGGTCATTGAGGAGTTGCTTGAGGT
GGAAGGGGAAGCGACGCAGCAACTGGGGCCTGGGGCCGACCAGGGCCAAGGAG
GGGGCGGCCCTATAGATGCGGCAGACTCGAAGGACGTTGACCTGTATGCCTCGC
GGGTGATGATCAGCAACCAAGTGCCTTTGGAGCCGCCGTTACTCTTTCTCTTGGA
GGAATACAAAAACTACCTGGATGCCGCCAACATGTCGATGCGTGTGCGGCGACA
CTCGGACCCCGCACGGCGGGGGGAGCTCAGCGTTTGTGACAGTATTAGCCAGTG
GGTGACAGCTGTGGACAAAAAGACGGCAATAGACATGTCGGGCCAGACGGTCA
CCGTTCTGGAGAAGGTCCCCGTGACTAATGGTCAGCTGAAGCAATACTTTTACG
AGACCAAATGCAACCCCTTGGGGTACACAAAGGAGGGCTGCCGAGGAATAGAC
AAGCGGCACTATAACTCGCAATGCCGGACAACCCAGTCTTACGTGCGAGCCCTT
ACCATGGATAGCAAAAGGAAGATCGGCTGGCGGTTTATACGGATAGACACTTCG
TGTGTATGCACATTGACCATTAAGAGGGGCAGATAGTGTACACAATGTATAGAT
TTTATTGAGAGTTCTAAAAAAAGAGAGAGAAAGAGAAAATATCTATTTGTAT
ATACATAACAGGGTAAATTATTCAGTCAGATAAAAATTTTATGGACTGCATGTA
AAAAAGAAAAAGTTTATACAGTAAGTGATACTACAGTCTATTTATTGAACATAT
TCATGACCTTGTAAACAATTAAAAAAAGATCTGATCAGTCA

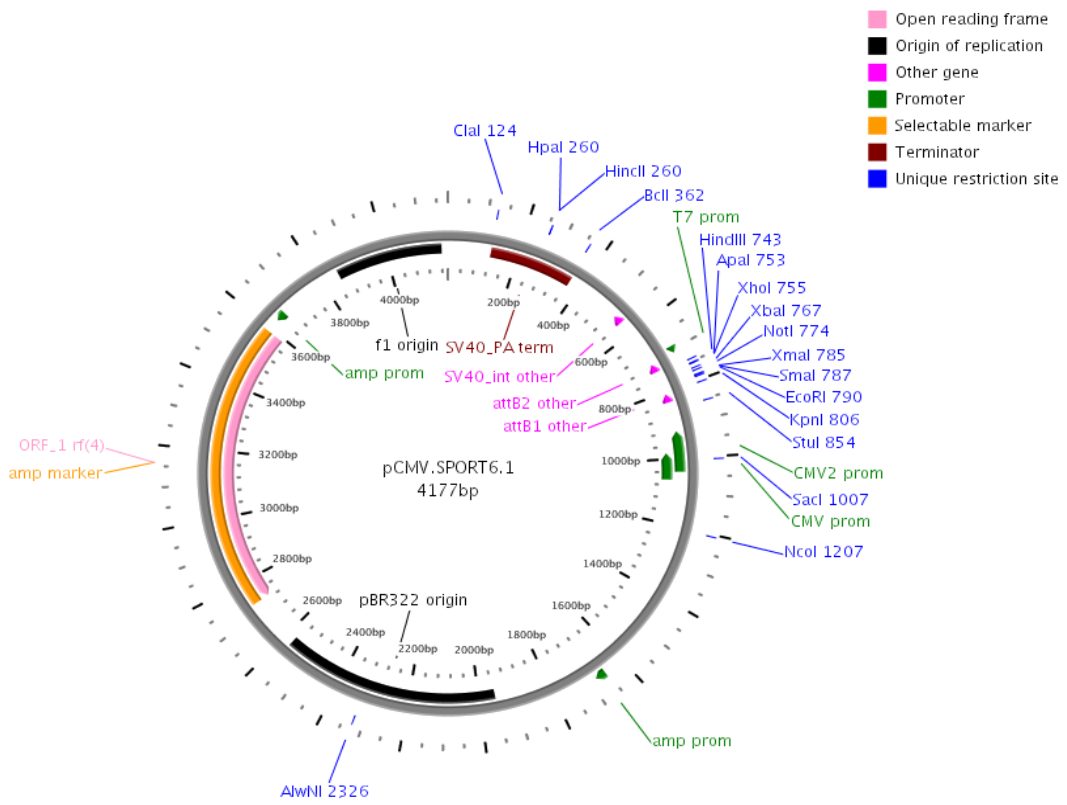


Fig. 6 Map of plasmid pCMV.SPORT 6.1

a) Transformation into thermocompetent cells

The transformation reaction was carried out following the supplier instructions. After heat shock, the bacteria were plated onto LB agar plates containing the appropriate antibiotic to select only the transformed bacteria. Transformants were screened, and the white colonies containing the insert were then cultivated.

Single bacterial colonies were picked and inoculated in LB medium containing the appropriate antibiotic plates. The bacteria were grown for one to three hours in an orbital rotator at 37°C.

b) DNA plasmid purification

Using the kit NucleoSpin® Extract II (MACHEREY-NAGEL GmbH & Co, Düren, Germany). DNA was purified from contaminations like salts and soluble macromolecular components, which were removed by a simple washing step with ethanolic Wash Buffer NT3. Pure DNA was finally eluted under low ionic strength conditions with slightly alkaline Elution Buffer NE.

c) Sequencing

Plasmidic DNA sequencing was carried out at the Laboratory of Genome analysis Inserm U1085, Université de Rennes 1 (IRSET). Sequence analysis was carried out using BLAST (Basic Local Alignment Search Tool).

d) Plasmid Linearization and DNA purification Machery protocoll

Digest 10 µg plasmid DNA using appropriate restriction enzymes (EcoRI and Not1). For making antisense probes, used a unique restriction site 5' to the insert which contain a sense 5'→3' sequence. For making sense (control) probes, used a unique restriction site 3' to the insert.

BDNF Sense probe: Linearized plasmid with Not1 and use SP6 RNA pol.
BDNF Antisense probe: Linearized plasmid with EcoRI and use T7 RNA pol.

1) Linearization reaction in a 1.5-ml microtube:

- 10× Buffer 10 µl

- Plasmid DNA 10 µg

- Restriction enzyme 5 µl (10–15 U/µl)

- Sterile water make to 100 µl and incubate 37 °C for 2 h

2) Plasmid DNA purification column Macherey

- Add H2O qs 100 µl

- Add 500 .mu.l of Buffer AW preheated to 50 °C Vortex and place on the column

- Centrifuge 60 sec. 11000 g. Remove the filtrate.

- Wash the column with 600 .mu.l of A4.

- Centrifuge 60 sec. 11000 g Remove the filtrate.

- Centrifuge 2 minutes to remove any residual trace buffer.

- Place 20 µl E. D. on the center of the column.

- Allow 1 min contact and centrifuge 1 min

- Nanodrope assay and agarose gel 1% Control

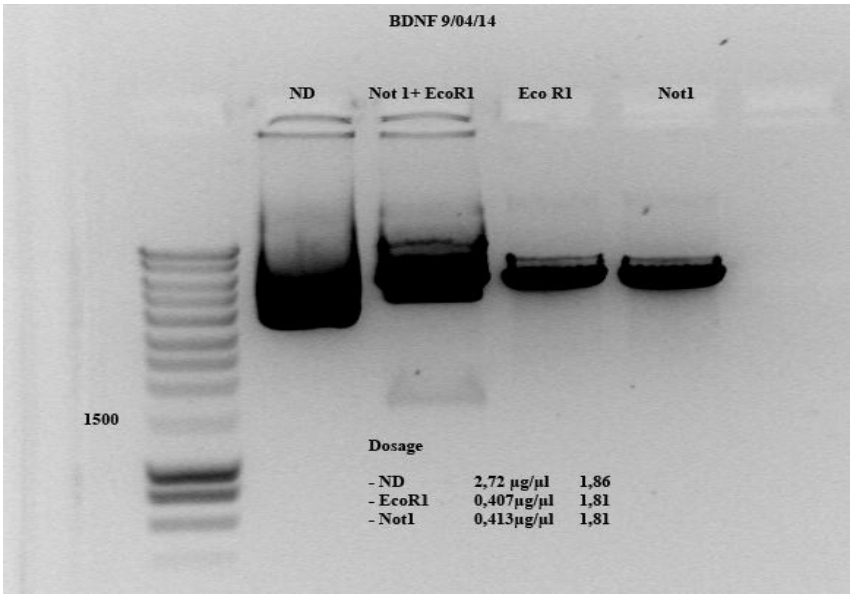


Fig. 7 Agarose Gel of plasmid linearized.

e) In vitro transcription of the probes

In vitro transcription of the probes following Roche protocol reaction:

- Linearized plasmid DNA 1,5 µg

- 10×DIG RNA labeling mix 3 µl

- 10× Transcription buffer 3 µl

- RNase Out (40 U/µl) 1,5 µl

- SP6 or T7 RNA pol. (20 U/µl; Roche) 1,5 µl

- Sterile water make to (21-x µl)

- Vortex and centrifuge

- Incubate for 2 h respectively; T7 RNA pol at 37°, and SP6 RNA pol at 42°C

- Add 3µl DNase, incubate 30 min at 37°C

f) Purification of the riboprobes

Probes purification using columns Nucleo Spin RNA Clean-up:

- Add the RNase-free water qs 100 µl (for one sample)

- Prepare a mix RA1/Ethanol 100 (300 µl. + 300 µl) for sample.

- Add 600 µl of the mix to 100 µl of each sample and vortex to mix. Place on the column placed on 2 ml microcentrifuge tube and centrifuge for 30 sec at 8000 g.

- Remove the filtrate and place the column in a new 2 ml. tube.

- Wash the column with 700 µl. Of Buffer RA3. Centrifuge 30 seconds at 8000 g.

- Remove filtrate and rewash with 350 µl. of Buffer RA3. Centrifuge 2 min at 8000g Put the open column in a 1.5 ml tube and wait 3 minutes. Elute with 30 µl. of RNase-free water. Centrifuge for 1 min at 8000 g. Assay and control agarose gel

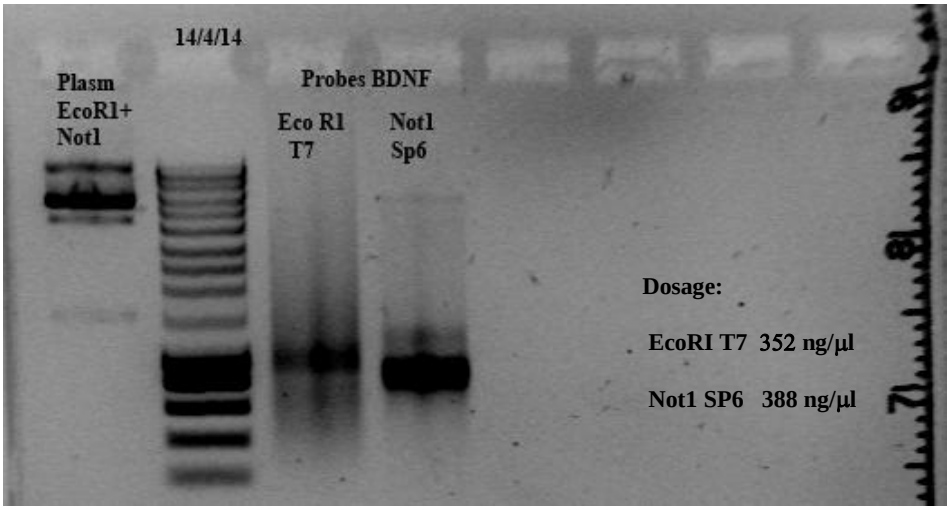


Fig. 8 Agarose gel of BDNF riboprobes.

3.7 In situ hybridization

For BDNF riboprobes synthesis, we used a pCMV-Sport 6.1 plasmid containing the full-length BDNF cDNA (Unigene DR.132862; Entrez Gene 58118). Antisense and sense riboprobes were generated with DIG RNA Labelling Mix (Roche Diagnostic, Indianapolis, IN) by in vitro transcription, using T7 polymerase (Roche-diagnostic) and SP6 polymerase (Roche-diagnostic) on plasmid linearized by EcoRI and Not1. To check the specificity of the staining, the sense and antisense riboprobes were always hybridized on adjacent sections. No staining was observed on sections hybridized with the sense riboprobes either in adults and larvae. Paraffin sections (7 □m) were deparaffinized with OTTIX and rehydrated through a series of graded ethanol (100– 30%). Sections were washed in PBS-NaCl (0.85%) and post-fixed for 20 minutes in PBS-PFA 4%. Tissue were rinsed in PBS and treated for 7 minutes with proteinase K (2mg/ml) diluted in PBS at 37°C. The reaction was stopped in PBS before post-fixation for 20 minutes in 4% PBS-PAF and the slides were rinsed 10 minutes in PBS and 10 minutes in standard saline citrate (SSC 2x). The sections were incubated overnight at 62.5°C in a moist chamber with the probes (1.5 mg/ml) diluted in hybridization buffer (formamide 50%; SSC 2X, Denhart 5 X, yeast tRNA 50 mg/ml, EDTA 4 mM, dextran sulfate 2.5%). On the following day, slides were rinsed with SCC 2x, SCC 2x/formamide 50%, SSC 0.2x and SSC 0.1x. Next, they were dipped in Tris-HCl/NaCl buffer (100 mM, Tris-HCl pH 7.5, 150 mM NaCl) and washed in the same buffer containing 0.1% Triton and 0.5% of milk powder. Sections were incubated overnight at room temperature with anti-digoxigenin alkaline phosphatase Fab fragments (1:2000, Roche Diagnostic). On the next day, slides were rinsed in Tris-HCl/NaCl buffer and washed three times with Tris-HCl 100 mM (pH 8) containing NaCl (100 mM) and MgCl2 (10mM). The hybridization signal was revealed with the HNPP/Fast-Red detection kit (Roche Diagnostic) for 6 or 12 hours according to the manufacturer's instructions. Sections were washed several times in PBS, mounted in Vectashield medium containing DAPI and coverslipped for microscopic analysis.

3.8 Immunohistochemistry

To get further insights in the identification of *bdnf* -expressing cells, sections were processed for immunohistochemistry just after in situ hybridization. To stop in situ hybridization reaction, sections were dipped 5 minutes in PBS-PAF 4%, washed in PBS and PBS/Triton (0,2%) to perform immunohistochemistry as follows: sections were incubated overnight at room temperature with different primary antibodies diluted in PBS containing 0.5% milk powder. Sections were washed three times in PBS-Triton 0.2% and incubated with goat anti-rabbit or anti-mouse Alexa Fluor 488 or 594 (1:200 Invitrogen Molecular Probes) for 2 hours. Tissue sections were washed in PBS-Triton 0,2%, and slides were mounted with the Vectashield medium containing DAPI for nuclei conterstaining (Vector Laboratories, Burlingame, CA).
Radial glial cells were identified with a rabbit anti- aromatase B (anti-zebrafish aromatase B, 1:200) and with a rabbit anti-BLBP (Brain Lipid Binding Protein, 1:100, Abcam, Reference 32423). The specificity of these antibodies was previously assessed in zebrafish. Three pan-neuronal markers were used to identify post-mitotic neurons. The labeling is processed with a mouse monoclonal anti-acetylated-tubulin (1:100, Sigma-Aldrich, clone 6-11B-1, Reference T 6793), with a mouse monoclonal anti-Huc/d (1:20, Molecular Probes, clone 16A11, Reference A21271) or with a mouse monoclonal anti MAP2 (Microtube-Associated Protein2, 1:100, Abcam, clone AP-20, Reference 11268). The specificity of these antibodies was previously assessed in zebrafish. Proliferative cells were visualized with a monoclonal antibody raised against PCNA (Proliferative Cell Nuclear Antigen, 1:100, Clone PC10, Dako, Reference M0879). The specificity of this antibody for PCNA has been validated in many vertebrate species including zebrafish.

3.9 Telencephalon lesion

Before surgery, adult male zebrafish were anesthetized with tricaine MS-222, 50 mg/ml in 100 ml of the aquarium H20. For the telencephalon injury, a sterile needle (BD Microlance 3; 27 G ½″; 0.3 mm × 13 mm) was inserted in one of the telencephalic hemispheres, guided by landmarks on the head, following a dorso-ventral axis. The localization of the injury site was performed as shown in (Fig.9 A-

B). After positioning the needle tip on the surface of the skull, pressure was applied vertically to achieve a penetration depth of 1.5 mm.

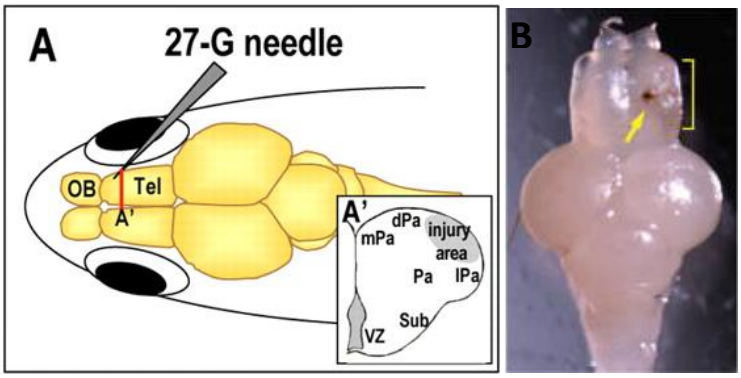


Fig. 9 (A) Scheme Lesion and (B) injured brain

3.10 Cell counting in zebrafish injured brain

BDNF positive cells were quantified using photographs of specific brain regions (regions with sky color). For each brain, the regions or nuclei of interest were located by identifying neuroanatomical structures with DAPI and counting proceeded in blind conditions.

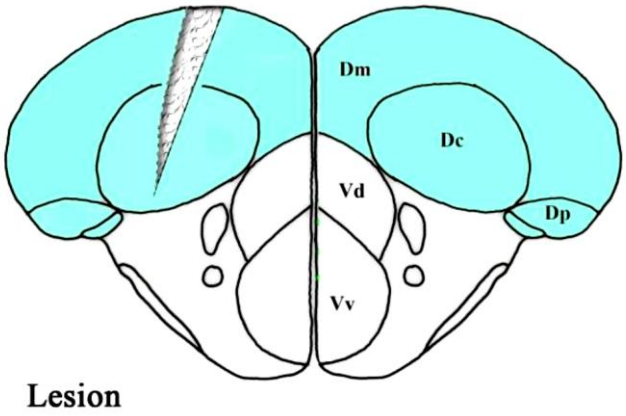


Figura 10 Scheme of telencephalon injured.

RESULTS

Bdnf messengers in the brain of 7 days old zebrafish larvae

To analyze in detail the distribution of *bdnf* transcripts in the brain of larva, I performed in situ Hybridization on series of transverse sections corresponding to the whole zebrafish brain (Fig 11). From anterior to posterior, *bdnf* messengers were detected in the the olfactory rosettes and in the anterior telencephalon (fig. 11A-D). At this level positive cells were mostly located into the dorsal and median parts of the dorsal telencephalon. More caudally, labeled cells were still observed in the dorsal telencephalon, preferentially in the central telencephalon and also in the the preoptic area (fig. 11E-H). *Bdnf* expression was particularly abundant in the thalamic area (fig 11I-J) and was also consistently observed in the optic tectum, particularly in the periventricular layer (fig 11K-L). More caudally, *bdnf* mRNA was also detected at different levels of the midbrain tegmentum, in particular in the reticular formation (fig 12H-J). The results concerning BDNF expression in the brain of larvae are summarized in Table1.

Attempts to identify the phenotype of these *bdnf*-positive cells were made by using double staining with either PCNA, aromatase B or Huc/d as markers of cell proliferation, radial glial cells or neurons respectively. Data showed that, in all studied regions, *bdnf* expressing-cells never correspond to PCNA-positive cells indicating that they are certainly post-mitotic (fig 12A-D). *Bdnf*-positive cells do not co-express with aromatase B attestesting that they do not correspond to radial glia progenitors (data not shown). Regarding the pan-neuronal marker Huc/d, although the coupling of immunohistochemistry with in situ hybridization resulted in some loss of *bdnf* messengers, it appears that in many regions such as the telencephalon, the optic tectum and the midbrain tegmentum, the signals generated overlap, suggesting that *bdnf*-expressing cells may rapidly gain at least a neuronal phenotype (fig 12E-J).

Table 1 Distribution of BDNF transcripts in larval zebrafish brain

Olfactofy bulb	+++
Pallium	++
Subpallium	+
Preoptic region	++
Habenula	+
Dorsal thalamus	+++
Dorsal part of posterior tuberculum	+++
Ventral part of posterior tuberculum	++
Intermediate hypothalamus	++
Rostral hypothalamus	++
Caudal hypothalamus	+++
Optic tect	+++
Tegmentum	+++

Bdnf messengers in the brain of adult zebrafish

After analysis of the *bdnf* mRNA distribution in larva zebrafish, I studied the distribution of BDNF transcript on transverse brain sections of sexually adult mature males and females from the olfactory bulbs to the medulla oblongata. The overall pattern of *bdnf* expression was identical in all animals and, in particular, there were no differences between sexes. For this reason, micrographs presented in fig 13 and 14 and data showed in table 2 refer to both male and female brains. The olfactory bulbs displayed few *bdnf*-positive cells in the glomerular cell layer and in the external and internal cell layers (data not shown). A strong hybridization staining was observed in a large number of small round cells localized in the dorsal telencephalon particularly in its medial, lateral and posterior divisions (fig 13A-C' and E). The ventral part of the telencephalon exhibited fewer and weakly labeled cells in the posterior zone and intensely stained cells in the entopedoncular nucleus (fig 13A-B, E). In the diencephalon, we observed an intense positive signal in the parvocellular (fig 13D-E), in magnocellular nuclei of the preoptic area and in the entopeduncular nucleus and in the suprachiasmatic nucleus (fig 13G). *Bdnf* was strongly expressed in cells of the habenula, specifically in its dorsal component (fig 13H-I) and in the ventrolateral and ventromedial nuclei of ventral thalamus (fig 13F-F'), but the intensity of labeling was weaker than in the dorsal thalamic nuclei. Transcripts were abundantly reported in the anterior, dorsalposterior and central posterior nuclei of dorsal thalamus (fig 14B-B', G). More ventrally, *bdnf* messengers were expressed in the posterior tuberal nucleus (fig 14D-D', F) and preglomerular nuclei. In the hypothalamus, *bdnf* messengers were abundantly expressed in the periventricular nucleus of its ventral and dorsal part (fig 14A-B', G) in the diffuse and central nuclei of the inferior lobe and in the mammilary body. Between diencephalon and mesencephalon, in the so-called synencephalon, the nucleus of the medial longitudinal fascicle displayed few *bdnf* positive cells, whereas *bdnf* messengers were highly expressed in the dorsal and ventral periventricular pretectal nuclei. In the mesencephalon, *bdnf*-positive cells were also observed in the torus longitudinalis. The periventricular gray zone of the optic tectum exhibited numerous cells expressing *bdnf* messengers (fig 14C-C', G). In the tegmentum, *bdnf* hybridization signal was observed in the central nucleus of the torus semicircularis

and in the interpeduncular nucleus. In the medulla oblongata, *bdnf* messengers were highly expressed in the secondary gustatory nucleus and in few cells of the vagal lobe. Finally, large *bdnf*-positive perikarya were seen in the superior and inferior reticular formation (fig 14E-E, H). In the table 2 the distribution of BDNF mRNA has been summarized in the brain of adult zebrafish.

Table 2 BDNF distribution in adult zebrafish brain (ISH)

Olfactory bulbs	GL	+
	ECL	+
	ICL	-
	LOT	+
	MOT	+
Dorsal telencephalic area	Dl	+
	Dm	++
	Dc	++
	Dd	+
	Dp	++
Ventral telencephalic area	End	+
	Vp	+
Area praeoptica	PPa	+
	PM	-
	PPp	+
	SC	-
Epithalamus	Had	+
	Hav	+
Dorsal thalamus	A	-
	DP	+

	CP		-
Ventral thalamus	VM		+
	VL		+
Posterior Tuberculum	PTN		+
	PGa		+
	PGl		+
	PGm		+
Hypothalamus	DIL		-
	Hv		+
	ATN		+
	LH		+
	CIL		+
	Hd		+
	CM		+
Synencephalon	NMLF		+
	PPv		+
	PPd		+
Tectum opticum	PGZ		++
	DWZ		-
	CZ		+
	SWGZ		-
	TL		+
Torus semicircularis	TSc		+
Tegmentum	NIn		-
	SRF		+
Cerebellum	valvula	GCL	+
Medulla oblongata	SGN		++
	IRF		++
	LX		+

Then, to identify the nature of *bdnf*-expressing cells in the brain of adult zebrafish, I performed *bdnf* in situ hybridization in combination with immunohistochemistry using antibodies directed against different types of markers (proliferation, neuronal or glial markers). As described above, *bdnf*-expressing cells were visualized close to the ventricular surface in many regions such as the telencephalon, the preoptic area, the hypothalamus, the thalamus, and the optic tectum. To investigate if cells undergoing proliferation can be *bdnf*-expressing cells, was performed PCNA labeling after *bdnf* in situ hybridization. PCNA-labeled cells were positioned along the ventricular cavities in the telencephalon (Fig. 15A-A'''), in the thalamus (Fig. 15B, B') and in the hypothalamus (Fig. 15B, B''). In those regions, can observe *bdnf*-expressing cells (in red in the images) very close to PCNA-labeled nuclei, but double staining studies indicated that *bdnf* mRNA were never expressed by proliferating cells.

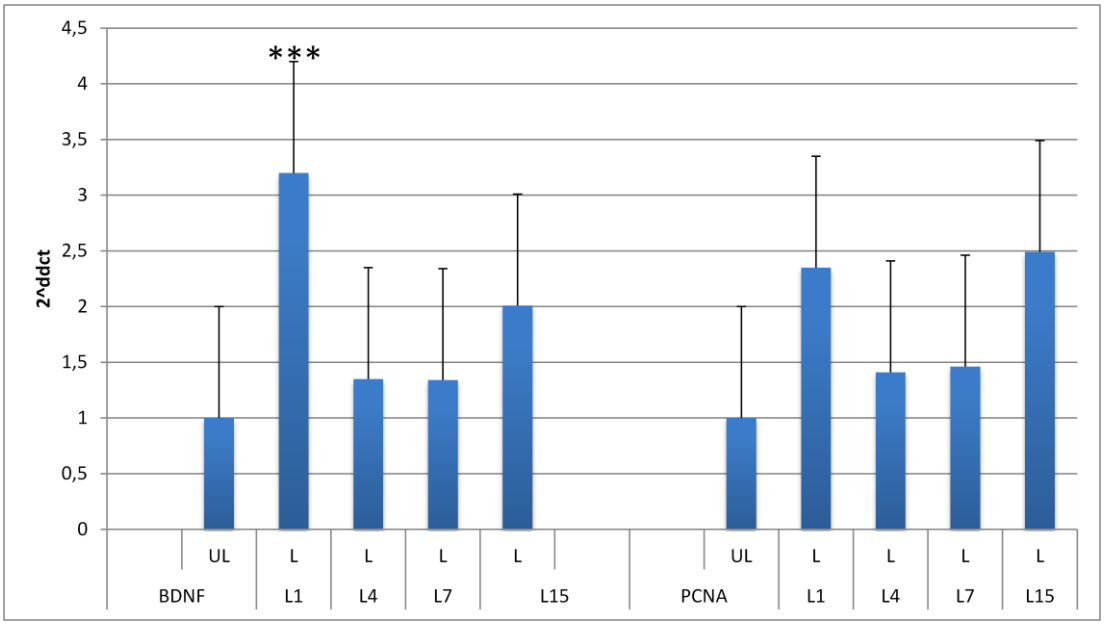
To test whether some of the *bdnf*-expressing cells could be radial glial cells, combined *bdnf* hybridization with immunohistochemistry against aromatase B and BLBP, two well-established markers of radial glial cells in fish. As shown in Figure 16, aromatase B-positive cells never co-expressed *bdnf* mRNA in the ventral telencephalon (Fig. 16A, A'), the preoptic area (Fig. 16B, B'), entopeduncular nucleus (Fig. 16B, B'') and the thalamus (Fig. 16C, C'). Analysis of sections from the entire brain using an Apotome-equipped Zeiss microscope did not provide any evidence for expression of *bdnf* mRNAs in radial glia (Fig. 16D-D''). Similarly, double staining with BLBP antibodies failed to demonstrate any expression of *bdnf* in the radial glial cells (Fig. 16E-E''). Antibodies against neuronal markers indicated that *bdnf* mRNA were expressed in cells with neuronal phenotype in all brain regions investigated. Immunohistochemistry using anti-MAP2 antibody indicated that *bdnf* is exclusively expressed in neuronal cells as shown in the telencephalon with confocal orthogonal projections (Fig. 17A, B). Immunohistochemistry using anti- acetylated-tubulin antibody confirmed that *bdnf* is exclusively expressed in neuronal cells in the telencephalon, in the habenula (Fig. 17C), the thalamus (Fig. 17D), the hypothalamus and the optic tectum (Fig. 17E, E').

BDNF expression in injured zebrafish brain

After collecting data regarding the BDNF expression in larval and adult zebrafish brain under normal physiological conditions, the research has been focused on the involvement of BDNF in regenerative processes of injuried telencephalon. For this purpose after anesthesia, a needle, guided by identified landmarks, was inserted in the telencephalon as previously described in Materials and Methods section. Then, zebrafish were kept alive for different periods of time, ranging from 1 to 15 days.

Quantitative real-time PCR

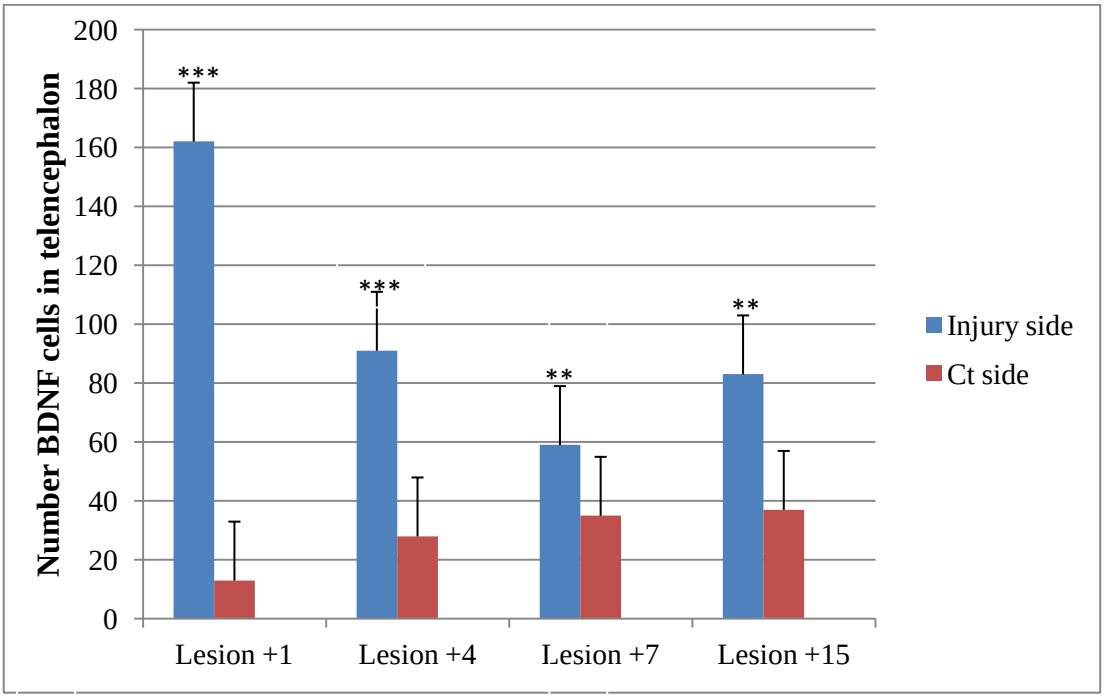
Quantitative PCR experiments showed that in lesioned telencephalon, compared to unlesioned one, the amount of BDNF mRNA noticeably increased after 1 day from lesion. Then, after 4 and 7 days from lesion, the BDNF mRNA values are slightly higher than value in unlesioned animals. Finally, after 15 days from lesion, BDNF messenger level increased again. Comparing the expression levels of BDNF mRNA with PCNA, a proliferative cell marker, the results showed a close relationship between the trend of levels.



Grf. 1 Effects of the mechanic injury on *bdnf* and PCNA expression in adult zebrafish telencephalon. Fold induction of *BDNF* and *PCNA* gene expression in injured compared to control telencephalon at different times post-injury. An asterisk indicates statistically significant differences ($P < 0.05$ by Student's t test).

In situ hybridization

In situ hybridization analysis clearly indicated an outstanding difference in number of *bdnf* expressing cells between unlesioned and lesioned side of zebrafish telencephalon, in injured being much more numerous for the entire time span from lesion. More in details, after 1day from lesion (Fig.18 A-C; A'-C'), a noticeable high number of BDNF positive cells occurred in the parenchyma around the lesion, while in the control side only a few cells showed BDNF mRNAs (Grf.2 a –c', 2 a). From 4 (Fig. 18 D-F; D'-F') to 7 days from lesion (Fig. 19 A-C; A'-C'), the number of *bdnf* expressing cells in the parenchyma around lesion decreased slightly, while in the parenchyma of the control side the number of *bdnf* expressing cells appear to increase. At 15 days from lesion *bdnf* expressing cells (Fig.19 D-F; D'-F'), in comparison to the previous group, decreased in unlesioned side and increased in lesioned side.



Grf.2 Image J and statistical analysis. An asterisk indicates statistically significant difference ($P < 0,05$). *Bdnf* expressing cells in unlesioned and lesioned side of telencephalon. After 1 day from lesion, high number of *bdnf* expressing cells in lesioned side, while only a few in unlesioned side of the telencephalon. After 4 days and 7 days from lesion *bdnf*-expressing cells progressively increased in unlesioned side and decreased in lesioned side. 15 days post-lesion *bdnf* expressing cells appeared in unlesioned side and increased in lesioned side.

Cell identification of BDNF expressing cells in injured brain

In order to identify the nature of *bdnf*-expressing cells in the injured brain of adult zebrafish, *in situ hybridization* and immunohistochemical reaction against proliferation (PCNA) and neuronal (acetylated-tubulin and Hu) markers were employed. PCNA-labeled cells were present in both the injury site and the proliferative ventricular regions facing the lesion (Fig. 20 A-F). In those regions, *bdnf*-expressing cells very close to PCNA-labeled nuclei were observed, but the double staining showed that BDNF mRNA was not expressed by proliferating cells. Conversely, *bdnf* expressing cells showed the presence of immunoreactivity to acetylated-tubulin (Fig. 21 A-D) and Hu (Fig. 22 A-F), both well known markers of postmitotic neurons.

Discussion

This survey reports, for the first time, the presence and distribution of *bdnf* messengers in the brain of larval and adult zebrafish in physiological condition and after mechanic injury of the telencephalon. In addition, BDNF expressing cells has been firstly characterized as postmitotic neurons.

BDNF mRNA localization throughout the brain of larval and adult zebrafish

In the brain of 7 days old larvae *bdnf* mRNAs are widely expressed. This finding is in agreement with the data previously obtained in zebrafish by RT-PCR and showing that *bdnf* expression is maternally inherited, drops down at 1 day post-fertilization (dpf) and then progressively increases from 1 to 8 dpf (De Felice et al., 2014). The results of the present research bring new information by showing that the strongest signal is observed in the dorsal telencephalon (pallium), the preoptic region, the thalamus and the optic tectum. In general, the expression as indicated by in situ hybridization is in good agreement with what has been shown by previous authors (De Felice et al., 2014). In this previous study, the distribution of *bdnf* messengers was addressed by in toto hybridization establishing that *bdnf* is strongly expressed in the pallium, hypothalamus, posterior tuberculum and optic tectum at 1, 2 and 3 dpf but there is no information on the precise structures expressing these messengers. In addition, at 7 dpf, the expression seems to be lower, possibly due to a problem of access of the probe to the brain (De Felice et al., 2014). My results show that in all cases, cells expressing *bdnf* mRNAs were located in the parenchyma and are identified as neurons. Furthermore, *bdnf*-expressing cells never exhibited PCNA, aromatase B or BLBP staining, indicating that *bdnf* is not expressed in radial glial progenitors. Although the function of BDNF is the developing brain of zebrafish was not addressed in the present study, these data suggest that it could be implicated in the differentiation and maintenance of newborn neurons, mainly in the forebrain.

In adult zebrafish, *bdnf* expression had a similar distribution than in the larvae with the most prominent staining in dorsal telencephalon, area preoptica, dorsal thalamus, posterior tuberculum, hypothalamus, synencephalon and optic tectum. A more

diffuse and weaker labeling was detected in other brain regions. The pattern of distribution of *bdnf* mRNA was relatively similar to that reported in the European eel and the Turquoise killifish (Dalton et al., 2009; D'Angelo et al., 2014) with some slight differences regarding the ventral telencephalon, diencephalon, tegmentum and rhomencephalon. Compared to the European eel and the turquoise killifish, *bdnf* expression in zebrafish is less expressed in the ventral telencephalon. By contrast, the staining in the diencephalon appeared more widespread than in the eel. Some slight differences also occurred in other diencephalic nuclei between zebrafish and turquoise killifish, consistently in the pretectum, which in zebrafish is reduced in its superficial component and the lack of nucleus corticalis and nucleus glomerulosus. Finally, the tegmentum and rhombencephalon of zebrafish showed fewer *bdnf*-expressing cells than eel and turquoise killifish. Thus, we can conclude that in zebrafish *bdnf* is expressed mostly in the forebrain and just in some areas of midbrain and hindbrain.

The localization of *bdnf* transcripts in adult zebrafish is similar in both sexes, despite the fact that sex hormones have been recognized to influences the expression and activity of BDNF through a combination of genomic and epigenetic mechanisms (Hill 2012; Carbone et al., 2013). In particular, during puberty BDNF protein expression was found to be higher in the frontal cortex of male mice, whereas in females expression levels where higher in the hippocampus (Hill et al., 2012). In this regard, preliminary experiments (not shown) based on RT-PCR in whole brains failed to show any significant effect of estradiol exposure (10^{-7}M to 10^{-10}M) on *bdnf* expression in 7 dpf zebrafish larvae.

Because in some regions, *bdnf*-expressing cells in adults were located close to the ventricular surface, have been examined whether such cells could correspond to radial glia cells. Indeed, these results and others have shown that in adult fish radial glial cells line entirely the ventricles (Menuet et al., 2005; Pellegrini et al., 2007). Furthermore, it is now recognized that radial glial cells are progenitors and sustain the constant growth of the brain throughout life (Pellegrini et al., 2007). To investigate if radial glial cells express or not *bdnf*, messengers, have been used two well-established markers of radial glia in fishes, aromatase B and BLBP (Menuet et al., 2005; Diotel et al., 2010). Interestingly, could not detect *bdnf*/aromatase B or *bdnf*/BLBP co-expression, suggesting that radial glial cells do not express *bdnf* under

physiological conditions. Similarly, double staining with the cell proliferation marker PCNA failed to show any co-expression of *bdnf* and PCNA in the same cells. In contrast, double staining with neuronal makers, notably Huc/d, MAP2 and acetylated-tubulin clearly identified *bdnf*-expressing cells as neuronal cells in the brain of adult zebrafish. This finding is consistent with the fact that BDNF is a factor of neuronal origin in mammals (Castrem et al., 1995; Smidth et al., 1996), birds (Yamaguchi et al., 2011; Dittrich et al., 2013), amphibians (Wang et al., 2005) and fish (Dalton et al., 2009; D'Angelo et al., 2014). However, in mammals, *bdnf* expression has been documented in glial cells under pathological conditions, in particular after brain lesions or around amyloid plaques (Burbach et al., 2004).

While three Trk receptors, TrkA, TrkB ands TrkC, have been identified in mammals, five receptors have been identified in fish (Martin et al., 1999) , due to the teleost specific genome duplication (3R) (Hoegg et al., 2004). In the zebrafish, one TrkA, two TrkB (TrkB1 and TrkB2) and two TrkC (TrkC1 and TrkC2) were characterized and shown to be differentially expressed in the developing brain, suggesting different functions during early neurogenesis (Martin et al., 1999). To date, there is very limited information on the expression of Trk in the brain of teleost fishes, due to the lack of immunological tools. Only TrkB receptors were investigated in the brain of the developing (Lum et al., 2001) and adult zebrafish (Abbate et al., 2014), but more detailed mapping are required. In the adult Turquoise killifish, immunoreactivity to TrkA, TrkB and TrkC was reported in different regions of the forebrain, such as the telencephalon, diencephalon (D'Angelo et al., 2012). Interestingly, radial glial cells lining the mesencephalic and rhombencephalic ventricles exhibit immunostaining for the 3 types of receptors (D'Angelo et al., 2012). In zebrafish, BDNF and TrkB are expressed in the developing lateral line system and appear to regulate the migration of the lateral line primordium and the generation of mechanoreceptors (Gasanov et al., 2015)

It is interesting to note that the distribution of *bdnf* messengers in the brain of zebrafish is quite similar to that reported in mammals, notably in rat, mouse and pig. Indeed, strong *bdnf* expression was reported in the cortex and hippocampus, two pallial structures. Because the telencephalon of fish develops by eversion, it is usually considered that the hippocampus equivalent in zebrafish is the dorsolateral region of the dorsal telencephalon (Mueller et al., 2011) in which abundant *bdnf*

messengers are detected. It is also noteworthy that the central part of the telencephalon, which exhibits a high density of *bdnf* mRNAs, is regarded as the presumptive equivalent of the isocortex of mammals, also strongly expressing *bdnf*. Similarly, the preoptic area and in particular the magnocellular neurons express *bdnf* messengers, similar to their mammalian counterparts, the paraventricular and supraoptic nuclei. Other structures exhibiting *bdnf* mRNAs in both fishes and mammals include the habenula, the thalamic region, the mediobasal hypothalamus and the optic tectum (inferior colliculus). These similarities suggest that BDNF functions are conserved between fishes and mammals and have emerged early in the vertebrate lineage. Studies in Drosophila have recently reported the existence of a neurotrophin-related gene (*DNT1*) (Zhu et al., 2008). According to this study, this gene would represent the ancestor of the neurotrophin family that diversified through whole genome duplications. Interestingly, in Drosophila, DNT1 exerts trophic and maintenance functions on neurons.

BDNF expression in injuried telencephalon of adult zebrafish

The injury in the dorsal telencephalon was obtained by a stab wound to mimic the cellular phenomena of adult traumatic brain injury (TBI), which is usually caused by an impact to the head that results in a mechanical insult to the brain. Particularly, the stab wound was made on telencephalon because it comprises the most studied neuronal stem cell niches (Grandel et al., 2006; Adolf et al., 2006; März et al., 2010; Ganz et al., 2010; Kishimoto et al., 2011) and its dorso lateral zone is retained to be equivalent to the medial pallium (hippocampus) of mammals (Meek and Nieuwenhuys 1998; Rodriguez et al. 2002; Salas et al. 2003; Broglio et al. 2005). The hippocampus contains one of the two constitutive neurogenic niches of mammals: the subgranular zone of the dentate gyrus (Altman, 1969; Kaplan and Hinds, 1977; Doetsch and Alvarez-Buylla, 1996; Alvarez-Buylla and Garcia-Verdugo, 2002; Kempermann, 2002; Taupin and Gage, 2002; Garcia et al., 2004; Sun et al., 2005; Chojnacki et al., 2009; Kaneko and Sawamoto, 2009).

The zebrafish telencephalon, along the entire studied period from the lesion, showed a general increase of *bdnf* expression, quantifiable both through BDNF levels in

extracts and BDNF positive cell numbers. Also several previous studies in rodents showed increase of *bdnf* expression following experimental TBI made by means of lateral fluid percussion (Hicks et al.,1997; Hicks et al.,1999a; Hicks et al.,1999b), lateral gas pressure (Yang et al.,1996) or penetrating TBI (Rostami et al 2014). However, as a result of different experimental approach and time window of 1-72 h or 1 day – 8 weeks from injury, BDNF increase appeared bilateral, ipsilateral or controlateral. Consistently, also in zebrafish the BDNF increase during the investigated 2 weeks showed variations in ipsilateral or controlateral side. These finding suggest that *bdnf* expression could finely modulated following tissue needs. In addition, the complexity of BDNF response is further suggested by the reported polymorphisms of human BDNF can affect the recovery of executive and cognitive functions of male Vietnam combat veterans with focal penetrating TBI (Krueger et al 2011;Rostami et al 2011). Upon injury in adult teleost fish, neuronal precursor cells in the ventricular zone of the injured hemisphere of telencephalon, compared with in the contralateral hemisphere, proliferate and migrate laterally, reaching the injury site by using radial glial fibers as a scaffold for their radial migration towards the injury site via the subpallium and pallium (Kishimoto et al 2012) and radial glial cells also divide to increase their generation of young neurons (Zupanc and Ott, 1999; Zupanc and Zupanc, 2006). Despite *bdnf* expression in mammalian glial cells has been documented under pathological conditions, such as after brain lesions or around amyloid plaques (Murer et al 1999; Tokumine et al 2003; Burbach et al 2004), it has been observed that in zebrafish telencephalon following injury *bdnf* expression was exclusively limited to neuronal populations, as suggested by co-localization of BDNF mRNA and the neuronal makers Huc/d and acetylated-tubulin. On the other hand, also in juvenile zebrafish and in unlesioned adult zebrafish brain BDNF mRNA was seen only in neurons of different regions. These findings are consistent with the general consensus that BDNF is a factor of neuronal origin in vertebrates (Castren et al 1995; Schmidt-Kastner et al 1996; Wang et al 2005; Dalton et al 2009; Quartu et al 2010; D'Angelo et al 2014). In zebrafish has been noticed the increase of BDNF expression following telencephalic injury concurrent to proliferation increase in the ventricular zone, thus suggesting close relationship between ventricular zone response and neuronal reaction both around lesion and in the controlateral side. Also in mammals brain injuries lead to increased proliferation

of progenitors in the subgranular and subventricular zones, and even appear to stimulate proliferation of quiescent neural progenitor cells in regions where adult neurogenesis is absent in the intact brain (for a review see Lin and Iacovitti 2015). However, regeneration of the adult mammalian brain is limited due to the failure of most of the new cells to develop further and/or to survive for sufficiently long periods of time. On the contrary, in zebrafish injury increases the constitutive neurogenesis, leading to effective production of neurons and repair in 3 weeks without remaining traces of the traumatic impact (Zupanc, 2006; Ayari et al., 2010; Kroehne et al., 2011; März et al., 2011; Baumgart et al., 2012; Kishimoto et al., 2012; Kizil et al., 2012a,c; Diotel et al., 2013; Edelmann et al., 2013; Kyritsis et al., 2013). Despite substantially similar response concerning *bdnf* expression after TBI in mammals and zebrafish, regenerative properties are dramatically different. The cause of such a disparity is not still evident; however, one of the reasons could be activation of peculiar molecular programs, which might have specific roles after injury or damage (Cosacak et al 2015).

Conclusion

In conclusion, this study presents a detailed pattern of *bdnf* expression in the brain of larval and adult zebrafish under physiological conditions and in injuried telencephalon of adult zebrafish. *Bdnf* transcripts are most abundant in forebrain regions, similarly to results reported in the mammalian brain. This study also bring evidences that *bdnf* messengers are expressed in neurons and not in radial glial cells or progenitor cells.

Moreover, the response concerning *bdnf* expression after telencephalic injury resulted substantial similar to that reported in mammals, indicating that BDNF is generally involved in the first response to brain damage. However, despite these comparable results, the regenerative properties of fish and mammals are dramatically different, suggesting the existence in fish of peculiar still unknown molecular programs, which might have specific roles after injury.

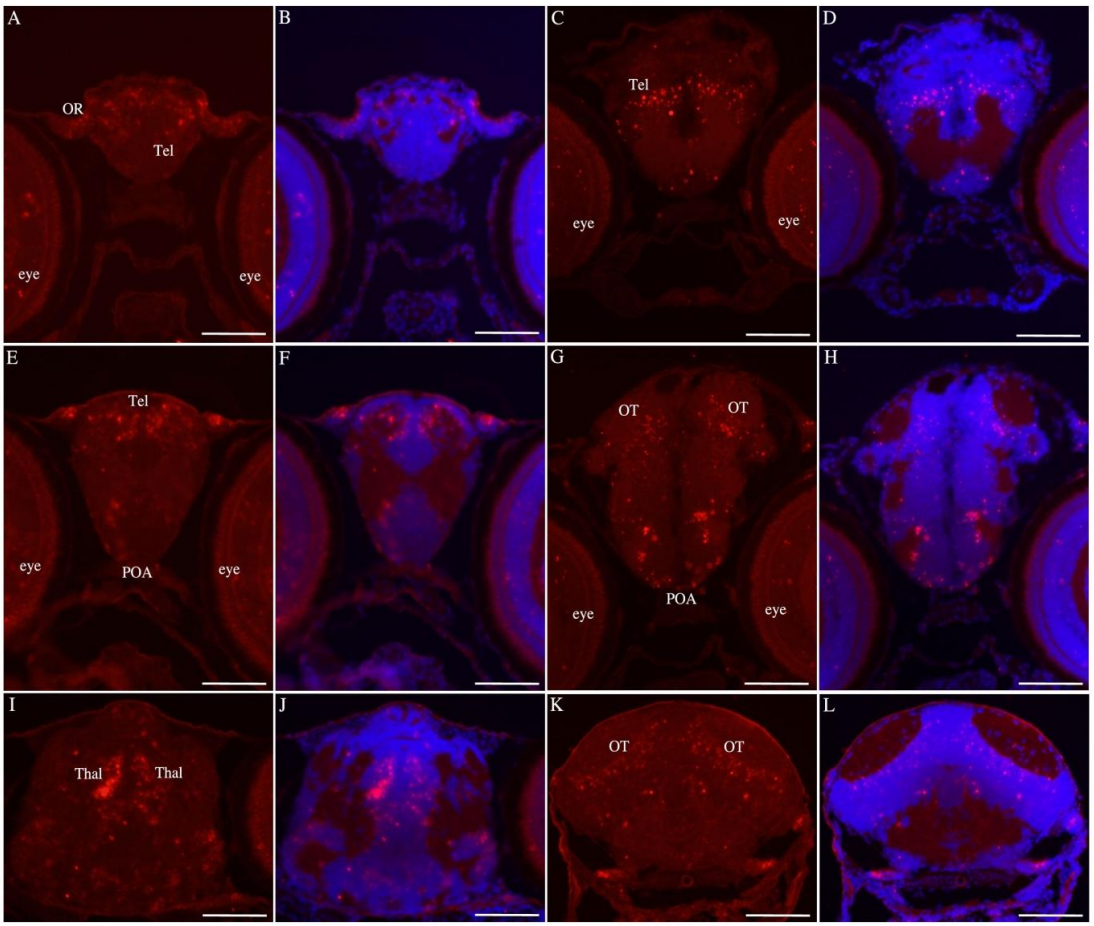


Fig. 11 *Bdnf* is expressed in the brain of 7 days old zebrafish. Olfactory rosettes (A-B), telencephalon(A-F), preoptic area (E-H), dorsal thalamus, (I-J), optic tectum and (G-H and K-L). Figures B, D, F, H, J and L show cell nuclei labeled with DAPI. OR: olfactory rosettes; POA: preoptic area; Tel: telencephalon; Thal: thalamus; OT: optic tectum. Scale bar: 120 μm

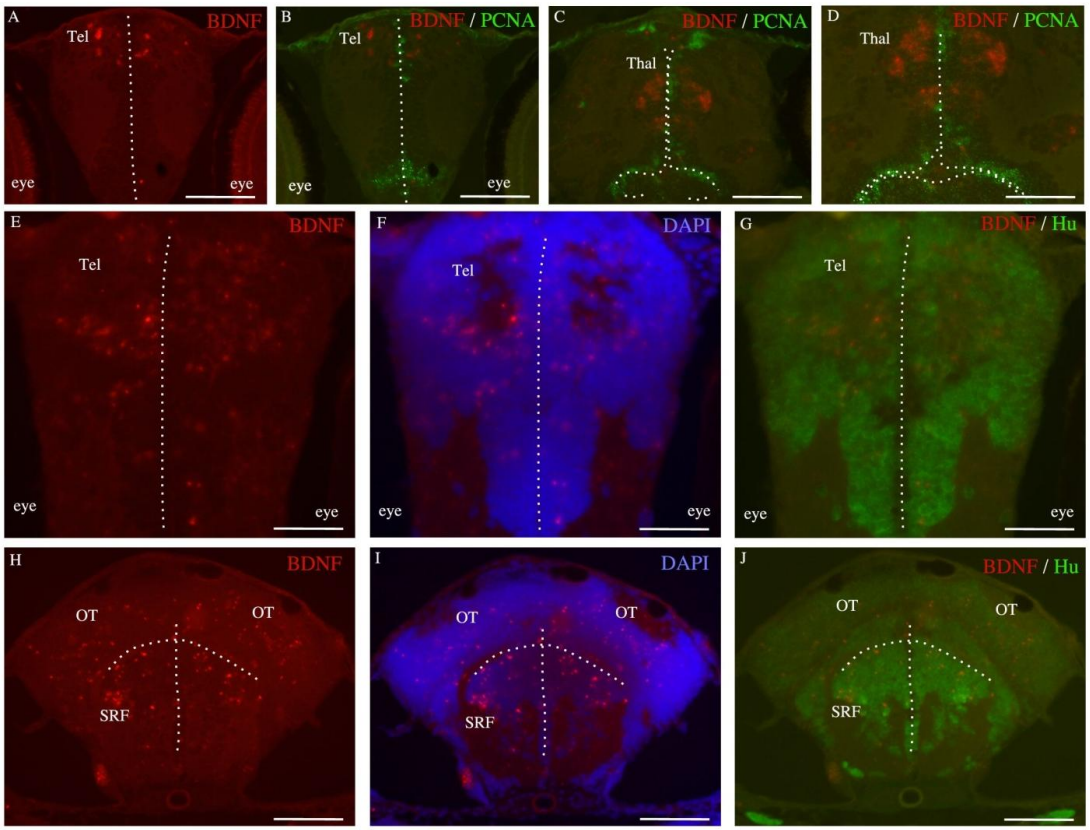


Fig. 12 Immunohistochemical characterization of *bdnf*-expressing cells in the brain of 7 days old zebrafish larvae. Double staining for *bdnf* mRNA (red) and PCNA protein (green) on cross-sections through the telencephalon (A-B) and the thalamus (C-D). Double staining for *bdnf* mRNA (red) and the neuronal marker Hu (green) in the thalamus (E,F and G), the optic tectum (H, I and J) and at the level of the superior reticular formation (H, I and J). In F and I cells nuclei are counterstained with DAPI. The dotted lines indicate the ventricles. OT: optic tectum; SRF: superior reticular formation; Tel: telencephalon; Thal: thalamus. Scale bar: 150 μm in H, I and J. 120 μm in A, B, C. Scale bar: 60 μm in D, E, F and G.

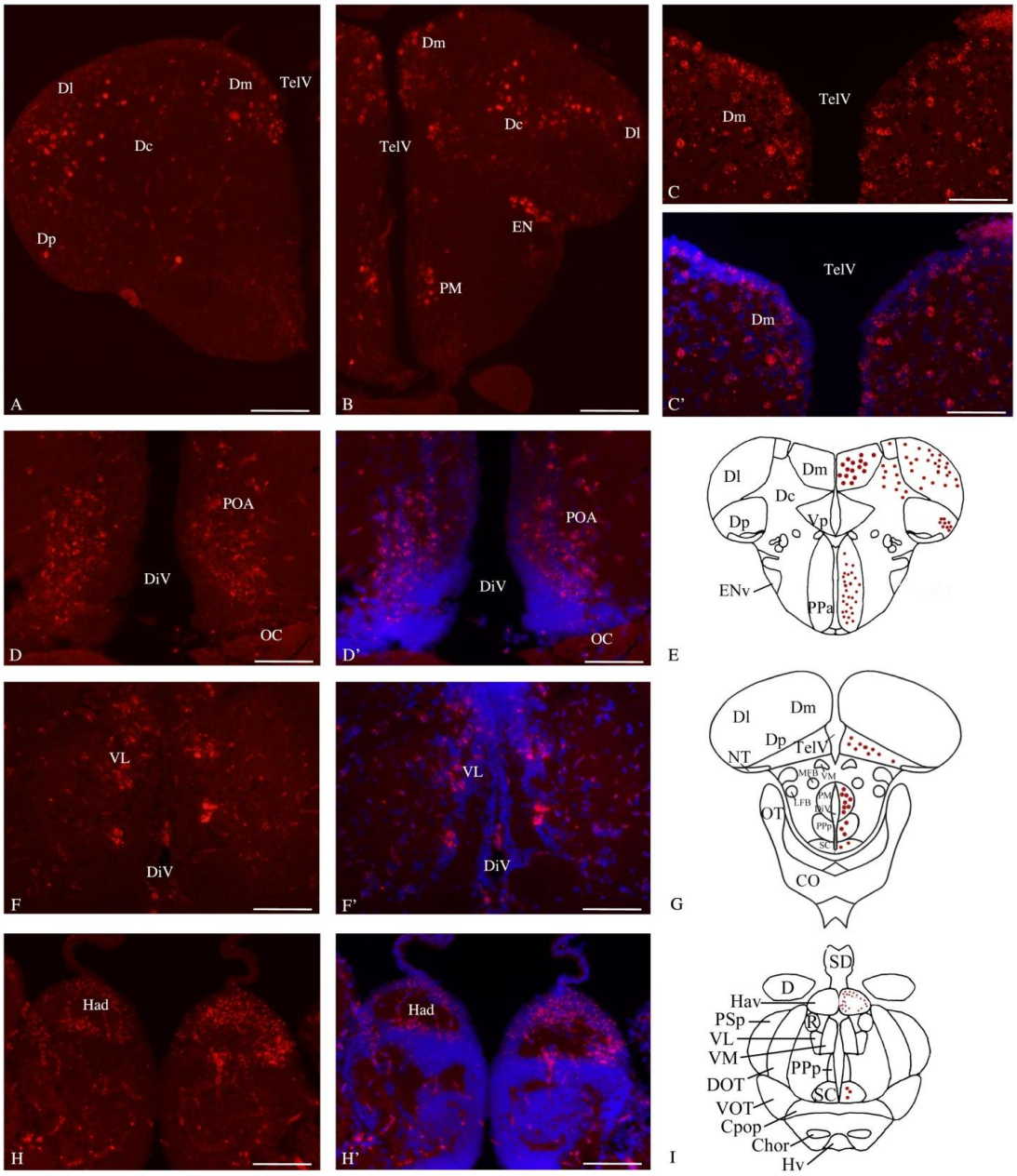


Fig. 13 *Bdnf* mRNA distribution (in red) in cross-sections through the adult zebrafish forebrain. Telencephalon (A, B, C, C' and E), preoptic area (B, D, D' and E, G), thalamus (F, F') and habenula (H, H' and I). In C', D', F', H' cell nuclei are labeled in blue with DAPI. E, G and I are representative sections taken from the zebrafish atlas (Wullimann et al., 1996). *Bdnf*-expressing cells are represented by red dots. Dc: central zone of the dorsal telencephalon; DiV: diencephalic ventricle; Dl: lateral zone of the dorsal telencephalon; Dm: medial zone of the dorsal telencephalon; Dp: posterior zone of the dorsal telencephalon; EN: endopedoncular nucleus; Had: dorsal habenular nucleus; PM: magoncellular preotpic nucleus; OC: optic chiasma; POA: preoptic area; TelV: telencephalic ventricle; VL: ventrolateral thalamic nuclei. Scale bar: 120 μm except in C and C': scale bar: 60 μm.

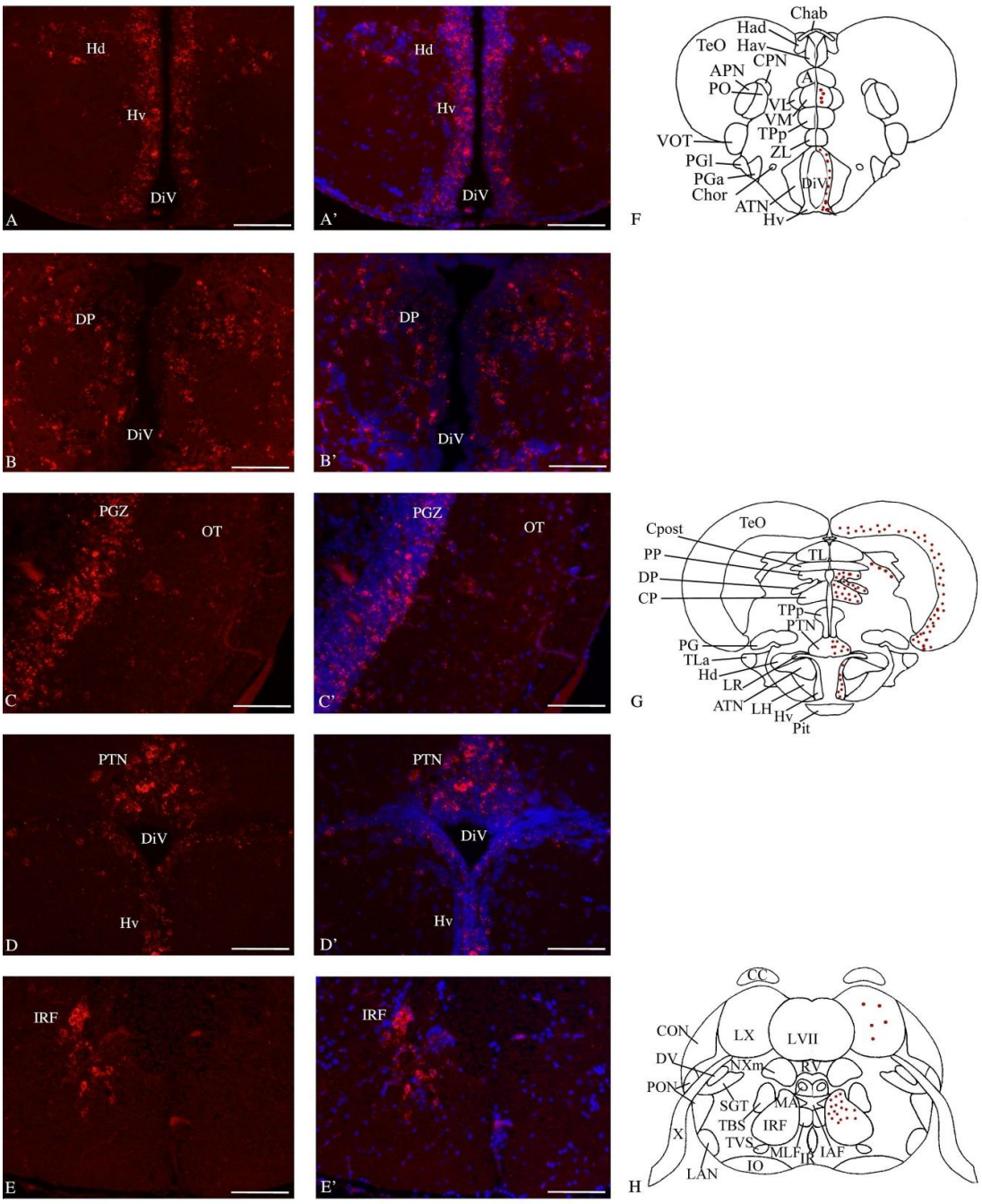


Fig. 14 *Bdnf* mRNA distribution (in red) in cross-sections through the adult zebrafish mid- and hindbrain. In the ventral hypothalamus (A, A', F and G), the dorsal thalamic region (B, B'), the optic tectum (C, C' and G), the posterior tuberal nucleus (D, D' and G) and medulla oblongata (E, E' and H). In A', B', C', D' and E', cell nuclei are labeled in blue with DAPI. F, G and H are representative sections taken from the zebrafish atlas (Wullimann et al., 1996). B*dnf*-expressing cells are represented by red dots. DiV: diencephalic ventricle, DP: dorsal thalamic nucleus; Hd: dorsal zone of the periventricular hypothalamus; Hv: ventral zone of the periventricular hypothalamus; IRF: inferior reticular formation; OT: optic tectum; PGZ: periventricular gray zone of the optic tectum. PTN: posterior tuberal nucleus. Scale bar: 120 μm.

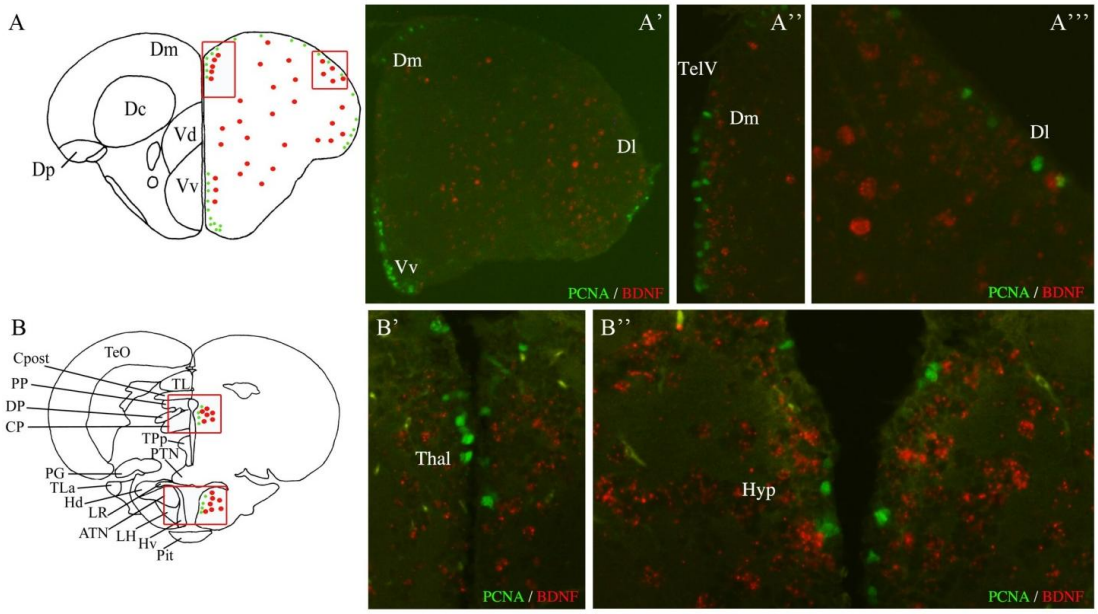


Fig. 15 Immunohistochemical characterization of *bdnf*-expressing cells. Double staining for *bdnf* mRNA (red) and PCNA protein (green) on cross-sections through the telencephalon (A to A'''), the thalamus (B and B') and the ventral hypothalamus (B and B''). A and B are representative sections taken from the zebrafish atlas (Wullimann et al., 1996). *Bdnf*-expressing cells are represented by red dots and PCNA-labeled cells are green dots. Dl: lateral zone of the dorsal telencephalon; Dm: medial zone of the dorsal telencephalon; Hyp: hypothalamus; TelV: telencephalic ventricle; Thal: thalamus. Scale bar = 200 µm in A'; 100 µm in A''; 50 µm in A'''.

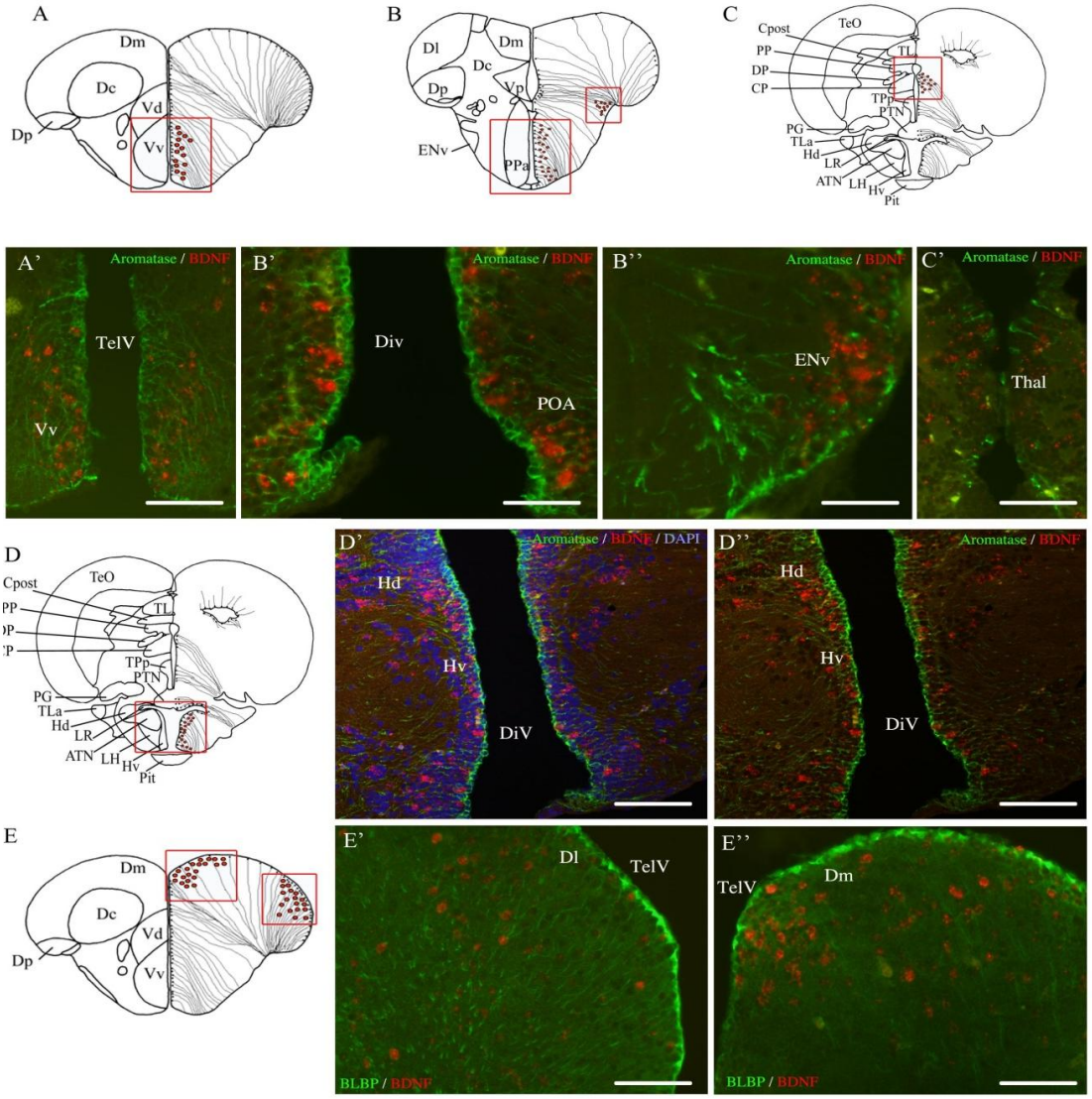


Fig. 16 Immunohistochemical characterization of *bdnf*-expressing cells. A, B, C, D and E are representative sections taken from the zebrafish atlas (Wullimann et al., 1996). *Bdnf*-expressing cells are represented by red dots and Aromatase B (A-D) or BLBP-labeled cells (E) are represented by black dots with thin lines indicating radial glia cytoplasmic processes. Double staining for *bdnf* mRNA (red) and Aromatase B protein (green) on cross-sections through the telencephalon (A-A'), the preoptic area (B-B'), the entopedoncular nucleus (B-B''), the thalamus (C-C') and the ventral hypothalamus (D-D''). Double staining for *bdnf* mRNA (red) and BLBP protein (green) on cross-sections through the telencephalon (E-E''). DiV: diencephalic ventricle; Dl: lateral zone of the dorsal telencephalon; Dm: medial zone of the dorsal telencephalon; ENv: endopedoncular nucleus; Hd: dorsal zone of the periventricular hypothalamus; Hv: ventral zone of the periventricular hypothalamus; POA: preoptic area; TelV: telencephalic ventricle; Vv: ventral telencephalic area. D' and D'' are obtained with an Apotome-equipped Zeiss. Scale bar = 60 μm in A', C', D', D'', E' and E''; 30 μm in B' and B''.

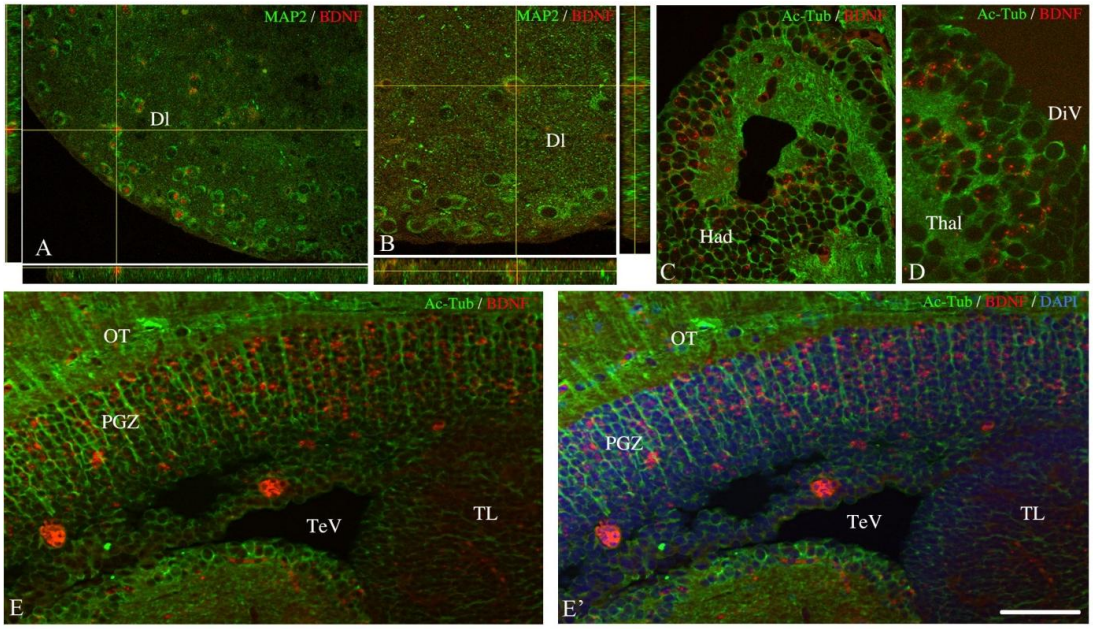


Fig. 17 Transverse sections at the level of the telencephalon showing co-expression of *bdnf* with neuronal markers. Orthogonal projections of Z-stacks (11 sections of 0.5 μm) evidencing the expression of *bdnf* (red) in neurons identified by MAP2 protein (green) (A and B). Double staining for *bdnf* mRNA (red) and acetylated-tubuline (green) in the habenula (C), the thalamus (D) and the optic tectum (E-E'). In E', cell nulei are labeled in blue with DAPI. Dl: lateral zone of the dorsal telencephalon; Had: dorsal habenular nucleus; OT: optic tectum; PGZ: periventricular gray zone of the optic tectum; TelV: telencephalic ventricle; Thal: thalamus; TL: torus longitudinalis. A, B, C and D were obtained with the confocal microscope. E and E' were obtained with the Apotome. Scale bar: 50 μm in E and E'; 40 μm in A and C; 25 μm in B and D.

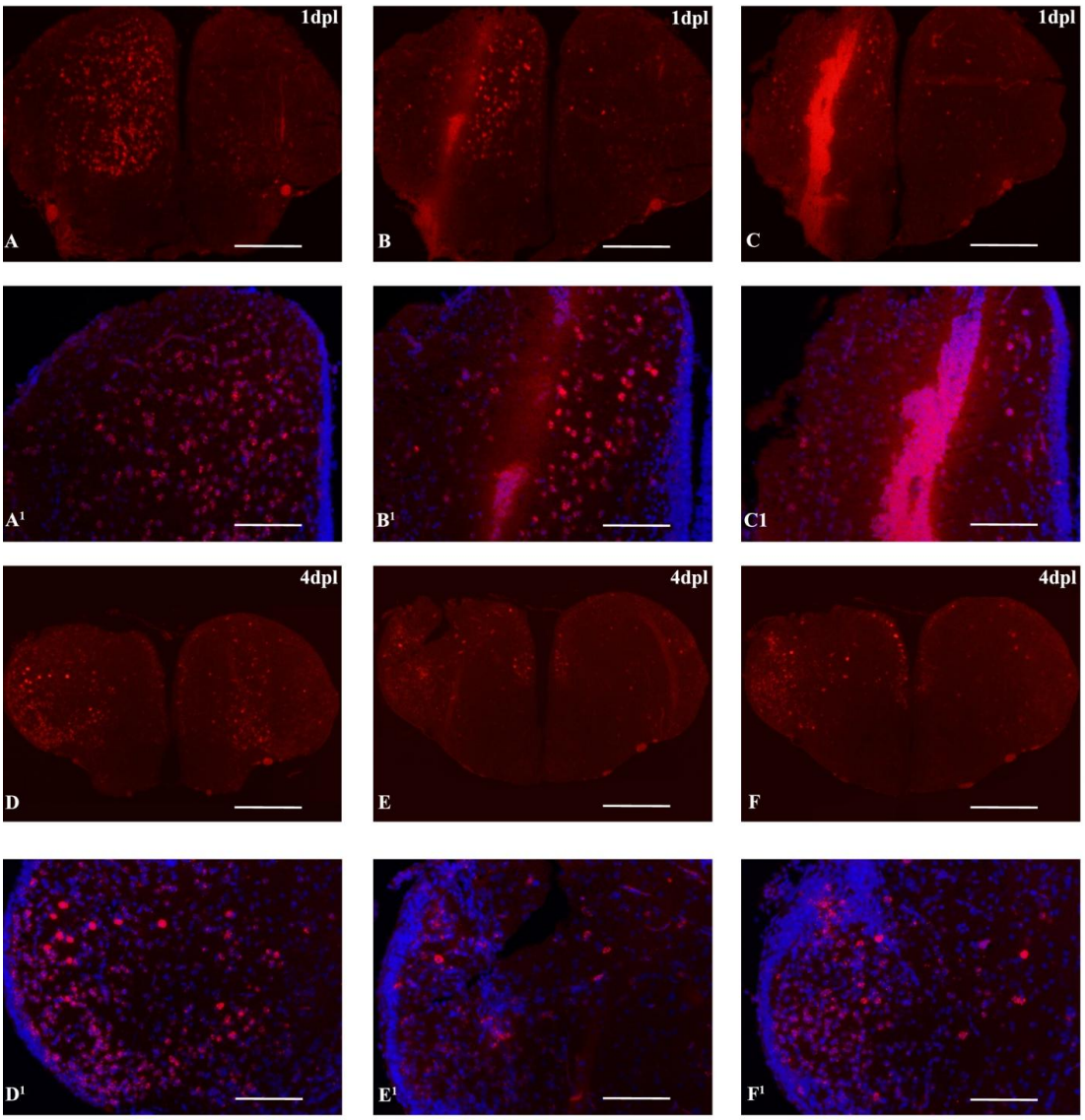


Fig. 18 In situ Hybridization. BDNF expression in injured brain at different times from lesion. (A-B-C). Serial section of the brain injured after 1 day from lesion, (A'-B'-C') cell nuclei in injured area are labeled with Dapi (blue). (D-E-F) BDNF messenger expressing on serial section of the telencephalon injured after 4 days from lesion, (D'-E'-F') cell nuclei are labeled with Dapi. Scale bar = 200 μm in A-B-C-D-E-F; 60 μm in A'-B'-C'-D'-E'-F'.

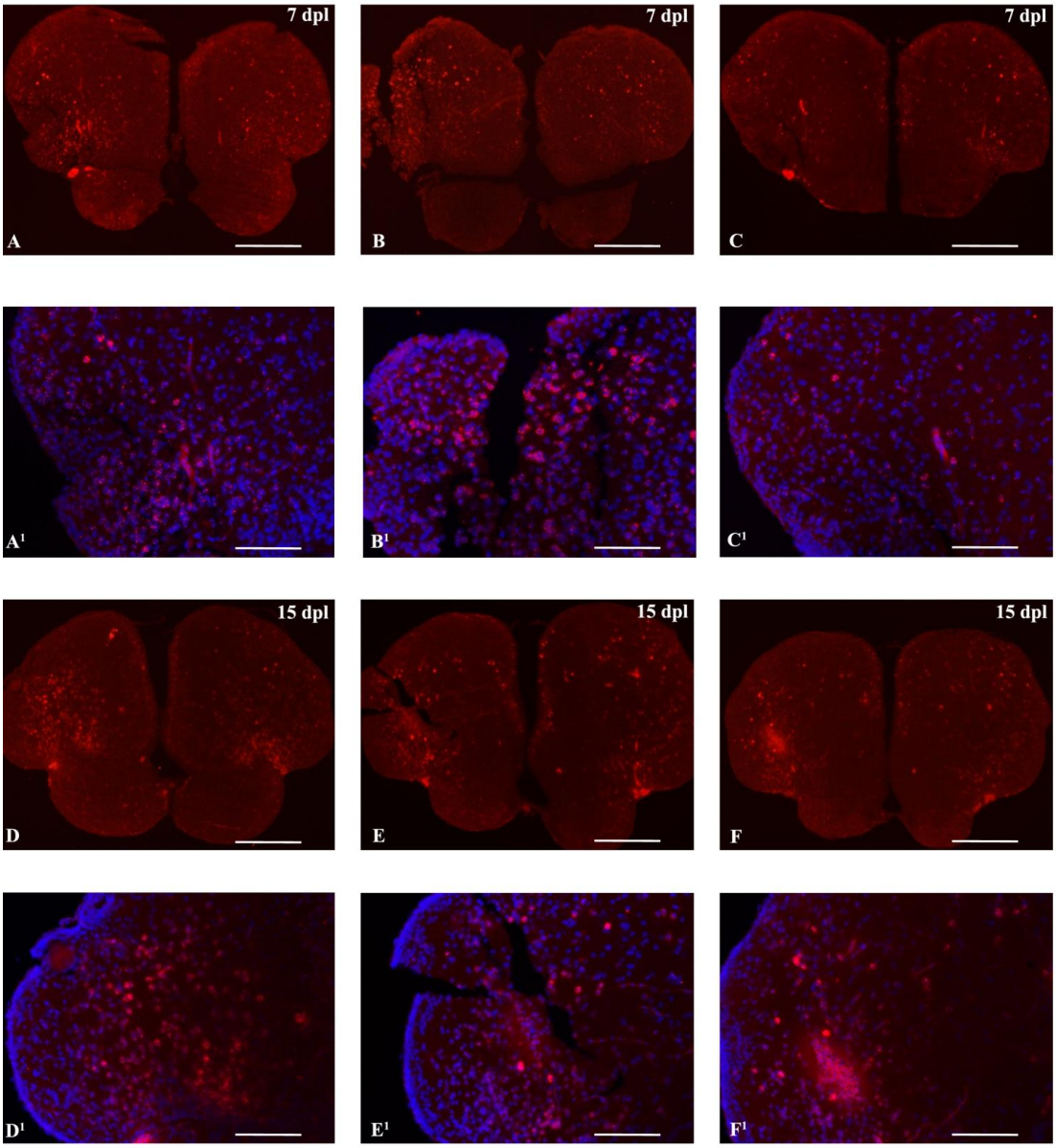


Fig 19 In situ Hybridization. BDNF expression in injured brain at different times from lesion. (A-B-C). Serial section of the brain injured after 7 day from lesion, (A'-B'-C') cell nuclei in injured area are labeled with Dapi (blue). (D-E-F) BDNF messenger expressing on serial section of the telencephalon injured after 15 days from lesion, (D'-E'-F') cell nuclei are labeled with Dapi. Scale bar = 200 μm in A-B-C-D-E-F; 60 μm in A'-B'-C'-D'-E'-F'.

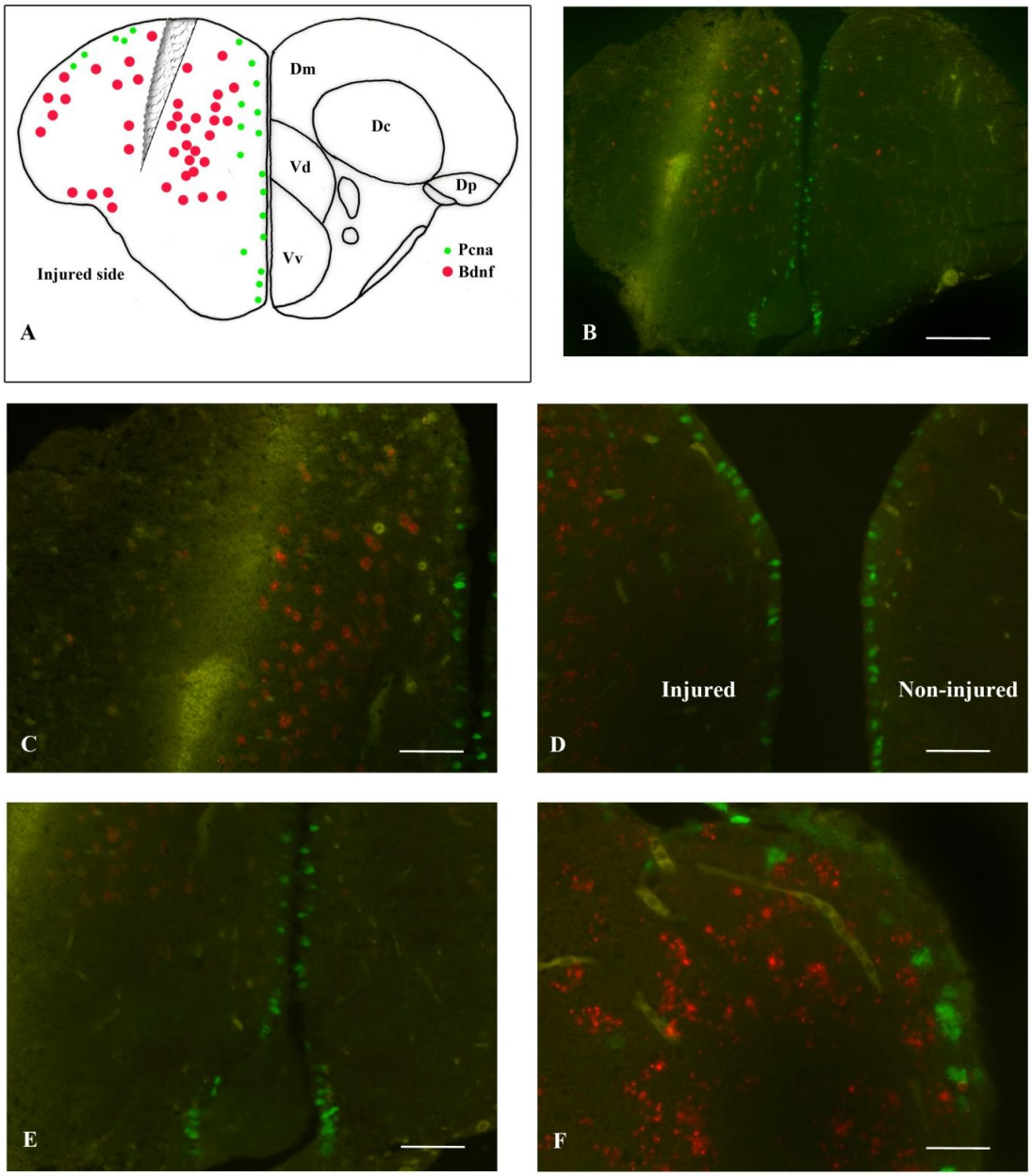


Fig. 20 Immunohistochemical characterization of *bdnf*-expressing cells in injured brain. Double staining for *bdnf* mRNA (red) and PCNA protein (green) on cross-sections through the telencephalon. (A) is a representative section of the telencephalon injured. (B-C-D-E-F) *Bdnf*-expressing cells are represented by red dots and PCNA-labeled cells are green dots. Dm: medial zone of the dorsal telencephalon; Dc: central zone of the dorsal telencephalon; Dp: posterior zone of the dorsal telencephalon. Scale bar: B 200μm; C-D-E 60μm; F 40μm

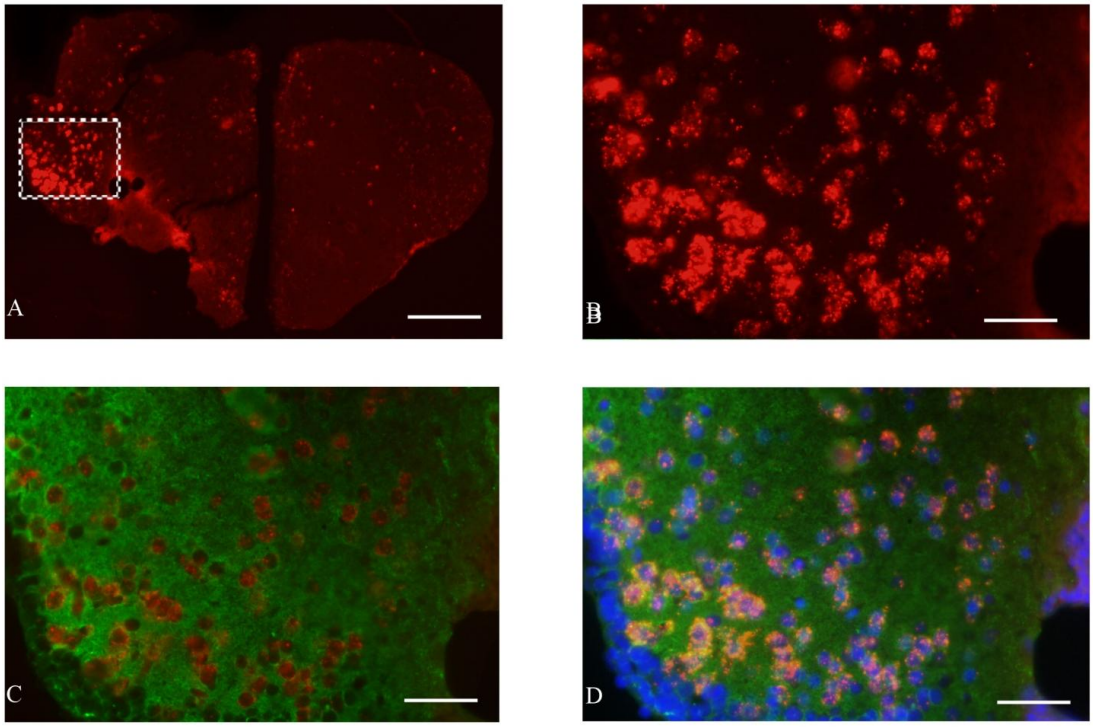


Fig. 21 (A) In situ hybridization of BDNF expression in telencephalon injured, (B) high magnification of the lesioned area, (C) Double staining for *bdnf* mRNA (red) and acetylated-tubuline (green). In (D), cell nulei are labeled in blue with DAPI. Scale bar: A 200μm; B-C-D 25μm

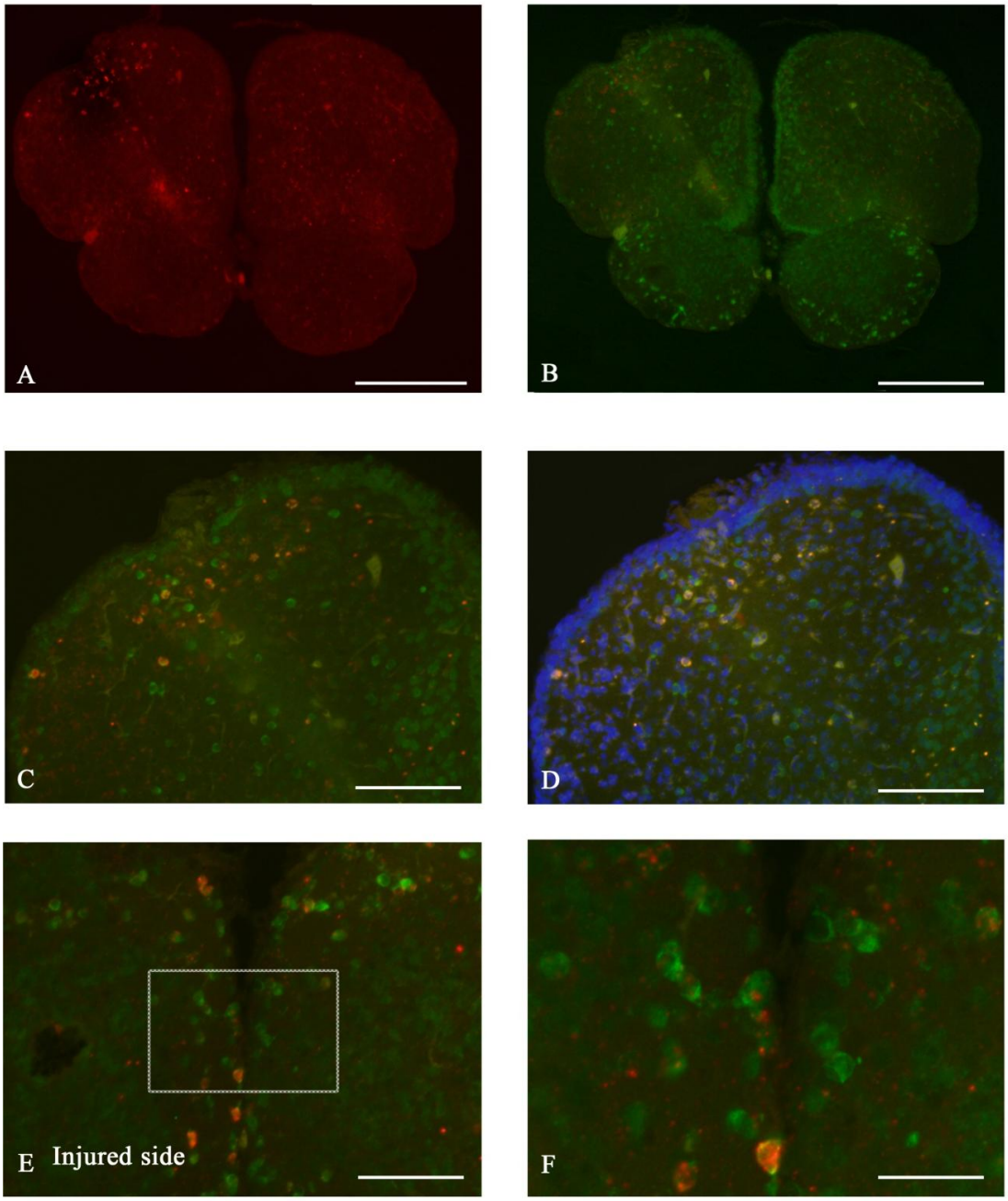


Fig. 22 Immunohistochemical characterization of *bdnf*-expressing cells in the brain of 1 days from lesion zebrafish. Double staining for *bdnf* mRNA (red) and the neuronal marker Hu (green) on cross-sections through the telencephalon (A-B). High magnification of the area lesioned (C) and (D) cell nuclei are counterstained with Dapi. (E) Duble staining and co-located for bdnf mRNA (red) and Hu green along medial zone of the dorsal telencephalon, particular (F) high magnification obtained with confocal microscope. Scale bar A-B 200μm ; C-D 50 μm; E 40 μm; F 20μm.

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